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Toxicology Letters

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Toxicology Letters serves as a multidisciplinary forum for research in all areas of toxicology. The prime aim is rapid publication of research letters with sufficient importance, novelty and breadth of interest. In addition to research letters, papers presenting hypotheses and commentaries addressing current issues of immediate interest to other investigators are invited. Mini-reviews in various areas of toxicology will also be published. A new feature is the provision of a forum for the discussion and interpretation of data published in the journal. Clinical, occupational and safety evaluation, legal, risk and hazard assessment, impact on man and environment studies of sufficient novelty to warrant rapid publication will be considered.

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Preface

EUROTOX 2002, the 40th Congress of the European Societies of Toxicology was organized at Budapest Convention Center in Budapest, Hungary from 15 to 18 September, 2002.

The scientific program of the congress consisted of four continuing education courses, ten symposia, five workshops, one plenary lecture, the Gerhard Zbinden memorial lecture and a EUROTOX-SOT debate with 77 invited speakers, as well as 349 posters. Six hundred and thirty six registered participants attended the congress.

The scientific program included many fields of toxicology, from basic processes such as genomics/proteomics or the importance of peroxynitrite, up to regulatory toxicology such as harmonization of test methods or inhalation toxicology. Emphasis was also given to hazard and risk communication, as well as to environmental pollution and biological monitoring. One of the most important issues in toxicology is the connection between animal studies and human data, which was demonstrated in the developmental neurotoxicity workshop and in the lectures on inhalation toxicology. The Gerhard Zbinden memorial lecture highlighted the importance of human toxicology.

The local organizers and the editors of the Proceedings would like to thank all the authors presenting their lectures at the congress and submitting their manuscripts.

The EUROTOX Proceedings 2002 comprises important areas of toxicology: regulatory toxicology, carcinogenic and genotoxic mechanisms, developmental neurotoxicology, inhalation toxicology, risk evaluation, ecotoxicology and food toxicology. The number of papers accepted for publication was 51. The articles reflect the rapid developments of the research methods and toxicological knowledge. We would like to thank all the authors who submitted their manuscripts in good time to ensure rapid publication of the Proceedings. Sincere thanks are also due to the Referees and Coeditors of the present EUROTOX Proceedings. We hope you will enjoy it.

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Review

From clinical to human toxicology: linking animal research and risk assessment in man

Jacques Descotes *

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Abstract

Since the 1960s, clinical toxicologists have primarily focused on acute poisonings. This proved very successful as the prognosis markedly improved with the use of resuscitation methods, evidence-based management and new antidotes. This latter area was the first major instance linking animal research and clinical toxicology, as illustrated with *N*-acetylcysteine or specific antibodies. Simultaneously the evolution of poison centers was a critical turning point as '2nd generation' centers are increasingly involved in risk assessment and toxicovigilance. Human toxicology is a broader area in that it is also involved in the toxicity evaluation of xenobiotics with the resulting need to link animal research and risk assessment to match the results of preclinical studies with clinical observations. However, this is not an easy task as experimental and clinical toxicologists seldom share ideas and expertise. Immunotoxicology is an example of this situation. Most of the available data on immunosuppression was obtained in animals and not in man, whereas allergic reactions have been extensively investigated in man, but overlooked in animals until recently. One of the major challenges facing toxicology is to bridge the gap between animal research and risk assessment in man. Human toxicology is expected to play a role in taking up this challenge.
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Keywords: Clinical toxicology; Poison center; Experimental toxicology; Risk assessment; Toxicovigilance; Immunotoxicology

1. Introduction

The Spanish-born French forensic toxicologist Orfila paved the way to modern toxicology by introducing the systematic comparison of data from human post-mortem examinations with the information gained by observing intoxicated animals and humans (Orfila, 1814). Despite this seminal work attempting to link animal and human data for the first time, toxicology rapidly split into two areas, namely experimental and clinical toxicology. Experimental toxicology has reached a very high level of sophistication and expertise (Klaassen, 2001), whereas clinical tox-

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icology is faced with the need to redefine its role and place as a specific subdiscipline of toxicology after a period of rapid development in the second half of the last century (Proudfoot, 1988).

This paper is intended to propose personal thoughts on the evolution of clinical toxicology which is expected not only to focus on the treatment of acutely poisoned patients, but also to become increasingly involved in the medical evaluation of toxic risks to human health in relation to chemical exposure, hence the proposed term 'human toxicology' (Descotes, 1996).

2. Clinical toxicology

Present-day clinical toxicology was born in the early 1950s when Scandinavian doctors introduced the recently discovered concepts of resuscitation to the field of human poisonings. This resulted, for example, in a dramatic drop (from ≈30 to 1%) in the death rate associated with barbiturate coma within a few years (Clemmesen, 1954). Since then, the primary focus of clinical toxicologists has consistently been the management of acute human poisonings (Lovejoy, 2000).

2.1. Clinical toxicology and the management of acute human poisonings

Following the introduction of the modern methods of intensive supportive care, clinical toxicologists further expanded their expertise by designing protocols based on clinical observations and analytical results to treat more effectively patients severely poisoned by a number of toxic substances. Typical examples include the identification of prognosis factors in digitalis poisonings, namely age above 55 years, preexisting cardiac disease and hyperkalemia (Dally et al., 1981), or the predictive value of plasma concentrations regarding the outcome of paraquat poisonings (Proudfoot et al., 1979).

Importantly, this demonstrates the continuing need for highly specialized clinical toxicology departments even though more and more physicians and general hospitals have the training and equipment required to treat a wider array of poisoned patients. Such specialized departments are indeed essential to maintain the expertise to treat rare and/or severe human poisonings, but also to design evidence-based management protocols, and finally to develop new antidotes. A major consequence of this is the increasing need for clinical toxicologists to establish close interactions between fundamental research, animal (preclinical) studies and clinical practice. The two following well-known examples are given to illustrate this need.

2.1.1. Paracetamol poisoning

Paracetamol (acetaminophen) is a minor analgesic known to be safe when used at the recommended therapeutic dose, but also a long-established cause of severe and even possibly lethal liver failure after ingestion of high doses. The biochemical basis of paracetamol hepatotoxicity was elucidated in the 1970s despite a few remaining uncertainties (Bessems and Vermeulen, 2001): briefly, the oxidation of paracetamol leads ultimately to the reactive intermediate *N*-acetyl-parabenzoquinone imine (NABQI), which is normally neutralized by glutathione. When the ingested dose of paracetamol exceeds a certain level (≈175 mg/kg), the glutathione body stores are depleted and NABQI can cause centrilobular hepatic necrosis. The regeneration of glutathione stores can be achieved by the administration of *N*-acetyl-cysteine (NAC), the precursor of glutathione. Based on this large body of experimental evidence obtained in vitro and in animal studies (Thomas, 1993), the efficacy of NAC, either orally (Smilkstein et al., 1988) or intravenously (Prescott et al., 1979) was conclusively established in poisoned patients, and NAC is nowadays unanimously recommended as the first-line treatment of paracetamol overdoses (Prescott, 2000).

2.1.2. Specific antibodies to treat acute poisonings

Another example of the link between animal and clinical studies leading to significant progress in the treatment of acute poisonings is the use of specific antibodies ('immunotoxicotherapy'). In the late 1970s American authors showed that anti-digoxin specific Fab antibody fragments can protect laboratory animals from experimental

intoxication (Ochs et al., 1978) as well as severely poisoned patients (Antman et al., 1990). Further non-clinical and clinical studies investigating the pharmacodynamic and pharmacokinetic effects of these antibodies proved instrumental to gain a better understanding of their mechanism of action and improve therapeutic regimens. The use of anti-digoxin specific antibodies is now approved in many countries for the management of severe digitalis poisonings.

Other specific antibodies have been produced and investigated, but none has so far reached the same level of recognition. They include antibodies against the antiarrhythmic drug ajmaline (Descotes et al., 1981), the anti-gout drug colchicine (Urtizberea et al., 1991) or tricyclic antidepressants (Heard et al., 1999). In the two later instances, efficacy was suggested in the clinical setting. However, not all toxicants can be effectively neutralized by specific antibodies, as exemplified by paraquat (Cadot et al., 1986). Despite limitations in the clinical efficacy and availability of specific antibodies (Bismuth et al., 1997), this approach is a good example of the necessary link between animal research and clinical toxicology to develop effective antidotal treatment for acute poisonings.

2.2. Clinical toxicology and poison information services

With significant advances in the treatment of acute poisonings, trained hospital departments gained wider recognition among physicians as well as the general population and received more and more phone queries for advice on the best ways to treat poisoned patients (Proudfoot, 1988; Lovejoy, 2000). This trend proved particularly marked in emergency departments from children's hospitals in the USA, and with the increasing number of phone calls and the obligation to respond to this demand, the first Poison Control Centre (PCC) ever was created in Chicago in 1953. A number of other PCCs were soon started in the USA, typically in children's hospitals. The trend was somewhat different in Europe where most PCCs were hosted by adult emergency departments from the 1960s and beyond. Despite differences in structure and organization, networks of PCCs were established in many countries, like the USA, the UK or France, whereas other countries, such as Sweden, Norway, Belgium, or Switzerland, partly because of a smaller population size, preferred national centers.

In their early days, PCCs were part of emergency departments and therefore were directly involved in the management of acutely poisoned patients. With the ever increasing number of phone calls received, PCCs had to establish a full poison information service requiring specific experience and know-how. Comprehensive and dedicated databases on the composition of commercial products and toxicity of their ingredients, as well as databases compiling clinical cases of poisonings were created in most PCCs. Efforts were also paid to improving the reliability and quality of answers given by PCCs; for example, the American Association of Poison Control Centers (1988) and the International Programme on Chemical Safety (1988) defined criteria to be met by PCCs.

In the 1990s, a shift became apparent with the proposed introduction of clinical toxicology centers (Vale and Meredith, 1993). Although the management of poisoned patients remained a central theme, other issues, such as prevention and education programs, or expert clinical advice to government bodies, were given greater consideration. At the present time, most PCCs are involved toward '2nd generation' PCCs which are no longer directly involved in the management of acutely poisoned patients, even though their primary role is still to provide expert advice on poisoning management via phone calls, but they also dedicate growing efforts to expanding product data and clinical case databases at both the national and international level, and to promoting new activities, such as toxicovigilance, the management of chemical catastrophe, or the surveillance of specific toxic exposures, e.g. lead in children.

3. From clinical to human toxicology

Despite the indubitable need for specialized clinical toxicology departments to treat severely

poisoned patients, there is also a growing interest in the evaluation of the potential adverse effects of chemicals to the health of exposed humans during manufacture, storage, transport and use.

Risk assessment is primarily derived from non-clinical toxicology and thus clinical toxicologists are rarely, if at all, involved in this process. As they receive a training essentially oriented toward medical intensive care, this training should be expanded to include other critical aspects required for the proper use and interpretation of non-clinical data. In doing so, clinical toxicologists will be more apt to share ideas and expertise with non-clinical toxicologists and to help bridge the present gap between clinical and non-clinical toxicology.

3.1. Risk assessment and human toxicology

Typically, the process of risk assessment consists of four subsequent phases, namely hazard identification, dose–response relationships, exposure evaluation and risk characterization.

3.1.1. Hazard identification

In the area of toxicology, hazard is synonymous with toxicity. In most instances, knowledge on the toxicity of xenobiotics is acquired through structure–toxicity relationships, in vitro assays and animal studies (Barlow et al., 2002).

Surprisingly and despite the seminal work of Orfila, clinical toxicology data and in particular data from PCC clinical databases are very rarely, if at all, used in hazard identification. A comparison of clinical and animal data could nevertheless be useful in a number of instances as acute poisonings represent nearly experimental conditions in which human beings are either inadvertently or voluntarily exposed to very high doses of a toxic substance. Such a comparison is also likely to highlight shortcomings in both clinical databases and animal toxicity studies, and thus lead to improvements in both.

3.1.2. Dose–response relationships

Information on this phase is essentially obtained from animal studies. However, the comparison of toxic effects in humans exposed to low or high doses for long or short periods of time is an example of such relationships. Thus, PCC clinical databases could be used to compare the results of dose–response relationship evaluation in animal toxicity studies and in man. The selection of doses in animal toxicity studies which is often based on theoretical, not to say traditional, rules could for instance benefit from such comparisons.

3.1.3. Exposure evaluation

It is widely recognized that human exposure cannot be fully and reliably evaluated by measuring only the levels of a given xenobiotic in biological fluids or the environment. The search for biomarkers of toxicity is essential and enormous efforts are being paid to this crucial area (Timbrell, 1998).

The identification and validation of reliable biomarkers require close interactions between fundamental research, animal and clinical studies. These interactions have already been set up in occupational toxicology (Mutti, 1999), but there is a need for similar interactions in other areas of toxicology.

3.1.4. Characterization of risk

The characterization of risk is the ultimate phase taking into account the data accumulated during the three previous phases. When the data available is limited, as is often the case, the characterization of risk largely relies on simulation models and uncertainty factors, and these would certainly benefit from the input of human toxicologists. For instance, data from PCC clinical databases could prove very useful for the determination of the NOEL in human beings.

Epidemiology is instrumental to characterize risk, but although convergences have been identified (Jaffery et al., 2002), it is uncertain that epidemiology can solve all problems regarding human toxicity in relation to chemical exposure (Taubes, 1995).

3.2. Toxicovigilance

In keeping with pharmacovigilance, the post-marketing surveillance of drug-induced adverse effects, toxicovigilance has recently been proposed

for identifying the adverse effects of chemical exposures in human beings. As indicated above, PCCs are more and more involved in toxicovigilance (Lall and Peshin, 1997).

In contrast to epidemiology, toxicovigilance is primarily based on the in-depth medical evaluation of case reports of human intoxications, either acute or chronic. This evaluation allows for the identification of the causal relationship between toxic exposures and pathological conditions on an individual basis (Evreux et al., 1987). Another approach is the systematic search for shifts in the recorded causes of poisonings in PCC clinical databases on a periodic basis, e.g. shifts due to changes in product formulation or use. Finally, toxicovigilance is also involved in the specific follow-up of groups of the general population exposed to given toxicants, e.g. lead in children. So far, toxicovigilance has not reached the same level of expertise and organization than pharmacovigilance. As this new approach is likely to improve the evaluation of toxic risks for human beings, e.g. risks for consumers, efforts should be paid to structure and develop this new activity at an international level.

4. Immunotoxicology as an example

The current status of immunotoxicology is an illustration of the above. Immunotoxic effects can be divided into four categories, namely immunosuppression, immunostimulation, allergy and autoimmunity. Overall, both animal and human data on a given category of immunotoxic effects are very seldom available simultaneously. The lack of human data is a major limitation to the validation of existing animal models and risk assessment strategies, whereas human data, when available, should serve as a basis to design more relevant new models, hence the need for clinical or human immunotoxicology to be paid much more attention (Descotes, 1988a,b).

4.1. Immunotoxicology and immunosuppression

During the 25 first years of its existence, immunotoxicology focused on immunosuppression to such an extent that immunosuppression and immunotoxicity are still mistakenly used interchangeably by many authors. In fact, the observation of adverse effects, e.g. infectious complications, soon after the introduction of potent immunosuppressive drugs into the clinical setting (Meyler, 1966) served as an impetus to a number of animal studies to investigate the unexpected immunosuppressive potential of medicinal products as well as industrial and environmental chemicals (Descotes, 1988a,b). Despite the continuing need to refine currently used animal models, it is arguable that the immunosuppressive potential of new chemical entities can be predicted to some extent. The implementation of recent immunotoxicity guidelines issued by major regulatory agencies, such as OECD, EPA, EMEA or FDA, should help avoid at least that unexpectedly potent immunosuppressive chemicals are released to the market.

Very little is known of the immunosuppressive potency in human beings of most chemicals so far tested in animals. The database on the immunotoxic effects of drugs and chemicals in man is very small. For example, dioxin is a potent immunosuppressant in rodents (Kerkvliet, 1995), but limited evidence is available that similar effects do occur in man. The same holds true for many metals, pesticides or medicinal products.

As far as medicinal products are concerned, the lack of human data is largely due to the fact that no immunological, either laboratory or clinical, endpoints are usually included in clinical trials. The issue of biomarkers of immunotoxicity is still a totally open question (Descotes et al., 1996). For instance, no immunological endpoints can be used to monitor the efficacy of immunosuppressive drugs. Nevertheless, that medicinal products can produce clinically significant immunosuppressive effects resulting in infectious complications has recently been demonstrated with reports of tuberculosis in rheumatoid patients treated with the anti-TNF monoclonal antibody infliximab (De Rosa et al., 2002).

As regards non medicinal products, immunotoxicity risk assessment is also in its infancy (Van Loveren et al., 1998). Obviously, the lack of human data is again a major limitation. The best

example so far is UV exposure. Based on the effect of UV exposure to the resistance of mice toward experimental infections and the estimated human UV exposure, it was possible to predict the risk of infections in man exposed to sunshine (Norval et al., 1999). Unfortunately, this is the only published example of a full application of immunotoxicity risk assessment in man although the methodology used could easily be applied to a wide array of drugs and chemicals.

4.2. Immunotoxicology and immunostimulation

Immunostimulation has long been merely considered as an immunological situation leading to allergy and autoimmunity. This view is no longer tenable and immunostimulation should be addressed as a specific immunotoxicological issue. Despite early reports showing that immunostimulating drugs can induce a particular pattern of adverse effects (Descotes, 1985), immunostimulation became a matter of concern only after the introduction of therapeutic cytokines into the clinical setting. The clinical experience showed that potent immuno-activating substances, e.g. therapeutic cytokines and monoclonal antibodies, can cause a number of immune-mediated adverse effects, such as more frequent auto-immune diseases and allergic reactions to unrelated allergens, flu-like syndromes and inhibition of hepatic drug metabolism (Vial et al., 2000).

However, very limited animal data is available on the unexpected immunostimulating effects of drugs and chemicals. There is a need to design and validate relevant animal models to predict the potential of new immunostimulating drugs to induce immune-mediated adverse effects. Experimental infection models are inadequate in this regard and animal models of experimental allergy or autoimmune disease are probably more relevant, but should be carefully standardized and validated. In addition, current models designed to predict immunosuppression may not predict immunostimulation as these models use doses and timing of antigen administration that are probably inappropriate. Thus, new experimental protocols should be investigated to ensure improved predictability.

4.3. Immunotoxicology and allergy

Until quite recently most immunotoxicologists did not consider allergies as an important issue. Although animal models of contact sensitization, a typical immunoallergic reaction, have long been in use (Maurer, 1983), allergic reactions in contrast to toxic effects were claimed to be unpredictable in animals. Accordingly, very limited efforts were being paid to designing predictive models of allergy so that the identification of the hypersensitivity potential of pharmaceuticals in laboratory animals is still a remote objective (Choquet-Kastylevski and Descotes, 1998).

Allergologists and medical immunologists nevertheless made significant progress in the understanding, diagnosis and management of drug and chemical allergies (Choquet-Kastylevsky et al., 2001). Based on the clinical experience, drug and chemical allergies are recognized as a significant cause of morbidity and they are also a major cause of drug withdrawal from the market. The clinical experience thus gained should serve as a basis to design new models intended to predict the risk of hypersensitivity reactions following drug treatments or chemical exposures.

4.4. Immunotoxicology and autoimmunity

Among the four categories of immunotoxic effects, autoimmunity is the least understood. Despite efforts to identify predictive animal models, the detection of drugs and chemicals which can induce autoimmune reactions, if at all possible (Descotes, 2000) and despite recent claims that drugs and chemicals might be a significant cause of autoimmunity, very few human data support this claim (Vial et al., 1997).

5. Conclusion

Clinical toxicology markedly contributed to significant improvements in the management of acute human poisonings and this certainly remains a central theme. However, efforts should also be focused on the evaluation of the adverse effects of chemical exposure to human health. So far, risk

assessment has been largely based on experimental data and limitations in this process have long been identified (Zbinden, 1990). There is a need to include clinical data more extensively. Human toxicology can help bridge the gap between animal research and risk assessment, but to achieve this new goal, the training of medical toxicologists should evolve and include forsaken areas of expertise, such as experimental toxicology and epidemiology. Closer interactions between experimental toxicologists and clinical toxicologists should also be established.

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Short communication

Mutual acceptance of data: harmonised test methods and quality assurance of data—the process explained☆

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Abstract

An essential aspect of the OECD is that it should not be considered a supranational organisation, but rather a center for discussion where governments express their points of view, share their experiences and search for common ground. This implies that decisions are made by consensus instead of majority. Once the Council, which is the highest authority of the OECD, adopts a formal Decision, such a decision is binding on all Member countries. The OECD Guidelines for the Testing of Chemicals, which are considered the leading international standard for safety testing, form an integrated part of such a binding Council Decision. An even more important part of that same Council Decision is that on Mutual Acceptance of Data, where it states that: 'Data generated in the testing of chemicals in an OECD Member country in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice (GLP) shall be accepted in other Member countries for purposes of assessment and other use relating to the protection of man and the environment.' In the various steps of the process of Test Guideline development, the National Co-ordinators of the Test Guideline Programme play an important role. The initiative to start the development of a particular guideline can be taken by the OECD Secretariat, by one or more Member countries or, most importantly, by the scientific community itself. Proposals, received by the Secretariat are discussed at the yearly Meeting of the National Co-ordinators. During these meetings, priorities for future activities are set and the approach that should be followed in dealing with the selected activities is discussed. Quite often, so-called Detailed Review Papers (DRP's) form the basis of a new or updated Guideline. These DRP's, which are either prepared by a Member country or by a consultant appointed by the Secretariat, describe the current 'state of the art' in scientific progress and technical possibilities of a well-defined area of research. After completion, either an expert meeting or a commenting round will be organised. All Member countries will have sufficient possibilities to express their views. When the DRP is acceptable to the experts of all Member countries, the next step is to actually develop a Test Guideline. Similar to the procedure followed for the DRP, the Test Guideline proposal will be circulated for comment to all Member countries and should reach the desks of relevant experts, nominated by their National Co-ordinator. Frequently, in addition to the commenting rounds, Test Guideline proposals are discussed in special expert meetings. Once the experts reach consensus on a particular Test Guideline, the proposal is put forward to the Meeting of the National Co-ordinators for approval. Since each guideline will form an

☆ Note: The opinions expressed in this paper have not been formally reviewed by OECD or its Member countries and should therefore be viewed as those of the author.

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integrated part of the earlier mentioned Council Decision, each new guideline also needs formal adoption by the Council before it becomes effective.
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Keywords: OECD; Test methods; Hazard assessment; GLP

1. Introduction

Our society's dependence on chemicals is a comparatively recent phenomenon. The chemical industry today is a US$1.55 trillion global industry, nearly four times as big as it was just 30 years ago. World-wide, the industry employs some 12 million people. The chemical industry in OECD Member countries accounts for 76% of world-wide production. Chemicals and related products represent 14% of total imports and exports of manufactured goods for OECD countries, and they make up 12% of Gross Domestic Product for some countries. The rapid growth of the chemical industry, which includes pharmaceuticals, pesticides and industrial chemicals, has also played a central role in the evolution of OECD countries' environmental protection policies.

The fast-paced expansion of the chemical industry has brought with it the possibility of the escalation of risks, endangering both human health and the environment. Maximising safety and minimising risk are therefore important aims for sustainable development for both governments and the chemical industry world-wide. The OECD has taken the lead in assisting countries in the development and co-ordination of chemical safety activities on an international basis. The forum provided by OECD enables countries to work together to discuss policies, clarify issues and protect the well-being of man and the environment, while giving due consideration to economic and trade concerns in its search for solutions.

The chemical industry is now in a period of strong globalisation and the manufacture and the use of chemicals is expanding rapidly outside the OECD. Increased trade liberalisations within the framework of the World Trade Organisation and regional agreements will undoubtedly lead to further growth in the trade of chemicals. Now that tariffs are going down across the globe, non-tariff barriers become the main impediments to trade. The chemical industry, which includes many multinational companies, recognises the considerable benefits derived from the OECD-wide harmonisation and it appreciates the cost-savings resulting from the limitation of non-tariff barriers to trade and avoidance of duplicative testing. The many practical products of OECD's work (such as the Mutual Acceptance of Data Scheme based on Council Decisions related to Test Guidelines and Good Laboratory Practice) support governments to develop their national programmes and to make the most efficient use of national resources by sharing the burden of work internationally. Many non-member countries, which are in the process of developing regulatory structures, look to the OECD policies and instruments as models and benefit from using the work of the Chemicals Programme.

2. The environment, health and safety programme

The OECD Environment, Health and Safety (EHS) Programme assists Member countries in developing common policies and instruments to guarantee a high level of environmental and health protection, while minimising duplicative work and distortions in trade. The specific objectives of the Environmental Health and Safety programme are to:

- assist Member countries in identifying, preventing and managing the risks of chemicals;
- promote the public's right to know about the potential risks of chemicals;
- prevent unnecessary distortions in the trade of chemicals;
- facilitate the optimal use of national resources available in government and industry for chemicals management;

- assist Member countries in working towards an integrated chemicals management approach that incorporates economic, social and environmental policy considerations, in support of achieving the objectives of sustainable development, and in particular those of UNCED's Agenda 21, Chapter 19;
- facilitate that globalisation of the chemical industry leads to positive impacts on human health and the environment in OECD member countries, as well as in non-OECD countries; and
- promote the development and implementation in Member countries of new and innovative technologies, policies and practices and prevent pollution from the manufacture, transport, use and disposal of chemicals.

The Programme achieves these objectives in three ways, namely: (i) through harmonisation of testing and assessment approaches; (ii) by sharing the burden of the work involved; and (iii) through outreach to Member and non member countries alike.

3. Testing and assessment

By harmonising national approaches to regulations related to chemicals, industry is not faced with a plethora of conflicting or duplicative requirements; governments are provided with a common basis for working with each other; and non-tariff barriers to trade are reduced. In this respect, testing methods are the cornerstone of any risk assessment procedure. Therefore, a major activity of OECD's Environment, Health and Safety (EHS) Programme is the development of harmonised test methods. The OECD Test Guidelines are considered the leading international standard for safety testing and the development of new Test Guidelines, as well as the updating of existing ones, are key to the work on testing and assessment. OECD Test Guidelines together with GLP Principles form an integrated part of the Council Decision on the Mutual Acceptance of Data (MAD) (OECD, 1981, 1997). It is stated in this Council Decision that: 'Data generated in the testing of chemicals in an OECD Member country in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice (GLP) shall be accepted in other Member countries for the purposes of assessment and other use relating to the protection of man and the environment'. Every year, many companies submit to governments notification or registration applications for thousands of new industrial chemicals, pesticides, pharmaceuticals and food additives. For many of these chemicals, an extensive set of safety tests is required. But, under the MAD Decisions, when data are developed in one country in accordance with the OECD Test Guidelines and GLP Principles, they are accepted for assessment purposes in all OECD countries. Therefore, a company is spared the added expense of re-testing a substance if it wishes to market that chemical in more than one country; and delays in marketing a new product and distortions in trade are avoided. The MAD Decision is a basic element of the EHS Programme and helps saving millions of dollars by avoiding duplicative testing and minimising non-tariff barriers to trade (OECD, 1998). Recently, additional Council Decisions related to Mutual Acceptance of Data allow adherence by non-member countries, in order to widen their field of application.

3.1. Good laboratory practice

The primary objective of the OECD Principles of GLP is to ensure the generation of high quality and reliable test data related to the safety of industrial chemical substances and preparations in the framework of harmonising testing procedures for the Mutual Acceptance of Data (MAD). The OECD Principles of GLP set out managerial concepts covering the organisation of test facilities and the conditions under which pre-clinical safety studies are executed. Their purpose is to ensure the generation of high quality and reliable test data (in vitro and in vivo) related to the safety of chemicals and preparations in the framework of the Mutual Acceptance of Data.

MAD also harmonises procedures of GLP compliance monitoring, ensuring that pre-clinical safety studies are carried out according to the

Principles of GLP and that countries can have confidence in the quality and rigour of safety tests. The 1989 Council Decision and Recommendation on Compliance with Good Laboratory Practice (OECD, 1989) requires the establishment of national compliance monitoring programmes based on laboratory inspections and study audits and recommends the use of the Guides for Compliance Monitoring Procedures for Good Laboratory Practice and the Guidance for the Conduct of Laboratory Inspections and Study Audits. Since 1997, a procedure through which non-OECD countries can adhere to the MAD system has been embodied in a Council Decision (OECD, 1997). A series of documents related to specific issues of GLP and compliance monitoring has been published (see Table 1).

Table 1
OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring

No. 1:	OECD Principles on Good Laboratory Practice
No. 2:	Revised Guides for Compliance Monitoring Procedures for Good Laboratory Practice
No. 3:	Revised Guidance for the Conduct of Laboratory Inspections and Study Audit
No. 4:	Quality Assurance and GLP (revised 1999)
No. 5:	Compliance of Laboratory Suppliers with GLP Principles (revised 1999)
No. 6:	The Application of the GLP Principles to Field Studies (revised 1999)
No. 7:	The Application of the GLP Principles to Short and Term Studies (revised 1999)
No. 8:	The Role and Responsibilities of the Study Director in GLP Studies (revised 1999)
No. 9:	Guidance for the Preparation of GLP Inspection Reports
No. 10:	The Application of the Principles of GLP to Computerised Systems (1995)
No. 11:	The Role and Responsibility of the Sponsor in the Application of the Principles of GLP
No. 12:	Requesting and Carrying Out Inspections and Study Audits in Another Country
No. 13:	The Application of the OECD Principles of GLP to the Organisation and Management of Multi-Site Studies

3.2. Test guideline development

The process of test guideline development comprises two parts. First, the need for a guideline should be defined. The various steps of this part of the process are indicated in Fig. 1. As clearly defined in this diagram, the National Co-ordinators of the Test Guidelines Programme have an important role in this process. The initiative to start the development of a particular guideline can be taken by the OECD Secretariat, by one or more Member countries, or, most importantly by the scientific community itself. Proposals received by the Secretariat include details of: (i) the regulatory need for the guideline; (ii) scientific consensus on the methodology; (iii) animal welfare considerations; and (iv) costs and country representatives willing to take the lead in the work. All proposals are discussed at the annual meeting of the Working Group of National Co-ordinators of the Test Guidelines Programme (WNT). During these meetings, priorities for future activities are set and the approach that should be followed in dealing with selected activities is discussed. Quite often, so called Detailed Review Papers (DRPs) form the basis of a new or updated guideline. These DRPs which are prepared either by a Member country or by expert consultants appointed by the Secretariat, describe the current 'state of the art' in scientific progress and technical possibilities of a well defined area of research. After completion, either an expert meeting or a commenting round will be organised. All Member countries will have sufficient opportunities to express their views. When the DRP is acceptable to the experts of all Member countries, the second part is the actual development of the test guideline. This part of the process of the test guideline development is indicated in Fig. 2.

Test Guideline proposals will first be reviewed and discussed by a small ad hoc expert group of internationally recognised key experts in the field. Following agreement on a proposal, it will be circulated for comment to relevant experts in all Member countries nominated by their National Co-ordinator. Frequently, in addition to the commenting rounds, test guidelines are discussed in special expert meetings. Once the experts reach

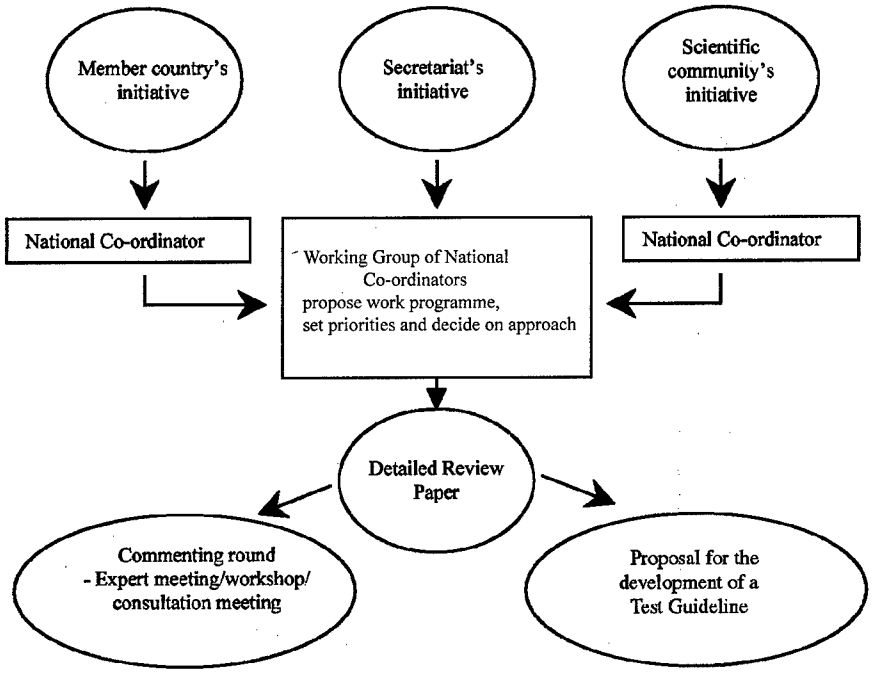

Fig. 1. Part 1 of the OECD Test Guideline Development: defining the need.

consensus on a particular test guideline, the proposal is put forward to the Meeting of the National Co-ordinators for approval. As each guideline will form an integrated part of the earlier mentioned Council Decision, each guideline also needs formal adoption by the Council before it becomes effective. Today, there are ≈100 Guidelines adopted by the Council. They cover the full breadth of endpoints essential for comprehensive hazard assessment including: physical chemical properties, effects on biotic systems (ecotoxicity), degradation and accumulation, and health effects. In the area of health effects, a variety of animal studies are available for acute, repeated dose and long-term toxicity, carcinogenicity, neurotoxicity, immunotoxicity and genetic and reproductive toxicity. A complete overview of all available Guidelines is given in Table 2. This information is regularly updated and is available, together with an overview of all draft Guidelines and Guidance Documents, on OECD's web pages.

3.3. OECD and animal welfare

With respect to animal welfare, the OECD has taken a rather pragmatic position. At the second High Level Meeting in 1982, bringing together ministers and other high level officials, the following statement was adopted: 'The welfare of laboratory animals is important. It will continue to be an important factor influencing the work of the OECD Chemicals Programme'. The progress in OECD on the harmonisation of chemicals control, in particular the agreement of mutual acceptance of data, by reducing duplicative testing, will do much to reduce the numbers of animals used in testing. Such testing cannot be eliminated at present, but every effort should be made to discover, develop and validate alternative testing systems'.

In 1987, when some existing guidelines on health effect were updated, animal welfare was indeed addressed. As a result, the number of animals

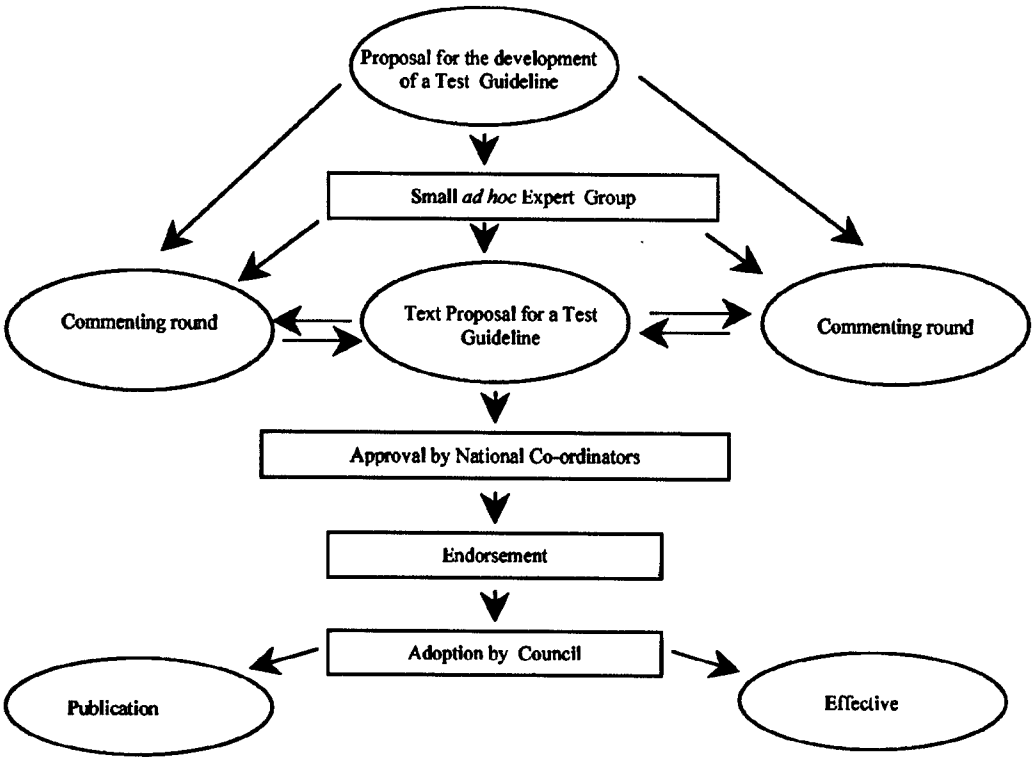

Fig. 2. Part 2 of the OECD Test Guideline Development: producing the product.

required for acute toxicity testing was reduced and the probability of severe animal suffering was diminished (Updated Test Guidelines 401, 402 and 405). At the same time, the updated Test Guideline on Acute Eye Irritation/Corrosion (Test Guideline 405) provided for the use of well validated alternative studies to identify corrosive or severe irritating substances. Now, substances recognised a such in alternative tests need not be further tested for eye irritation, it being presumed that such substances will produce similarly severe effects on the eyes in a live animal test. Because the High Level Meeting in 1982 had recommended that alternative methods be validated before they be applied in hazard identification schemes, there was a need to define the scientific criteria for validation of alternative methods.

The preferred approaches to validation were discussed in September 1996 at an OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods, held in Solna, Sweden (OECD, 1996). That meeting reached consensus on an internationally acceptable approach and strategy for the validation of new test methods, taking into account available approaches as recommended by the European Commission's centre for the Validation of Alternative Methods (ECVAM, 1995) and leading centres in the US (CAAT, ICCVAM) (Goldberg et al., 1993; ICCVAM, 1996). In March 2002, the Solna Workshop was followed-up by an OECD Conference in Stockholm with the objective to provide further practical guidance on how to apply the harmonised principles and criteria for validation and regulatory acceptance for all new and updated test methods (OECD, 2002).

4. Harmonised hazard and risk assessment

To achieve consistency in hazard and risk assessments of chemicals and pesticides, validated

Table 2
OECD guidelines for testing of chemicals

No.	Title	Original adoption	No. of updates	Most recently updated
101	UV-VIS Absorption Spectra	12 May 1981	0	–
102	Melting Point/Melting Range	12 May 1981	1	27 July 1995
103	Boiling Point	12 May 1981	1	27 July 1995
104	Vapour Pressure	12 May 1981	1	27 July 1995
105	Water Solubility	12 May 1981	1	27 July 1995
106	Adsorption/Desorption Using a Batch Equilibrium Method	12 May 1981	1	21 January 2000
107	Partition Coefficient (*n*-octanol/water): Shake Flask Method	12 May 1981	1	27 July 1995
108	Complex Formation Ability in Water	12 May 1981	0	–
109	Density of Liquids and Solids	12 May 1981	1	27 July 1995
110	Particle Size Distribution/Fibre Length and Diameter Distributions	12 May 1981	0	–
111	Hydrolysis as a function of pH	12 May 1981	0	–
112	Dissociation Constants in Water	12 May 1981	0	–
113	Screening Test for Thermal Stability and Stability in Air	12 May 1981	0	–
114	Viscosity of Liquids	12 May 1981	0	–
115	Surface Tension of Aqueous Solutions	12 May 1981	1	27 July 1995
116	Fat Solubility of Solid and Liquid Substances	12 May 1981	0	–
117	Partition Coefficient (*n*-octanol/water), HPLC method	30 March 1989	0	–
118	Determination of the Number–Average Molecular Weight and the Molecular Weight Distribution of Polymers using Gel Permeation Chromatography	14 June 1996	0	–
119	Determination of the Low Molecular Weight Content of a Polymer Using Gel Permeation Chromatography	14 June 1996	0	–
120	Solution/Extraction Behaviours of Polymers in Water	14 June 1996	1	21 January 2000
121	Estimation of the Adsorption Coefficient (K_{oc}) on Soil and on Sewage Sludge using High Performance Liquid Chromatography (HPLC)	22 January 2001	0	–
201	Alga, Growth Inhibition Test	12 May 1981	1	7 June 1984
202	*Daphnia* sp. Acute Immobilisation Test and Reproduction Test	12 May 1981	1	Currently being updated and will be restricted to the acute test. For the reproduction test, see Guideline 211
203	Fish, Acute Toxicity Test	12 May 1981	2	17 July 1992
204	Fish, Prolonged Toxicity Test: 14-Day Study	4 April 1984	0	–
205	Avian Dietary Toxicity Test	4 April 1984	0	–
206	Avian Reproduction Test	4 April 1984	0	–
207	Earthworm, Acute Toxicity Tests	4 April 1984	0	–
208	Terrestrial Plants, Growth Test	4 April 1984	0	–
209	Activated Sludge, Respiration Inhibition Test	4 April 1984	0	–
210	Fish, Early-Life Stage Toxicity Test	17 July 1992	0	–
211	*Daphnia magna* Reproduction Test	21 September 1998	0	–
212	Fish, Short- term Toxicity Test on Embryo and Sac-fry Stages	21 September 1998	0	–
213	Honeybees, Acute Oral Toxicity Test	21 September 1998	0	–
214	Honeybees, Acute Contact Toxicity Test	21 September 1998	0	–

Table 2 (*Continued*)

No.	Title	Original adoption	No. of updates	Most recently updated
215	Fish, Juvenile Growth Test	21 January 2000	0	–
216	Soil Microorganisms: Nitrogen Transformation Test	21 January 2000	0	–
217	Soil Microorganisms: Carbon Transformation Test	21 January 2000	0	–
301	Ready Biodegradability; 301A: DOC Die-Away Test; 301B: Co2 Evolution Test; 301C: Modified MITI Test (I); 301D: Closed Bottle Test; 301E: Modified OECD Screening Test; 301F: Manometric Respirometry Test	12 May 1981	1	17 July 1992
302A	Inherent Biodegradability: Modified SCAS Test	12 May 1981	0	–
302B	Inherent Biodegradability: Zahn-Wellens/EMPA Test	12 May 1981	1	17 July 1992
302C	Inherent Biodegradability: Modified MITI Test (II)	12 May 1981	0	–
303	Simulation Test–Aerobic Sewage Treatment; A: Activated Sludge Units; B: Biofilms	12 May 1981	1	22 January 2001
304A	Inherent Biodegradability in Soil	12 May 1981	0	–
305	Bioconcentration: Flow-Through Fish Test	12 May 1981	1	14 June 1996
306	Biodegradability in Seawater	17 July 1992	0	–
307	Aerobic and Anaerobic Transformation in Soil	24 April 2002	0	–
308	Aerobic and Anaerobic Transformation in Aquatic Sediment Systems	24 April 2002	0	–
401	Acute Oral Toxicity	12 May 1981	1	Date of Deletion: 20 December 2002
402	Acute Dermal Toxicity	12 May 1981	1	24 February 1987
403	Acute Inhalation Toxicity	12 May 1981	1	–
404	Acute Dermal Irritation/Corrosion	12 May 1981	2	24 April 2002
405	Acute Eye Irritation/Corrosion	12 May 1981	2	24 April 2002
406	Skin Sensitisation	12 May 1981	1	17 July 1992
407	Repeated Dose 28-Day Oral Toxicity Study in Rodents	12 May 1981	1	27 July 1995
408	Repeated Dose 90-Day Oral Toxicity Study in Rodents	12 May 1981	1	21 September 1998
409	Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents	12 May 1981	1	21 September 1998
410	Repeated Dose Dermal Toxicity: 90-Day	12 May 1981	0	–
411	Subchronic Inhalation Toxicity: 90-Day	12 May 1981	0	–
412	Repeated Dose Inhalation Toxicity: 28/14-Day	12 May 1981	0	–
413	Subchronic Inhalation Toxicity: 90-Day	12 May 1981	0	–
414	Prenatal Developmental Toxicity Study	12 May 1981	1	22 January 2001
415	One-Generation Reproduction Toxicity	26 May 1983	0	–
416	Two-generation Reproduction Toxicity Study	26 May 1983	1	22 January 2001
417	Toxicokinetics	4 April 1984	0	–
418	Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure	4 April 1984	1	27 July 1995
419	Delayed Neurotoxicity of Organophosphorus Substances: 28-Day Repeated Dose Study	4 April 1984	1	27 July 1995
420	Acute Oral toxicity–Fixed Dose Procedure	17 July 1992	1	17 December 2001
421	Reproduction/Developmental Toxicity Screening Test	27 July 1995	0	–
422	Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test	22 March 1996	0	–
423	Acute Oral Toxicity–Acute Toxic Class Method	22 March 1996	1	17 December 2001
424	Neurotoxicity Study in Rodents	21 July 1997	0	–

Table 2 (*Continued*)

No.	Title	Original adoption	No. of updates	Most recently updated
425	Acute Oral Toxicity: Up-and-Down Procedure	21 September 1998	1	17 December 2001
429	Skin Sensitisation: Local Lymph Node Assay	24 April 2002	0	–
451	Carcinogenicity Studies	12 May 1981	0	–
452	Chronic Toxicity Studies	12 May 1981	0	–
453	Combined Chronic toxicity/Carcinogenicity Studies	12 May 1981	0	–
471	Bacterial Reverse Mutation Test	26 May 1983	1	21 July 1997
472	Genetic Toxicology: *Escherichia coli*, Reverse Assay	26 May 1983	0	Date of deletion: 21 July 1997 (Method merged with TG 471)
473	In Vitro Mammalian Chromosome Aberration Test	26 May 1983	1	21 July 1997
474	Mammalian Erythrocyte Micronucleus Test	26 May 1983	1	21 July 1997
475	Mammalian Bone Marrow Chromosome Aberration Test	4 April 1984	1	21 July 1997
476	In Vitro Mammalian Cell Gene Mutation Test	4 April 1984	1	21 July 1997
477	Genetic Toxicology: Sex-Linked Recessive Lethal Test in *Drosophilia melanogaster*	4 April 1984	0	–
478	Genetic Toxicology: Rodent dominant Lethal Test	4 April 1984	0	–
479	Genetic Toxicology: In Vitro Sister Chromatid Exchange assay in Mammalian Cells	23 October 1986	0	–
480	Genetic Toxicology: *Saccharomyces cerevisiae*, Gene Mutation Assay	23 October 1986	0	–
481	Genetic Toxicology: *Saccharomyces cerevisiae*, Mitotic Recombination Assay	23 October 1986	0	–
482	Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells In Vitro	23 October 1986	0	–
483	Mammalian Spermatagonial Chromosome Aberration Test	23 October 1986	1	21 July 1997
484	Genetic Toxicology: Mouse Spot Test	23 October 1986	0	–
485	Genetic Toxicology: Mouse Heritable Translocation Assay	23 October 1986	0	–
486	Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vivo	21 July 1997	0	–

methods are required. Many methods still need to be developed, however, particularly in the area of environmental assessment. By working together in OECD, Member countries share the burden of developing these methods. It has been agreed with WHO that OECD will take the lead in the environmental assessment area field and will work with WHO in the human health assessment area.

4.1. Good assessment practices

The objective of the work on Good Assessment Practice is to encourage the mutual use of assessment reports of chemicals and pesticides between countries; as a further step the mutual acceptance of such reports may be possible. If mutual use and acceptance of assessments is to be a reality, then countries and other organisations must be familiar and have confidence in the way in which the assessments are made. It is also important that the assessment reports are transparent so that the reasoning behind any decisions made is clear to the reader. The three activities going on in OECD in this respect are as follows.

4.1.1. Compendium of assessment methods

The development of the compendium of assessment methods available in Member countries and international organisations; the compendium will enable those involved in risk assessment to become acquainted with methods used elsewhere.

4.1.2. Harmonisation of assessment reports

Work on the harmonisation of assessment reports is taking place in a practical way within the Existing Chemicals Programme (for industrial chemicals) and the Pesticide Programme (for pesticides)

4.1.3. Harmonisation of terminology in hazard/risk assessment

This work is undertaken as a joint project with IPCS and involves as well terminology of generic terms (e.g. risk characterisation), as harmonisation of technical terms.

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Review

Testing and assessment strategies, including alternative and new approaches

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Abstract

The object of toxicological testing is to predict possible adverse effect in humans when exposed to chemicals whether used as industrial chemicals, pharmaceuticals or pesticides. Animal models are predominantly used in identifying potential hazards of chemicals. The use of laboratory animals raises ethical concern. However, irrespective of animal welfare it is an important aspect of the discipline of toxicology that the primary object is human health. The ideal testing and assessment strategy is simple to use all the available test methods and preferably more in laboratory animal species from which we get as many data as possible in order to obtain the most extensive database for the toxicological evaluation of a chemical. Consequently, the society has decided that certain group of chemicals should be tested accordingly. However, realising that, this idea is not obtainable in practice because there are more than 100 000 chemicals which are potential for human exposure, so the development of alternative testing and assessment strategies has taken place in the recent years. The toxicological evaluation should enable the society to cope with the simultaneous requirement of many chemicals for different uses and of the absence of health problems involved with their use. Thus, the regulatory toxicology is a cocktail of science and pragmatism added a crucial concern for animal welfare. Test methods are most often used in a testing sequence as bricks in a testing strategy. The main key driving forces for introducing assessment and testing strategies e.g. using a limited number of tests and/or alternative test methods are: (a) animal welfare considerations; (b) new scientific knowledge i.e. introducing tests for new endpoints and tests for better understanding of mode of action; and (c) lack of testing capacity/reduction of required resources economically as well as time wise.
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Keywords: Testing; Assessment; Strategies; Chemicals; Human health

1. Introduction

The object of toxicological testing is to predict possible adverse effect in humans when exposed to, e.g., chemicals whether used as industrial chemicals, pharmaceuticals or pesticides. Animal

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models are predominantly used in identifying potential hazards of chemicals. The use of laboratory animals raises ethical concern. However, irrespective animal welfare is an important aspect of the discipline of toxicology the primary objective is human health.

The ideal assessment and testing strategy is simply to use all the available test methods and preferably in more laboratory animal species getting as many data as possible in order to obtain the most extensive database for the toxicological evaluation of a chemical. Consequently, the society has decided that certain group of chemicals should be tested accordingly. However, realising that this idea is not obtainable in practice having more than 100 000 chemicals for which there are a potential for human exposure the development of alternative testing and assessment strategies has taken place in the recent years. The toxicological evaluation should enable the society to cope with the simultaneous requirement of many chemicals for different uses and of the absence of health problems involved with their use. Thus the regulatory toxicology is a cocktail of science and pragmatism added a crucial concern for animal welfare and the latter considered an essential part of life quality.

Test methods are almost never used alone in an isolated way but in a testing sequence as bricks in a testing strategy. Testing and assessment strategies/data requirements differ for the different chemicals according to their uses. For some of the chemicals like food additives and plant protection products the entire toxicological testing package is required. The regulatory authorities will only occasionally accept that individual tests are waived and only when there is a sound scientific justification for not testing. The philosophy behind the requirement is for the former group that there exists a direct exposure via the mouth, and for the latter group that the chemicals in question by definition are toxic. For pharmaceuticals, the data requirement that needs to be considered is somewhat parallel to that of the two groups mentioned above. However, the data requirement and consequence of the testing strategy is more flexible based upon the nature of the drug, the duration of intended treatment, and the target patient population. For example, a potential life-saving anti-cancer agent may require only limited testing, whilst life-time exposure to a CNS drug could require a complete package of extensive toxicology test. The same philosophy is reflected in the test guidelines for pharmaceuticals. Thus the ICH (International Conference on Harmonisation of Technical Requirements for registration of pharmaceuticals for human use) guidelines are much more flexible than the corresponding OECD (Organisation for Economic Co-operation and Development) test guidelines for testing chemicals and act more in line with the so called OECD guidance documents. Another major group of chemicals in EU the industrial chemicals are tested according to a tonnage driven testing programme. This testing and assessment strategy are to some extent based upon the fact that not all chemicals need to be accompanied by the same high amount of data as the general exposure measured maybe rather low. Nevertheless, parallel to the tonnage figure indications from the data derived from the base set of testing can trigger a further testing programme.

The main key driving forces for introducing testing and assessment strategies using a limited number of tests and/or alternative test methods as an alternate for using data from the entire testing package are: animal welfare considerations; new scientific knowledge i.e. introducing tests for new endpoints and tests for better understanding of mode of action; lack of testing capacity/reduction of required resources economically as well as time wise.

Examples illustrating the three main key driving forces are given below. However, it should be recognised that there are overlaps i.e. that some of the examples presented are pertaining to more than one of the sections.

2. Animal welfare considerations

Experience and fundamental similarities in cell structure and biochemistry between animals and humans provide a general valid basis for the use of laboratory animal models for prediction of likely effects of chemicals on the human population (Meyer, 1993). However, the use of laboratory

animals raises ethical concern. Consequently, many resources are invested in order to observe the 'three Rs' (Replacement, Reduction, Refinement) concerning the use of laboratory animals in toxicological testing.

The classical example of animal welfare considerations is the introduction of three new test guidelines for acute oral toxicity, i.e., 'Fixed Dose Method', 'Acute Toxic Class Method' and 'Up and Down Procedure' as alternative tests to classical test for acute oral toxicity (OECD, 2002a). Determination of the LD_{50} is an integral part of most regulatory systems. However, a less precise toxicity quantification provided by the alternative tests is sufficient in the most cases and consequently these tests will eventually replace the classical one in the risk assessment of chemicals. Development of these tests to cover dosing routs other than the oral route is in progress.

The proposals for the OECD test guidelines 'Acute Dermal Irritation/Corrosion' and 'Acute Eye Irritation/Corrosion' are another more recent example (OECD, 2000a,b,c,d). In observing animal welfare, an attachment for each of the two proposed guidelines are introduced as a new element presenting a Stepwise/Tiered Testing Strategy for Eye Irritation and Corrosion and Skin Irritation and Corrosion, respectively. These testing strategies take advantage of the existing information on the test substance and enable the regulatory authorities to use the data for hazard classification. The proposed testing strategies rely on data/indication(s) showing the respective harmful effects. Consequently, the testing of chemicals in laboratory animals is limited to those for which there are no indications of harmful effects.

Step five in the 'Tiered Testing and Evaluation of Dermal Corrosion and Irritation Potential' in the above-mentioned attachment to the OECD proposal for 'Acute Dermal Irritation/Corrosion Test Guideline' suggests to test in a valid and accepted in vitro corrosion test. Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material. Consequently, the concern for pain and suffering of laboratory animals constitute a pressure to develop test guidelines that does not require the use of live animals for the assessment of skin corrosivity. Currently there are no internationally accepted and validated in vitro methods of dermal corrosion, but a validation study on several methods has been completed. OECD has recently circulated two draft proposals for a new guideline: 'In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)' (OECD, 2002b), and 'In Vitro Skin Corrosion: Human Skin Model Test' (OECD, 2002c) for member state comments. A positive response in one of these tests will imply no further testing. Thus these tests are used as supplement to the existing animal tests.

The OECD draft proposal for a 'New Guideline: 432. In Vitro 3T3 NRU phototoxicity test' also recently circulated for comments is an another example on animal welfare consideration as well as an example on introducing tests for new endpoints. This test has been shown to be predictive of acute phototoxicity effects in animals and humans in vivo whereas the test is not designed to predict other adverse effects that may arise from combined action of a chemical and light e.g. photogenotoxicity, photoallergy or photocarcinogenicity. The test does not allow an assessment of phototoxic potency. Of other limitations are mentioned indirect phototoxicity, effects of metabolites and effects of mixtures (OECD, 2002d).

ECVAM (ECVAM, 2002) has very recently submitted a report with the title: 'Alternative (non-animal) methods for chemical testing: Current status and future prospects' for publication. The principle aim of this report is to summarise the current status of alternative tests for contributing to the assessment of potential toxicological (human health) effects that are currently required by European Union legislation on chemicals.

Recently OECD has launched a Draft New Guideline 429: 'Skin Sensitisation: Local Lymph Node Assay (LLNA)' (OECD, 2001a). This test guideline provides an alternative method for the present OECD guideline 406 (OECD, 2002a) for the use in the identification of skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. Thus the test is introduced as a complete standalone test. This does not necessarily imply that in all cases the LLNA should be used in place of guinea pig tests, but rather that the assay is of

equal merit and may be employed as an alternative in which generally positive and negative results no longer require further confirmation. The LLNA provides certain advantages with regard to both animal welfare and scientific progress and as such the introduction of LLNA could constitute an example of another main key driving force for introducing testing and assessment strategies namely introducing tests for new endpoints and tests for better understanding of mode of action (see below). However, despite the advantages of the test compared to the traditional existing test, it is emphasised that there are certain limitations that may necessitate the use of the classical guinea pigs tests (e.g., false negative findings with certain metals).

Data on dermal absorption are essential when assessing the exposure of workers among others farmers applying plant protection products. In year 2000, OECD circulated two new 'Draft Guidelines on Skin Absorption' one of which is an in vitro method (OECD, 2002e). The EU Scientific Committee on Plants (SCP) has just released an opinion: 'Opinion on The Draft Guidance on Dermal Absorption' (SCP, 2002). In that, the SCP states that in vitro human skin absorption alone would be sufficient to determine the dermal absorption percentage to be used for risk assessment. The SCP is aware that there are ethical and technical issues regarding the use of human skin that need to be resolved. The absorption through rat skin is generally higher than through human skin. So, when only rat skin data are available, the most conservative approach would be to assume that human skin absorption would be equal to rat skin absorption. This opinion is based upon that there is no biological reason why absorption through skin in vivo should be significantly different from absorption through the same appropriately prepared viable skin in vitro. As for the LLNA, this does not necessarily imply that in all cases the in vitro test for skin absorption should be used in place of the corresponding in vivo test, as the latter in addition to the dermal absorption rate gives information on toxicokinetics that might be relevant when assessed in comparison with type of effects and their mechanism/mode of action caused by the compound.

To minimise the use of animals in evaluating safety, a tiered strategy for testing food additives has been suggested (Knight and Breheny, 2002). The initial stages of the strategy involve the use of physiologically based pharmacokinetic (PBPK) modelling to identify metabolites produced and their potential target organs. PBPK modelling will also assess the concentrations likely to be encountered in humans. This is particularly important, as the demonstration of an effect of a substance at high concentrations in an in vitro assay may not necessarily imply that the same effects would be observed in vivo. After identification of the potential target organs, a battery of cell lines could be used to assess the genotoxicity and tissue specific toxicity of the substance. Short- and long-term genotoxicity and non-genotoxicity studies would be carried out concurrently. Should any undesirable results be obtained, the substance should be rejected. If no cytotoxic or genotoxic effects are observed, testing will continue further for neurotoxicity, immunotoxicity, reproductive toxicity and acute toxicity. Concerning the derivation of an acceptable daily intake (ADI) in vitro metabolism and mechanistic studies in isolation have limited value. However, in conjunction with detailed short- and long-term in vivo studies, they have a potential to be used in the calculation of both inter-species and inter-individual variability in the toxicokinetics and toxicodynamics of food additives and therefore may assist in removing some of the uncertainty involved in extrapolating toxicity from the most sensitive species to humans (Walton et al., 1999).

3. New scientific knowledge i.e. introducing tests for new endpoints and tests for better understanding of mode of action

It has been shown that environmental chemicals may cause serious effects on human reproductive organs. In order to detect chemicals having this intrinsic effect and evaluate the safety of those, several rodent experimental models have been proposed. Thus OECD are in the process of

developing two test guidelines for identification of hormonal disruption: 'The Rodent Uterotropic Assay' and 'The Rodent Hershberger Assay' for the evaluation of the ability of a chemical to show biological activities are consistent with the agonism or antagonism of natural oestrogens or natural hormones with masculinising effects known as androgens (e.g. testosterone propionate), respectively (OECD, 2001c,d). Additionally, there is a simultaneous on-going activity in OECD with a third test, 'The Enhanced OECD Test Guideline 407, 28-day Repeated-Dose Oral Toxicity Study in Rodents' (OECD, 2000e). Recently, as a part of the international validation project of this Enhanced OECD Test Guideline 407, data from a study with an exogeneous androgen agonist, 17α-methyltestosterone has been reported (Okazaki et al., 2002). These data confirm the utility of the Enhanced OECD Test Guideline 407 while pointing to the existence of a no-observed-adverse-effect-level, the most sensitive parameters being organ weights and histopathological examinations of sexual organs. The three tests mentioned above form together with other tests like e.g. in vitro tests for receptor binding, the one- and not at least two-generation reproductive toxicity studies and the 'Developmental Neurotoxicity Study', existing information collection and analysis and structure activity considerations, a base for a testing and assessment strategy for endocrine disruption as a consequence of exposure to chemicals (OECD, 2002f).

With regard to the development of a testing strategy using a tiered approach in which screening methods delineating the system/organ specific toxicity are performed early in the testing sequence, the present OECD Test Guideline 407, 'the 28-day Repeated-Dose Toxicity Study' (OECD, 2002a) is a result of a previous amendment with the objective of obtaining additional information from the animals used in the study. Thus the present test guideline should identify chemicals with a neurotoxic potential, which may warrant further in-depth investigation of this aspect. In addition, the method may give an indication of immunological effects and reproduction organ toxicity. Irrespective of the limitations of the ability of the OECD 407 to detect the effect on reproduction, the latter among other is important for industrial chemicals for which only data from base set studies are required i.e. absence of data from specific tests on reproductive toxicity.

The last example given here is the development of a test guideline, 'Developmental Neurotoxicity Study' (OECD, 1999). A number of chemicals are known to produce developmental neurotoxic effects in humans and other species, and as such the assessment and evaluation of chemicals for the potential developmental neurotoxicity is important and not yet sufficiently covered by data from the existing test guidelines. The US Environmental Protection Agency (USEPA) is requiring registrants to conduct developmental neurotoxicity studies for a number of neurotoxic pesticides (EEA, 2002). In EU, the Scientific Committee for Food (SCF) recommended that appropriate experts should address this issue with the view to setting criteria to decide when developmental neurotoxicity studies in the future are necessary (EU SCF, 1998).

The applications of new techniques such as genomics and proteomics to mechanism-based toxicological research and biomarker identification and the use of transgenic animals could be included as examples in this section as well. However, these rather new approaches will be presented in the following section of the paper.

4. Lack of testing capacity/reduction of required resources economically as well as time wise

The principal objective of carcinogenicity testing is to identify substances that may cause an increase in human cancer at any site by any mechanism. The ultimate test for carcinogenicity is studies like the OECD 451 (OECD, 2002a). This is a costly and time-consuming test, and it is virtually impossible to test all chemicals in such studies. On the other hand, we want to be sure, that we do not release chemicals with a carcinogenic potential. Few years ago EU set up an assessment strategy for new and existing chemicals in which data on mutagenicity is an essential element. Many compounds shown to be mutagenic in whole animals have not been tested for carcinogenicity but, of

those that have, the vast majority are carcinogenic in laboratory rodents. It therefore seems reasonable and prudent to include results from short-term mutagenicity tests in assessment of putative carcinogenicity. Consequently, the assessment strategy implies that a positive in vivo mutagenicity test is sufficient for classifying a chemical as human carcinogen.

Where possible, the animal models used for carcinogenicity testing should be biologically appropriate for the assessment of possible human risk. However, the selection of test species is usually limited, by practical considerations, to laboratory rats and mice. There is an on-going discussion in the scientific community about whether the use of both rodent species is necessary. It has been stated, based upon a survey of databases covering studies with pharmaceuticals, that in general the rat is more sensitive than mouse i.e. a higher proportion of pharmaceuticals tested caused tumours in the rat than in mouse (Van Oosterhout et al., 1997). Carcinogenic risk assessment on the basis of a life span study in a single rodent species in combination with short-term genotoxicity tests and mechanistic information has also been suggested (Van Oosterhout et al., 1997). Accordingly, a long-term study in rat supplemented by a short- or medium-term in vivo rodent test, such as a model of initiation–promotion, or a carcinogenesis model using neonatal or transgenic mice, have been proposed as an alternative to life-span bioassays in two rodent species for human pharmaceuticals (ICH, 1997). However, such an approach has yet to be validated and is not therefore recommended for e.g. food additives and pesticides. Use of the male rat and the female mouse has also been suggested as an alternative to a standard two species, two sex bioassay (Ashby, 1996). Reduced bioassay testing may well be sufficient for the detection of genotoxic carcinogens as these are more often found to give rise to tumours in more than one species, in more than one sex and more than one site, compared to non-genotoxic carcinogens (Ashby and Tennant, 1991; Ashby and Paton, 1993). However, to optimise the likelihood of detecting non-genotoxic carcinogens, testing in more than one species may be more appropriate. Until there is an international consensus on newer approaches for carcinogenicity risk assessment, both sexes of rats and mice should normally be used for the testing of food additives and pesticides, unless specific considerations suggest otherwise.

Concerning non-genotoxic carcinogens some tests could, based on current understanding, serve as indicators of some events in the process of carcinogenesis caused by non-mutagenic effects. In vitro cell transformation assays like the 'Syrian Hamster Embryo cells (SHE) assay' or test for 'Gap Junctional Intercellular Communication' has among others been proposed as non-mutagenicity tests for the risk assessment of chemicals. One proposed application of these tests could be screening for non-mutagenic, tumourigenic effects, which would be carried out simultaneously with mutagenicity tests. Thus, in vitro evidence on both mutagenicity and 'promoter'-type effects would be available when the need for further testing in the carcinogenicity test is considered. The SHE assay is the best-validated test of the two mentioned tests so far. However, at present none of these tests have been developed into internationally accepted test guidelines (Anonymous, 1997; OECD, 2001b).

The successes of large-scale genome sequencing programmes such as the human genome project have stimulated the development of new technologies that facilitate the simultaneous measurement of thousands of biological variables in test material. These technologies, termed genomics, transcript profiling (transcriptomics), proteomics and metabonomics are rapidly developing and may enable researchers to study and describe biological events at the level of genetic material (genomes) and its expression in organisms (ECETOC, 2001). DNA array technology makes it possible to rapidly genotype individuals or quantify the expression of thousands of genes on a single filter or glass slide, and holds enormous potential in toxicologic applications (Rockett and Dix, 1999; Hamadeh et al., 2001; Knight and Breheny, 2002).

cDNA microarrays are powerful tools for associating gene expression with observed pathology. Data obtained from cDNA microarray gene expression studies can help to explain many aspects of an observed pathology and can reveal specific gene signalling pathways that are key

players in the toxic response. For example, cDNA micorarray has been used to study gene expression in the liver of peroxisome proliferator- or Phenobarbital-treated rats (Hamadeh et al., 2001). The exposure of rodent hepatocytes to the non-genotoxic carcinogen, phenobarbitone, has been studied by using microarray and gel-based expression technologies with the result that in excess of 300 genes were found whose expression is modulated by this reagent (ECETOC, 2001). Long- and short-term effects, scored pharmacologically and toxicologically, together with the archived expression patterns, would generate a very dynamic database that could be continuously populated with new data (Hamadeh et al., 2001). Micoarrays may be able to identify differences in gene expression at various doses, and enable the prediction of the potential mode of action of a compound before chronic bioassays is conducted (Knight and Breheny, 2002). Changes in gene expression at subclinical or sub-pathologic doses, that are predictive of the toxicity of a particular chemical or class of chemicals, can be identified. These changes in gene expression can then be monitored throughout a dose–response study. It is feasible to associate a set of gene expression changes with tumour development and then monitor that specific suite of genes, thus leading to a better definition of the shape of the dose–response curve at low doses. Arsenic is an example compound for which this approach maybe useful (Hamadeh et al., 2001; Knight and Breheny, 2002).

Perhaps the greatest hurdle for the application of arrays is the actual interpretation of data in relation to their toxicological significance (Rockett and Dix, 1999). Lack of reference data could easily lead to miss- or over-interpretation and subsequently to undue concern by regulatory agencies. Therefore, there is an urgent need for the chemical industry as a whole to collaborate with academia and regulators in the development and sharing of such data sets (Hamadeh et al., 2001). The new information obtained can be meaningfully used only when understanding the physiological pathways that lead to toxicity, so we can filter out relevant changes in gene/protein expressions (in reality, only a small proportion of the gene/protein expression changes can be interpreted, as many gene/protein functions are unknown). By use of pattern recognition and multivariate analytical tools: fingerprints that are correlated with specific toxicities should be established (possibly even in absence of mechanistic understanding) (Fry and George, 2001). Thus the new technology promises a lot. However, even though the new tool is now available to the toxicologist and more data are coming, examples on the implication for regulatory toxicology has yet to be seen. Concerning one specific area, the assessment of food safety related to genetically modified foods, the microarrays are currently used to develop a nonbiased system for detection of altered gene expression in genetically modified crop varieties in comparison to the parent line (Kuiper et al., 2001).

The computerised determination of Quantitative Structure–Activity Relationship (QSAR) has been used for many years in design of potentially useful chemical agents, but it is only in recent years that these techniques have been used in toxicology. QSAR evaluations use the known biological activity of a set of chemicals to establish mathematical relationships between their activities and chemical structure. An alternative approach, using structure–activity relationship (SAR), uses computerised methods to identify fragments of molecules that are known to be associated with particular biological properties.

QSAR can be used in certain circumstances for notification of new substances in EU, and in particular, to refine the risk assessment. Furthermore, if further testing is needed, QSAR may also be used to optimise the testing strategies (Knight and Breheny, 2002; EC, 1996). In a quite recent report of a US Food and Drug Administration (USFDA), QSAR system has been presented as a tool with a broad application in situations in which rapid and reliable decision support information of toxicology data are needed and toxicology data that are either unavailable or inadequate. The report foresee the use of the QSAR system within areas of cosmetic ingredients, nutritional supplements, herbal medicines and what is currently considered as grandfathered i.e. food additives such as spices and flavours (Matthews and Contrera, 1998). Validated QSARs are not currently

available for toxicity endpoints (Knight and Breheny, 2002).

The Danish Environmental Protection Agency (2001) has recently published an Advisory list for self-classification of dangerous substances. The substances have been identified by means of computer models, the so-called QSAR models. The list is intended as an aid to producers and importers for their self-classification, and the list is not binding. Using these QSAR models, the Danish EPA has examined approximately 47 000 chemical substances, identifying 20 624 substances which are deemed to require classification for one or more of the following dangerous properties: acute oral toxicity, skin sensitisation, mutagenicity, carcinogenicity, and danger to the aquatic environment.

According to EU's classification criteria, classification should be carried out on the basis of the knowledge available. However, the Danish EPA estimates that for approximately 90% of all substances, only few or no test results from animal testing, are available on any dangerous properties to humans or the environment. In addition to results from animal testing, the criteria for classification of chemicals also provide opportunities for using alternative methods. QSAR modelling is such an alternative method to assess the potential danger of chemical substances. For several years, the Danish EPA has carried out work to develop and apply QSAR models in order to predict the properties of chemical substances. The models used here are now so reliable that they are able to predict whether a given substance has one or more of the properties selected with an accuracy of approximately 70–85%. If data, however, from suitable animal testing are available for some of the substances found in the Advisory list for self-classification of dangerous substances, these data should be employed for self-classification in preference to the recommendations of this list. The report emphasises that the list should be used with precautions as among others, it is not an exhaustive list of all dangerous substances in EU, the number of dangerous properties included is limited and limited models are used. The list, only containing positive predictions, cannot be used to 'free' substances for having intrinsic dangerous properties.

A specific alternative approach different from the above mentioned is to reduce the toxicological testing and the use of data from these tests by employing a limit of exposure under, theoretically, no toxicity is expected and consequently no testing needed. Thus the concept of Threshold of Toxicological Concern (TTC) has been proposed for chemical substances present in the diet (ILSI, 1999). The use of TTC could lead to more effective use of toxicological testing and evaluation resources, reduction in use of animals and providing a simple basis for regulation according to this proposal.

5. Conclusions

In general, an alternative approach e.g. an alternative test could be introduced either as a supplement to or a replacement of an already existing test. In the latter case the regulatory authority should require that the alternative test give both a reliable 'yes' and a reliable 'no'. If that cannot be achieved do we then want to reduce the strength of prediction—do we then want to 'compromise' in order to achieve our goals with respect to animal welfare, etc?

The toxicology is steadily moving from being a discipline where the main object is to describe all possible toxicity and determine ' No Observed Adverse Effect Levels' towards focusing more on the detection of and the mechanism behind specific adverse effects. The regulatory authorities will be faced with the challenge that more data come from specific tests involving more sophisticated techniques sometimes requiring specialists for interpretation. Concerning risk assessment, it will be a challenge to decide when to take the consequences of more knowledge e.g. about the relevance of findings in laboratory animals for man and consequently in some cases reduce the uncertainty factor. Some of the new tests will serve as very early screening tests indicating a more specific potential hazard of the chemical. Where a new chemical belongs to a group of already known chemicals it could lead to a reduced testing

programme or even no further testing at all. In addition some of the new techniques could add to the information of dose–response at low doses e.g. for cancer and as such improve the database for the risk assessment.

The development of new test methods based upon more knowledge of the mode of action of the different toxicological effects could lead to a more optimal but demanding testing strategy based upon an initial common basic test programme for all substances followed by an individual testing strategy specifically addressing the problems/abilities of that one chemical in a kind of tailored approach.

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Review

Validation and acceptance of new and revised tests: a flexible but transparent process

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Abstract

Scientific validation is a necessary prerequisite for the regulatory acceptance of new toxicological tests, and for acceptance as Organisation for Economic Co-operation and Development Test Guidelines. Validation is defined as the determination of the reliability and relevance of a test method for a particular purpose. Reliability refers to the reproducibility of the test within and among laboratories. The relevance of a test addresses how well it measures or predicts what it is supposed to measure or predict. There are no fixed values for how reliable or relevant a test should be. Acceptable values depend on the type of test, the inherent variability in the biological systems used, and what regulatory decisions the test will be asked to support. The validation of in vitro and in vivo tests should follow the same general principles. The validation process should be flexible, according to the type of test being evaluated and its proposed uses. If a test is designed to replace a test currently in use, its reliability and relevance should be measured against that reference test or against the effect that the test is designed to predict. For the evaluation of health effects where adequate human data are available, the performance of the new test should be measured against the relevant effect in humans, rather than a surrogate species. All proceedings from the validation process and the subsequent peer-review of the method should be transparent, i.e., publicly available. It is also recommended that the peer-review of the validation study be accessible to the interested public.
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Keywords: Test validation; Reliability; Relevance; Transparency; Regulatory acceptance

1. Introduction

Scientific validation has become a prerequisite for acceptance of a new or significantly revised method as an Organisation for Economic Co-operation and Development (OECD) Test Guideline (OECD, 2001). It is also required for regulatory acceptance of new methods by US (NIEHS, 1997; USC, 1993, 2000), and other (EEC, 1976) regulatory agencies. Test validation is currently defined as the determination of the reliability and relevance of a test method for its intended purpose

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(Balls, 1995; Balls et al., 1990, 1995; NIEHS, 1997; OECD, 2001; Zeiger and Stokes, 1998). Although the requirement for formal test validation is new, the principles of test validation are not, and are, essentially, the scientific principles of test reproducibility and test validity.

Traditionally, new tests have been adopted for regulatory use based on the history of use and experience with the test, and confidence that it would provide sufficiently accurate data to support a regulatory decision. In addition to this approach, there have been official or unofficial sponsored or directed inter-laboratory collaborative (or round-robin) studies to evaluate the performance of tests of interest to the scientific and regulatory communities. For the most part, the design and performance of these efforts have been informal or guided by general scientific principles, and not subject to any specific, formal procedures or oversight, but are influenced by the nature of the test and the question(s) being asked of it.

Recently, however, guidance documents have been developed for the design and performance of validation studies for tests planned or proposed for formal guideline development and/or regulatory agency acceptance (NIEHS 1997, 1999; OECD, 2001). These documents generally require standardised information about the test, a structured study design and management system, and a formal peer-review process.

The importance of retaining the flexibility of the scientific process for designing validation studies is well recognized. It is possible to maintain design flexibility yet provide scientific rigor to the system so that the regulatory agencies will have sufficient, good-quality information about the test and its performance characteristics so that they can use data from the test to support regulatory decisions. This manuscript will briefly describe the currently used formal procedures for determining the reproducibility and validity of a test, and the areas in a validation study where it is important to maintain flexibility and transparency.

Detailed documents describing test validation principles and procedures have been published by the US Interagency Committee to Coordinate and Validate Alternative Methods (ICCVAM) (NIEHS, 1997, 1999) and by the OECD (2001).

2. Test validation—the scientific processes

Validation is defined by the OECD, ICCVAM, and ECVAM as the assessment of the reliability and relevance of a test for its intended purpose (Balls et al., 1990, 1995; NIEHS, 1997; OECD, 2001). Validation is a scientific process designed to determine a test's intra- and inter-laboratory reproducibility (reliability) and its ability to measure the effect it is designed, or proposed, for (relevance).

The determination of a test's reliability and relevance should, ideally, include a determination of its limitations and strengths. Not all tests are equally responsive to all structural and functional classes of chemicals. For example, a test may be highly effective with chemicals that are directly active, but may not be able to approximate in vivo metabolism, or a cell line may not be effective against substances with detergent properties because of their effects on the cell membrane.

A test may be proposed as a definitive test for effects for which no prior test existed, as a replacement or substitute test for an existing, accepted test, as a screening test for prioritisation of future testing, or as a component of a formal test battery. Although scientifically rigorous validation studies are needed for all types of test, the extent and design of the validation study may be different according to the type of test and its proposed use.

Validation has become a prerequisite for regulatory acceptance of a new or revised test, or for its incorporation into a Test Guideline. It is a basic tenet of the process that, while validation is strictly an objective scientific exercise, the regulatory acceptance of a validated test is the responsibility of the regulatory organisation. This means that the regulatory agency has the sole authority to determine whether the operating characteristics of a test are sufficient for that agency's purposes. As a result, a test may be accepted for specific purposes by some agencies, but not accepted by other agencies with different mandates or requirements.

2.1. The process of validation—reliability

The reliability (reproducibility) of a standardised test protocol can be determined in a number of ways depending on the test and its proposed uses. The numbers and types of chemicals to be tested in order to evaluate a test's reproducibility, and the numbers of laboratories participating in the study, will vary according to the type of test (e.g., in vitro, acute in vivo, longer term in vivo), and the extent of information already available about the test. The measure of a test's reproducibility can be combined with the determination of its relevance, so that the test substances that are appropriate for measuring a test's predictive capability can also be used to measure its reproducibility. To maintain objectivity in the testing and analyses of the test results, it is recommended that the test substances be coded and tested and evaluated blind. It is also important that the substances selected encompass a wide range of activity, from the strongly active to inactive, in order to model the types of substances that will be tested if the test is approved for use.

There is no predetermined value for test reproducibility that can be recommended. The reproducibility of a biological test will depend on many factors, including the biological variability inherent in the biological system and unavoidable sampling variability. An acceptable value for reproducibility would also be a function of the proposed use(s) of the test data and, where appropriate, the reproducibility of the existing test that the new test is designed to replace.

2.2. The process of validation—relevance

Similar to reproducibility, the relevance of a test can also be determined in a number of ways. For example, the majority of new tests are developed to predict or model an in vivo effect. In these situations, the in vivo effect, whether it be in humans or laboratory animals, serves as the reference standard against which the test performance is measured. It is not sufficient for a test to be able to predict/model only active substances, it must be able to distinguish between active and inactive substances. For this reason, the substances tested in the validation program must cover a full range of activities, ideally representative of the classes or types of substances for which the test will be used, and coded substances should be used wherever possible.

As with the concept of reproducibility, there are no predetermined values for judging a test's relevance. The effectiveness of a test will depend on the inherent variability and limitations of the test, itself, and of the effect that the test is designed to predict or model. An acceptable value for relevance will also depend on the proposed use(s) of the test and the effectiveness of the test (if any) that it is designed to replace.

2.3. Flexibility

The flexibility (described above) in the criteria for determining the reliability and relevance of a test ensures that the test will be evaluated by criteria appropriate for the test and its proposed uses, rather than by a predetermined, standard procedure that does not take into consideration the mechanism of the test, its proposed uses, and logistic (e.g., costs; animal use; test complexity) and scientific (e.g., mechanistic limitations; prior information) relationships. Despite the flexibility inherent in the system being described, the quality of the science will not be compromised if the validation procedure and protocols are properly designed.

3. Test validation—the administrative processes

3.1. Peer-review of the validation study

After completion of a validation study, the results of the study should be peer-reviewed by uninvolved experts who do not have a financial, or other significant, interest in the test. It is not always possible to find scientists who are expert or knowledgeable about a specific method who do not have a bias regarding that method or its proposed use. However, it is generally agreed that such a bias does not necessarily mean that an individual cannot objectively evaluate the results of a validation study, and make recom-

mendations for the use of the method. It is important that the potential biases of the reviewers be known and balanced within the review panel. Although the reviewers may have individual biases, it is possible for the panel, as a whole, be unbiased.

The results of the peer-review, and the panel's questions for clarification, are presented to the individual or group managing the validation study for possible rebuttal or additional studies or analyses. The peer-review panel's role is to determine whether the results of the validation study support the proposed use of the test, whether additional laboratory work or data analyses is necessary prior to drawing a conclusion, or whether the test is not adequate for its proposed use. If the test is judged to be acceptable, the panel's conclusions, and all supporting information, are made available to the regulatory authorities to support the use of the test.

3.2. Transparency

The decision as to whether or not a test is validated for a specific regulatory use, or for support of a test guideline, affects many parties, including regulatory agencies, test developers/owners, regulated industries that would be using the test, and the scientific community. 'Transparency' is simply the concept that all decisions, and the information available to the people making the decisions, also be available to all interested parties. This should also include the deliberations of the peer-review panel and its recommendations.

The test protocols and data used to support decisions on test validation and the recommendation for regulatory acceptance should be publicly available. The participating laboratories should also be identified; this can be done without linking specific data sets to individual laboratories. If the peer-review process cannot be open to public, the summary report given to the peer-review panel, the panel's report and recommendations, and the responses to the recommendations should be made public.

4. Conclusions

Because different legislative and regulatory mandates require different criteria for test data, the validation process must be separated from the regulatory acceptance of the test. A test suitable for one organisation or use may not be suitable for others. The purpose of the validation process is to provide the regulatory community with sufficient information to support a decision to incorporate the test into a Guideline or testing requirement.

The validation process and the test validation criteria must be sufficiently flexible to take into account the nature of the specific test, the available information about the test, its mechanistic relationship to the effect of concern and biological relationship to the species of concern, and the proposed uses of the test. This flexibility in the validation process and criteria should not compromise the scientific quality of the study.

All stages in the validation of a new test should involve the people (regulators, managers, scientific community, and the public) who will use the test or be affected by its use. The validation process, including the impartial review of the validation study and recommendations, should, therefore, be open to public (including general scientific) scrutiny.

Validation does not imply the permanent approval of a test or its use. The recommended uses of the test, and its validity, should be reviewed and reconsidered as experience with the test and new data become available.

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Review

The leading role and responsibility of the international scientific community in test development

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Abstract

The possibility that one or more environmental factors may be affecting adversely the endocrine systems of humans and wildlife has been the subject of international study for the past 7 years. Irrespective of which factors are the most important contributors to the perceived problem; the decision has been taken to evaluate synthetic chemicals for such chemical toxicities. This decision requires access to reliable and relevant endocrine disruption (ED) assays. However, few such tests currently exist, albeit many candidate assays are currently under development. Faced with this situation, the US Environmental Protection Agency has taken the lead in supporting the development and validation of appropriate ED assays. Two of these assays, the rodent uterotrophic assay and the Hershberger anti-androgen assay, are in the final stages of validation, a project conducted under the auspices of the Organisation for Economic and Commercial Development. This article describes the particular scientific issues associated with the validation of these two assays and alerts to the continuing need to consider new assay protocols as they become available.
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Keywords: Endocrine disruption; Low-dose; Uterotropic assay; Genistein; Bisphenol-A

1. Introduction

The schematic shown in Fig. 1 serves to illustrate several sources of uncertainty in experimental endocrine disruption (ED), uncertainties that require international cooperation if they are to be resolved without recourse to a variety of individual national approaches. The first four columns in Fig. 1 illustrate that while there are many human and rodent carcinogens, and several such non-carcinogens, there is no equivalent database for EDs. Thus, it is not possible to evaluate the value of rodent ED data by reference to their correlation with human ED data. Further, there are no generally acknowledged chemicals that are devoid of ED activity, a fact that prevents the specificity of newly developed ED assays from being assessed. The next two columns in Fig. 1 illustrate pressures that are being applied to all branches of toxicology, including ED. These are that current perceptions of the no observed adverse effect level (NOAEL) for chemicals is coming under scrutiny from three directions—would a larger study define an effect not seen in

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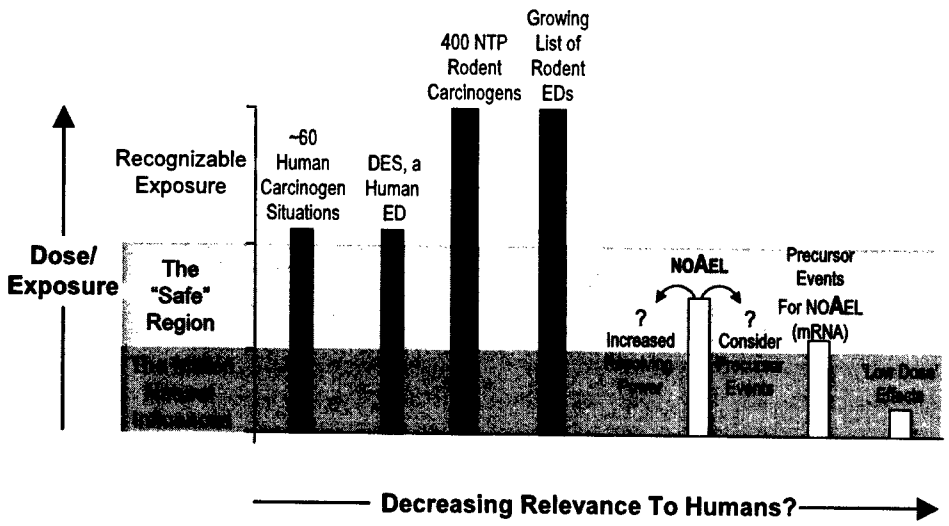

Fig. 1. Basis for considering an effect to be observed at a 'low dose'. NOAEL, no observed adverse effect level; DES, diethylstilbestrol; NTP, US National Toxicology Program.

a smaller study, would the NOAEL be lowered if precursor events to the adverse response were to be monitored, and can the term 'adverse' be described adequately? In the field of ED there is also the additional concern that some chemicals may produce effects at dose levels far below the NOAEL value (final column in Fig. 1; vom Saal et al., 1997; Almstrup et al., 2002). These several concerns require urgent attention; in particular, agreement is needed on the toxicological significance of changes in mRNA levels induced by a chemical at dose levels below the currently accepted NOAEL value. Perhaps the single most important theme to emerge to date in the study of ED is the need to acquire appropriate control and test data before major regulatory decisions are taken on ED testing strategies (Sharpe et al., 1998; Ashby, 2000; Ganmaa et al., 2001).

2. The rodent uterotrophic assay

This assay has been in use for over 70 years and underwent its first modern evaluation in 1996 (Shelby et al., 1996). The history and the data available for this assay have been reviewed as part of the Organisation for Economic and Commercial Development (OECD) validation initiative (Owens and Ashby, 2002). The endpoint of the assay is estrogen-induced growth of the uterus in either sexually immature rodents (whose uterus has yet to develop) or in ovariectomised rodents (whose uterus has regressed with the loss of ovarian estrogen drive). Changes in the weight of the uterus, although a simple and non-adverse endpoint, can be indicative of other and adverse properties of a chemical. This was illustrated by Newbold et al. (2001) who demonstrated that dose levels of the synthetic estrogen diethylstilbestrol (DES) and the phytoestrogen genistein, that gave equivalent uterotrophic effects, also gave equivalent carcinogenic responses in the mouse uterus (Fig. 2).

When the OECD uterotrophic validation commenced it was known that the assay was conducted according to different protocols in different countries. Some use the immature rodent and oral administration of the test agent, others used subcutaneous (sc) injection of the test agent, and others used the ovariectomised model with either a short (3 day) or a longer (7 day) exposure period. The data shown in Fig. 3 illustrate that these protocol variables can affect the outcome of an assay. In Fig. 3 it is clear that genistein gives a stronger assay response using the sc route than the oral route of administration. Further, the rat is

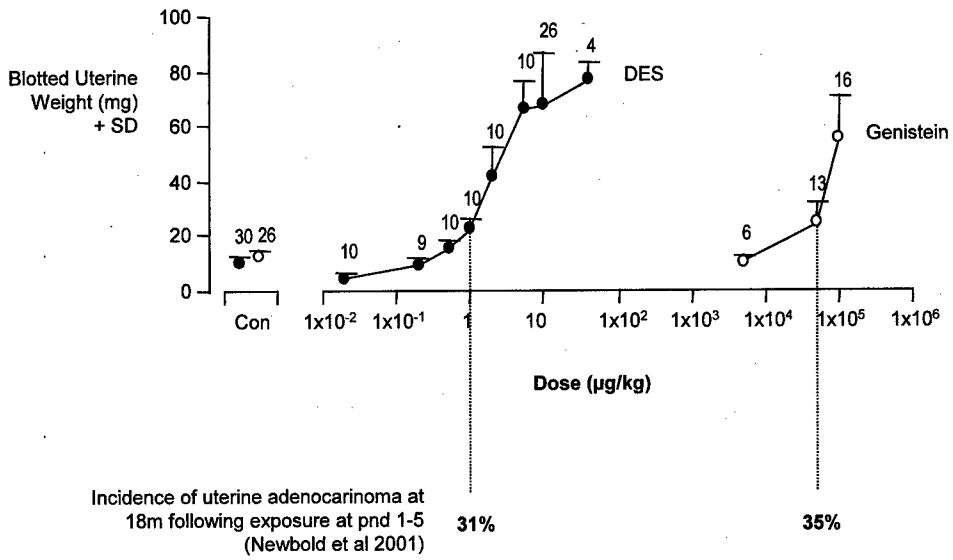

Fig. 2. Dose levels that yield equal levels of activity for DES and genistein in the immature mouse uterotrophic assay (Odum et al., 2002; Ashby, 2000) are also associated with the induction of equal incidences of uterine adenoma in the mature mouse when exposed on postnatal days 1–5 (Newbold et al., 2001).

marginally more sensitive to this agent than is the mouse (c.f. the case of Bisphenol-A, BPA; Tinwell et al., 2000). It was therefore decided to evaluate the general significance of the main of these protocol variables. Four protocols were evaluated in the rat—the use of immature animals exposed by either sc injection or oral gavage, and the use of ovariectomised animals exposed for either 3 or 7 days. When evaluating the potent estrogen ethinyl estradiol each of the test protocols gave similar responses (Kanno et al., 2001). However, when the data for a range of both potent and weak chemical estrogens were assessed it became clear that each of the test protocols performed best for individual chemicals, but that no single protocol was uniformly superior (Kanno et al., in press(a) and Kanno et al., in press(b)). As part of those studies it was also established that the phytoestrogen contents of the many rodent diets employed by the participating laboratories had no major effect on assay sensitivity (Owens et al., in press). Part of the studies conducted involved the use of coded

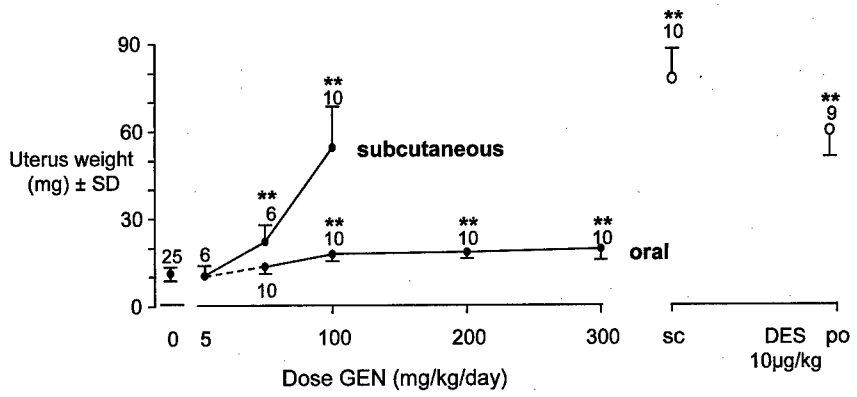

Fig. 3. Relative activity of genistein and DES in the immature mouse uterotrophic assay using either oral or sc routes of exposure (Ashby, 2000).

chemicals, and several detailed dose response studies were conducted. When all of these data are taken into account the rodent uterotrophic assay can be considered to be essentially validated and ready for routine use. The final stage of the validation process is for an independent panel to review the data and for a test protocol guideline to be issued by the OECD.

It is important to note that the uterotrophic assay does not provide evidence of an adverse effect, it merely alerts to the potential of a chemical to produce adverse ED effects in appropriate studies. This point is illustrated by the fact that one of the test chemicals employed in the OECD validation study, BPA, gave uniformly positive uterotrophic immature rat assay responses (Ashby and Tinwell, 1998), yet has subsequently been found to give negative results in definitive 2-generation (Ema et al., 2001) and 3-generation (Tyl et al., 2002) rat multigenerational assays (the recently updated OECD test guideline 416). Thus, in this case, the rodent uterotrophic assays could be considered to have produced 'false positive' results. However, that conclusion would not be universally owned because some investigators do not consider the multigenerational assay to be the 'gold standard' assumed by other investigators—again, this question requires international discussion and agreement before real progress can be made in the assessment and regulation of endocrine-active chemicals. As part of that necessary discussion it remains to be decided if it is critical to include evaluations involving exposure in utero when assessing the ED-potential of a chemical (Odum et al., 2002).

A further area of uncertainty associated with the routine use of the uterotrophic assay concerns the significance of precursor events observed in the absence of an increase in uterine weight. The data shown in Fig. 4 illustrate one such case, and further instances are certain to occur as genomic data become available. On the one hand it could be said that the gravimetric endpoint of the uterotrophic assay is insensitive when compared to changes in mRNA levels associated with precursor events to uterine growth. On the other hand it could be argued that there is a need to observe a phenotypic change in the test animals before the toxicological consequences of a chemical treatment can be fully assessed. Resolution of this question can only be achieved at the international level.

Assessment of the possible anti-estrogenic properties of a chemical can be achieved by negation of the uterotrophic response induced by a reference estrogen such as estradiol (Kanno et al., 2001). However, an alternative protocol has been described wherein the test agent delays the onset of puberty (Ashby et al., 2002a). It is difficult to consider the validation of such alternative assay protocols, but that does not remove the need to consider their validation. In the absence of such international initiatives assay development and refinement will cease.

3. The castrated male rat anti-androgen assay (Hershberger assay)

Despite initial concern only with environmental estrogens, consideration is now being given to the potential importance of synthetic anti-androgens (Kelce et al., 1997; Kelce and Wilson, 1997). The primary assay for anti-androgens is that described by Hershberger et al. (1953), which underwent a preliminary evaluation in 2000 (Ashby and Lefevre, 2000a). The basis of the assay is that castrated sexually mature male rats undergo regression of androgen-sensitive tissues (prostate, seminal vesicle and levator-ani muscles, among other tissues). These tissues are restored to their original weight upon treatment with testosterone, and that growth can be blocked by the concomitant administration of an anti-androgen. The OECD validation of this assay has progressed to the point where agreement has been reached on the optimum dose level of testosterone to be used and work is now commencing on the testing of reference agents. To date the assay has performed well, and it is expected that it will be successfully validated by 2003.

A disadvantage of the assay is the need for surgical castration, a potentially stressful operation requiring technical skill. Attention is therefore also being given to the possibility of using pubertal male rats (Monosson et al., 1999; Ashby and

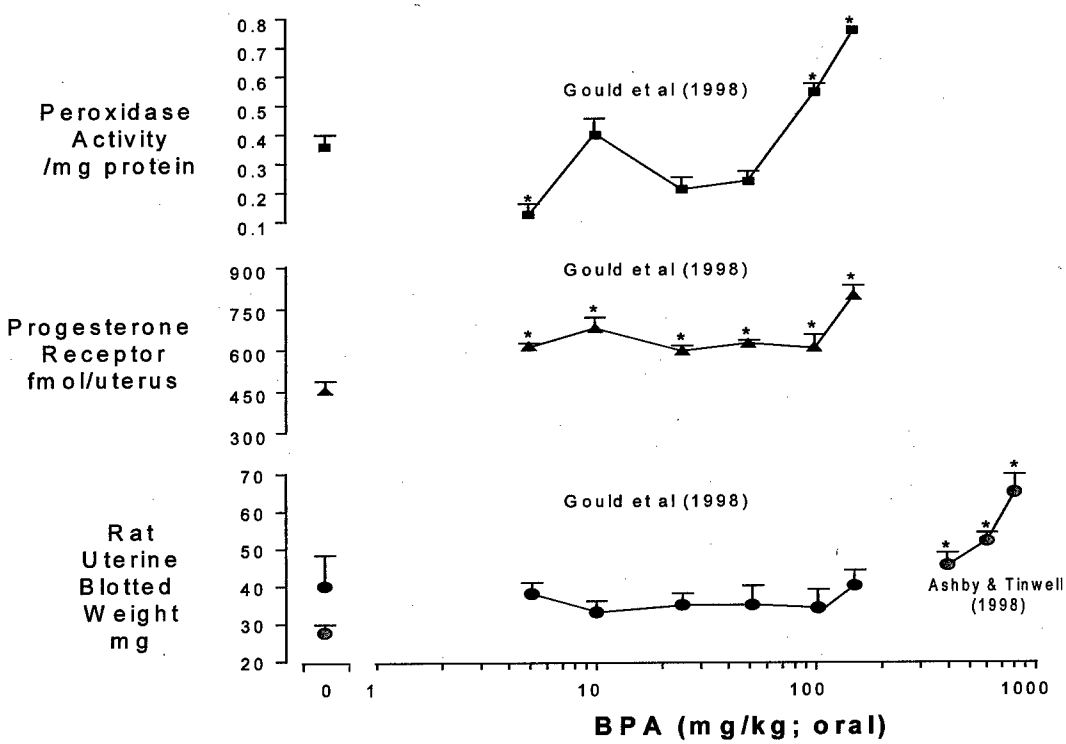

Fig. 4. Reported activities of BPA in the immature rat uterus by Gould et al., 1998.

Lefevre, 2000b), mature male rats (O'Connor et al., 1999) or androgen-stimulated pubertal male rats (Ashby et al., 2002b) when screening for anti-androgens. It is obviously important that the validation of the Hershberger assay should not act as a brake on the development and validation of such alternative assays, but that could happen in the absence of purposeful attempts to evaluate such new assays at the international level.

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Review

Genotoxicity—threshold or not? Introduction of cases of industrial chemicals

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Abstract

Many industrially and environmentally important industrial carcinogens display effects that lead them to be viewed and regulated as 'genotoxic compounds'. Some of these chemicals cause experimental tumours only at high or toxic doses. The current view is that non-threshold principles should be applied for risk assessments and to define permissible exposure values. The toxicological impact of underlying mechanisms is frequently not well investigated and understood. The classification of carcinogens is now in a state of discussion. In Germany, the 'MAK-Commission' has issued new recommendations to distinguish between 5 groups of proven and suspected carcinogens. This proposal includes a category of 'substances with carcinogenic potential for which genotoxicity plays no or at most a minor role'. Another category comprises 'substances with carcinogenic and genotoxic potential, the potency of which is considered so low that, provided that the MAK-value is observed, no significant contribution to human cancer risk is to be expected'. There is also a number of apparently genotoxic carcinogens where the existence of 'practical thresholds' is at least debated. One outstanding example is vinyl acetate, which must be viewed against the background of discussions on other industrial high-volume chemicals like formaldehyde, acrylonitrile, acrylamide and trichloroethylene. Main arguments in favour or against thresholds of carcinogenicity of these individual compounds are summarised. Current instruments of regulation should be adjusted to allow adequate consideration of carcinogenic effects of chemicals that are practically relevant at high doses only. Also, research into this field is encouraged.
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Keywords: Genotoxicity; Threshold effects; Dose–response; Formaldehyde; Vinyl acetate acrylonitrile; Acrylamide; Trichloroethylene

1. Introduction

The classification of carcinogens is currently in a state of discussion (Seeley et al., 2001). In Germany, the Senate Commission of the DFG for the Investigation of Health Hazards in the Work Area ('MAK-Commission') has issued new recommen-

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dations to distinguish between 5 groups of proven and suspected carcinogens (Neumann et al., 1998). Heretofore, the Labelling Guide of the European Union distinguishes only 3 groups (categories 1, 2, 3). The new proposal was preceded by discussions for about 10 years (Bolt et al., 1988). It includes a Category 4 of 'substances with carcinogenic potential for which genotoxicity plays no or at most a minor role. No significant contribution to human cancer risk is expected, provided that the MAK value is observed'. A new Category 5 comprises 'substances with carcinogenic and genotoxic potential, the potency of which is considered so low that, provided that the MAK-value is observed, no significant contribution to human cancer risk is to be expected'. The distinction of these two new categories means that the classification of carcinogens should be based more on mechanisms by which carcinogenic effects are elicited and should consider aspects of potency. Concepts underlying present research strategies go even further, as it should probably not only be distinguished between 'genotoxic' and 'non-genotoxic' carcinogens, but within the group of 'genotoxic' carcinogens between compounds characterised by 'threshold' and 'non-threshold' effects. The new proposal for classification of carcinogens (Neumann et al., 1998) has led to similar considerations on germ-cell mutagens (Adler et al., 2000). Also in this respect, a distinction between 'threshold' and 'non-threshold' effects could probably be made in the future.

Relevant research is being discussed at the current EUROTOX Workshop of the Speciality Section Carcinogenesis. Micheline Kirsch-Volders will present new insights into indirect mechanisms of genotoxicity. The Workshop will highlight advanced examples that contribute to this discussion (Ricarda Thier). There are now a number of apparently genotoxic carcinogens where the existence of 'practical thresholds' is at least debated. Matthew Bogdanffy will elaborate on the outstanding example of vinyl acetate (see also: Bogdanffy, 2002). Finally, the regulatory recognition of indirect genotoxicity mechanisms in the European Union will be questioned by Iona Pratt.

The general topic must be viewed against the background of current and past discussions on a number of industrial high-volume chemicals, such as formaldehyde, acrylonitrile, acrylamide and trichloroethylene. Main arguments in favour or against thresholds of carcinogenicity of these compounds will be summarised here.

2. Formaldehyde

Toxicological effects of formaldehyde have been widely discussed for many years (BMJFG, 1984; Bolt, 1987; Imbus, 1988). The primary focus was on the pathogenesis and interpretation of nasal tumours found in long-term rodent bioassays. The genetic toxicology of formaldehyde has been reviewed (Ma and Harris, 1988). Formaldehyde is mutagenic in different test systems, especially when high concentrations act directly on cells (gene and chromosome mutations). Positive cell transformation assays have been reported in vitro. After inhalation of the compound local DNA adducts were observed in the nasal mucosa of rats, in the absence of simultaneous systemic genetic effects (Casanova-Schmitz et al., 1984).

Experiments on induction of sister chromatid exchange in human lymphocyte cultures (Kreiger and Garry, 1983) have demonstrated no significant response below an apparent 'threshold' of 5 μg/ml culture medium. In accordance with this, the cytotoxic dose–response curve showed a biphasic pattern with a marked increase of slope at about 10 μg formaldehyde/ml. Studies on unscheduled DNA synthesis in primary cultures of human bronchial epithelial cells (Doolittle et al., 1985) have demonstrated no response to formaldehyde. Damage induced by formaldehyde in DNA of different human cell culture systems comprises DNA-protein cross-links (DPX) and DNA single-strand breaks; these lesions undergo efficient repair by complex mechanisms (Grafstrom et al., 1984).

The bioassay on which discussions have focused was performed on male and female F-344 rats and $B_6C_3F_1$ mice that were exposed to 2.0, 5.6 or 14.3 ppm formaldehyde for 6 h/day and 5 days/week over 24 months (Swenberg et al., 1980; Kerns et al., 1983). Of concern was the induction of squamous cell carcinomas in the nasal passages,

occurring primarily in rats exposed at the highest concentration. No morphological changes were observed at 2 ppm, whereas 5.6 and especially 14.3 ppm led to rhinitis and to dysplasia and metaplasia of the nasal epithelium. Two (male) mice of the highest dose group developed squamous cell carcinoma (not statistically significant). The histological changes of dysplasia and metaplasia were more marked in rats. Changes were also observed at 2 ppm in this species. At 5.6 ppm one male and one female rat, but at 14.3 ppm nearly half of all rats developed squamous cell carcinomas. At the highest dose dysplasia and metaplasia of the trachea were also noticed.

This has triggered in-depth investigations of the quantitative coherence of primary DNA-formaldehyde interaction, resulting DPX, cell replication and local tumour formation at the nasal epithelium. The formation of DPX in rodent nasal respiratory mucosa (Casanova-Schmitz and Heck, 1983) was regarded as an important biochemical primary lesion in the pathogenesis of formaldehyde-induced tumours (Lam et al., 1985).

The general paradigm of a sub-linear dose–response of formaldehyde has been experimentally supported on different levels of the toxicological chain of events:

(1) The covalent binding of labelled formaldehyde to DNA of respiratory rat mucosa was not linear with the exposure concentration, but increased steeply between 2 and 6 ppm (Casanova-Schmitz et al., 1984). This has been put forward as an important argument against the applicability of linear (or quasi-linear) risk estimates for formaldehyde.

(2) The dose–response of incorporation of ^{14}C-formaldehyde into DPX of rat nasal mucosa has clearly demonstrated a non-linearity at formaldehyde doses below 5 ppm (Casanova et al., 1989). Effects of pre-exposures of rats on nasal DPX formations were visible, but were observed at high doses only and therefore considered of only marginal importance for risk extrapolations (Casanova et al., 1994).

(3) Formaldehyde exposure led to an increase in the rate of cell turnover in the respiratory mucosa (the tissue from where the nasal squamous cell carcinomas originate). This increase was non-linear with formaldehyde concentration, being undetectable at 2 ppm, highly significant at 6 ppm, then decreasing at 15 ppm due to cytotoxicity (Swenberg et al., 1983). Such increased cell replication would substantially increase the number of non double-stranded DNA sites available for reaction with formaldehyde (Casanova et al., 1994).

Possible human risk estimates for formaldehyde on the basis of the animal experiments have been discussed. The high incidence of experimental tumours in rats at 14.3 ppm, together with the mutagenic properties and the possibility of DNA interaction have been main arguments in favour of a substantial human carcinogenic risk that is calculated from the experimental tumour data by conventional risk extrapolation models.

On the other hand, the following arguments have been used against application of a linear risk extrapolation for formaldehyde to humans (BMJFG, 1984):

- species differences in target tissue doses due to differences in respiration physiology;
- the highest dose in the bioassay producing excessive toxicity and mortality;
- local cell and tissue lesions being necessary precursor stages before tumours develop;
- dose–response of cell proliferation leading to higher proportions of single-stranded DNA which is susceptible to the generation of DPX;
- non linear dose–response of covalent formaldehyde-DNA interaction;
- endogenous formation of formaldehyde and physiological formaldehyde levels;
- rapid detoxification of formaldehyde, if present in doses which are not excessive;
- formaldehyde seems not to act systemically.

Recommendations of various official bodies, on national and international levels, have mostly considered these arguments. In general, the concept that humans are less susceptible than test animals, especially rats, has widely been accepted (Squire and Cameron, 1984). Genotoxicity of formaldehyde was also viewed to be confined mainly to higher dose levels where increased

cytotoxicity and regenerative cell division are found (Lutz, 1986).

As a result of the experimental studies on formaldehyde, it was recommended that low-concentration ($\leqslant 2$ ppm airborne exposure) extrapolation, where no tissue damage is observed, be uncoupled from the responses at high concentrations ($\geqslant 6$ ppm). At high concentrations epithelial degenerations, regenerative cell replication, and inflammation appear as essential driving forces in formaldehyde carcinogenesis (Morgan, 1997).

3. Acrylonitrile

Acrylonitrile is carcinogenic to rats following either oral administration or inhalation. Common target organs identified were the central nervous system (brain and spinal cord), Zymbal gland, gastrointestinal tract (tongue, non-glandular stomach and small intestine) and mammary gland (for review of details, see IARC, 1999). A high incidence of astrocytomas in the brain and spinal cord was the most consistent finding. In a 2-year inhalation study (6 h/day, 5 days/week) in rats, the lowest exposure associated with an increased incidence of astrocytomas was 20 ppm (Quast et al., 1980).

Acrylonitrile appears as weakly mutagenic in vitro, indicative of a genotoxic potential. However, these findings are not reliably reflected in the in vivo situation, possibly due to the detoxification of the epoxide metabolite via a glutathione conjugation pathway that may not exist in test systems in vitro. Nevertheless, the overall body of evidence on the mutagenicity of acrylonitrile in vitro leads to the conclusion that acrylonitrile should be regarded as a genotoxic carcinogen, although non-genotoxic mechanisms of tumour induction in experimental animals may also be involved (Whysner et al., 1998). Acrylonitrile has been shown to be weakly mutagenic, primarily through its oxidative metabolism. Acrylonitrile itself hardly, if at all, interacts with DNA. Its epoxide (cyanoethylene oxide, 'glycidonitrile'), however, is a direct acting mutagen, which binds with DNA with a much greater affinity than acrylonitrile (Peter et al., 1983). Adducts on guanine have been detected at very low levels in the liver of rats treated with cyanoethylene oxide, but the significance of these adducts for the carcinogenic process is not clear. Acrylonitrile, at a nearly lethal dosage, has been found to interact with DNA in the liver and stomach but no DNA adducts have been detected (Whysner et al., 1998). This may point to an epigenetic rather than a genetic mechanism involved in the induction of astrocytomas in the brain of rats exposed to acrylonitrile. Recent research has suggested a role of secondary processes in consequence of oxidative stress (Kamendoulis et al., 1999).

In general, the mechanisms of the experimental tumour formation following exposure to acrylonitrile are not fully understood. Although the primary oxidative metabolite, cyanoethylene oxide, appears clearly genotoxic, acrylonitrile may also act via non-genotoxic mechanisms of carcinogenicity. Such argumentations, mainly focussed on the experimental brain tumours, were based on the following (Chapman et al., 2002):

1) absence of DNA adducts in brain tissue after acrylonitrile exposure and absence of DNA repair on the basis of slide autoradiography,
2) oxidative DNA damage in astrocytes exposed to acrylonitrile but not in primary hepatocytes, with an apparent threshold response,
3) reversible loss of gap junction intercellular communication in astrocytes exposed to acrylonitrile, but not in primary hepatocytes.
4) The available long-term bioassays by inhalation and by the oral route (gavage and drinking water studies) have been analysed as to the dose-dependence of tumours at the main target sites, using the Kaplan-Meier probability model. It has been concluded that the shape of the different dose–response curves was sub-linear.
5) The genotoxicity in vivo, at low levels of exposure, is not straightforward.

However, as acrylonitrile appears from the rodent bioassays as a pluripotent (multi-organ) carcinogen, and a substantial impact of genotoxicity cannot be ruled out, it appears prudent to consider a non-threshold mechanism.

Acrylonitrile has been subject of quite a number of epidemiological studies in exposed workers. A meta-analysis of 25 studies of acrylonitrile workers has indicated no excess for lung, brain and prostate cancer (Collins and Strother, 1999). In a cohort study by the US NCI and NIOSH, however, some excess of lung cancer was noted in the highest quintile of cumulative exposure to acrylonitrile (Blair et al., 1998). In this case, it has been counter-argued that 67% of workers in the highest quintile originated from one plant, with a prior history of exposure to asbestos involved (Chapman et al., 2002). Having reviewed 18 published cohort studies, Sakurai (2000) arrived at the conclusion that, although there was no adequate evidence in humans for carcinogenicity of acrylonitrile, the possibility of a causal association between high exposure and lung cancer in humans could not be excluded.

Based on this considerable number of reported epidemiological studies the carcinogenic potential in humans appears to be weak (if any). By contrast, the preponderant health risks of industrial handling of acrylonitrile appear connected with its very high acute toxicity, in combination with its clear potential of skin penetration, which leads to a high danger of even fatal accidents (Thier et al., 2000). It has been concluded that current Occupational Exposure Limits in Western countries offer adequate protection against health effects other than carcinogenicity (Sakurai, 2000).

In industrial practice, preventive strategies may reasonably be based on analysis of acrylonitrile adducts to blood proteins (haemoglobin and/or albumin, Thier et al., 1999, 2002).

4. Acrylamide

In long-term experiments on the carcinogenicity of acrylamide female rats were treated daily with 1.0 and 3.0 mg/kg b.w., and male rats with 0.5 and 2.0 mg/kg b.w. acrylamide. In groups treated with the highest doses peripheral neurotoxic symptoms were found together with an increase in tumour incidence. In the female animals an increase of mammary adenomas and thyroid tumours occurred, in the male animals the incidence of scrotal mesotheliomas and thyroid tumours was elevated. The majority of the reported tumours, however, was benign. Significantly increased mammary gland tumours were fibroadenomas, and most of the observed thyroid tumours were adenomas as well, i.e. benign tumours. Scrotal mesotheliomas represent a type of tumour that is not found in humans, but not unusual in rats exposed to toxic substances (for details, see IARC, 1994). There have been very detailed discussions on mechanisms and types of dose–response concerning scrotal mesotheliomas (Crump Group, 1999a), mammary fibroadenomas (Crump Group, 1999b) and thyroid follicular cell tumours (Crump Group, 2000).

The general impression is that a cancerogenic potential of acrylamide is clearly visible, but is of a low order. Concerning other endpoints of toxicity, the neurotoxicity of acrylamide in humans and animals is clearly predominant. Cancerogenic effects occur only at or just below the range of toxic dosing.

Two epidemiological cohort studies (Sobel et al., 1986; Collins et al., 1989) did not show increased cancer mortalities of acrylamide-exposed workers.

Mechanistic studies have described interesting effects of acrylamide on motor protein systems, namely on the kinesin-based microtubule motility in vitro (Sickles et al., 1996). It was hypothesised that kinesin and microtubules are covalently modified by acrylamide resulting in reduced affinity for each other, and this was viewed as a mechanistic factor in axonal degeneration produced by acrylamide. But such mechanisms are likely also relevant for chromosomal genotoxicity, affecting the process of chromosomal segregation. Therefore, more detailed research is needed in this direction to draw practical conclusions (Stone et al., 2000). Ricarda Thier, this volume, will further highlight this.

Very recently and based on the available experimental data, an expert panel convened by the German Federal Agency of Consumer Health Protection and Veterinary Medicine (BGVV, 2002) has issued the opinion that, despite acrylamide being a genotoxic carcinogen, a non-linear relation between dose and carcinogenic effect appears likely.

5. Trichloroethylene

The discussion on renal carcinogenicity of trichloroethylene covers epidemiological, mechanistic and metabolic aspects in view of human risk assessment; this has been reviewed in detail (Brüning and Bolt, 2000). On the basis of animal studies trichloroethylene had been classified by IARC (1995) as 'probably carcinogenic to humans (Group 2A)'. Mice orally administered with trichloroethylene, both with and without stabilisers, exhibited increases in incidence of benign and malignant liver tumours. In rats renal cell tumours were increased in males, and an increased incidence of interstitial cell tumours of the testes was reported. Studies on the metabolism of trichloroethylene in rodents and in humans supported the role of bioactivation reactions for the development of tumours following exposure to trichloroethylene. Similar to acrylonitrile and acrylamide, trichloroethylene is metabolised by two competing pathways. For trichloroethylene, oxidation by cytochromes P-450 (mainly CYP2E1) is the major route of metabolism, and conjugation with glutathione by glutathione-*S*-transferases is a quantitatively minor pathway. Evidence for the existence of the glutathione-dependent pathway also in humans has been obtained by the identification of *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine and *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine in the urine of humans exposed to trichloroethylene (Brüning et al., 1998).

Earlier epidemiological cohort studies addressing the carcinogenicity of trichloroethylene with respect to the renal or urothelial target sites have been evaluated by Weiss (1996), where no clear evidence for an elevated renal or urinary tract cancer risk in trichloroethylene-exposed groups was visible. However, a study by Henschler et al., (1995) supported a nephrocarcinogenic effect of trichloroethylene in humans. They conducted a cohort study of 169 male workers having been exposed to unusually high levels of trichloroethylene in Germany. The results of this study were extensively discussed; criticism was mainly based on the choice of the study group, which had been recruited from personnel of a company in which a cluster of 4 renal tumours was observed previously. Another case-control study was conducted by Vamvakas et al. (1998) in the same region as the study of Henschler et al. (1995). Patients were nephrectomised because of renal cell cancer was included, and the diagnosis was confirmed histopathologically. This study confirmed the results of the previous cohort study, supporting the concept of involvement of prolonged and high-dose trichloroethylene exposures in the development of renal cell cancer. Both studies, by Henschler et al., (1995) and by Vamvakas et al., (1998), had been conducted in a region in Germany with a particular industrial structure where numerous small and medium-sized companies of the iron and metal industry had been established over the past 150 years. For decades, these companies had used trichloroethylene as main degreasing agent without any precautionary measures; the working conditions prevailing in the past in this industry would by no means be acceptable today. Further investigations on patients with renal cell carcinoma and with histories of high trichloroethylene exposures, on the basis of excretion of marker proteins in the urine, pointed to existence of toxic damage to the proximal renal tubules by trichloroethylene (Brüning et al., 1999). Hence, the hypothesis of implication of a glutathione transferase-dependent bioactivating pathway of trichloroethylene, established in experimental animals, seems at least plausible also for humans. Apparently, the occurrence of renal cell carcinomas accompanied by damage to tubular renal cells follows high-dose exposures to trichloroethylene.

Development of renal cell carcinomas has also been related to mutations in the von-Hippel-Lindau (VHL) tumour suppressor gene. Renal cell carcinoma tissues of persons with histories of prolonged high-dose exposure to trichloroethylene were investigated for the occurrence of mutations of the VHL tumour suppressor gene. VHL gene mutations were found in the majority of renal cell tumours associated with high-level exposure to trichloroethylene. Moreover, a specific mutational hot spot at the VHL nucleotide 454 occurred in 39% of these patients, and this mutation was also present in adjacent non-neoplastic kidney parenchyma in a few patients. This was addressed as a

unique mutation pattern of the VHL tumour suppressor gene (Brauch et al., 1999).

A synopsis of all experimental, clinical and epidemiological data suggested that reactive metabolites of trichloroethylene, with likely involvement of dichlorovinylcysteine, exert a genotoxic effect on the proximal tubule of the human kidney. This constitutes a tumour-initiating process of genotoxic nature, the initial genotoxic effect being apparently linked with mutational changes in the VHL tumour suppressor gene. However, there is compelling evidence that the full development of a malignant tumour requires continued promotional stimuli. Repetitive episodes of high peak exposures to trichloroethylene over a prolonged period of time apparently lead to nephrotoxicity, visualised by the excretion of tubular marker proteins in the urine (Brüning and Bolt, 2000).

This critical process of development of tubular damage by trichloroethylene should follow a 'conventional' dose-dependence, with the implication of a practical threshold. Such a view is much corroborated by the fact that the occurrence of human renal cell cancer is obviously confined to cases of unusually high trichloroethylene exposures in the past, with special characteristics of very high and repetitive peak exposures. Based on these argumentations, the Committee on Dangerous Chemicals (AGS) in Germany has considered the possibility of deriving a practical threshold for the carcinogenic effect of trichloroethylene in humans. However, before further continuing on this avenue, the following experimental studies were deemed necessary (AGS, 2001):

- assessment of dose–response of trichloroethylene genotoxicity in vivo (model of Schiestl et al., 1997), with inclusion of controls receiving an oxidative metabolite (trichloroethanol or chloral hydrate),
- experimental study in the most sensitive model (male rats; Mensing et al., 2002) of the dose–response of nephrotoxicity upon trichloroethylene inhalation, with determinations of expressions of beta-lyase in relevant organs/tissues,
- implementation of suitable health surveillance programmes in occupationally exposed populations, also comprising biological monitoring strategies.

6. Conclusion

Current instruments of regulation should be adjusted to allow adequate consideration of such carcinogenic effects of chemicals that are practically relevant at very high doses only. Experimental and human research into this field should be encouraged.

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Review

Regulatory recognition of indirect genotoxicity mechanisms in the European Union

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Abstract

The European Union (EU) system for the regulation of chemicals includes approval systems for pharmaceuticals, pesticides and biocides, requirements for hazard classification and for risk assessment of industrial chemicals. Regulators have traditionally used the commonly accepted categorisation of chemicals into genotoxic (DNA-reactive) or non-genotoxic agents in their decision-making processes, and have generally considered that there is no threshold level for the former group. The recognition that a number of genotoxic agents operate by indirect genotoxicity mechanisms such as induction of aneuploidy, oxidative stress, inhibition of DNA synthesis or cytotoxicity presents new problems for the regulator. The dose–response relationship for a number of such agents is generally accepted to show a threshold, however, the degree of acceptance of the threshold effect differs in different EU regulatory systems. The classification system for mutagens is based primarily on intrinsic hazard rather than risk, and the classification criteria do not allow for a less stringent classification for chemicals operating by a threshold mechanism. In contrast, regulatory approval systems for plant protection products and therapeutic agents are based on a risk assessment approach, in which a demonstrated threshold effect for a genotoxic agent is likely to be an important factor in reaching a decision concerning authorisation of the product.
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Keywords: Regulatory toxicology; Indirect genotoxicity; Threshold effects

1. Introduction

The European Union (EU) has comprehensive systems for the regulatory control of chemicals, encompassing classification and labelling, limit setting, emission controls, approval systems for medicines, plant protection products, biocides and industrial chemicals, specific workplace legislation, etc. The key task of the regulators involved in such systems is to ensure as far as possible the protection of human health and/or the environment, a task which (arguably) demands an inherently conservative approach to decision-making where there is any element of doubt, in other words application of the precautionary principle.

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In relation to mutagenic/genotoxic and carcinogenic chemicals, a key issue for regulatory scientists has been whether there is a safe level of exposure that can be used in decision-making and risk management. In terms of assessing the risk of such chemicals for human health, regulators have generally adopted the principle that a threshold exists for non-genotoxic carcinogens, but that there is no safe level of exposure to an in vivo mutagen or to a genotoxic carcinogen. This assumption is based on the assumption that any damage to DNA has the potential to induce a mutation and consequently a threshold level cannot be defined. This disregards the existence of normal cellular defense mechanisms, e.g. metabolic pathways that can rapidly detoxify reactive genotoxic chemicals before they can reach critical cellular targets or DNA repair mechanisms. The recognition that a significant number of chemicals having genotoxic properties operate by indirect rather than direct mechanisms of genotoxicity presents new challenges for the regulator. The mechanisms involved include aneugenic activity, oxidative stress, inhibition of enzymes involved in DNA synthesis such as topoisomerase or cytotoxicity (Scott et al., 1991). It is widely accepted that genotoxic effects arising via such mechanisms show a threshold (ECETOC, 1997), the effect being expressed only when cellular defense mechanisms are overwhelmed and/or a critical level of multiple target sites in the cell are occupied (Parry et al., 2000). The biological relevance of such threshold effects for humans is, however, still a matter of debate among regulatory scientists. This paper explores how indirect genotoxicity mechanisms are treated in the EU regulatory systems for:

1) classification and labelling of mutagens and carcinogens,
2) approval of pharmaceuticals, plant protection substances and biocides,
3) risk assessment of new and existing chemicals under Regulation 793/93 and Directive 67/548/EEC,
4) establishment of occupational exposure limits (OEL).

As will be seen, the regulatory approach may differ, dependent on whether the system is based on intrinsic hazard or a consideration of risk and benefit.

2. Recognition of indirect genotoxicity mechanisms in the classification of mutagens and carcinogens

The EU system for classification and labelling of chemicals is laid down in Annex VI of Directive 67/548/EEC, the so-called Labelling Guide (European Communities, 1997). The criteria for classifying chemicals as mutagenic or carcinogenic were developed initially in the period 1985–1989 and have not changed since then. The system is primarily based on intrinsic hazard, despite the statement in the Annex that 'the object of classification is to identify all the physicochemical, toxicological and ecotoxicological properties of substances and preparations which may constitute a *risk* during normal handling or use'. Carcinogens or mutagens operating via indirect mechanisms of genotoxicity, for which a threshold effect has been demonstrated, may be considered an issue of risk rather than hazard, but as will be seen, the criteria for classification do not accommodate this.

2.1. Mutagens

The criteria for classification as mutagenic place chemicals in one of three categories, category 1 being used for chemicals known to be mutagenic in humans (there are no chemicals in this category), category 2 for chemicals that should be regarded as if they are mutagenic in humans, while category 3 is used for chemicals of concern but for which the evidence is not sufficient to place them in categories 1 or 2. The criteria, shown in Table 1, do not include consideration of threshold effects.

The criteria for classification of a mutagen, whether in category 2 or category 3, require evidence of in vivo mutagenicity, and chemicals are not normally classified as mutagenic based on in vitro data alone. Thus, substances that are (indirectly) genotoxic in vitro only, and which are very unlikely to give a positive result in vivo

Table 1
EU criteria for classification of chemicals as mutagenic

Category 1	Category 2	Category 3
Positive evidence from human mutation epidemiology studies will be needed. Examples of such substances are not known to date. It is recognised that it is extremely difficult to obtain reliable information from studies on the incidence of mutations in human populations, or on possible increases in their frequencies	Positive results from assays showing (a) mutagenic effects, or (b) other cellular interactions relevant to mutagenicity, in germ cells of mammals in vivo, or (c) mutagenic effects in somatic cells of mammals in vivo in combination with clear evidence that the substance or a relevant metabolite reaches the germ cell	Assays showing (a) mutagenic effects or (b) other cellular interaction relevant to mutagenicity, in somatic cells in mammals in vivo. The latter especially would normally be supported by positive results from in vitro mutagenicity assays.

because the in vitro toxicodynamics will not be reproduced in vivo will not be classified. The criteria thus can be considered to cater for threshold effects where the mutagenic response is only expressed at cytotoxic concentrations, where characteristically steep dose–response curves are seen, extremes of pH, osmolality or when e.g. metabolic detoxification pathways are saturated, etc. (Kirkland, 1992; Kirkland and Müller, 2000). Positive in vitro results are, however, interpreted as an indication that the chemical may have intrinsic genotoxic properties, whether direct or indirect, and are taken into account in decision-making in e.g. classification of a chemical as carcinogenic or in the approval systems for pharmaceuticals, plant protection products, etc., discussed below.

What, however, is the regulatory position regarding substances that are in vivo mutagens but for which a threshold has been postulated, based on the established mechanism of (indirect) genotoxicity? Much attention has focussed on the interpretation of the classification criteria in relation to aneugenic chemicals such as the benzimidazole fungicides, benomyl and carbendazim, and other mitotic spindle poisons such as colchicines. These non-DNA reactive chemicals have a strong affinity for fungal tubulin, resulting in disruption of microtubule assembly and consequent inhibition of fungal mitotic spindle function (Davidse and Flach, 1977; Ireland et al., 1979). Binding to mammalian tubulin has been demonstrated, but the affinity is much lower (Albertini et al., 1993). Additionally, it is generally accepted that interaction with multiple binding sites is required before spindle function is inhibited, resulting in a threshold dose–response curve (Parry et al., 1994). A threshold mechanism of action has, therefore, been proposed for this class of compounds (Elhajouji et al., 1995, 1997).

The relevance of the aneuploidy induced by aneugens such as benomyl and carbendazim for humans has been debated by several expert bodies. The European Commission Group of Specialised Experts in the fields of Carcinogenicity, Mutagenicity and Reprotoxicity (Specialised Experts) were asked in 1999 for their advice on the classification of carbendazim and benomyl. At that stage, both compounds were classified as

category 3 mutagens. Based on new studies published since the original decision on classification, the Specialised Experts considered that there was sufficient evidence that carbendazim and benomyl could interact with the microtubules of the spindle apparatus in germ cells. They recognised that a plausible biological mechanism with a threshold had been demonstrated in somatic cells, however, they considered that there was a lack of knowledge about the interaction of known mitotic spindle poisons with the protein structures and processes involved in meiotic chromosome segregation. They considered that mechanistic considerations in somatic cells could not, therefore, be extrapolated to meiotic cells. Despite the demonstration of a threshold in somatic cells (e.g. Elhajouji et al., 1995; Bentley et al., 2000), they considered that the concept of a threshold for induced aneuploidy in germ cells was as yet a hypothetical one. They, therefore, stated that the criteria for classification as category 2 mutagens were fulfilled, that threshold arguments should not have any impact on the classification, and that the biological consequences of aneugenic effects in germ cells are of a potentially serious nature. Benomyl and carbendazim have, therefore, been reclassified as Category 2 mutagens, based on this advice. This example shows that the criteria for classification of in vivo mutagens, unlike the criteria for classification of carcinogenic substances discussed below, do not allow regulators sufficient flexibility to take into account indirect genotoxicity mechanisms of debatable relevance for humans.

The issue of whether in vivo somatic cell mutagens can reach the germ cells in vivo and thus should be classified as category 2 mutagens (see criteria for classification as category 2 in Table 1 above) has also frequently led to debate. A number of chemicals have been classified in category 3 rather than category 2 on the basis that there is no evidence that the agent can reach the germ cells other than by routes of administration that are considered to be irrelevant for man, such as intraperitoneal injection. Examples include the protein synthesis inhibitor cycloheximide and the chemical hydroquinone. Mechanisms suggested for the genotoxicity of hydroquinone include the formation of reactive oxygen species, and positive in vivo results are only seen at high dose levels, thought to be related to saturation of detoxification pathways. Thus both agents can be considered to operate by indirect mechanisms of genotoxicity for which thresholds can be demonstrated, and this has been used to classify less stringently than might be expected. Another substance for which there has been extensive debate on mutagenicity (and carcinogenicity) is the chlorinated solvent trichloroethylene. Trichloroethylene was reviewed by the Specialised Experts in 2000. The majority of experts considered that, in a weight of evidence approach, positive results in vivo supported the assumption that trichloroethylene exerts genotoxic activity. While indirect mechanisms of genotoxicity have been suggested, the experts considered that there was sufficient evidence of interaction of metabolites of trichoroethylene with DNA in somatic cells of mammals in vivo. Trichloroethylene has, therefore, been classified as a category 3 mutagen. In contrast, clearly genotoxic agents such as propylene oxide have been classified as category 2 mutagens despite the fact that reactive compounds like propylene oxide are unlikely to reach the germ cells in vivo at pharmacodynamically-relevant concentrations, and there is little evidence for the germ cell mutagenicity of such compounds. A number of investigators (e.g. Parry, 2000) have argued that such agents should be more appropriately classified as category 3 mutagens on the basis that the risk of germ cell mutagenicity for humans is extremely low at relevant exposure concentrations and by relevant exposure routes.

These debates have led to the suggestion (Parry, 2000; Crebelli, 2000) that the EU criteria for classification of mutagens need to be amended to take into account clearly demonstrated threshold effects. The Specialised Experts, at the time of their review of benomyl and carbendazim, were also asked whether there was need to revise the criteria in Annex VI of Dir. 67/548/EEC for the classification of aneugens. There was some (not universal) support for introduction of risk and potency as modulators of classification of mutagens, however, the experts considered at that time (1999) that it would be premature to discuss a

revision of the criteria. Revision is theoretically possible as part of the revision of the EU legislation on chemicals which is planned over the next 2 years, in order to implement the proposals in the Commission's White Paper on a Strategy on a future Chemicals Policy (European Commission, 2001). Reconsideration is more likely when and if the EU implements the Globally Harmonised System for Classification and Labelling (GHS). The GHS provides improved classification criteria for mutagens, since only substances for which there is direct evidence of effects on germ cells will be classified for mutagenicity, somatic mutagens being encompassed within the criteria for carcinogenicity. The GHS criteria also state that 'The classification of individual substances should be based on the total weight of evidence available, using expert judgement'. This could afford regulators the requisite degree of flexibility to take mechanistic considerations into account when classifying mutagens.

2.2. Carcinogens

The debate about the relevance of threshold effects in the classification of mutagens in the EU may also impact on the classification of carcinogens, since one of the aims of genotoxicity testing is to provide information that can be used in the assessment of the carcinogenicity of a chemical. As indicated above, the EU criteria for classification of carcinogens do afford some flexibility in classification when it is considered that the mechanisms involved are of debatable relevance for humans. Evidence of carcinogenicity in two animal species will normally result in classification as a category 2 carcinogen, however, the criteria outline a number of arguments which can be used to decide whether a chemical should be placed in category 2 or category 3, including the following of relevance to a discussion of chemicals operating by an indirect mechanism of genotoxicity:

- carcinogenic effects only at very high dose levels exceeding the 'maximal tolerated dose',
- existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g. hormonal effects on target organs or on mechanisms of physiological regulation, chronic stimulation of cell proliferation).

Thus, the concept of thresholds for the induction of cancer are accepted by EU regulators, and are becoming increasingly important with the recognition that the process is multi-stage, despite the long-held tenet that interaction of one molecule of a carcinogen with DNA is sufficient to result in cancer. In practice, however, evidence of genotoxicity in in vitro and in vivo short-term tests coupled with carcinogenicity in two species is likely to result in classification as a category 2 genotoxic carcinogen. Classification of a threshold carcinogen in category 3 rather than category 2 would be normally only considered when one or other of the above arguments applies and there is 'lack of genotoxicity in short-term tests in vivo and in vitro'. However, where there is experimental carcinogenicity data on an aneugen, it has generally been accepted by the regulatory community that aneuploidy in somatic cells should result in classification as a category 3 carcinogen rather than category 2, as aneuploidy has been suggested to be a late stage (not an initiating step) phenomenon involved in tumour formation.

Similarly, the criteria outline arguments which can be used to decide whether a chemical should be classified as a category 3 carcinogen or attract no classification, including the following:

> a substance should not be classified in any of the categories if the mechanism of experimental tumour formation is clearly identified, with good evidence that this process cannot be extrapolated to man.

Again, if the data support classification as a category 3 carcinogen (some evidence from appropriate animal studies, but insufficient to place the substance in Category 2), and there is also evidence of genotoxicity, albeit that this is thought to occur by an indirect mechanism of genotoxicity and there is a threshold below which the genotoxic/carcinogenic effect will not be seen, regulators will be reluctant to accept that these effects cannot be extrapolated to humans.

3. Recognition of indirect genotoxicity mechanisms in the approval of plant protection substances, biocides and pharmaceuticals

The various approval systems for pharmaceuticals, plant protection substances and biocides in the EU are grounded in the risk assessment process, with consideration of the risks versus the benefits of the particular product under evaluation.

3.1. Plant protection substances and biocides

The preceding section has shown that regulatory scientists have been reluctant to take indirect genotoxicity mechanisms into consideration in the EU classification system, as evidenced by the examples of benomyl and carbendazim. It is of interest, therefore, to examine how these two fungicides have fared in the regulatory approval system for plant protection products under Directive 91/414/EEC. In relation to the specific issue of carbendazim, the Scientific Committee on Plants (SCP) was asked for its views on the following question: 'Can the Committee comment on the advisability of establishing an Acceptable Daily Intake (ADI) and Acceptable Operator Exposure Level (AOEL) having regard particularly to the results of mutagenicity, carcinogenicity and reproductive data for the active substance?'

Their conclusion was as follows:

> 'It has been firmly established that direct binding of carbendazim to tubulin is required for the toxic effects of the methylbenzimidazoles. Through this mechanism, carbendazim and its precursors provoke toxic effects on reproduction and induce numerical changes in chromosomes (aneuploidy) in mammalian cells in vitro and in bone marrow and male and female germ cells of rodents dose in vivo. There is no evidence for the induction of gene mutations in vitro, for structural chromosomal damage in vitro or in vivo or for interaction with DNA of liver cells of rats dosed in vivo with carbendazim. Thus, interactions of carbendazim and its precursors with biological material is of a nature that is entirely consistent with a dose level being identifiable as having no toxicological effect'.

Carbendazim thus, remains under consideration as a systemic fungicide in the EU, for inclusion in Annex 1 of Directive 91/414/EEC through the ECCO Peer Review process, despite its in vitro and in vivo genotoxicity, regulators having accepted that the threshold effects demonstrated in experimental systems are relevant in assessing the risk of the chemical for man and the environment. Although benomyl is no longer under consideration for inclusion, this is not because of the possible genotoxic properties but rather that further data required to support the authorisation was not provided. In general, it appears that classification as a category 2 mutagen or carcinogen is not a barrier to approval for use under Directive 91/414/EC, provided a threshold mechanism of action can be demonstrated which is considered not to be relevant for humans. The same is likely to apply to biocides in the approval scheme under Directive 98/8/EC. It has to be recognised, however, that this is a position which is not supported by a number of EU Member States, who consider that any substance classified as category 2 (mutagen, carcinogen or reproductive toxicant) should not receive such approval, based on the precautionary principle.

3.2. Pharmaceuticals

In the approval process for pharmaceuticals, there is also acceptance of the tenet that there is a threshold for chemicals showing indirect genotoxicity, and a number of such agents have been approved for therapeutic use on the basis of the risk:benefit ratio for the target patient population. Müller and co-workers (Müller and Kasper, 2000) presented a number of examples of such therapeutic agents approved by the German regulatory authorities in the period 1990–1997. The authors noted that apparent thresholds were most consistently associated with agents producing clastogenesis in in vitro chromosomal aberration tests, negative results being generally obtained in other tests in the standard genotoxicity test battery for

pharmaceuticals. They identified three major therapeutic groups showing such a pattern of genotoxicity: nucleoside/nucleotide analogues, topoisomerase or gyrase inhibitors and cytostatics. In these cases, the mechanism of action of the drug, which is also the indirect mechanism of genotoxicity, is well understood, the existence of a threshold has been demonstrated, and in some cases also a greater affinity for the macromolecular target in the target organism or cell, e.g. viral phosphorylase or bacterial gyrase. There was regulatory acceptance of the mechanism of genotoxicity and the fact that an acceptable margin of safety could be demonstrated for humans, resulting in approval of the therapeutic agent in question. In the case of the cytostatic drug taxol and taxol derivatives, the mechanism of action is via interaction with cellular tubulin, blocking spindle formation and thus cell division, as already discussed for benomyl and carbendazim.

Muller and Kasper also cited the example of paracetamol, hepatic metabolism of which produces an electrophilic quinine-imine which is detoxified at normal therapeutic levels by conjugation with glutathione, but which is clastogenic in vitro and in vivo at high levels of exposure, due to the fact that cellular defense mechanisms are overcome. There is general acceptance that paracetamol is safe for human use at normal therapeutic levels (Bergman et al., 1996), despite its genotoxic profile, because of the existence of a threshold. Mechanistically, this is analogous to the situation with phenol, discussed in Section 5 below.

4. Recognition of indirect genotoxicity mechanisms in the risk assessment of new and existing chemicals under Regulation 793/93 and Directive 67/548/EEC

The risk assessment process for industrial chemicals and biocides under the Existing Chemicals Regulation, 793/93 and the notification scheme for new chemicals under Directive 67/548/EEC involves a comparison of the exposure level or levels at which no toxic effects are expected to occur with the exposure level to which the key target population are likely to be exposed. If the anticipated exposure of the exposed population exceeds the determined No-observed-Adverse-Effect-Level (NOAEL), the conclusion is reached that there is a potential risk, and a risk reduction strategy must be developed. The Technical Guidance Documents for risk assessment (European Commission, 2002) state that 'genotoxicity refers to potentially harmful effects on genetic material, which may be mediated directly or *indirectly*', and that 'Unless a *threshold mechanism of action is clearly demonstrated*, it is generally considered prudent to assume that thresholds cannot be identified in relation to mutagenicity, genotoxicity and genotoxic carcinogenicity, although a dose–response relationship may be shown under experimental conditions'. However, the possibility of establishing such a threshold mechanism of action is clearly documented e.g. 'for induction of aneuploidy', and the TGD states that 'In such cases, the dose–response assessment (in particular, a N(L)OAEL value, if obtainable) can be used in the risk characterisation...'. The TGD represents the harmonised approach of the EU regulatory community to risk assessment of industrial chemicals and biocides and the above, therefore, indicates that there is regulatory recognition of indirect genotoxicity mechanisms and the possibility of threshold effects in the risk assessment process, at least for aneugens. However, the consequences of this for the risk characterisation part of the risk assessment process are as yet unclear, since to the best of the author's knowledge, no substance that has been demonstrated to have genotoxic properties operating solely by an indirect mechanism, with a proposed threshold, has been completely evaluated by the EU Technical Meeting on Risk Assessment. Discussions are in progress on propan-1-ol, a chemical that has aneugenic activity (Crebelli et al., 1989) but is generally negative in other genotoxicity assays, and for which further information on genotoxicity is currently being sought.

In the EU risk assessment process under Regulation 793/93, there is clear regulatory acceptance that non-genotoxic carcinogens can show a threshold effect and that many different mechanisms of action are involved in non-genotoxic carcinogenicity. There is a differentiation between threshold

and non-threshold carcinogens in risk characterisation, which in turn influences the regulatory approach to risk reduction. The issue is whether the same approach would be taken for a chemical considered to be genotoxic via an indirect mechanism. The decisions made in relation to the classification of benomyl and carbendazim might suggest that this is unlikely to be the case. Taking cadmium as an example, the current evaluation of cadmium metal and cadmium oxide within the risk assessment group has indicated that the genotoxicity involves at least three possible and non-mutually exclusive mechanisms: (1) direct DNA damage, (2) oxidative damage and (3) inhibition of DNA repair (Hartwig, 1994). Although it is noted in the cadmium risk assessment that 'if it could be demonstrated that the genotoxic effect of cadmium compounds is fully mediated through a mechanism such as inhibition of DNA repair enzymes, it would be reasonable to assume, from a theoretical perspective, that a threshold relationship applies' (Kirsch-Volders et al., 2000), the fact that direct DNA damaging properties have been demonstrated has led to the conclusion in the risk assessment that cadmium is a direct acting genotoxic substance and that it is prudent to consider that there is no threshold exposure level below which effects will not be expressed.

5. Recognition of indirect genotoxicity mechanisms in the establishment of occupational exposure limits

The final area to be considered in relation to regulatory recognition of indirect genotoxicity mechanisms showing a threshold response is the establishment of OELs for chemicals in workplace air. The EU 'Scientific Committee on Occupational Exposure Limits' (SEG/SCOEL) has published its opinion (European Commission, 1999) on the methodology for the derivation of these OEL, setting out the general principles and approaches taken by the Committee in reaching its decisions. The core principle established by the Committee is that OELs should be divided into two categories, depending on the scientific basis on which they are established:

- 'Health-based' OELs, established in those cases where a review of the total available scientific data leads to the conclusion that it is possible to identify a clear threshold dose below which exposure to the substance in question is not expected to lead to adverse effects.
- 'Pragmatic' OELs, established for substances causing certain adverse effects, in particular genotoxicity, carcinogenicity and respiratory sensitisation, for which it may not be possible based on present knowledge to define a threshold of activity. Such OELs will be established at levels considered to carry a sufficiently low level of risk.

The SCOEL documentation states that 'some mutagens cause mutations in germ cells which can be transmitted to the offspring. There is no commonly accepted threshold of activity for substances causing such heritable damage and the role of SCOEL in respect of such substances will be similar to that described for genotoxic carcinogens'. This might suggest that SCOEL would be unlikely to set a health-based OEL for a substance operating by an indirect mechanism of genotoxicity, but for which there was evidence of in vivo mutagenicity and effects on germ cells, even if a threshold effect has been postulated. SCOEL have, however, recently reconsidered the case of phenol, for which they had already recommended an Indicative Occupational Exposure Limit (IOELV) of 2 ppm. Phenol is generally negative in in vitro bacterial mutagenicity tests (IARC, 1999), but produces mutations, sister chromatid exchanges and micronuclei in cultured mammalian cells. It binds to cellular protein, but not to DNA and inhibits cell-to-cell communication (IARC, 1999). It is also weakly clastogenic in vivo (Shelby et al., 1993; Marrazzini et al., 1994). Chromosomal aberrations in spermatogonia of mice were also produced at doses levels producing significant toxicity (Bulsiewicz, 1977). Phenol and several of its metabolites have been demonstrated to inhibit cellular topoisomerases, thus affecting DNA unwinding during replication and transcription, an effect which is probably key in their genotoxic profile (Chen and Eastmond, 1995), although DNA adducts have been reported in vivo, but

only after extremely high doses. SCOEL concluded that the genotoxic potential of phenol in vivo is weak, markedly dose-dependent and probably metabolism-dependent, with rapid conjugation of the most critical metabolites (e.g. 1,4-benzoquinone) by cellular glutathione. They considered that a threshold mechanism can be assumed for the genotoxicity of phenol and that at low concentrations no genotoxic potential is likely. They, therefore, reconfirmed their earlier decision to assign an OEL to the substance.

6. Conclusions

Regulatory acceptance of indirect mechanisms of genotoxicity differs in the different areas of chemical regulation at EU level. The classification system under Directive 67/548/EEC, covering industrial chemicals, biocides, plant protection substances and active ingredients used in cosmetics and medicinal products is based primarily on intrinsic hazard rather than risk. It is widely accepted that the dose–response relationships for many substances having genotoxic properties involving an indirect mechanism of genotoxicity show a threshold, yet the criteria for classification of mutagenic substances do not provide flexibility in the classification of such substances, as is the case for carcinogens. In addition, in relation to acceptance that such agents show a threshold response, there are somewhat differing perspectives on this within the different EU Member States. As stated at the beginning of this paper, the key task of the regulator is to ensure as far as possible, the protection of human health and/or the environment, a task which (arguably) demands an inherently conservative approach to decision-making where there is any element of doubt, and it is clear from recent classification decisions that have been made that many EU regulators involved in the classification of mutagens (and carcinogens) are currently not sufficiently convinced by the threshold argument to depart from this approach. Despite this, there is, however, a growing body of opinion that threshold effects should be taken into account in the criteria for classification of mutagens. This will require a shift from the current intrinsic hazard-based decision-making process to one that better reflects risk at 'normal handling and use' exposure levels.

In contrast, those regulators involved in processes which require an assessment of risk before the regulatory decision-making process have been more prepared to recognise that some indirectly genotoxic agents do show a threshold and that this can be taken into account in the final decision. Notably, a number of plant protection products and, in particular, pharmaceuticals have been authorised for use via the EU approval systems which exist for such agents, despite the existence of a positive genotoxicity profile. This has been on the basis that the mechanisms of genotoxicity are clearly understood, are considered to show a threshold below which the effect will not be seen, and/or there is mechanistic evidence that these effects cannot be extrapolated to humans. The European Commission's Technical Guidance on risk assessment for industrial chemicals and biocides also makes provision for consideration of a threshold-type effect in characterising the risk presented by such substances, and this in turn may influence the decision-making process in relation to risk reduction.

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Review

Indirect mechanisms of genotoxicity

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Abstract

Indirect mechanisms of genotoxicity correspond to interactions of mutagens with non-DNA targets, and are expected to show threshold concentration–effect response curves. If these thresholds can be proven experimentally they may provide a third alternative for risk assessment, besides the No Effect Level/Safety Factor approach and the low dose linear extrapolation method. We contributed significantly to the in vitro assessment of thresholds in human lymphocytes exposed to the spindle inhibitors nocodazole and carbendazim showing dose dependency and existence of lower thresholds for induction of non-disjunction as compared to chromosome loss. Micronuclei correlated with p53-independent or p53-dependent apoptosis and elimination of aneuploid cells. Extrapolation from in vitro threshold values to the in vivo situation remains unsolved. Comparing the in vitro threshold values for griseofulvin in human and rat lymphocytes with in vivo NOAEL/LOAEL in bone marrow/gut/erythrocytes suggests that the in vitro human system is the most sensitive. The threshold for induction of non-disjunction in in vitro maturing, nocodazole-exposed mouse oocytes was in the same low range. Regulators (UK Committee on Mutagenicity, http://www.doh.gov.uk/com/com.htm) considered the importance of thresholds for indirect mechanisms of genotoxicity. Acceptance of a non-linear extrapolation for mutagens requires mechanistic studies identifying the mutagen/target interactions. Moreover appropriate risk evaluation will require additional studies on individual susceptibility for indirect mutagenic effects and on interactions of aneugens in complex mixtures.
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Keywords: Genotoxicity; Thresholds; Indirect mutagens; Risk assessment

1. Introduction

Indirect mechanisms of genotoxicity can be defined as interactions with non-DNA targets leading to genotoxic effects. This covers essentially lipid peroxidation and protein adducts. As far as proteins are concerned, recent research focused on inhibition of repair enzymes (e.g. OGG_1, XPD, Ni), on cell cycle control proteins (e.g. p53, Rb, cyclins), on apoptosis related gene products (e.g. p53, bax, bcl-2), on nuclear lamins, on defence proteins against oxidative damage (glutathione), on metabolization enzymes, and tubulines of the

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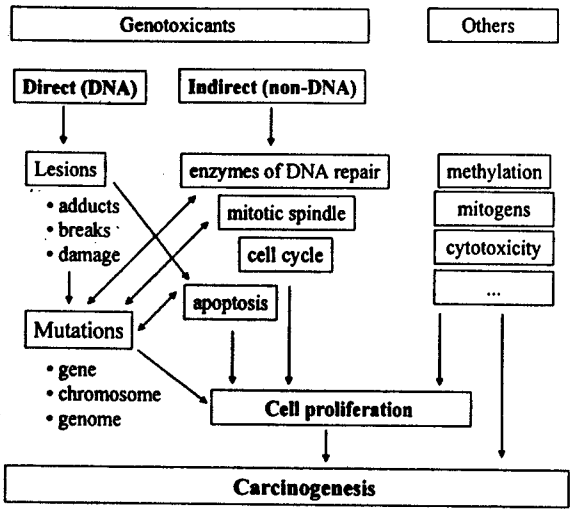


Fig. 1. Overview of mechanisms by which direct and indirect acting genotoxicants and other molecules can play a role in carcinogenesis (Adapted from Kirsch-Volders et al., 2000).

mitotic/meiotic spindle. Fig. 1 shows the mechanisms by which genotoxicants (directly and indirectly acting) and other molecules play a role in carcinogenesis.

As described earlier by us (Kirsch-Volders et al., 2000; Elhajouji et al., 1995, 1997) it is theoretically expected that in contrast to DNA-binding mutagens, mutagens which induce their genotoxic effects through non-DNA binding show threshold concentration–effect response curves.

The objective of this paper is to review the contributions of our laboratory to the assessment of thresholds from the theoretical point of view and in practical for a specific type of indirect acting mutagens, microtubule inhibitors. The implication of this new approach of risk assessment for the regulation of chemicals will be illustrated with examples of the conclusions presented by the UK Committee on Mutagenicity.

2. Theoretical basis for the existence of thresholds as response to indirect mutagens

The definition of a threshold can lead to confusion. One should indeed distinguish the definition as made earlier by several authors.

2.1. Absolute threshold

A concentration of a chemical below which a cell would not 'notice' the presence of this agent. In other words, the chemical is present but does not interact with a cellular target (Seiler, 1977; Dinman, 1972; Henschler, 1974; Claus, 1974).

2.2. Apparent threshold

A concentration of a test chemical which causes no mutagenic effect due to an immeasurable (zero) concentration at the target (Seiler, 1977). An apparent threshold may be due to rapid degradation, pharmacokinetics of a chemical, or other factors that limit its exposure to the target.

2.3. Statistical threshold

The lowest concentration of a chemical that induces a statistically significant increase in the endpoint being measured.

2.4. Real (or biological) threshold

A concentration of chemical which, although present at the target in finite amounts, does not produce any damage through its inability to perform, at or below this concentration, the necessary biochemical reactions (Seiler, 1977). In other words, the chemical is present, and can interact with the target, but no adverse consequence is induced.

The theoretical basis for the existence of this real biological threshold was analysed in three steps (Kirsch-Volders et al., 2000).

2.4.1. Number of targets for mutagens

A schematic drawing of the simplest relationships between a mutagen (M), a target (T) and the measured endpoint (E) is presented on Fig. 2. To illustrate the result in dose–effect response curves obtained from the theoretical M–T–E relationship drawings modelizing the single hit, single target and single hit, multiple target/multi-hit, single target (Fig. 3a and b) are shown.

Theoretically, as far as the single hit, single target model is concerned, it is known that one

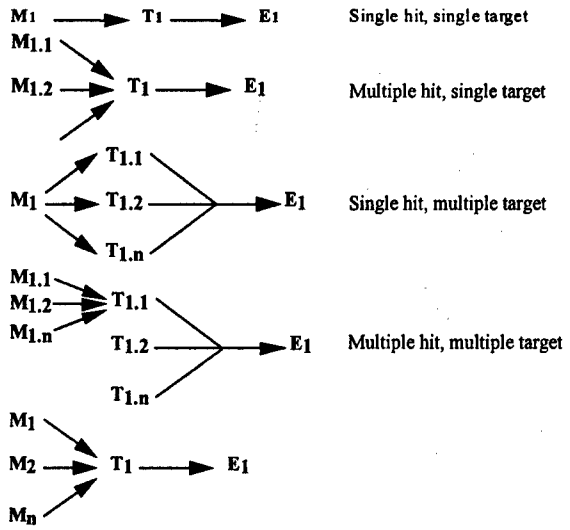


Fig. 2. Relationships between a mutagen (M), a target (T) and the measured endpoint (E). Different molecules of the same mutagen are labelled $M_{1.1}$ to $M_{1.n}$.

base change can lead to a mutation, or one 'hit' resulting in a base change can lead to a mutation. In terms of probability, already a single molecule of a mutagen (or a carcinogen) (gene mutagen) entering the body could—although with an exceedingly small, but finite probability—induce mutation and cancer. Therefore thresholds for mutagens (and carcinogens) with such a mechanism of interaction cannot exist.

For the calculation of the curves (Fig. 3a and b) representing the single hit, multi-target/multi-hit, single target interaction, two possible mathematical models were applied: either x^i (i is the number of hits) or $p = 1 - e^{-u}\Sigma_{i=0}^{x} u^i/i!$, where $\Sigma_{i=0}^{x} u^i/i! = 1 + u + u^2/2 + u^3/6 + \ldots + u^x/x!$. In the latter model, u is assumed to be proportional to the concentration of mutagen and i the number of hits. The first approach assumes that all events are independent of each other and does not take into account molecular interaction and survival. The second approach fitted well with the data observed for aneuploidy induction in human lymphocytes exposed in vitro to spindle poisons (Elhajouji et al., 1995, 1997). Both figures clearly indicate that with increasing i values (hits or targets), the curve is smoothing down and at lower concentrations suggest progressively a threshold-type response. The question so far becomes whether with $i > 1$, there exists a real threshold or not, at low doses.

Practically the data can also be compared to a simplified threshold model with a piecewise linear regression analysis (or breakpoint regression). The equation used by us (Elhajouji et al., 1995) is a sum of a constant function (if the concentration is lower than the breakpoint) and a linear function (if the concentration is higher than the breakpoint).

2.4.2. Identity of the targets

Progressing from theoretical approaches to cell reality, it is necessary to identify the targets, which follow the theoretical interaction schemes M–T. Table 1 presents some examples, which of course are not exhaustive.

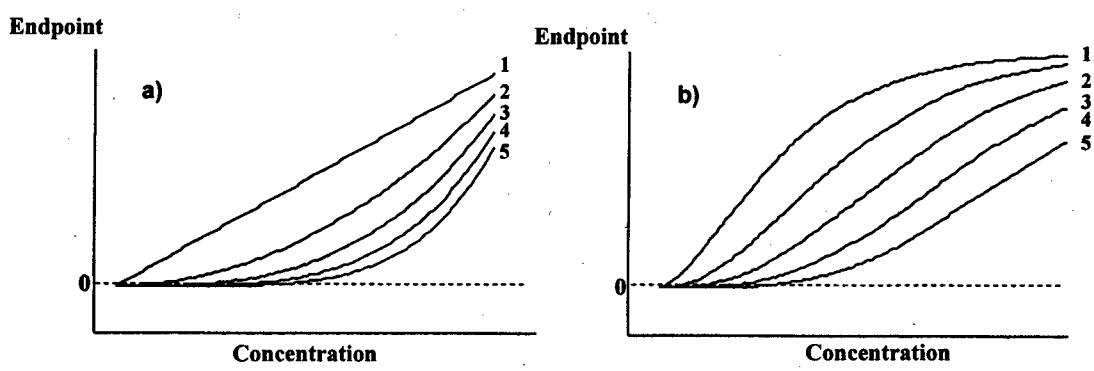


Fig. 3. Dose–response curves obtained for single hit, single target or single hit, multiple target/multi-hit, single target interactions according to two different mathematical models: either (a) x^i (i is the number of events) or (b) $p = 1 - e^{-u}\Sigma_{i=0}^{x} u^i/i!$, where $\Sigma_{i=0}^{x} u^i/i! = 1 + u + u^2/2 + u^3/6 + \ldots + u^x/x!$. 1, 2, 3, 3, 4, 5: number of hits.

Table 1
Examples of targets following the different types of mutagen–target interaction

Mechanisms	Identity of target
Single hit, single target	DNA
Single hit, multiple target	α- and β-tubulins, topoisomerase
Multiple hit, single target	K^+ and Ca^{2+} channels
Multiple hit, multiple target	Spindle, nuclear membrane

2.4.3. Type and sensitivity of endpoints

The choice of the endpoint is a determining step in mutagenicity studies. There exists a variety of well developed test systems that might be used as an endpoint. The simplest system is when the endpoint corresponds to the target (e.g. analysis of DNA adducts, DNA breakage or modified bases e.g. 8-hydroxyguanine). In this case one may consider a direct relationship between the biochemical or physical interaction of the compound with the target and the effect (endpoint).

At cellular level when the endpoint is different from the target, three different levels can be considered depending on the steps that separate the initial interaction of the chemical agent with the target and the final measurable endpoint:

a) *Proximal:* the endpoint is close but still different from the target, e.g. gene mutations, chromosome structural mutations or genome mutations. These proximal endpoints are very important for risk assessment of adverse mutagenic effect and cancer.
b) *Intermediate:* the endpoint is separated by some steps from the target, e.g. analysis of repair and metabolic pathways.
c) *Distal:* the endpoint is a result of complex alterations of cellular activities after the initial interaction of the compound with the target, e.g. analysis of apoptosis, necrosis and cell survival.

At the level of the tissue and the organism, important differences are expected between different tissues of a given species and between species.

3. In vitro demonstration of thresholds for spindle inhibitors

To assess experimentally in vitro the existence of thresholds we selected aneugenic compounds binding specifically to β-tubulin and inhibiting tubulin polymerisation such as nocodazole, a chemotherapeutic drug or carbendazim, a pesticide (Elhajouji et al., 1995, 1997, 1998). Due to the high specificity of nocodazole for its molecular target: Arg 390 of β-tubulin, it was assumed that there is no other target and that all toxic effects observed were related to this specific interaction with microtubules.

Two endpoints reflecting aneuploidy were studied in vitro in human lymphocytes: chromosome loss and non-disjunction. To assess chromosome loss the detection of centromere-positive versus centromere-negative micronuclei (MN) by fluorescence in situ hybridisation (FISH) with a general alphoid centromeric probe was performed on cytochalasin-B blocked binucleates resulting from cultures exposed to the spindle poisons for subtoxic concentration-range finding. In a second step the same technology was applied on F.A.C.S Vantage™ flow-sorted MN induced by the mutagens at concentrations ranging from low to subtoxic to assess the threshold concentrations. Fig. 4 shows the thresholds for induction of chromosome loss. As far as chromosome non-disjunction is concerned, the same compounds were investigated on cytokinesis-blocked binucleated lymphocytes in combination with FISH using chromosome specific centromeric probes for chromosome 1 and chromosome 17. This protocol allowed the accurate evaluation of non-disjunction since artefacts were excluded from the analysis as only binucle-

Table 2
Threshold values for chromosome non-disjunction

Chemical	Chromosome loss (μM)	Chromosome non-disjunction (μM)
Colchicine	0.033	0.02
Mebendazole	0.29	0.23
Carbendazim	2.47	2.85
Nocodazole	0.053	0.032

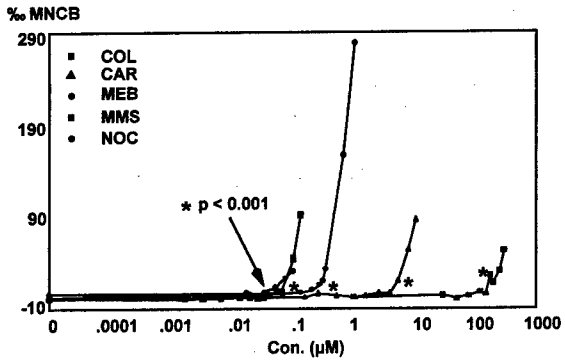


Fig. 4. Thresholds for induction of chromosome loss by aneugens as determined on flow-sorted MN painted by FISH with a general alphoid centromeric probe. COL, colchicine; CAR, carbendazim; MEB, mebendazole; MMS, methanesulfonate; NOC, nocodazole.

ates with the correct number of hybridization signals were taken into account.

We showed dose dependency of the aneugenic effects and the existence of thresholds for the induction of chromosome non-disjunction and chromosome loss by these spindle inhibitors (lower for non-disjunction than for chromosome loss) (Table 1and Table 2).

Since genotoxic events may lead to apoptosis, it was necessary to determine whether below the threshold values determined for chromosome loss and non-disjunction aneugens are really non-genotoxic or, alternatively, induced aneuploid cells which were selectively eliminated by apoptosis. If this would be the case, the defined thresholds would not be applicable on cells unable to undergo apoptosis. We investigated whether apoptosis was induced directly or indirectly as a response to aberrant chromosome segregation below the thresholds for the induction of chromosome loss and non-disjunction. To answer that question human lymphocytes were exposed in vitro to five concentrations of nocodazole and five concentrations of carbendazim: the threshold concentrations for chromosome non-disjunction and chromosome loss, two concentrations below the lowest threshold and one concentration between the two threshold values. The choice of these concentrations was based on our previous studies on threshold values for the two aneugens. The induction of apoptosis was analysed by annexin-V and the frequencies of chromosome non-disjunction and chromosome loss were estimated in cytokinesis-blocked human lymphocytes in combination with FISH, the same methodology which was used in our previous studies (Elhajouji et al., 1995, 1997). In order to check whether aneuploid cells are preferentially driven to apoptosis, we sorted by magnetic annexin beads apoptotic and viable cells from the same cultures and measured micronucleus induction, chromosome loss (MNCen+) or chromosome non-disjunction in the apoptotic and viable fractions.

Our results suggested that elimination of micronucleated cells or cells with chromosome non-disjunction does occur by apoptosis. A higher frequency of micronucleated cells was found among apoptotic cells than among viable cells, as shown in Fig. 5. The frequency of micronucleated binucleates was significantly increased in the fraction containing apoptotic cells, corresponding to selective sorting of micronucleated lymphocytes for both nocodazole and carbendazim. Cells bearing non-disjunction seemed to be a less strong trigger for apoptosis. In Fig. 6 it can be observed that for all concentrations tested the ratio between apoptotic and viable cells is close to one and similar for all concentrations tested. These results suggest that the trigger for disappearance of these cells is most probably not aneuploidy but the presence of a micronucleus. Therefore the presence of MN in a cell correlates with apoptosis and contributes to the elimination of aneuploid cells (Decordier et al., 2002). For this reason, when risk assessment is performed on the basis of thresholds, one should take into account individual susceptibility related to the presence or absence of apoptosis related genes.

4. Thresholds in mitotically dividing somatic cells and oogenesis

We raised some concern about the notion that dose–response to aneugens is similar between somatic and meiotic cells (Parry et al., 2000) since mammalian oocytes possess unusual spindles without pairs of centriols that organize the two spindle poles in somatic cells (Eichenlaub-Ritter et al.,

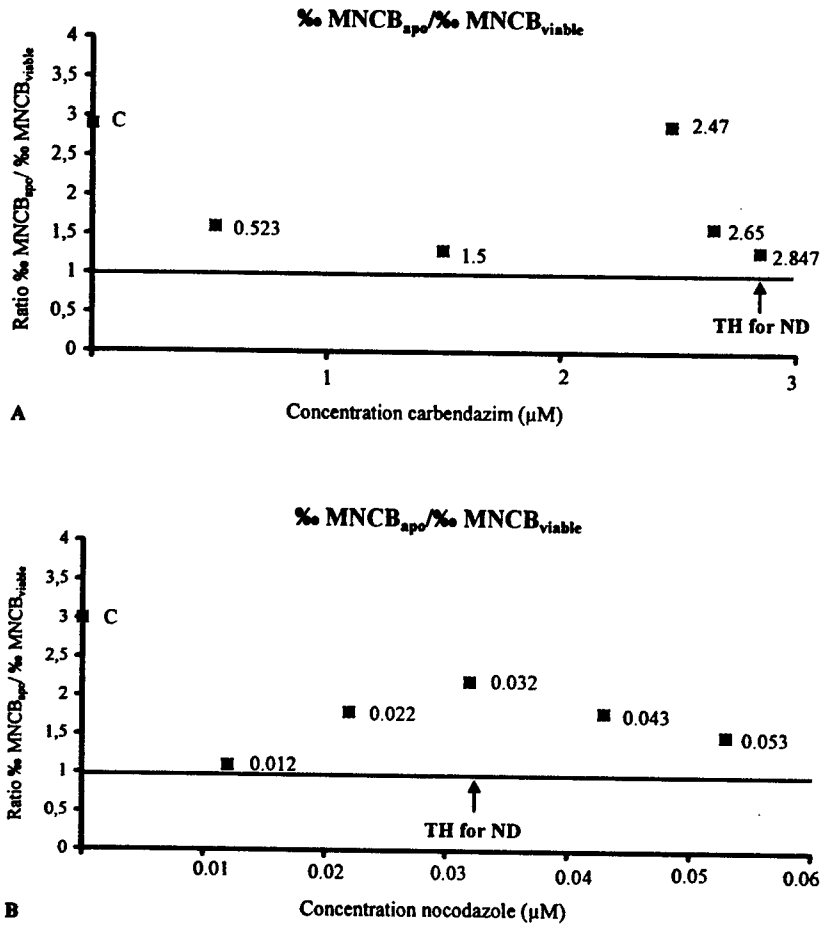


Fig. 5. Induction of micronucleated cytokinesis-blocked lymphocytes (‰MNCB) in the apoptotic versus viable cells, induced by carbendazim (A) and nocodazole (B). The ratio between frequencies of ‰MNCB in the apoptotic fraction versus the viable fraction was calculated for each studied concentration. A ratio equal to one expresses the same probability of finding that class of cells among viable and apoptotic cells. A higher ratio value indicates that those cells preferentially undergo apoptosis. The threshold value for non-disjunction (TH for ND) is indicated by the arrow. The actual tested concentrations are reported close to each experimental point.

1996; Eichenlaub-Ritter, 2002). In vertebrate oocytes, bivalent chromosomes recruit multiple microtubule organizing centres to organize an anastral, barrel-shaped spindle (Yin et al., 1998a; Eichenlaub-Ritter, 2002). Recent studies in the mouse suggest that checkpoints sensing unaligned chromosomes (Gardner and Burke, 2000; Shonn et al., 2000; Nasmyth, 2001) may be less effective in mammalian oogenesis as compared to mitosis and male meiosis (Yin et al., 1998b; Hodges et al., 2002). This and other unique features of oogenesis could contribute to the high susceptibility to errors in chromosome segregation at meiosis I and II, which is causal to the exceptionally high aneuploidy rate in human oocytes (e.g. Sandalinas et al., 2002), aneuploidy in preimplantation embryos and the high risks for implantation failure, spontaneous abortion and trisomy of the human conceptus (for further discussion, see Eichenlaub-Ritter, 2002).

In order to assess whether response is different, and oocytes are more susceptible to chemically-induced aneuploidy as compared to somatic cells, we recently analysed meiotic progression, spindle morphology and aneuploidy of mouse oocytes which matured spontaneously in vitro in absence

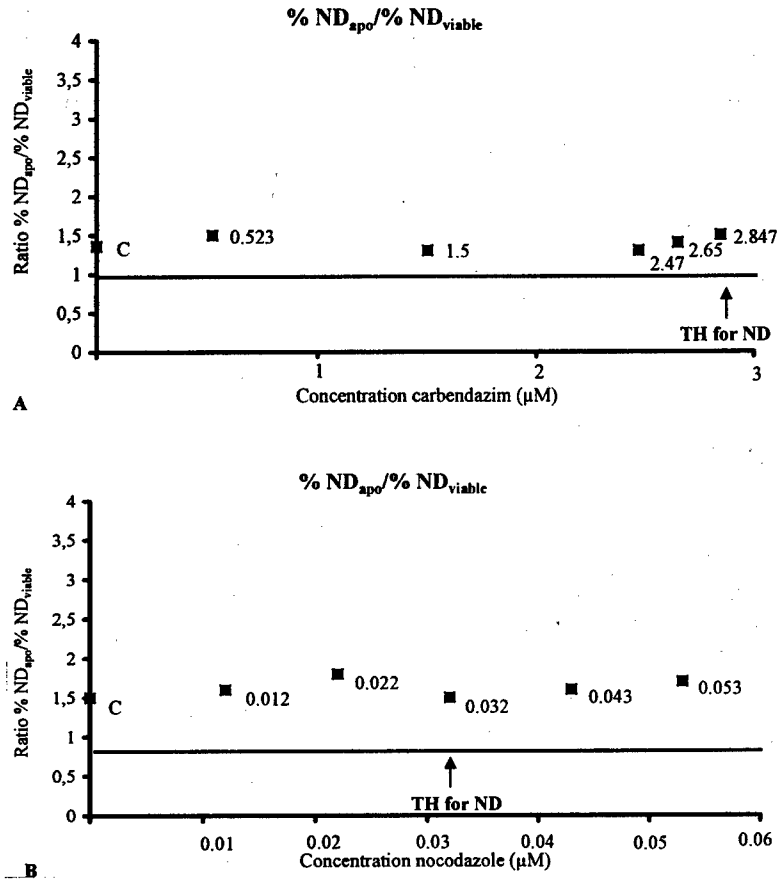
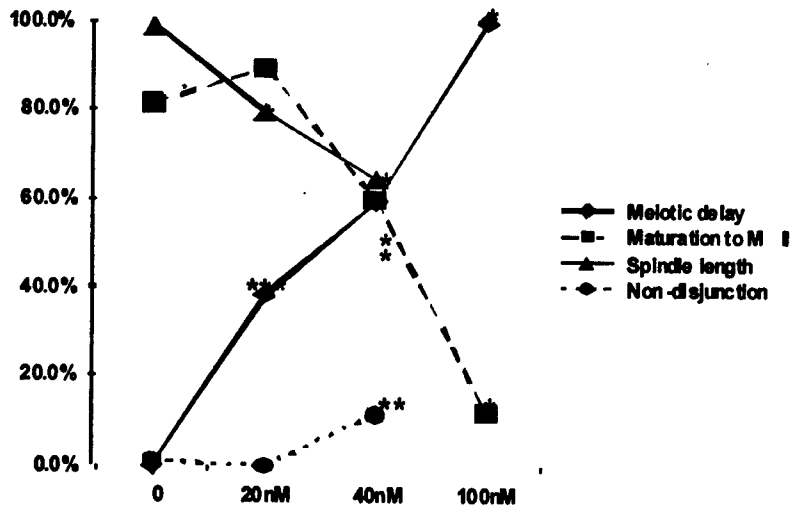


Fig. 6. Induction of chromosomal non-disjunction for the total genome (% Tot. ND) in the apoptotic versus viable cells, induced by carbendazim (A) and nocodazole (B). The ratio between frequencies of chromosomal non-disjunction (% Tot. ND) in the apoptotic fraction versus the viable fraction was calculated for each studied concentration. A ratio equal to one expresses the same probability of finding that class of cells among viable and apoptotic cells. A higher ratio value indicates that those cells preferentially undergo apoptosis. The threshold value for non-disjunction (TH for ND) is indicated by the arrow. The actual tested concentrations are reported close to each experimental point.

of follicle cells from G2-phase (dictyate stage) to metaphase II without or with nocodazole (Eichenlaub-Ritter et al., 2002; Shen et al., 2001). Spindle formation and morphology was analysed non-invasively in living oocytes by enhanced polarizing microscopy (PolScope Sun et al., 2001), and oocytes were spread and C-banded after 16–18 h of culture (Eichenlaub-Ritter et al., 2002). We observed that spindle pole-to-pole distance at metaphase II was significantly reduced already by 20 nM nocodazole by approximately 20% (Eichenlaub-Ritter et al., 2002), and spindle length shortened dose-dependently by exposure to higher nocodazole concentrations (Fig. 7). However, hyperploidy was not increased by maturation in 20 nM nocodazole suggesting the presence of a threshold. A significant increase in hyperploidy rate from 0.5% in controls to 5.7% ($P < 0.01$) and a significant increase in diploid metaphase II oocytes (from 0.5 to 4.4%, $P < 0.05$) were detected in mouse oocytes exposed to 40 nM nocodazole (Shen et al., 2002). Therefore, the biological threshold for induction of non-disjunction in in vitro maturing, naked mouse oocytes exposed to the cytostatic nocodazole appears to closely resemble that of in vitro cultured human lympho-



Significant difference t o control; *p<0.001; **p<0. 01; ***p<0.05

Fig. 7. Dose–response of in vitro maturing mouse oocytes to nocodazole. Exposure to 20 nM nocodazole during maturation induces significant meiotic delay (–●–) (assessed by relative numbers of oocytes undergoing cytokinesis at 11 h of culture in nocodazole group relative to controls) at 20, 40 and 100 nM nocodazole, while maturation to metaphase II (–●–) (assessed by formation of the first polar body after 16 h of culture) is significantly reduced only by ⩾40 nM nocodazole (n = 115, 80, 73 and 62 oocytes for control, 20, 40, and 100 nM nocodazole, respectively). Although there is a dose-dependent nocodazole-induced reduction in spindle length at metaphase II examined already at 20 nM (–●–) (n = 100, 20, and 65 oocytes for control, 20 and 40 nM nocodazole, respectively), non-disjunction (–●–) (conservatively calculated as 2 × hyperploidy rate) is significantly increased by ⩾40 nM nocodazole (n = 197, 141, and 160 oocytes for control, 20 and 40 nM nocodazole, respectively) but not by 20 nM nocodazole.

cytes (32 nM). Meiotic delay as assessed by reduced anaphase I and cytokinesis at 11 h of culture was found for ⩾20 nM nocodazole while maturation to metaphase II as assessed by first polar body formation at 16 h of culture was significantly decreased by ⩾40 nM nocodazole but not by 20 nM nocodazole (Fig. 7). This suggests that a block in cytokinesis may prevent the genesis of aneuploid oocytes, which can cause monosomy or trisomy in the embryo, comparable to the preferential elimination of micronucleated lymphocytes by apoptosis. Oocytes, which fail to undergo cytokinesis and first polar body formation, have a high risk to develop into digynic triploid embryos upon fertilization, which cannot develop to term. The present preliminary data therefore suggest that isolated, in vitro maturing oocytes do not respond more sensitive to exposures to a classical aneugen like nocodazole as compared to mitotically dividing cells, in spite of the differences in spindle formation and cell cycle regulation. However, spindle pole-to-pole distance is about 20–25 μm in mouse oocytes, but only about 11–14 μm in the human oocyte (Shen et al., 2001). Shortening of the spindle of human oocytes by low concentrations of nocodazole might therefore have a more dramatic effect in humans when more distal endpoints (like non-disjunction) are assessed. For this reason, species-, meiosis- and gender-specific susceptibility and the expression of checkpoint genes causing meiotic arrest have still to be considered in risk assessment, and further studies are required.

5. Extrapolation from in vitro threshold values to the in vivo situation

The differences between in vitro and in vivo responses point to a major difficulty in the interpretation of thresholds type effect in risk assessment. Indeed, testing strategies in mutagenicity often include a stepwise procedure, in vitro to in vivo. It is assumed that effects occurring in vitro

do not necessarily also occur in vivo. The value of mutagenicity testing in vivo for risk assessment would be in question, if we have to assume that in principle an in vivo no-effect-level does not exist for a 'single hit–single target' chemical (in vitro). The widely accepted 'conservative' view on genotoxic chemicals assumes that an effect with such a chemical at a certain dose has to be extrapolated back to zero without thresholds. But it does not imply the contrary i.e. that if no effect is found in vivo, one has to expect an adverse effect from a single molecule entering the body. In fact, at the present time, the strongest data establishing a mechanistically relevant threshold for mutagenic endpoints (chromosome loss and chromosome non-disjunction) were obtained only in vitro in human lymphocytes for spindle poisons by Elhajouji et al. (1995, 1997) and Marshall et al. (1996).

Recently, interesting attempts to compare the in vitro threshold values for griseofulvin in human and rat lymphocytes with in vivo NOAEL/LOAEL in bone marrow/gut/erythrocytes were performed by Vanhauwaert et al. (2001) (Fig. 8). In Fig. 8 different situations for exposure to griseofulvin (rat/mouse/human; in vitro/in vivo) and different endpoints (mutagenesis assessed by micronucleus induction and induction of hyperploidy) are compared in different cell types. Pharmacokinetic studies allowed interesting extrapolations from in vitro studies to the in vivo situation, both in rat and human (Kolachana and Smith, 1994). Moreover Vanhauwaert et al. (2001) demonstrated an in vivo LOAEL in gut for a concentration which was ineffective in bone marrow. The lowest 'calculated' or NOAEL observed for the aneugen griseofulvin, in vivo is 100 mg/kg.

6. Implication for regulation of chemicals

If thresholds can be proven experimentally in vitro/in vivo for indirectly acting mutagens, this will have important implications for risk assessment. Up to now there are two principal methods of assessing risk of exposure to chemicals.

The No Effect Level/Safety Factor approach used in most assessments of chemical toxicity. The low dose linear extrapolation method used for genotoxic carcinogens, the justification for selecting the latter method is often expressed as

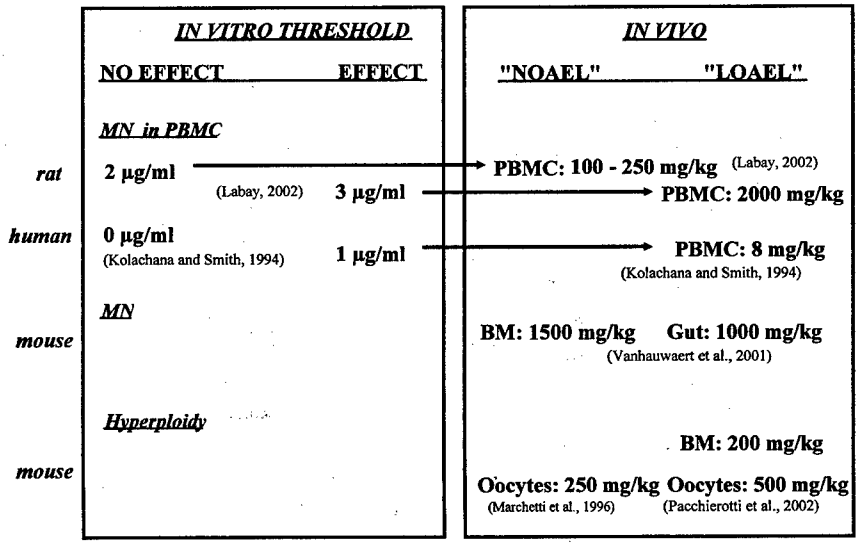


Fig. 8. Comparison of in vitro 'no effect/effect' thresholds and in vivo 'NOAEL/LOAEL' for mutations induced by griseofulvin given orally.

the absence of threshold for genotoxic carcinogens, requiring linear extrapolation.

A third method, the non-linear extrapolation—if mechanistic evidence is provided—could be considered for indirect mutagens, as shown by us for spindle inhibitors.

Regulators (see e.g. the UK Committee on Mutagenicity, http://www.doh.gov.uk/com/com.htm) already considered the importance of thresholds for indirect mechanisms of genotoxicity for the spindle inhibitors benomyl, carbendazim, and thiophanate-methyl. The Committee agreed on the following conclusions:

(1) The aneuploidy induced by methyl benzimadazole carbamates (specifically benomyl, carbendazim and thiophanate-methyl) which act by inhibiting spindle formation is a threshold related effect. There is a sound scientific basis to assume that these chemicals have a threshold of action in both somatic and germ cells. The Committee did not agree with the interpretation reached by the European Commission's Group of Specialised Experts in fields of carcinogenicity, mutagenicity and reprotoxicity at its meeting of the 1–2 September 1999 particularly with regard to the finding by de Stoppelaar et al. (1999) of diploid sperm in rats. The Committee considered the finding of diploid sperm to be an expected effect of carbendazim on male germ cells undergoing meiosis and entirely consistent with the known effects of this chemical on microtubule formation.

(2) The Committee concluded that de Stoppelaar et al. (1999) had not adequately demonstrated a lower threshold for aneuploidy in male germ cells of the rat compared to somatic cells. The Committee agreed that a more appropriate study in somatic cells for comparison with germ cells in the rat would be an investigation of the dose–response for the formation of MN containing aneuploid chromosomes in polychromatic erythrocytes obtained in bone marrow smears from rats using a similar treatment protocol to that used by de Stoppelaar et al. The Committee agreed to review the subject when appropriate studies had been undertaken.

(3) The Committee agreed that the approach used for risk assessment of MBCs by regulatory authorities for pesticides and veterinary medicines and the strategy outlined in the Pesticide Safety Directorate position paper on the role of aneuploidy in the risk assessment of agricultural pesticides were acceptable but would need to be reviewed should a marked difference in sensitivity to aneuploidy induced by these chemicals be reported between germ cells and somatic cells.

(4) The Committee agreed that these conclusions were only relevant to aneuploidy inducing chemicals acting by spindle inhibition. The risk assessment (i.e. consideration of thresholds in somatic and germ cells) of aneuploidy inducing chemicals acting via other mechanisms needed to be considered on a case by case basis.

The same Committee also considered two compounds which are directly acting on DNA but might eventually be considered as having threshold response curves since their capacity to interact with DNA is dependent of protective mechanisms, namely hydroquinone and phenol, and

1) the Committee concluded that by the oral route there was potential for a threshold of activity based on the protective mechanisms such as rapid conjugation and detoxification via glutathione pathway and catalase.
2) However, there is insufficient evidence to support a threshold approach to risk assessment for inhalation or dermal exposure to hydroquinone.
3) The Committee concluded that the available data showed that occupational exposure to hydroquinone was associated with a mutagenic hazard but it was not possible to quantify the risk.

7. Conclusions

The objective of this paper was to summarize the knowledge about threshold dose–responses related to indirectly acting mutagens for which the mechanistic background is clear. Extending this concept to mutagens which have DNA as a target is suggested by some investigators considering the modulating effect of metabolism and

protective mechanisms. The latter should still be considered with much precaution.

Our data showed that consideration of thresholds for indirectly acting mutagens is a relevant issue. From the scientific point of view, it must be emphasized that

1) without mechanistic studies identifying the mutagen/target interactions, no threshold argument should be accepted for regulatory purposes
2) data on individual susceptibility (genotype/phenotype) are missing for these mutagenic effects induced through indirect mechanisms
3) further studies are needed for the evaluation of complex mixtures.

In the regulation of chemicals, the paradigm is that genotoxicity has a non-threshold mode of action. It is obvious that there are exceptions which, however, need specific justification (Madle et al., 2000). In practice, there are only few examples where clear evidence is available for a threshold mode of action, e.g. carbendazim as a spindle inhibitor and phenol due to endogenous protection by the glutathione pathway.

Finally it should be noted that in practice, risk assessment of genotoxic substances is closely related to the evaluation of carcinogenicity (Madle et al., 2000). This perspective puts emphasis on the fact that tumour development is a multifactorial process which involves a sequence of various alterations (e.g. toxicity, cell proliferation and mutagenicity) and therefore probably not dependent on a threshold effect.

Acknowledgements

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Interaction of metal salts with cytoskeletal motor protein systems

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Abstract

Interactions of chemicals with the microtubular network of cells may lead to genotoxicity. Micronuclei (MN) might be caused by interaction of metals with tubulin and/or kinesin. The genotoxic effects of inorganic lead and mercury salts were studied using the MN assay and the CREST analysis in V79 Chinese hamster fibroblasts. Effects on the functional activity of motor protein systems were examined by measurement of tubulin assembly and kinesin-driven motility. Lead and mercury salts induced MN dose-dependently. The no-effect-concentration for MN induction was 1.1 μM $PbCl_2$, 0.05 μM $Pb(OAc)_2$ and 0.01 μM $HgCl_2$. The in vitro results obtained for $PbCl_2$ correspond to reported MN induction in workers occupationally exposed to lead, starting at 1.2 μM Hg(II) (Vaglenov et al., 2001, Environ. Health Perspect. 109, 295–298). The CREST Analysis indicate aneugenic effects of Pb(II) and aneugenic and additionally clastogenic effects of Hg(II). Lead (chloride, acetate, and nitrate) and mercury (chloride and nitrate) interfered dose-dependently with tubulin assembly in vitro. The no-effect-concentration for lead salts in this assay was 10 μM. Inhibition of tubulin assembly by mercury started at 2 μM. The gliding velocity of microtubules along immobilised kinesin molecules was affected by 25 μM $Pb(NO_3)_2$ and 0.1 μM $HgCl_2$ in a dose-dependent manner. Our data support the hypothesis that lead and mercury genotoxicity may result, at least in part, via disturbance of chromosome segregation via interaction with cytoskeletal proteins.
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Keywords: Lead; Mercury; Genotoxicity; Micronucleus test; Cytoskeletal motor protein; Tubulin

1. Introduction

The classification of carcinogens, as well as of germ cell mutagens, is in a state of present discussion. In particular, in Germany the Senate Commission of the DFG for the Investigation of Health Hazards in the Work Area (MAK-Com-

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mission) has issued new recommendations to distinguish between 5 groups of proven and suspected carcinogens (Neumann et al., 1998). The new classification system introduced in Germany in 1998 has been the result of a continuing discussion over about 10 years (Bolt et al., 1988).

This new classification includes as Category 4 'Substances with carcinogenic potential for which genotoxicity plays no or at most a minor role. No significant contribution to human cancer risk is expected, provided that the MAK-value is observed.' Moreover, the new Category 5 comprises 'substances with carcinogenic and genotoxic potential, the potency of which is considered so low that, provided that the MAK-value is observed, no significant contribution to human cancer risk is to be expected.'

The distinction of these two new categories means that the classification of carcinogens, in future, should be based much more on mechanisms by which carcinogenic effects are elicited experimentally. In principle, the concept goes even further, as it should probably not only be distinguished between 'genotoxic' and 'non-genotoxic' carcinogens, but within the group of 'genotoxic' carcinogens between those characterised by 'threshold' and 'non-threshold' effects.

Quite a number of organic industrial chemicals and inorganic compounds display chromosomal effects, which lead them to be viewed as 'genotoxic compounds'. Such chemicals may induce experimental tumours at high doses. Non-threshold principles are still applied in defining permissible exposure values by most regulatory boards. Examples for these compounds include a wide variety of chemicals, such as carbonyl compounds (aldehydes, ketones), compounds with activated double bonds (e.g. allylic compounds), and chemicals strongly interacting with functional groups of proteins (e.g. heavy metal ions like Pb^{2+} and Hg^{2+}).

Current research shows that an important target of macromolecular interaction of such compounds within target cells are cytoskeletal proteins (including tubulin, kinesin, dynein) which are involved in motor processes in eukaryotic cells, such as cell division in general and chromosomal segregation in particular. Especially, the motor functions of the spindle apparatus are affected by distinct proteins, i.e., tubulin and specific motor proteins (kinesin, dynein). The toxicological impact, the underlying mechanisms, and the dose–response characteristics of these macromolecular interactions represent in one of the mechanisms of 'genotoxic' response to chemicals, and are heretofore insufficiently investigated and understood.

This study aims at elucidating basic mechanisms of interactions of lead and mercury with cytoskeletal proteins as the key macromolecules of interaction with foreign chemicals leading to chromosomal genotoxic damage (e.g. aneuploidy, MN formation, etc.). It is supposed that for such interactions thresholds may be defined which could allow derivation of no-observed-adverse-effect-levels. This would be fundamental for setting health-based environmental and occupational standards in the future. Among inorganic chemicals, the effects of mercury and lead salts on functional activity of tubulin and kinesin were investigated by measurements of tubulin assembly and kinesin-driven motility and dose–response relationships determined. In addition, the cytotoxic and genotoxic potential was studied employing the neutral red assay and the MN test with CREST analysis.

2. Material and methods

2.1. MN assay and CREST analysis

Interactions of chemicals with cytoskeletal macromolecules are reflected by the micronucleus (MN) assay. Aneugenic compounds cause spindle or cinetochore damage and lead to the formation of MN containing complete chromosomes. Clastogens can induce structural chromosome breaks. Lead and mercury salts have been investigated regarding their ability to induce MN in V79 hamster fibroblasts. Distinction of aneugenic and clastogenic mechanisms was achieved with CREST analysis.

The MN assay was performed according to Matsuoka et al. (1992). Briefly, V79 hamster lung fibroblasts were incubated for 1.5 cell cycles

(i.e. 18 h) with the respective metal salts. The cells were fixed, stained and 4000–28 000 cells scored for each value. Test substance concentrations examined in the MN assay were generally below the cytotoxicity threshold as determined with the neutral red assay. Distinction between aneugens and clastogens was achieved by CREST analysis (Miller and Adler, 1990). A positive CREST reaction denotes that the MN consists of one or more complete chromosomes and that the compound has primarily aneugenic effects. Vincristine and methanemethylsulfonate served as aneugenic and clastogenic control substances, respectively.

2.2. Tubulin assembly assay

The tubulin assembly assay is based on the observation that tubulin polymerises and depolymerises depending on the ambient temperature. Microtubules of mammalian cells are commonly known to be sensitive to cold. That means that they disintegrate at temperatures below 10 °C. On the other hand, microtubules can be reconstituted from tubulin. Physiological temperatures lead to polymerisation of the tubulin dimers and thus to microtubule formation. This process is reversible at cooler temperatures. Tubule assembly were observed spectrophotometrically (360 nm) during temperature cycles (4 °C→37 °C→4 °C). The absorbance increases due to the formation of microtubules. If tubulin assembly is inhibited, e.g., by $PbCl_2$, the absorbance at 37 °C is reduced compared to the control sample. Precipitation of tubulin with $PbCl_2$ is reflected by increased absorbance at 37 °C compared to the control sample. Such precipitation is not completely reversible, resulting in higher absorbances at 4 °C compared to control samples (Böhm et al., 1984; Unger et al., 1990).

Tubulin was isolated from porcine brain (Shelanski et al., 1973). Standard procedure was performed as described previously (Böhm et al., 1984; Unger et al., 1990) with minor modifications. DTT was replaced by sodium azide and removal of Ca^{2+} ions allowed the use of EGTA-free buffer systems. The plateau level of turbidity was detected after 20 min of incubation at 37 °C, before the reversibility of the assembly was verified at 4 °C.

2.3. Gliding assay

The effects of lead and mercury salts on the cytoskeleton were further studied by a motility assay. The kinesin gliding assay mimics intracellular movement and transport processes in vitro by gliding of taxol-stabilised microtubules across a kinesin-coated glass surface.

The microtubule gliding assay was essentially performed according to Böhm et al. (2000). Microtubule gliding was examined in a narrow flow chamber formed between a glass slide and a coverslip spaced by two strips of double-sided adhesive tape. The wet chamber was precoated with kinesin (0.15 mg/ml) in imidazole buffer. Then the assay mixture—containing the taxol-stabilised microtubules in sample buffer—was dropped onto the slide with the immobilised kinesin and covered by a coverslip. The gliding activity was monitored by video-enhanced differential interference contrast microscopy, using an Axiophot microscope (Zeiss) equipped with the image processing system Argus 50 (Hamamatsu). Gliding velocities were determined from video records by measuring the distance the microtubules migrated within a defined time.

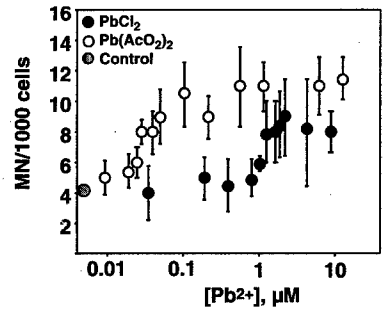

Fig. 1. Dose-dependent induction of MN in V79 cells by lead(II). Cells were incubated for 1.5 cell cycles (18 h) with the respective salts. Each value represents 4000–28 000 scored cells.

3. Results

3.1. MN assay and CREST analysis

Lead chloride and lead acetate induce, dose-dependently, MN in V79 cells. Fig. 1 presents the no-effect-concentration of lead chloride as 1.1 μM $PbCl_2$ and of lead acetate as 0.05 μM Pb(II). The CREST analysis verified an aneugenic effect of Pb(II), which was already anticipated by MN size evaluation (data not shown).

Mercury chloride and mercury nitrate induced MN dose-dependently starting at the same concentration of 0.01 μM mercury(II). Maximal MN induction was seen at 0.1 μM (Fig. 2). CREST analysis revealed aneugenic and clastogenic mechanisms of MN induction by mercury ions (data not shown).

3.2. Tubulin assembly assay

Lead chloride affected dose-dependently tubulin assembly (Fig. 3). The same effects were found for $Pb(NO_3)_2$ and $Pb(OAc)_2$. All tested lead(II) salts exhibited the same dose–response relationship. The effect can therefore be attributed to the Pb^{2+} ion. Three main ranges of influence can be distinguished: at low concentrations (up to 10 μM) no significant influence can be detected. In a range of 20–60 μM the lead salts showed an inhibitory effect on the assembly of tubulin. With increasing lead concentration (> 70 μM) the tubulin tends to aggregate with the lead ions and finally precipitates, as shown by a rise in turbidity at the plateau levels at 20 min (polymerisation) and 40 min (depolymerisation). The no-observed-effect-concentration of lead(II) has been established at 10 μM.

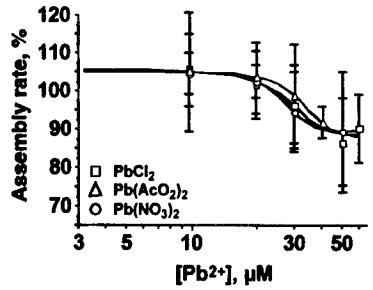

Fig. 3. Dose–response relationship of inhibition of tubulin polymerisation by lead salts. Relative absorbance of the tubulin assembly mixture (compared to control) during incubation of tubulin with various concentrations of lead(II) salts for 20 min at 37 °C.

Mercury(II) inhibits microtubule assembly at concentrations above 1 μM. The inhibition is complete at about 10 μM. In this range the polymerisation is fully (up to 6 μM) or partially (~ 6 × 10 μM) reversible. Higher doses of mercury(II) cause the formation of protein–mercury-aggregates, as shown by complete irreversible polymerisation at 100 μM mercury(II). As for lead the inhibition is independent of the anion chloride or nitrate. Both mercury(II) salts exhibit the same dose–response relationship (Fig. 4). The no-observed-effect-concentration for microtubule assembly inhibition is 1 μM mercury(II), the IC_{50} is 5.8 μM.

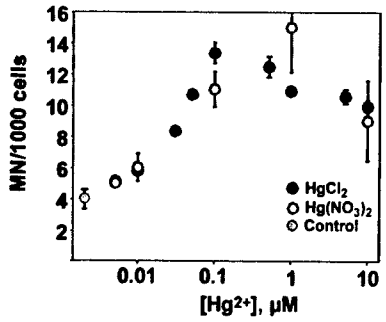

Fig. 2. Dose-dependent induction of MN in V79 cells by mercury(II). Cells were incubated for 1.5 cell cycles (18 h) with the respective salts. Each value represents 4000–28 000 scored cells.

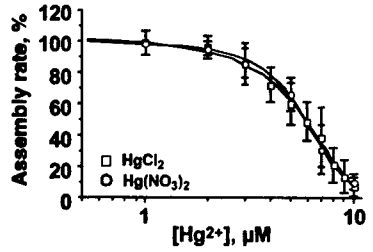

Fig. 4. Dose–response relationship of inhibition of tubulin polymerisation by mercury salts. Relative absorbance of the tubulin assembly mixture (compared to control) during incubation of tubulin with various concentrations of mercury(II) salts for 20 min at 37 °C.

3.3. Gliding assay

Fig. 5 demonstrates the dose-dependent effect of lead nitrate on the gliding velocity of taxol-stabilised microtubules on a surface coated with kinesin. For calculation of the mean gliding velocities the migration of at least 15 individual microtubules was measured. Not shown in the graph above is the velocity without addition of lead (control value), which in this experiment was determined to be about 414 nm/s. $Pb(NO_3)_2$ affects the gliding velocities of microtubules in a clearly dose-dependent manner, starting with concentrations above the no-observed-effect-concentration of 10 μM and reaching complete inhibition of motility at about 500 μM. $HgCl_2$ affects the gliding velocities of taxol-stabilised microtubules also in a dose-dependent manner, starting at a concentration of 0.1 μM and reaching complete inhibition of motility at about 1 μM (Fig. 6).

4. Discussion

The concept underlying the present study is that genotoxicity as represented by aneugenic MN may be mediated through interactions with proteins of the cytoskeleton, i.e., with tubulin and/or the motor protein kinesin. These proteins are involved in the activity of the spindle apparatus of the cell and thereby in cell division. Such protein interactions should be characterised by conventional dose–response relationships, which may enable the definition of thresholds for genotoxicity of these compounds. Definition of such thresholds could then provide arguments for more adequate regulations, both at national and EU levels.

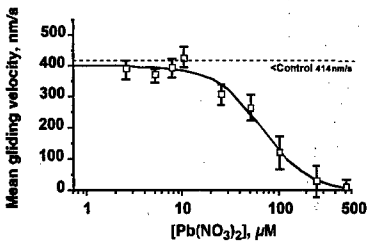

Fig. 5. Dose-dependent inhibition of microtubule motility by lead nitrate.

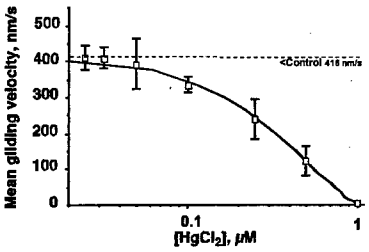

Fig. 6. Dose-dependent inhibition of microtubule motility by mercury chloride.

Several lead and mercury salts were subjected to the MN assay including CREST analysis to establish their genotoxic potential. Interactions of these salts with motor protein systems were determined by measurements of tubulin assembly and kinesin-driven motility.

Lead salts induced MN in V79 hamster fibroblast dose-dependently. Sizes of the MN and CREST analyses indicate aneugenic mechanisms of $PbCl_2$ and $Pb(OAc)_2$. The no-observed concentration of $PbCl_2$ was approximately 20 times higher than that of $Pb(OAc)_2$. No such differences between salts were seen in the cell free tubulin assay. Thus, the difference may be due to, at least in part, variance of intracellular accumulation of lead facilitated via pH differences. However, further studies are necessary to elucidate the mechanism responsible for this observation. Lead salts inhibited tubulin assembly and microtubule motility starting at about a concentration of 20 and 25 μM Pb^{2+} ion, respectively. The tubulin assay showed the same dose–response-curve for all three lead salts ($PbCl_2$, $Pb(OAc)_2$, and $Pb(NO_3)_2$) investigated suggesting that the lead ion is responsible for the observed effects.

Inorganic lead compounds show clastogenic potential (DFG, 2002). It induced chromosomal aberration in vitro and in vivo. No direct interaction of lead with DNA has been observed (Valverde et al., 2001) and it has been suggested that the mechanism is indirect (Hartwig et al., 1990). The data presented here show that lead interferes with motor protein systems. The inhibition of tubule formation and kinesin motility may affect chromosomal segregation during cell division and result in induction of MN. The lowest observed effect concentration detected in this study is

consistent with recent in vivo studies. Vaglenov et al. (2001) evaluated 103 workers exposed to lead. Formation of MN in lymphocytes was dose-dependent and found to start at blood levels of 1.2 μM lead.

Mercury salts induced MN also dose-dependently, based on aneugenic and clastogenic effects. Dose-dependency of mercury effects was observed in the tubulin assembly and kinesin motility assays as well and was alike for all salts in the respective assays. Induction of MN started at concentrations about 10 nM, inhibition of tubule assembly at 2 μM and inhibition of kinesin motility at 1 μM mercury ion concentration. The observed effects are, therefore, merely an effect of the mercury ion and independent of the respective anion. This is in line with our hypothesis.

Induction of MN by mercury has been observed in in vitro and in vivo studies. Unfortunately, only in an in vitro study using peripheral human lymphocytes a dose-dependent increase of MN frequency was observed (Ogura et al., 1996). Although several studies report an increase in MN frequency in exposed workers, no association of MN frequency and urinary mercury levels were found (Queiroz et al., 1999; Shamy et al., 1995; Anwar and Gabal, 1991). These studies support our findings that mercury salts induce MN. The lack of correlation with urinary mercury excretion levels in the in vivo studies suggests that this may not be the right parameter for accurate estimation of target concentration or that confounding factors were not considered.

Interestingly, both cell free systems the tubulin assembly and the kinesin motility assays are less sensitive compared to the MN assay. This holds true for lead and mercury salts.

This study strived at resolving basic mechanisms of interaction of lead and mercury salts with cytoskeletal proteins, interactions that may lead to chromosomal genotoxic damage, i.e., aneuploidy resulting in MN formation. It is supposed that for such interactions thresholds may be defined. This would be fundamental for setting health-based environmental and occupational standards.

In conclusion, lead and mercury induce MN at concentrations that are of concern in occupational environments. Our data support the hypothesis that interactions of lead and mercury salts with motor protein systems are responsible for these observed genotoxic effects. However, whether other factors play an important mechanistic or modulating role cannot be excluded and needs further investigation. Until the remaining questions have been resolved satisfactorily deductions of permissible occupational and environmental exposure from these studies have to be handled cautiously.

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Review

Differentiating between local cytotoxicity, mitogenesis, and genotoxicity in carcinogen risk assessments: the case of vinyl acetate

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Abstract

Understanding the mode of action of carcinogens is critical to scientifically assessing exposure-related risk. Regulatory hazard classification schemes and dose–response assessment paradigms generally require basic knowledge of genotoxic potential to guide decisions on which scheme or paradigm is most appropriate. Although convention suggests that classification and dose–response assessment of genotoxic chemicals should be assessed using conservative assumptions of no threshold, several examples, such as vinyl acetate, exist that challenge this assumption. Vinyl acetate is carcinogenic at portals of entry (nasal cavity and upper gastrointestinal tract). Local metabolism of vinyl acetate produces DNA-reactive acetaldehyde but also produces acetic acid and protons, which contribute to intracellular acidification, cytotoxicity and cell proliferation. This paper reviews their relative contributions to the overall mode of action. Elevated cellular proliferation, well understood to be a risk factor for carcinogenesis, is observed at concentrations associated with tumor formation. Cytotoxicity and compensatory tissue regeneration is one pathway for stimulating cellular proliferation while intracellular acidification is a mitogenic stimulus. Both of these pathways may be operative in nasal tissues while mitogenic proliferation alone appears to be induced in the upper gastrointestinal tract. Using a physiologically-based pharmacokinetic model, quantitative relationships between critical tissue dosimeters and tissue responses are developed to assess the relative importance of genotoxicity and cell proliferation in the overall mode of action of vinyl acetate. This approach supports the concept that intracellular acidification is the sentinel response that precedes cytotoxicity and cellular proliferation. Secondarily, the carcinogenic potential of vinyl acetate is expressed only when tissue exposure to acetaldehyde is high and when cellular proliferation is simultaneously elevated. This mode of action suggests that exposure levels that do not increase intracellular acidification beyond homeostatic bounds will be adequately protective of adverse downstream responses including cancer. These mechanistic insights provide the scientific basis for a cancer classification that incorporates thresholds for cytotoxic and/or mitogenic cell proliferation secondary to intracellular acidification.
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Keywords: Vinyl acetate; Cancer risk assessment; Cytotoxicity; Mutagenesis; Mitogenesis; Cell proliferation

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1. Introduction

A key element of chemical risk management is the evaluation and classification of new and existing substances with carcinogenic, mutagenic, or reproductive/developmental effects. Different chemical classification schemes exist globally as a component of chemical risk assessment, some based on intrinsic toxicological properties and others based on the magnitude of the potential adverse effects at occupationally- or environmentally-relevant exposure concentrations.

In the European Union, the Dangerous Substances Directive (Directive 67/548/EC, 1967) and its subsequent amendments address classification and labeling of chemicals with human health and environmental concerns. EU classification and labeling are intended to inform both workers and consumers as well as the general public. Classifications are based on a substance's inherent toxicity compared with specific criteria. Thus, when toxicity testing reveals the substance produces certain adverse toxicological effects, specific hazard classifications and specific labeling requirements apply to both substances and preparations containing those substances. For example substances 'regarded as if they are carcinogenic to man' carry a category 2 cancer classification and would require labeling of preparations as toxic providing they contain more than 0.1% of the substance; the classification criteria are fully described in Directive 1999/45/EC (1999). Separate from this hazard-based classification is a quantitative risk assessment performed by member states. This risk assessment incorporates exposure- and dose response-characterization for workers, consumers and the general population. At the conclusion of the risk assessment, decisions are made regarding the need for further information or whether risk reduction measures are necessary in the exposed populations.

In the US, the Environmental Protection Agency (EPA) does not employ a similar hazard-based cancer classification process. Instead, EPA has adopted a risk based approach which relies on hazard (e.g. cancer) identification, tumor dose response characteristics, nature (magnitude and duration) of human exposure, and integration into a substance risk characterization. These elements are addressed in recent EPA guidance on carcinogen risk assessment guidelines (US EPA, 1999). The EPA recognized that gaps may exist in the toxicology database for specific substances and thereby allows the use of conservative, default assumptions in calculating risk. These default assumptions represent inferences in general scientific knowledge and are intended to bridge uncertainties in existing data for potential human health effects. EPA encourages pursuit of mechanistic, pharmacokinetic/dynamic research that can be applied for chemical risk assessments and, where appropriate and justified, be used to replace default assumptions. No universal guidance is offered on how much certainty or proof is necessary to justify departure from using a default.

Information on the mode of action (e.g. genotoxic potential) can influence the ultimate classification and levels of acceptable risk. In the EU, genotoxic carcinogens for which a clear threshold for response does not exist may be subject to different dose extrapolation methodologies to predict responses at very low doses. For genotoxic carcinogens, carcinogenicity is assessed using the T25 method which is defined as the chronic daily dose in mg/kg per day that produces tumors in 25% of the animals after accounting for background tumor incidence (Dybing et al., 1997). In contrast, for nongenotoxic carcinogens, cancer risk is calculated from NOAELs or LOAELs with application of uncertainty factors to address uncertainties in data quality and human variability (Moolenaar, 1994). Carcinogenic effects brought about by secondary mechanisms, rather than through direct interaction of chemical (or metabolite) with DNA, could warrant a different risk assessment paradigm. An example is the increase in mutations caused by target tissue proliferation (EEC Annex VI to Directive 67/548/EC, 1967, as amended).

Central to a discussion of the carcinogenicity and approach to risk assessment of vinyl acetate is whether the observed dose–response is attributable to genotoxic or cytotoxic responses, or a combination of both. Differentiating between these two primary modes of action can have important implications for assessing human cancer

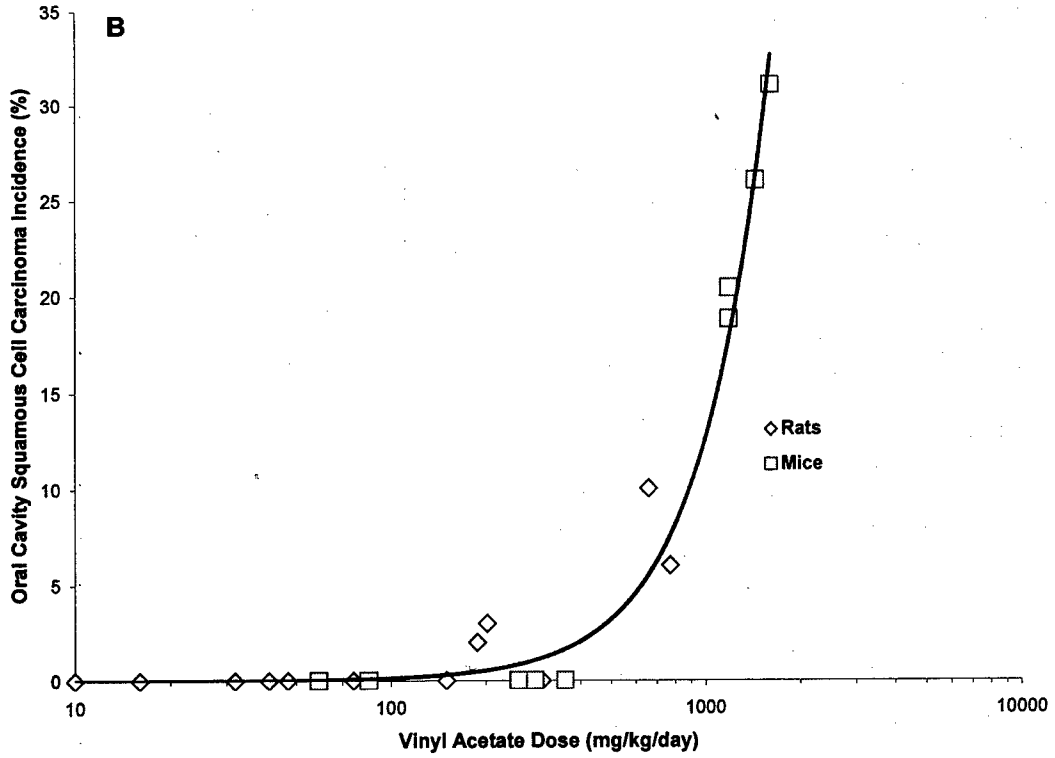

Fig. 1. Composite incidence of oral cavity squamous cell carcinomas in rats and mice chronically exposed to vinyl acetate in the drinking water. Data were obtained from Bogdanffy et al., 1994; Maltoni et al., 1997; Japanese Bioassay Research Center, 1997.

risk because of the widely held beliefs that genotoxic modes of action are without dose thresholds whereas carcinogenic responses driven by cytotoxicity and cellular proliferation are considered to have thresholds below which carcinogenic risk is zero or at least of negligible concern. However, as the molecular basis for chemical carcinogenesis becomes more widely understood, it is less clear that there are distinct differences between these two mechanisms.

This paper reviews the mode of action of vinyl acetate with particular emphasis on data that elucidate relative contributions of primary genotoxicity and secondary mechanisms as drivers for carcinogenesis in upper respiratory and gastrointestinal tract epithelium. Mechanistic research on vinyl acetate carcinogenesis and pharmacokinetics provides an example how this information can be used to improve the scientific basis underlying the risk assessment process.

2. Vinyl acetate cancer bioassays

Epidemiological studies that include vinyl acetate exposures are generally considered insufficient as a basis for establishing carcinogenic potential in humans (IARC, 1995). Vinyl acetate is a nasal carcinogen in rats, but not mice, by the inhalation route and is carcinogenic to the upper gastrointestinal tract of both rats and mice when given in the drinking water. Twelve nasal tumors were observed by inhalation in 120 male and female rats of which five were benign (Bogdanffy et al., 1994). In contrast, most of the tumors of the upper gastrointestinal tract were malignant squamous

cell carcinomas (Bogdanffy et al., 1994a; Japanese Bioassay Research Center, 1997; Maltoni et al., 1997)[1]. By the inhalation or oral routes of exposure, all tumors were confined to the site of contact (i.e. portal of entry) and occurred only at high concentrations that appear to exceed traditional definitions of a maximum tolerated dose (MTD); the minimally effective concentrations were 600 ppm in air and 5000 ppm in drinking water. The dose–response curves for carcinogenicity are highly non-linear with clear breaks in the shape of the curves above 200 mg/kg/d orally and and 200 ppm by inhalation (Figs. 1 and 6) suggesting that biological defense mechanisms are overwhelmed above these concentrations.

Understanding the biochemical and molecular causes for the observed dose-dependent nonlinearities is critical to the overall assessment of carcinogenic risk in terms of both hazard identification (i.e. relevance of these response to humans), regulatory classification and low dose risk assessment.

3. Vinyl acetate mode of action

As knowledge of the mechanisms by which chemicals cause toxicity continues to develop, approaches to assessing health risks from exposures to chemicals are relying less on default assumptions regarding the relevance of bioassay results to humans. Mechanistic toxicology is an important component of understanding (a) the relevance to human diseases, such as cancer, of observations made during high exposure animal bioassays, and (b) the relationship between risk and low-level exposures that are more likely to occur in humans. That is, regulatory decision-making is becoming more dependent on an understanding of a chemical's mode of action and less dependent on default assumptions of relevance and low-exposure risk relationships. The mode of action for vinyl acetate has been studied extensively and will be described here with special emphasis on the role of metabolism, cytotoxicity, genotoxicity, alterations of the homeostatic balance of intracellular pH, cellular proliferation and expectations of the interdependence of these factors in the ultimate expression of cancer.

3.1. Metabolism of vinyl acetate

Vinyl acetate is hydrolyzed to acetic acid and vinyl alcohol, which readily rearranges to acetaldehyde. This conversion has been shown to be dependent on carboxylesterase and is catalyzed by extracts of nasal respiratory and olfactory tissue, oral cavity mucosa, lung and liver (Bogdanffy and Taylor, 1993; Bogdanffy et al., 1998; Simon et al., 1985; Morris et al., 2002). The histochemical localization of carboxylesterase has been described in the nasal cavity and upper gastrointestinal tract of rats and mice (Bogdanffy et al., 1987; Robinson et al., 2002). Tissues rich in carboxylesterase are expected to be susceptible to the toxic effects of vinyl acetate. Nasal respiratory epithelial cells and olfactory sustentacular cells lining the airway are rich in carboxylesterase activity. Olfactory sensory cells and basal cells are devoid of activity. Glandular regions of respiratory mucosa contain little activity while that of the olfactory mucosa stains intensely for carboxyelsterase. In the oral cavity, carboxylesterase activity was confined to the stratified squamous epithelium. Generally, staining was highest within the stratum corneum particularly in areas of keratinisation. Within the stratum spinosum carboxylesterase activity was lower, and there was little or no staining in the basal cell layer. Thus, metabolic activation of vinyl acetate is highly compartmentalized. Those regions and cell types possessing carboxylesterase activity are correlated to sites of carcinogenic action.

Acetaldehyde is further metabolized by aldehyde dehydrogenase which has also been localized

[1] Maltoni et al. (1997), reported squamous cell carcinomas of the upper gastrointestinal tract of mice at concentrations of 5000 ppm, but not 1000 ppm. Using standard estimates of drinking water consumption, it appears that doses of the 5000 ppm group exceed OECD guidelines for upper limit testing of 1000 mg/kg (OECD, 1981). However, this study is difficult to interpret because of an unusual study design and incomplete reporting, and may have been confounded by contaminants present in the test sample. As a result, we have not relied extensively on these data.

histochemically within nasal epithelial cells and the metabolic rates have been quantified (Casanova-Schmitz et al., 1984; Bogdanffy et al., 1986). The oxidation of acetaldehyde is NAD^+-dependent and liberates one proton. At physiological pH, acetic acid formed would be ionized bringing to three the total number of protons liberated within the cell during the complete conversion of vinyl acetate to acetic acid. Acetic acid is then introduced into the tricarboxylic acid cycle upon condensation with Co-A by acetyl CoA-synthetase (Knowles et al., 1974).

From these metabolic pathways it is clear that three cellular toxins are produced during the enzymatic oxidation of vinyl acetate: acetaldehyde, acetate, and protons. By assessing the contributions of each of these metabolites to the total pathogenesis of vinyl acetate toxicity in the upper respiratory and gastrointestinal tracts, an interesting mode of action common to both tissues has developed that is informative to human health risk assessments. Together with the pharmacokinetic determinants of the mode of action, the predominance of genotoxic, cytotoxic or mitogenic drivers of vinyl acetate carcinogenesis can be evaluated quantitatively and their contributions to the assessment from both hazard identification and dose–response perspectives can be assessed objectively.

3.2. Role of acetaldehyde in vinyl acetate carcinogenesis

When vinyl acetate is assessed in standard tests for point mutations (e.g. Ames and other bacterial assays) the results are largely negative (IARC, 1995). The genetic toxicology profiles of both vinyl acetate and acetaldehyde indicate that both have clastogenic activity. The principal difference in the spectrum of genotoxic activity is that mammalian carboxylesterases are necessary for vinyl acetate to express clastogenic potential, i.e. formation of chromosomal aberrations (Norppa et al., 1985; He and Lambert, 1985; Dellarco, 1988). Important to the shape of the cancer dose–response curves for vinyl acetate is that the formation of such aberrations requires at least two independent DNA lesions prior to mutagenic fixation via replication. This sequence would lead to a non-linear response with dose, if those lesions were independently produced (Rhomberg et al., 1990). The clastogenic activity is most likely related to the weak activity of acetaldehyde as a DNA-protein crosslinking (DPX) agent (He and Lambert, 1990; Kuykendall and Bogdanffy, 1992a,b; Kuykendall et al., 1993; Lambert et al., 1985; Lam et al., 1986).

The ability of vinyl acetate to induce DPX focused on two different in vitro systems. Using isolated plasmid DNA and histone protein, acetaldehyde was shown to induce DPX (Kuykendall and Bogdanffy, 1992a). Vinyl acetate was also shown to induce DPX, but only when a source of carboxylesterase (nasal tissue homogenate) was included in the reaction mixture (Kuykendall and Bogdanffy, 1992b). Simultaneous inclusion of an esterase inhibitor along with the nasal tissue homogenate caused inhibition of DPX formation. DPX formation by both acetaldehyde and vinyl acetate was also demonstrated using isolated rat nasal respiratory and olfactory epithelial cells. Again, DPX formation was inhibited if the cells were pretreated with an esterase inhibitor (Kuykendall et al., 1993). These data suggest that the clastogenic effects of vinyl acetate are a result of carboxylesterase-mediated hydrolysis of vinyl acetate and acetaldehyde-induced formation of DPX.

Whether or not the primary genotoxic activity of vinyl acetate is entirely attributable to acetaldehyde is not clear. It is possible that protons generated during the ionization of metabolically formed acetic acid or during oxidation of acetaldehyde might also contribute to the expression of genotoxicity, i.e. clastogenesis. Morita (1995) has shown that low pH (pH 6.6) leads to chromosomal aberrations and sister chromatid exchanges (SCE) in Chinese hamster ovary cells and that these effects are S phase-dependent. Thus, cells in a highly replicating population might be extra sensitive to low pH-induced clastogenesis. Neutralization of the media abolished the clastogenic activity (Morita et al., 1990). These observations supported the work of Sipi et al. (1992) who showed that addition of organic acid metabolites from a variety of vinyl esters to the culture media reduced the pH of the media 0.5–1.0 units and facilitated induction of SCEs in whole blood

human lymphocytes. However, these authors studied vinyl acetate specifically and also noted that vinyl acetate-induced SCE results could not be explained solely by the acetic acid-induced reduction in media pH.

Our work with isolated plasmids and histone protein shows that low pH (≤ 6.5) can facilitate DPX formation. A tighter association of histone protein with DNA at low pH likely facilitates intermolecular crosslinking between lysine residues and guanosine (Kuykendall and Bogdanffy, 1994). Thus, it appears that simultaneous reduction in cellular pH and elevated exposure of proliferating cells to acetaldehye can act in a combinatorial manner to yield chromosomal breakage, potential rearrangements and ultimately mutation. Coupled with a state of induced cellular proliferation it is likely that these conditions contribute to the genotoxic events required for vinyl acetate-induced neoplasia.

For purposes of both hazard identification and dose–response assessment, it is also important to consider the concentration of acetaldehyde required to produce a clastogenic response in vitro. Although SCE is arguably an irrelevant endpoint for assessing genotoxicity it provides at least a very conservative basis for assessing minimally effective clastogenic concentrations (Preston, 1999). The minimally effective concentration of acetaldehyde for induction of SCE in human lymphocytes is 3.9 μg/ml (Obe et al., 1979). Chromosomal aberrations in human lymphocytes were noted at concentrations of 7.8 μg/ml (in Fanconi's anemia cells) and higher (15.6 μg/ml in normal human lymphocytes). As discussed later, these concentrations are significantly above background blood concentrations in humans and within the range of plasma levels encountered during ethanol intoxication. Exposures to vinyl acetate likely to result in these levels of acetaldehyde in nasal and oral cavity tissues will be discussed later in the context of pharmacokinetics and dosimetry.

3.3. Role of cytotoxicity in vinyl acetate carcinogenesis

The cytotoxic activity of vinyl acetate, acetaldehyde and acetic acid were studied in an in vitro system utilizing nasal turbinates (Kuykendall et al., 1993). The results showed that inhibition of tissue carboxylesterase blocks the cytotoxic effects of vinyl acetate. Acetic acid at the levels produced in nasal tissues by metabolism of vinyl acetate was also cytotoxic whereas comparable levels of acetaldehyde were not cytotoxic. Although the significance was not fully appreciated in these early studies, the concentrations of vinyl acetate required to induce cytotoxicity under the conditions of the in vitro assay were high (>25 mM) suggesting a weak cytotoxic effect. Nevertheless, these experiments demonstrate the key role of acetic acid formation in the induction of a cytotoxic response in nasal turbinates. Acidic metabolites have been implicated in the nasal toxicity of other inhaled esters (Trela and Bogdanffy, 1991).

In vivo, olfactory degeneration is observed in rats and mice exposed for a lifetime to ≥ 200 ppm vinyl acetate. Degeneration, necrosis and regenerative hyperplasia of the respiratory epithelium of the nasal mucosa is observed in rats exposed for up to 4 weeks. Although the response of olfactory epithelium is unequivocal, the cytotoxic response of respiratory epithelium has only been observed at 1000 ppm. Lifetime exposure of rats to 600 ppm is without cytotoxic effect on the respiratory epithelium.

Efforts to show that vinyl acetate is cytotoxic to oral cavity mucosa suggest the oral cavity, like nasal respiratory epithelium is fairly resistant to cytotoxicity. Morris et al. (2002) have shown that exposure of the oral cavity of rats for 10 min to 24 000 ppm of vinyl acetate in water results in increased extravasation of Evans blue from the vasculature into the oral cavity. Evans blue binds to plasma albumin; its transfer is an indicator of irritation. However, measures of acid phosphatase release from mucosal tissue into the oral cavity, a marker of direct cytotoxicity, were negative. Additionally, Valentine et al. (2002) reported no histopathologic changes in oral cavity mucosa following up to 92 days of administration of vinyl acetate at drinking water concentrations as high as 24 000 ppm.

3.4. Cellular proliferation in vinyl acetate carcinogenesis

Cytotoxicity can be a critical factor in the induction of cancer because of the compensatory or reparative proliferation induced during the wound healing process and the increased risk of mutation imposed by error-prone DNA replication during S-phase synthesis. The role of cytotoxicity in the carcinogenic process has long been appreciated, and has been illustrated, for example, by the case of chloroform-induced hepatocellular lethality, regeneration and carcinogenesis (Butterworth and Bogdanffy, 1999). Augmentative, also known as mitogenic, proliferation has also been recognized as a driver for DNA replication that can enhance carcinogenic risk (Cohen and Ellwein, 1991; Ames and Gold, 1991; Michalopoulos, 1991). Mitogenic proliferation is commonly associated with tumor promoting agents such as phorbol esters and other xenobiotics (Iverson, 1988; Columbano et al., 1991) but could also be secondary to local inflammation, where macrophages release cytokines, growth factors and other signals for cellular replication. Apoptosis, a counter balancing force in tissue growth dynamics, may result in selective growth advantage of preneoplastic foci or may offset elevated rates of proliferation resulting in a constant tissue mass. Common to all of these mechanisms of proliferation is the triggering of intracellular signal transduction pathways that ultimately result in the cell entering S-phase. Regardless of whether the cell birth rate (i.e. mitogenesis) is counter-balanced by an increase in the cell death rate (e.g. by cytotoxicity or apopotosis), what is most important to understanding mode of action is that a chemical exposure induces an elevated state of proliferation increasing the chance of mutation.

Whether induced by a mitogenic signal or by cytotoxicity, tumor dose–response curves for proliferation-driven carcinogenesis tend to be sublinear with dose and show highly nonlinear time-to-tumor profiles with the majority of tumors occurring late in life. Limited two stage growth modeling of the vinyl acetate-induced nasal tumor incidence data, the late-life dependency of tumor formation and the highly nonlinear and steep dose–response relationship collectively suggest a mechanism that is more reliant on induction of cellular proliferation than induced mutations (Bogdanffy et al., 2001). Although time-to-tumor data are not available for the JBRC drinking water study, there was no effect of vinyl acetate on survival suggesting that these tumors did not develop early in life.

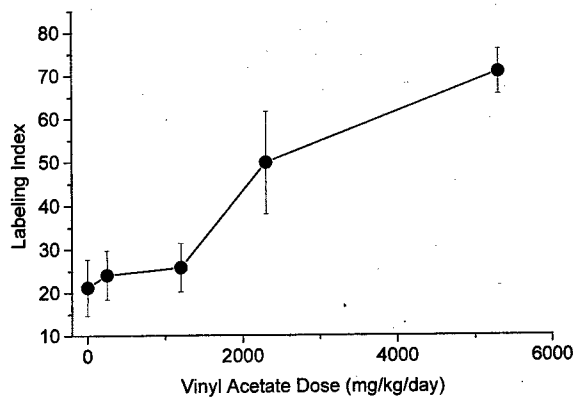

Fig. 2. Cell proliferation in mouse oral mucosa following 92 days of exposure to vinyl acetate in drinking water. Data for oral cavity region V (mandibular) are presented. Data are expressed as labeled cells/1000 mucosal basal cells (mean ± S.D.). (From Valentine et al., 2002). Responses of the 10 000 and 24 000 ppm groups (2300 and 5300 mg/kg/d, respectively) are statistically different from controls ($P < 0.05$).

We set out to determine the state of proliferation of nasal and oral cavity epithelium under bioassay conditions. Inhalation exposures produced a state of induced proliferation in both respiratory and olfactory epithelium of the nasal cavity following just a single 6-h exposure (Bogdanffy et al., 1997). Interestingly, rates of proliferation were elevated in respiratory epithelium of rats exposed to 600 ppm but these exposure conditions were not associated with histopathological evidence of cytotoxicity. At 600 ppm cytotoxicity was present only in the olfactory regions of nasal tissue. From these observations, it is clear that cellular proliferation is induced by vinyl acetate in nasal epithelium, despite the presence or absence of cytotoxicity, and that cellular proliferation is likely required for tumorigenesis (Fig. 6).

Cellular proliferation, and not cytotoxicity per se, as a necessary event in the mode of action of

vinyl acetate carcinogenesis is supported by the results of the JBRC bioassay and the very recent studies of cell proliferation in oral cavity mucosa. In the JBRC study, squamous hyperplasia and basal cell activation of the oral cavity and esophagus were observed. To study the active process of cell proliferation, rats and mice were administered vinyl acetate for up to 92 days in drinking water at levels studied in 2-year bioassays and up to 24 000 ppm, the water solubility limit. Similar to the results obtained for nasal respiratory epithelium, vinyl acetate induced proliferation in the absence of histopathologically detectable cytotoxicity (Fig. 2). The lack of histopathologic change in oral mucosa supports the weak response of biochemical markers of cytotoxicity observed by Morris et al. (2002). Taken together, these observations suggest that an alternative stimulus for cellular proliferation may be operative in nasal respiratory and oral cavity epithelia. It may also be possible that this alternative mechanism is operative in olfactory epithelium but is overshadowed by the additional compensatory proliferative stimulus induced by cytotoxicity. Regardless of the mechanism for induction it is clear that cellular proliferation is induced in nasal respiratory and upper gastrointestinal tract epithelial tissues and that this step is critical to the complete expression of the carcinogenic potential of vinyl acetate.

3.5. Intracellular acidification, mitogenesis and cytotoxicity

An alternative hypothesis for the cell proliferation stimulus in respiratory epithelium has recently emerged (Bogdanffy, 2002) that might clarify the mode of action in the upper gastrointestinal tract and nasal respiratory epithelium. Literature reports suggest that reductions in intracellular pH (pH_i) can induce mitogenesis in the absence of cytotoxicity. Alterations in pH_i are involved in stimulation of cell growth and transformation. Decreasing pH_i through addition of potassium ferricyanate or addition of sodium propionate elicited a mitogenic response in PC12 cells (Thomas et al., 1996). These authors suggested that proliferation occurs upon activation of a mitogen-activated protein kinase through pH_i reduction. Syrian Hamster Embryo cells, cultured at pH 6.7, show a marked increase in lifespan, compared with those cultured at pH 7.3, as measured by the number population doublings that occur before cellular senescence (Kerckaert et al., 1996). The higher proton burden of the intracellular environment has been shown to displace Ca^{2+} from intracellular binding sites (Batlle et al., 1993). Ca^{2+} displaced from the growth and differentiation factor protein blocks the intracellular signaling that leads to differentiation (Isfort et al., 1993). Blockage of the differentiation pathway could promote sustained proliferation, expansion of the undifferentiated cell population, and clonal expansion of spontaneous- or chemical-induced mutants.

The pH_i-dependent mechanism of mitogenesis is particularly relevant to the vinyl acetate-induced oral cavity and esophageal tumors observed in the JBRC lifetime drinking water bioassay. Gastroesophageal reflux disease, a condition known as Barrett's esophagus, has been established as a strong risk factor for esophageal adenocarcinoma (Lagergen et al., 1999). Intracellular acidification of esophageal epithelial cells, from exposure to gastric HCl, activates mitogen-activated protein kinase pathways, stimulates cellular proliferation and decreases apoptosis (Tobey et al., 1998; Souza et al., 2002). Basal epithelial cells of rabbit esophagus have also been shown to proliferate in response to treatment with acidic solutions (Carpizo et al., 1998). It is plausible that intracellular acidification from vinyl acetate metabolism could activate these very same pathways thereby promoting a proliferative response without overt cytotoxicity. The hypothesis that intracellular acidification is mitogenic in nasal or oral cavity mucosal cells suggests further experimentation. Nevertheless the proposal is supported by the literature and could provide a fundamental linkage to many tumor promotion mechanisms.

Based on the expectation that the normal proton burden of carboxylesterase-containing tissues is elevated and pH_i decreased when exposed to vinyl acetate, and the observation that cell culture medium turns acidic when nasal explants are incubated in the presence of vinyl acetate (Kuykendall et al., 1993), we measured pH_i of hepato-

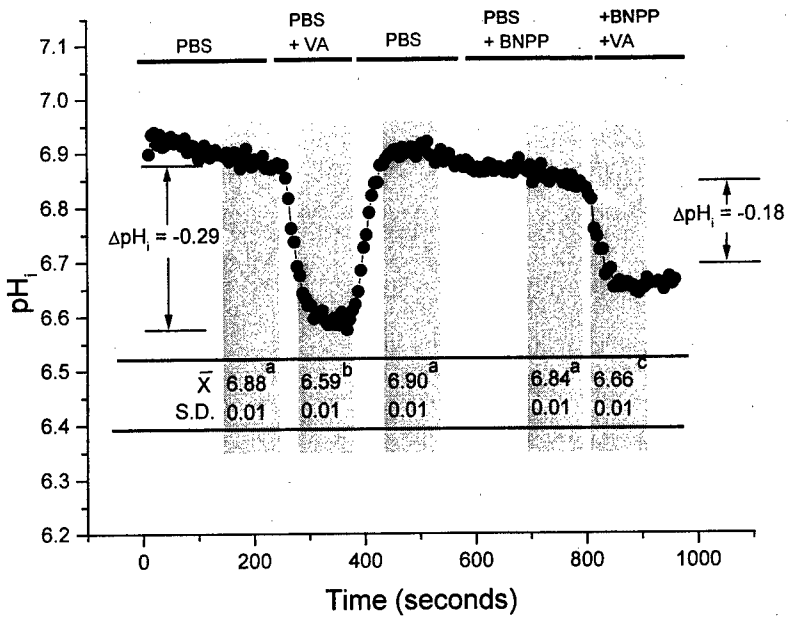

Fig. 3. Effect of bis(*p*-nitrophenyl)phosphate (BNPP), a carboxylesterase inhibitor (200 μM) on vinyl acetate (200 μM)-induced changes in pH_i. Hepatocytes, used a model cell, were exposed sequentially to phosphate buffered saline (PBS), vinyl acetate in PBS, PBS, BNPP in PBS, and then vinyl acetate+BNPP in PBS. Shaded areas indicate the period during which data were averaged and compared. Mean values with differing superscripts are statistically different from each other. These data provide proof of concept that intracellular acidification is a sentinel pharmacodynamic response of cells to vinyl acetate exposure. (From Bogdanffy, 2002).

cytes in vitro under dynamic steady state conditions of vinyl acetate exposure (Bogdanffy, 2002). Hepatocytes were used as a model system because they are readily obtained in large numbers and levels of carboxylesterase are quantitatively similar to that of nasal mucosa. Exposure of hepatocytes to vinyl acetate (10–1000 μM) caused rapid and sustained reductions of approximately 0.03–0.65 pH units which rapidly returned to control levels when vinyl acetate exposure was stopped (Fig. 3). Furthermore, carboxylesterase inhibition attenuated the vinyl acetate-induced acidification. There was no evidence of cytotoxicity at these concentrations. These results provide proof of concept that intracellular acidification is a sentinel pharmacodynamic response of cells to vinyl acetate exposure and raise the possibility that intracellular acidification is a mitogenic signal and that some tissues, such as olfactory epithelium, may be particularly sensitive to its cytotoxic effects (Fig. 4).

4. Considerations of pharmacokinetics and dosimetry in assessing vinyl acetate mode of action

Physiologically-based pharmacokinetic (PBPK) modeling is a useful tool for both hypothesis generation and risk extrapolation. As a hypothesis generating tool, mathematical descriptions of physiological and biochemical processes believed to be important to the overall mode of toxic action can be written, parameterized with experimentally-derived kinetic constants, and used to simulate relationships between external dose versus target tissue dose or various measures of dose versus target tissue dynamic responses. Comparison of the model simulations to experimental observation over a range of dose levels provides guidance regarding the proposed mode of action. As a risk extrapolation tool, PBPK models allow simulations of predicted dosimetry in humans under various exposure scenarios where experimental data are not available. PBPK modeling provides a more sound basis for inter-species dosimetry

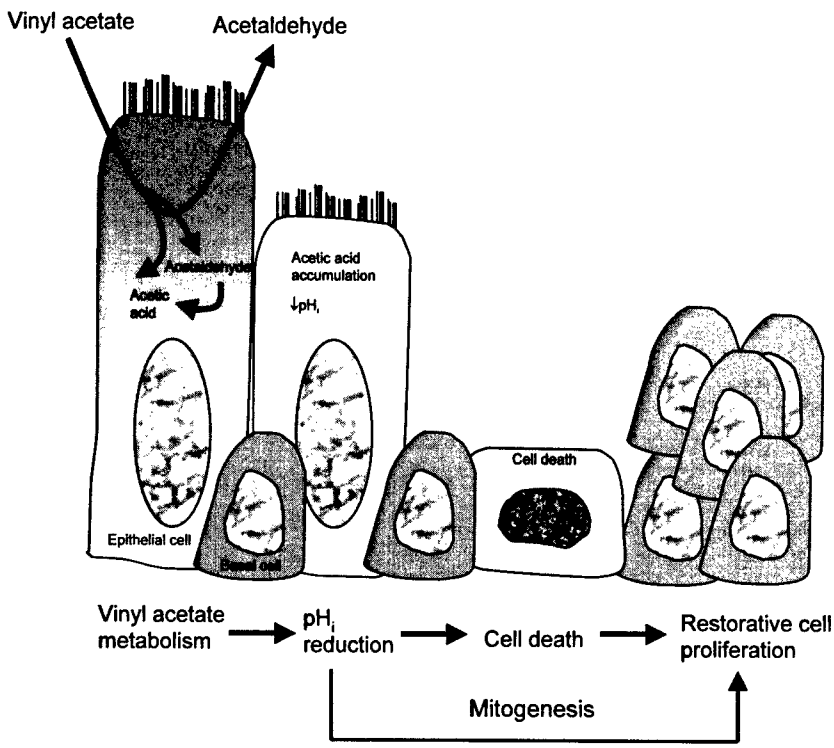

Fig. 4. Schematic diagram illustrating the proposed mode of action for vinyl acetate in nasal olfactory tissue. A parallel mode of action has been proposed for nasal respiratory epithelium and the upper digestive tract except that cytotoxicity does not appear to be a significant factor driving cellular proliferation. Rather, mitogenic effects of intracellular acidification appear to promote cellular proliferation in those tissues.

extrapolation than default approaches (Andersen, 2003).

4.1. Physiologically-based modeling of vinyl acetate

PBPK models describing the dosimetry of vinyl acetate and metabolites believed to be critical to the non-carcinogenic and carcinogenic responses in upper respiratory tract and gastrointestinal tract epithelium have been developed (Bogdanffy et al., 1999). These models are similar in that they describe the processes of absorption, metabolism and elimination of vinyl acetate from the target tissues. Dosimeters related to the mode of action such as tissue exposure to vinyl acetate, acetaldehyde, acetic acid and protons (i.e. intracellular acidification) are predicted. A simplified description of the upper respiratory tract model is presented in Fig. 5. Using the PBPK models as a tool, questions can be formulated quantitatively regarding expected levels of tissue exposure to cytotoxicants (e.g. acetic acid and protons), mitogens (e.g. intracellular acidification) and genotoxicants (e.g. acetaldehyde). Model predictions can then be compared with experimental observations of cytotoxicity, mitogenesis, and genotoxicity to shed light on the likely contribution of these factors to the overall mode of action.

4.2. Mode of action-based kinetic modeling supports five steps required for carcinogenesis

The mode of action research can be summarized as requiring five critical steps that are necessary for the carcinogenic potential of vinyl acetate to be expressed (Figs. 4–6). Using the PBPK model, quantitative estimates can be made of tissue exposure to vinyl acetate, metabolites, or protons, which can be expressed as pH_i. The five critical steps and the tissue dosimetry and responses are

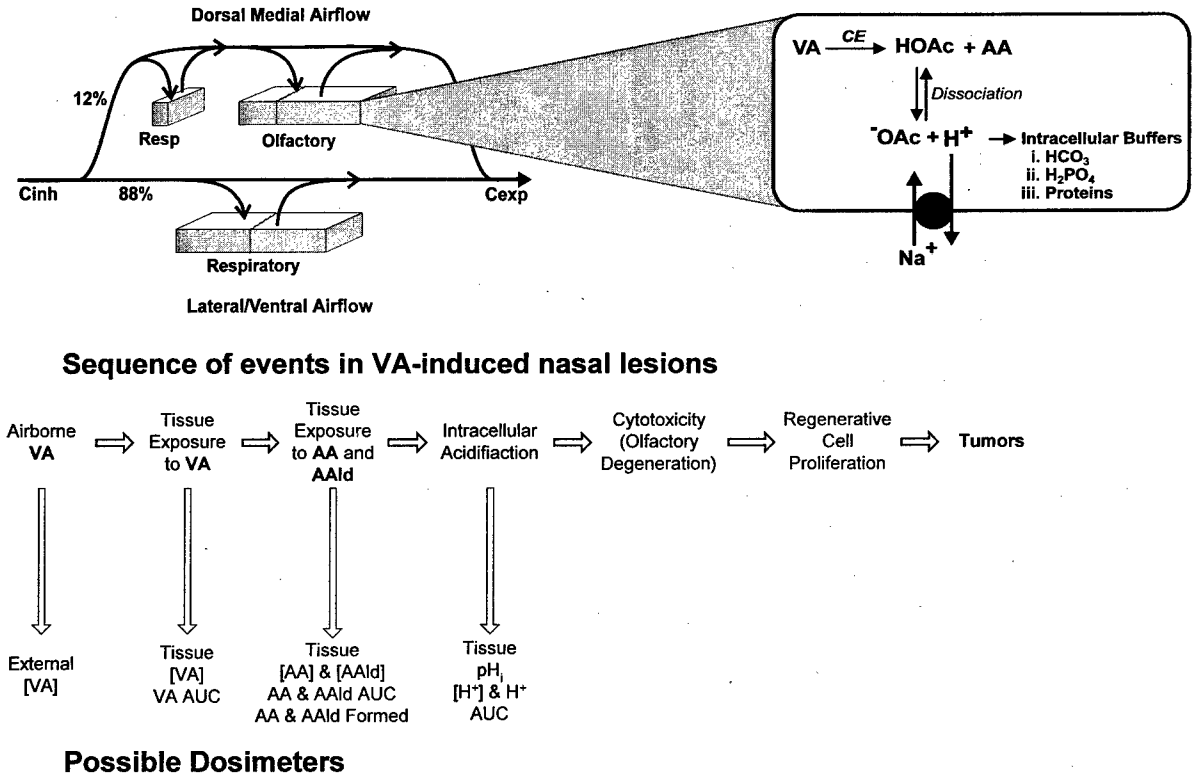

Fig. 5. Vinyl acetate dosimeters estimated by the PBPK/pharmacodynamic model in relation to the continuum of responses that begin with intracellular acidification and culminate in nasal tumor formation. VA, vinyl acetate; AA, acetic acid; AAld, acetaldehyde; AUC, area under the curve. (From Bogdanffy et al., 2001).

illustrated for the upper respiratory tract in Fig. 6. The threshold for pH_i reduction in neuronal cells that does not induce cytotoxicity in vitro is 0.15 pH units (Nedergaard et al., 1991). The lowest concentration of acetaldehyde that has been shown to induce SCE in vitro in Chinese hamster ovary (CHO) cells is 3.9 μg/ml (Obe et al., 1979). The PBPK model, developed to simulate tissue dosimetry (Bogdanffy et al., 1999), predicts that in rat nasal olfactory tissue, 50 ppm exposure will induce a 0.08 unit reduction in pH_i and a basal cell acetaldehyde concentration of 1.7 μg/ml. Fifty ppm is a NOAEL, and the pH_i reduction and basal cell acetaldehyde levels are below their thresholds. As the air concentration increases to 200 ppm, pH_i is predicted to be reduced by 0.25 pH units, a value slightly above the threshold, and cytotoxicity (i.e. olfactory degeneration) occurs. However, the cell proliferation response at 200 ppm is weak (step 3, Fig. 6). At this concentration, levels of acetaldehyde (5.4 μg/ml), slightly in excess of the threshold for genotoxicity, are achieved in tissues. Thus, at 200 ppm there is minimal pH_i reduction above the threshold, minimal exposure above threshold levels of acetaldehyde, and slightly enhanced cellular proliferation. At 200 ppm, one nasal tumor was observed which was not statistically significant. At 600 ppm, pH_i is predicted to be reduced by 0.49 pH units (step 1), basal cell acetaldehyde levels are predicted to be above threshold at 12.4 μg/ml (step 2), and cellular proliferation is more than 2 fold above control (step 3). At 600 ppm, all of the critical steps in the mechanism of carcinogenesis are active and tumors appear (steps 4 and 5). This sequence of events and the physiological modeling suggest that all the conditions necessary for a complete carcinogenic mechanism are in place only when critical

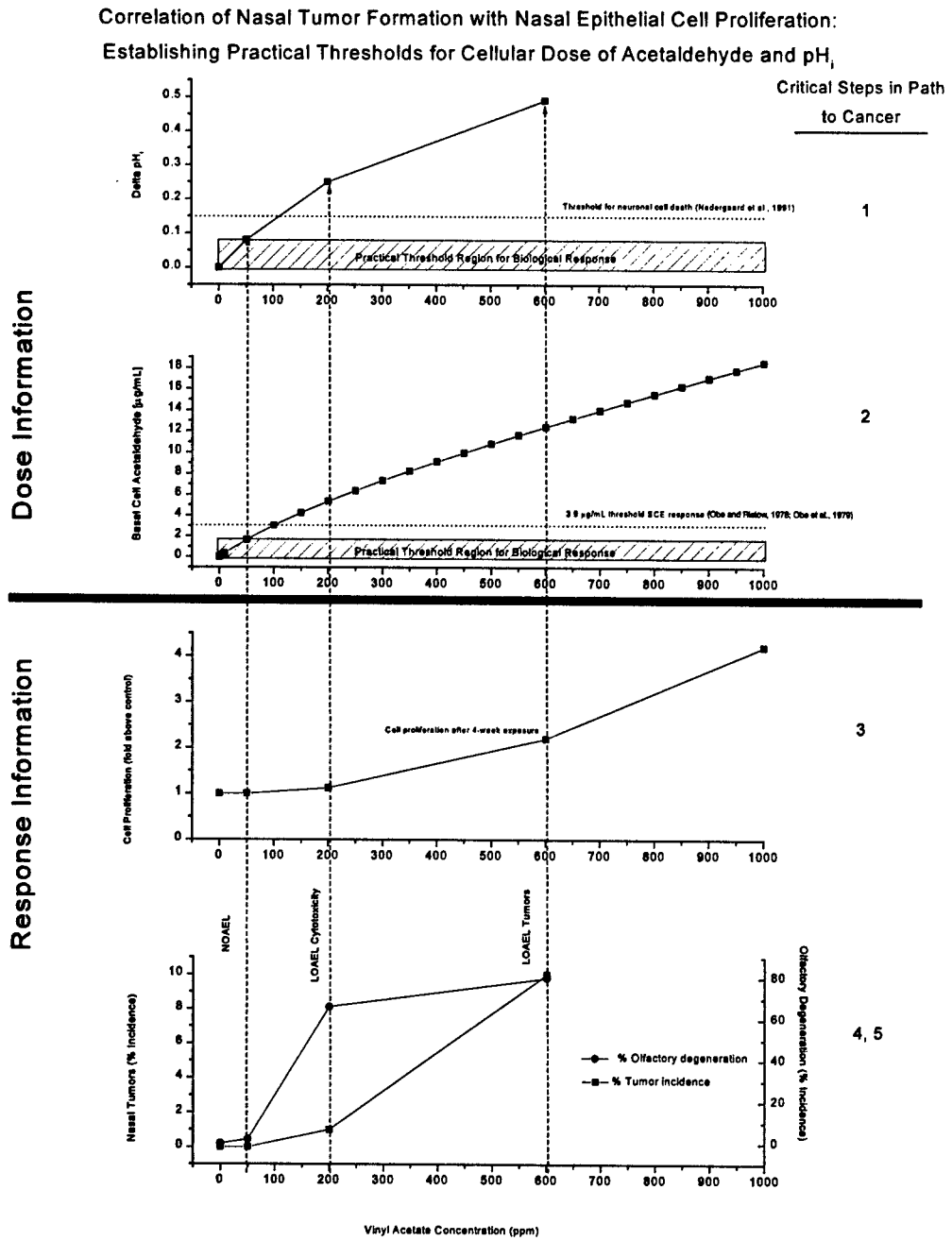

Fig. 6

exposure concentrations are achieved and threshold levels for pH$_i$ reduction, cytotoxicity, cellular proliferation, and acetaldehyde exposure are exceeded. A similar quantitative description of these processes is being developed for the upper gastrointestinal tract.

Fig. 6. Composite presentation of dose and response data for the five critical steps on the pathway to carcinogenesis illustrated for nasal olfactory tissue. Panel 1 shows predicted pH_i changes in olfactory epithelium in relation to changes in pH_i that are cytotoxic to neuronal cells in vitro. pH_i reduction is proposed to be the critical step leading to cytotoxicity. Panel 2 shows the predicted steady state concentration of acetaldehyde at the basal cells in relation to in vitro doses that produce SCE. Panel 3 shows the cell proliferation response which is elevated at 200 ppm (statistically significant at 600 ppm), the concentration at which ΔpH_i exceeds threshold. Panels 4 and 5 show histopatholgical changes. Olfactory degeneration (cytotoxicity) is observed at 200 ppm. Because acetaldehyde levels are only slightly above thresholds, there is no significant tumor response. At 600 ppm all thresholds are exceeded, cell proliferation is significantly enhanced and a significant incidence of nasal tumors is observed.

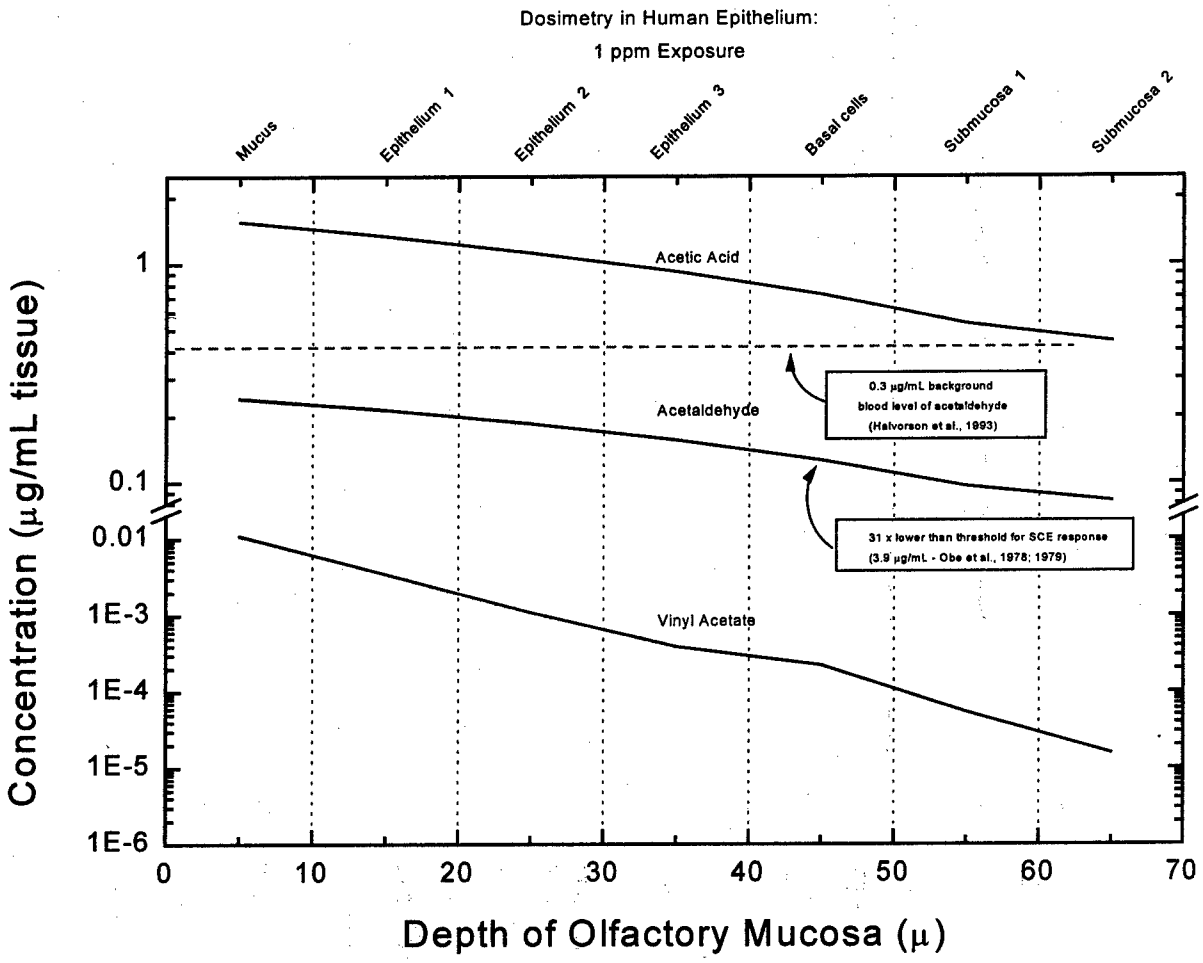

Fig. 7. Predicted steady state concentrations of acetaldehyde, acetic acid, and vinyl acetate throughout the olfactory nasal mucosa of humans exposed continuously to 1 ppm vinyl acetate. Concentrations of acetaldehyde at the basal cell layer are critical for consideration because basal cells are the progenitor cells for the epithelium and are the target cell for carcinogenesis. The figure illustrates that at 1 ppm, basal cell acetaldehyde concentrations are predicted to be approximately three times lower than background blood acetaldehyde levels and more than 31 times lower than the lowest concentration shown to induce sister chromatid exchanges (SCE). SCE are a sensitive marker of genetic damage but of questionable relevance (Preston, 1999). The margin of safety relative to the more appropriate endpoint of chromosomal aberrations is much greater. Obe et al. (1979) report the lowest level to induce SCE in normal human lymphocytes or lymphocytes from Fanconi's anemia patients to be 15.6 or 7.8 µg/ml, respectively. The margin of safety below the chromosomal aberrations endpoint is 124 times.

4.3. Role of background acetaldehyde

Acetaldehyde is present in blood and tissues as a normal metabolic product and from exposures to acetaldehyde-containing foods and alcoholic beverages. Acetaldehyde is produced intracellularly as the product of threonine metabolism (Lehninger et al., 1993; Pronko et al., 2002). Circulating levels of acetaldehyde measured in individuals that do not consume alcoholic beverages are approximately 0.3 μg/ml. It stands to reason that practical thresholds of exposure to acetaldehyde must exist and that the aim of risk management practices should be to minimize exposures to chemicals that are additive to these background levels. In this regard, the German MAK recently reviewed occupational exposures to ethanol and concluded that a workplace 8 h time-weighted average of 500 ppm would be adequately protective of health effects and that the associated blood levels lie within the range of the standard deviation of the endogenous blood burden (Greim and Reuter, 2001). Unfortunately the available data did not allow estimation of blood or tissue burdens of acetaldehyde. Considering lifetime ambient exposures to vinyl acetate, the nasal PBPK model predicts that continuous exposure of humans to 1 ppm results in steady state acetaldehyde concentrations at the level of the basal cells of olfactory mucosa (the progenitor cells for carcinogenesis in the most sensitive nasal tissues) that are three times lower than background blood acetaldehyde levels (0.3 ± 0.03 μg/ml), 31 times lower than the lowest concentration shown to induce sister chromatid exchanges, and 124 times lower than the concentration that induces chromosomal aberrations (Fig. 7). This analysis suggests that practical thresholds must exist below which the cancer risk is negligible.

5. Conclusions

Developing a sound understanding of mode of action of chemical carcinogens is of paramount importance to scientifically based risk assessments and regulatory activities such as hazard classification, product safety labeling and setting occupational and ambient exposure limits. For carcinogenicity risk assessments, information on critical elements of a mode of action including genotoxicity, cytotoxicity, and cell proliferation and the development of quantitative dose–response relationships between these effects, tissue dosimetry and tumor outcome facilitates decisions regarding which of the elements are the primary drivers for carcinogenicity. These elements have been described for vinyl acetate and quantitative relationships among them assessed using a PBPK-based description of dosimeters for inhalation exposure. For the inhalation route of exposure intracellular acidification of target sites within the nasal epithelium appears to be a sentinel pharmacodynamic change. Secondary downstream responses such as mitogenesis, cytotoxicity, and cell proliferation are ultimately consequential. These secondary responses occur in the presence of high tissue exposures to acetaldehyde, but are experienced only under bioassay conditions where they eventually lead to tumorigenesis. It follows that decisions aimed at protecting the tissue from intracellular acidification will be protective of all the downstream effects of vinyl acetate inhalation exposure, including cancer. An analogous approach can be developed for assessing risk of exposure to vinyl acetate via oral ingestion.

Acknowledgements

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Review

Genetic and epigenetic damage induced by reactive nitrogen species: implications in carcinogenesis

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Abstract

Chronic infection and inflammation are recognized risk factors for human cancer at various sites. Infection and inflammation can activate and induce a variety of oxidant-generating enzymes, including NADPH oxidase and inducible nitric oxide synthase. Reactive oxygen and nitrogen species produced by such enzymes react with each other to generate new and more potent reactive species. These oxidants not only can damage DNA and induce mutations, but also can activate oncogene products and/or inactivate tumor-suppressor proteins, thus contributing to most processes of carcinogenesis. Appropriate treatment of inflammation should be further explored for chemoprevention of human cancers, especially those associated with chronic inflammation.
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Keywords: Infection; Inflammation; Cancer; Reactive nitrogen species; DNA damage; Inducible nitric oxide synthase

1. Chronic inflammation and cancer

About 16% (1 450 000 cases) of the worldwide incidence of cancer in 1990 has been estimated to be attributable to infection with various viruses, bacteria and parasites (Pisani et al., 1997). Chronic inflammation such as gastritis and hepatitis, often caused by chronic infection, and that induced by chemical and physical agents (e.g. cigarette smoking and asbestos exposure), and inflammatory reactions of uncertain etiology (e.g. ulcerative colitis, pancreatitis etc.) are also recognized risk factors for human cancers. Thus, a significant portion of the global cancer burden is attributable to chronic infection and inflammation.

In inflamed tissues, a variety of inflammatory cells, including neutrophils, basophils, eosinophils, macrophages and lymphocytes, are recruited and activated to produce potent oxidants primarily to attack and destroy invading microorganisms and foreign bodies. However, if infection is not resolved rapidly, or if control of the immune

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response is not well regulated, inflammation becomes chronic, which often causes extensive tissue damage, due to continuous production of excess active proteolytic enzymes and potent oxidants. Various oxidant-generating enzymes are induced and activated under such conditions, including NADPH oxidase and xanthine oxidase, which produce superoxide anion ($O_2^{\bullet -}$). Hydrogen peroxide (H_2O_2), produced from $O_2^{\bullet -}$, reacts with reduced transition metals (either free or bound to macromolecules such as DNA) to generate highly toxic hydroxyl radical ($HO^\bullet$) or metal-peroxide complexes (Me-OOH). $O_2^{\bullet -}$ also reacts with $NO^\bullet$ rapidly (reaction rate, 6.7×10^9–1.9×10^{10} M^{-1} s^{-1}) to form peroxynitrite anion ($ONOO^-$), which is a highly reactive nitrating and oxidizing species (Beckman and Koppenol, 1996; Ducrocq et al., 1999).

Inducible nitric oxide synthase (iNOS) also contributes to cytotoxicity of phagocytes. This enzyme is induced in a variety of human cells and tissues upon stimulation with lipopolysaccharide, cytokines and interferon-γ (Alderton et al., 2001; Bogdan, 2001). The enzyme utilizes L-arginine, NADPH and O_2 as substrates, producing nitrogen oxide, $NADP^+$ and citrulline. However, much controversy exists regarding whether NOS synthesizes nitric oxide ($NO^\bullet$) or not. Recent studies suggest that NOS does not produce $NO^\bullet$, but produces nitroxyl anion (NO^-) especially in the absence of the cofactor tetrahydrobiopterin (BH_4) (Rusche et al., 1998; Adak et al., 2000) and NO^- is then converted to $NO^\bullet$ by superoxide dismutase (SOD) or other electron acceptors (Schmidt et al., 1996; Hobbs et al., 1994). BH_4-free NOS can also reduce oxygen to form $O_2^{\bullet -}$ (Alderton et al., 2001; Huisman et al., 2002), whereas in the presence of BH_4 NOS produces $NO^\bullet$ using L-arginine as substrate (Rusche et al., 1998). Thus under certain circumstances NOS can produce both $NO^\bullet$ and $O_2^{\bullet -}$, which react with each other to form peroxynitrite (Fig. 1).

$NO^\bullet$, NO^- and peroxynitrite have significantly different reactivity towards many biological molecules (Beckman and Koppenol, 1996; Ducrocq et al., 1999; Ma et al., 1999). Several studies have shown that $NO^\bullet$ generated from $NO^\bullet$ donors is anti-inflammatory and cytoprotective, because

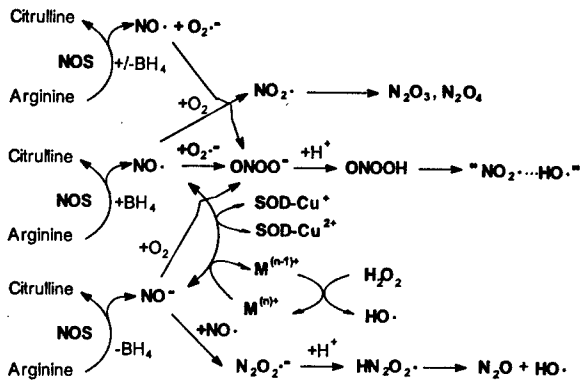

Fig. 1. BH_4-dependent formation of RNS by NOS.

$NO^\bullet$ can form complexes with transitional metals, inhibiting Fenton-type reactions (Yoshie and Ohshima, 1997) and also terminate radical chain propagation reactions of lipid peroxidation by radical–radical reactions between $NO^\bullet$ and lipid hydroperoxy radicals ($LOO^\bullet$) and alkoxyl radicals ($LO^\bullet$) (Rubbo et al., 1995). On the other hand, NO^- generated from an NO^- donor (Angeli's salt) exerts strong cytotoxic effects (Wink et al., 1998), possibly producing $HO^\bullet$ or generating peroxynitrite (Ohshima et al., 1999b). Overexpression of iNOS is often associated with inflammation and inhibition of iNOS leads to anti-inflammatory and cytoprotective effects in many experimental systems (Hobbs et al., 1999). This can be explained if NOS synthesizes cytotoxic NO^- and/or peroxynitrite, but not protective $NO^\bullet$, under pathophysiological conditions in which the level of BH_4 is reduced due to oxidative stress (Nakamura et al., 2001; Huisman et al., 2002).

2. DNA damage by RNS

$NO^\bullet$ can be converted, in the presence of oxygen, into the strong nitrosating agent N_2O_3, which can deaminate various DNA bases (e.g. guanine to xanthine and oxanosine, adenine to hypoxanthine, cytosine to uracil, 5-methylcytosine

to thymine). N_2O_3 can react with secondary amines to form carcinogenic *N*-nitrosamines, which can damage DNA by alkylation. Increased formation of nitrosamines has been reported to occur in vivo in experimental animals with acute and chronic inflammation as well as in human subjects with infection and inflammation (Ohshima and Bartsch, 1999).

Peroxynitrite anion ($ONOO^-$), a highly reactive species formed by diffusion-controlled coupling of $NO^\bullet$ with $O_2^{\bullet -}$ (Beckman and Koppenol, 1996; Ducrocq et al., 1999) can damage DNA by nitration, nitrosation and oxidation (Szabo and Ohshima, 1997). Treatment of isolated DNA and RNA with authentic peroxynitrite led to dose-dependent formation of 8-nitroguanine (Yermilov et al., 1995; Masuda et al., 2002). On the other hand, peroxynitrite induced only small increases in levels of some oxidized bases, including highly cytotoxic base-propenals (base-CH=CH–CHO) and 8-oxoguanine (Yermilov et al., 1995; Douki and Cadet, 1996). One possible explanation is that oxidized bases such as 8-oxoguanine are further oxidized by peroxynitrite to ring-cleavage products (Niles et al., 1999).

NO^-, generated by NOS in the absence of BH_4, can produce oxidants, possibly highly toxic hydroxyl radicals ($HO^\bullet$), which may be generated from the rapid reaction between NO^- and $NO^\bullet$ (reaction rate, $1.7 \times 10^9\ M^{-1}\ s^{-1}$) (Ohshima et al., 1999b). More recently, it has been shown that strong oxidants are generated from the NO^--generating agent, Angeli's salt, especially in the presence of H_2O_2 plus Fe(III)–EDTA or Cu(II) (Chazotte-Aubert et al., 1999). $NO^\bullet$ released from diethylamine-NONOate had no such effect. Distinct effects of $HO^\bullet$ scavengers and patterns of site-specific DNA cleavage caused by Angeli's salt alone or by Angeli's salt with H_2O_2 plus metal ion suggest that NO^- acts as a reductant to catalyze formation of $HO^\bullet$ from H_2O_2 plus Fe(III) and formation of Cu(I)–peroxide complexes with similar reactivity to $HO^\bullet$ from H_2O_2 and Cu(II). NO^- may be formed in vivo under a variety of physiological conditions, including by NOS. As stimulated immune cells including neutrophils and macrophages can produce H_2O_2, one can expect that, during an inflammatory process, the formation of both NO^- and H_2O_2 would dramatically enhance anti-microbial and tumoricidal activity.

Peroxynitrite and NO^- can also induce DNA strand breakage (Ohshima et al., 1998), which activates the nuclear enzyme poly-ADP ribosylase. Rapid activation of this enzyme results in depletion of its substrate, NAD^+, followed by prevention of ATP synthesis, leading to acute cell dysfunction and cell death (Szabo and Ohshima, 1997).

3. Mutations caused by RNS

The NOx-mediated deamination of DNA bases leads to a variety of mutations (Tannenbaum et al., 1994), the majority of point mutations being G:C to A:T transitions. Deamination of 5-methylcytosine at CpG by NOx results in formation of thymine, inducing G:C to A:T transitions at CpG, one of the most frequently detected mutations in the *p53* gene and the *Hprt* locus. However, it has been reported that 5-methylcytosine in codon 248 of the *p53* gene, which is one of the hot spots for G:C to A:T mutations at CpG sites, is not deaminated when human bronchial epithelial cells are exposed to an $NO^\bullet$-releasing compound (Felley Bosco et al., 1995). Recently, the G:C to A:T mutation at codon 248 of the *p53* gene was observed when cells were exposed to both an $NO^\bullet$-releasing compound and an $O_2^{\bullet -}$-generating hypoxanthine/xanthine oxidase system (Souici et al., 2000), suggesting that peroxynitrite may induce such a mutation. It is interesting to note that peroxynitrite can mediate both nitration and nitrosation (deamination) reactions (Masuda et al., 2000).

In addition to various in vitro studies showing that RNS are genotoxic, several in vivo systems also clearly show mutagenic effects of activated neutrophils and macrophages. RNS formed by activated macrophages were responsible for an increased mutation frequency observed in pUR288 transgenic C57BL/6 and SJL mice (Gal and Wogan, 1996). This increase was prevented by administration of *N*-methylarginine, an inhibitor of NOS. Recent studies have shown that iNOS of neutrophils infiltrating into tumors induced muta-

tions in the *Hprt* locus of tumor cells (Sandhu et al., 2000). The authors proposed that genotoxic RNS produced by tumor-infiltrating immune cells contribute to the burden of genetic abnormalities associated with tumor progression (Sandhu et al., 2000).

4. Epigenetic damage induced by RNS

RNS react with proteins to modify amino acid residues by oxidation, nitrosation and nitration. Modified forms of proteins with altered structure and function accumulate during aging, oxidative stress and some pathological conditions (Ischiropoulos, 1998; Davis et al., 2001). Tyrosine residues in protein react with various RNS to form 3-nitrotyrosine (NTYR) (Ohshima et al., 1990; Ischiropoulos, 1998; Ohshima et al., 1999a). Myeloperoxidase and eosinophil peroxidase can also nitrate tyrosine to form NTYR using H_2O_2 and nitrite (NO_2^-) as substrates (Eiserich et al., 1998; Wu et al., 1999). Other types of protein modification include interactions of RNS with thiols, metals and radical residues (Davis et al., 2001).

Alterations of protein structure and function induced by RNS may contribute to carcinogenesis. Many studies with antibodies against NTYR-containing proteins have shown that levels of nitrated proteins are elevated in inflamed tissues, including gastric mucosa of patients with *Helicobacter pylori*-induced gastritis (Pignatelli et al., 2001a; Li et al., 2001), as well as in plasma of lung cancer patients and cigarette smokers (Pignatelli et al., 2001b). Various proteins and enzymes have been reported to be nitrated, and this modification often results in loss of enzymic activity (Ischiropoulos, 1998; Davis et al., 2001). Mammalian cells incubated with excess NO• accumulate p53 tumor-suppressor protein but concomitantly this p53 loses its capacity to bind to its DNA consensus sequence (Calmels et al., 1997). This could be due to modification(s) of the p53 protein by NO•, including formation of disulfide bonds through *S*-nitrosylation (Calmels et al., 1997) and/or nitration of tyrosine residues (Chazotte-Aubert et al., 2000). Conversely, NO• is capable of activating the protooncogene c-Ha-ras p21 protein via *S*-nitrosation (Lander et al., 1995). These post-translational modifications of p53 and ras p21 proteins may be a consequence of overproduction of NO• in inflamed tissues. Inactivation of p53 through mutations occurs only in one-half of human cancer. Mutations of the *ras* gene are also found only in some tumors. In view of these observations, one can hypothesize that in some tumors carrying wild-type *p53* and *ras* genes, epigenetic events, such as inactivation of p53 protein and activation of ras p21 protein by overproduction of NO•, may play an important role in carcinogenesis.

Other roles of NO• and/or other reactive species in carcinogenesis include inhibition of important enzymes such as various DNA repair enzymes (Jaiswal et al., 2001) and caspases and other pro-apoptotic enzymes, which protect cells from apoptotic cell death (Mannick et al., 1994). NO• and/or other reactive species, however, can also activate other enzymes, such as telomerase, through which cells acquire replicative potential (Vasa et al., 2000), DNA methyltransferase, which suppresses gene expression (e.g. tumor-suppressor genes) (Hmadcha et al., 1999), and metalloproteases, which facilitate invasion by cancer cells into surrounding tissues (Okamoto et al., 2001). NO• can also activate the enzyme cyclooxygenase-2 which plays pivotal roles in the progression of a variety cancers through its participation in prostaglandin synthesis (Mei et al., 2000). In addition, stimulation of angiogenesis (Garcia-Cardena and Folkman, 1998) and suppression of immunity by inhibiting lymphocyte proliferation (Lejeune et al., 1994) are also mediated by RNS.

5. Conclusion

RNS not only can damage DNA and induce mutations, but also can participate in most carcinogenic processes by activating oncogene products and/or inactivating tumor-suppressor proteins. Thus, inflammation stimulates normal cells to initiate, grow and progress towards malignancy. Appropriate treatment of inflammation (e.g. by inhibition of iNOS induction, inhibition of iNOS activity, scavenging peroxynitrite and other RNS)

should be further explored for chemoprevention of human cancers, especially those associated with chronic inflammation.

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Review

Multiple pathways of peroxynitrite cytotoxicity

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Abstract

Peroxynitrite is a reactive oxidant produced from nitric oxide (NO) and superoxide, which reacts with a variety of biomolecules including proteins, lipids and DNA. Peroxynitrite is produced by the body in response to a variety of toxicologically relevant molecules including environmental toxins. It is also produced by the body in response to environmental toxins, as well as in reperfusion injury and inflammation. Here we overview the multiple pathways of peroxynitrite cytotoxicity. Initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane Na^+/K^+ ATP-ase activity, inactivation of membrane sodium channels, and other oxidative protein modifications contribute to the cytotoxic effect of peroxynitrite. In addition, peroxynitrite is a potent trigger of DNA strand breakage, with subsequent activation of the nuclear enzyme poly-ADP ribosyl synthetase or polymerase (PARP), with eventual severe energy depletion and necrosis of the cells. Studies conducted with peroxynitrite decomposition catalysts suggest that neutralization of peroxynitrite is of significant therapeutic benefit after exposure to various environmental toxins as well as in a variety of inflammatory and reperfusion disease conditions.
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Keywords: Nitric oxide; Superoxide; Mitochondria; Inflammation; Free radicals; Environmental toxins

1. The reactivity of peroxynitrite

Simultaneous generation of nitric oxide (NO) and superoxide favors the production of a toxic reaction product, peroxynitrite anion ($ONOO^-$) (Beckman et al., 1990; see for reviews Pryor and Squadrito, 1996; Szabó, 1996). In in vitro systems, the ratio of superoxide and NO determines the reactivity of peroxynitrite: excess of NO reduces the oxidation elicited by peroxynitrite (Rubbo et al., 1994; Miles et al., 1996). The end-products of specific oxidative processes triggered by peroxynitrite can be detected in vivo, suggesting in vivo formation of peroxynitrite. The oxidant reactivity of peroxynitrite is mediated by an intermediate with the biological activity of hydroxyl radical (Pryor and Squadrito, 1996).

The decomposition of peroxynitrite to nitrate is intimately coupled with the oxidation chemistry of this species, and both reactions have been the subject of recent investigations and intense debate. Peroxynitrite and its conjugate acid are strong

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oxidants, capable of effecting one- and two-electron reactions akin to those of $HO^{\bullet}$, nitrogen dioxide (NO_2), and nitrosonium cation. Oxidations of thiols (Radi et al., 1991), sulfides (Padmaja et al., 1996), transition metal complexes (Goldstein and Czapski, 1995), halide ions (Goldstein and Czapski, 1995), ascorbate (Barlett et al., 1995), olefins, benzenes, phenols (Halfpenny and Robinson, 1992; Ischiropoulos et al., 1992), and other aromatics by peroxynitrite have been described.

Peroxynitrite is a particularly effective oxidant of aromatic molecules and organosulfur compounds that include free amino acids and peptide residues. Cysteine and glutathione, which are significant components of antioxidant reservoirs, are converted to disulfides. Methionine is converted to sulfoxide or is fragmented to ethylene and dimethyldisulfide. Dimethyl sulfoxide is oxidized to formaldehyde. Tyrosine and tryptophan undergo one-electron oxidations to radical cations, which are competitively hydroxylated, nitrated, and dimerized (Ischiropoulos et al., 1992; Ramezanian et al., 1996). The formation of nitrotyrosine is particularly favorable, and the appearance of this product in biological samples is taken as diagnostic of exposure to peroxynitrite. Purine nucleotides are vulnerable to oxidation and to adduct formation (Douki and Cadet, 1996). For a more detailed review on the chemistry, decomposition and reactivity of peroxynitrite, peroxynitrous acid and its activated isomer (see Pryor and Squadrito, 1996; Groves, 1999).

In in vitro systems, peroxynitrite is highly reactive. Its reported activities include a rapid oxidation of sulfhydryl groups and thioethers, as well as nitration and hydroxylation of aromatic compounds, including tyrosine, tryptophan and guanine. While the reaction with the sulfhydryl groups is likely to represent a direct reaction of peroxynitrite, the tyrosine nitration probably occurs through a NO_2^+-like intermediate. The detection of 3-nitrotyrosine by analytical and immunological techniques has established that a marked increase in tyrosine nitration occurs in a wide variety of disease states (Greenacre and Ischiropoulos, 2001). Other reactions such as a reaction catalyzed by myeloperoxidase may also contribute to the net tyrosine nitration seen in pathophysiological states (Halliwell, 1997; Eiserich et al., 1998).

The various reactions of peroxynitrite when occurring during the reaction of peroxynitrite with enzymes, macromolecules and lipids, have been shown to influence cellular functions. For instance, tyrosine nitration may lead to dysfunction of nitrated proteins, as has been shown or suggested in the case of superoxide dismutase, cytoskeletal actin, neuronal tyrosine hydroxylase, cytochrome P450 and prostacyclin synthase (overviewed in Greenacre and Ischiropoulos, 2001). Oxidation of critical sulfhydryl groups is responsible for the inhibition of mitochondrial and cytosolic aconitase and other critical enzymes in the mitochondrial respiratory chain (Hausladen and Fridovich, 1994). There is also evidence that peroxynitrite can cause covalent modification of an active site thiol of glyceraldehyde-3-phosphate dehydrogenase (Mohr et al., 1994) and in creatine kinase (Konorev et al., 1998). Peroxynitrite-mediated nitration of myofibrillar creatine kinase activity may lead to contractile dysfunction of the heart (Mihm et al., 2001). Peroxynitrite-modified cellular proteins are subject to accelerated degradation via the proteosome (Grune et al., 1998).

Peroxynitrite has been shown to inhibit a variety of ion pumps including calcium pumps (Klebl et al., 1998), calcium-activated potassium channels and also membrane Na^+/K^+ ATP-ase activity (Muriel and Sandoval, 2000). These effects are likely to contribute to a global dysregulation of ion balance and a variety of related cellular functions in peroxynitrite-challenged cells.

The reaction of peroxynitrite with lipids leads to peroxidation (malondialdehyde and conjugated diene formation) and formation of nitrito-, nitro-, nitrosoperoxo- and/or nitrated lipid oxidation adducts (Rubbo et al., 1994).

It has been recently discovered that peroxynitrite potently oxidizes various biomolecules. Peroxynitrite-mediated oxidation of tetrahydrobiopterin (BH_4) to quinonoid 5,6-dihydrobiopterin has been demonstrated in vitro. A large proportion of the quinonoid isomer readily loses its side chain to form 7,8-dihydropterin which is not a cofactor for NO synthase. Thus, in endothelial

cells and other cell types, pathophysiologically low levels of BH_4 can promote a cycle of its own destruction mediated by NO synthase-dependent formation of peroxynitrite (Milstien and Katusic, 1999). This mechanism might contribute to vascular endothelial dysfunction induced by oxidative stress in various diseases. In vitro it has been reported that reaction of NADH with authentic peroxynitrite resulted in the formation of NAD^+ and superoxide and, thus, of hydrogen peroxide (Goldstein and Czapski, 2000). This reaction can both induce an imbalance in cellular pyrimidine nucleotide levels, as well as a positive feedback cycle of cytotoxic oxidant generation. Peroxynitrite mediated oxidation of catecholamines (Kerry and Rice-Evans, 1998) has also been described and may contribute to a variety of CNS and cardiovascular pathologies.

It is important to note that peroxynitrite can inhibit superoxide dismutase (Ischiropoulos et al., 1992; MacMillan-Crow et al., 1998; Yamakura et al., 1998), glutaredoxin (Aykac-Toker et al., 2001) and other antioxidant molecules and systems. Peroxynitrite-mediated depletion of one of the key cellular antioxidants, glutathione (Cuzzocrea et al., 1998) can lead to positive feedback cycles of intracellular oxidant generation and exacerbation of the oxidative cellular injury.

Oxidative stress may cause tissue injury through activation of the precursors of matrix metalloproteinase (proMMPs). Recent work suggests that the activation of proMMPs is triggered by peroxynitrite generation, via an extensive *S*-glutathiolation reaction (Okamoto et al., 2001). By inhibiting this reaction, peroxynitrite decomposition catalysts may reduce MMP activation, an important mechanism of tissue injury in inflammation and reperfusion.

An important interaction of peroxynitrite occurs with nucleic acids, with the production of 8-hydroxydeoxyguanosine or 8-nitroguanine. Peroxynitrite can also cause DNA cleavage in solutions of end-labelled DNA restriction fragments and can initiate DNA nicking in the supercoiled plasmid pBR322 (overviewed in Szabó and Ohshima, 1997). Peroxynitrite-induced DNA single strand breakage can activate the nuclear enzyme poly(ADP-ribose) polymerase, which can trigger a cellular suicide pathway (Szabó et al., 1996, 1997; overviewed in Virág and Szabó, 2002).

2. The cytotoxicity of peroxynitrite

Peroxynitrite is more cytotoxic than NO or superoxide in a variety of experimental systems. In fact, recent studies suggest that, peroxynitrite, and not NO, may be the ultimately cytotoxic species in many conditions. In cells exposed to authentic peroxynitrite or to compounds that simultaneously generate NO and superoxide, marked changes in the level of cellular energetics and DNA integrity occur (overviewed in Szabó, 1996).

In addition to being a terminal mediator of cell injury—also enhances and triggers a variety of pro-inflammatory processes. For example, peroxynitrite enhances the expression of ICAM-1 and *P*-selectin in human endothelial cells (Zingarelli et al., 1998), and it mediates the cytokine-induced IL-8 expression in human leukocytes (Zouki et al., 2001). In human neutrophils, peroxynitrite triggers the down-regulation of L-selectin expression, and up-regulation of CD11b/CD18 expression (Zouki et al., 2001). These effects are likely to be mediated, at least in part, by the ability of peroxynitrite to trigger and enhance nuclear factor kappa B (NF-κB) mediated pro-inflammatory signal transduction pathways (Matata and Galinanes, 2001). These alterations can culminate in a global dysregulation of cellular signal transduction pathways.

In pathophysiologically relevant situations—e.g. in macrophages that produce NO and superoxide, and thus peroxynitrite from endogenous sources—DNA strand breakage also occurs, and the time course of the strand breakage parallels the time course of NO and peroxynitrite production (Zingarelli et al., 1996). Moreover, in immunostimulated macrophages, (5-hydromethyl)uracil; 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-oxoguanine formation have been reported, indicating both oxidative and deaminative DNA injury (Rojas-Walker et al., 1995). In motor neurons, both axotomy and peroxynitrite exposure

leads to a time-dependent accumulation of DNA single strand breaks (Liu and Martin, 2001).

DNA single strand breakage, initiated by endogenous or exogenous peroxynitrite, is a potent trigger of PARP activation (overviewed in Virág and Szabó, 2002), which is a major contributor to cell necrosis under conditions of severe oxidative stress.

While exposure to high concentrations of peroxynitrite leads to rapid cell death, associated with rapid energetic derangements, lower concentrations of peroxynitrite, after several hours, can lead to apoptotic cell death which is dependent on cytochrome *c* release from the mitochondria and activation of caspases 3, 2, 8 and 9 (Virág et al., 1998, Zhuang and Simon, 2000).

In various tissues and organs, peroxynitrite elicits a variety of alterations. Table 1 overviews some of the peroxynitrite-mediated deleterious molecular, subcellular and cellular pathophysiological alterations. Peroxynitrite has been implicated in the pathogenesis of a wide variety of diseases and toxicologically relevant conditions. In the following sections, we will restrict our overview to a few selected conditions induced by environmental toxins (Chapter 3), ischemia–reperfusion (Chapter 4) and inflammatory conditions (Chapter 5).

3. The toxicological relevance of peroxynitrite

Recent work demonstrates the formation of peroxynitrite, and its potential pathogenetic relevance in cells or animals exposed to various environmental toxins. For example, in vitro studies by Liu et al. concluded that peroxynitrite formation contributes to the in vitro cytotoxicity induced by peroxyacetyl nitrate, an ubiquitous air pollutant (Liu et al., 1999; Lin et al., 2000). Similarly, benzene-induced cytotoxicity may involve peroxynitrite (Tuo et al., 1998). It has been suggested that the increased production of peroxynitrite during chronic inflammation combined with benzene exposure may increase the carcinogenicity of benzene by a mechanism that includes the formation of metabolites from the chemical reaction between benzene and peroxynitrite.

Table 1
Selected cytotoxic processes initiated by peroxynitrite

Action	Mechanism
On the molecular level	
Cytosolic enzyme inhibition	Oxidation, nitration
Membrane pump inhibition	Oxidation, nitration
Antioxidant enzyme inhibition	Oxidation, nitration
Signal transduction pathway disturbances	Oxidation, nitration
DNA injury	Oxidation, nitration, deamination, adduct formation
Surfactant protein damage	Nitration
Metalloproteinase activation	*S*-glutoxidation of pro-metalloproteinases
Antioxidant enzyme depletion	Glutathione, cysteine oxidation
Inhibition of BH4-dependent enzymes	BH4 oxidation
Inhibition of NAD-dependent enzymes	NAD oxidation
Lipid peroxidation	Peroxidation, lipid peroxide chain reactions
On the subcellular level	
Mitochondrial dysfunction	Inhibition of cytochromes, NADH-COQ1, etc.
NAD depletion	PARP activation, direct NAD oxidation
Upregulation of adhesion receptors	NF-κB activation
DNA fragmentation	DNA injury, caspase activation
Calcium dysregulation	Dysfunctional calcium pumps and cell energetics
On the cellular level	
Necrosis	Mitochondrial injury, energetic collapse, oxidation, nitration, antioxidant depletion, calcium dysregulation
Apoptosis	Mitochondrial injury, DNA injury, caspase activation, signal transduction disturbances, calcium dysregulation

Peroxynitrite formation has been implicated in various forms of pulmonary injury induced by respirable mineral dusts or asbestos fibers (Choe et al., 1998; Zhu et al., 1998; Tanaka et al., 1998; Morio et al., 2001), diesel exthaust particles (Bai et al., 2001) and ozone, bleomycin (Yamazaki et al., 1998). With respect to asbestos, studies in Chinese

hamster cells have demonstrated a link between peroxynitrite formation and mutagenicity (Park and Aust, 1998).

The toxicity of nitroarene 1,3-dinitrobenzene (1,3-DNB), a cerebellar neurotoxin in rats, is an interesting example of how peroxynitrite formation can mediate cytotoxicity in response to neurotoxins. 1,3-DNB is metabolized by the NADPH-cytochrome P450 reductase in liver. In a manner similar to NADPH-cytochrome P450 reductase, the neuronal NO synthase can interact with 1,3-DNB and generate superoxide anion radical. Therefore, NO, L-citrulline, and superoxide are simultaneously produced by the neuronal NO synthase in the presence of 1,3-DNB and other nitroarenes. The simultaneous production of NO and superoxide leads to peroxynitrite, and the resulting nitrosative stress plays a role in the cerebellar neurotoxicity of 1,3-DNB (Miller, 2002). There is also evidence that peroxynitrite formation in the hepatocytes may contribute to the hepatotoxic effects of various drugs and xenobiotics (Jaeschke et al., 2002).

4. The pathophysiological relevance of peroxynitrite: reperfusion injury

There is experimental evidence demonstrating the protective effect of peroxynitrite decomposition catalysts in various models of reperfusion injury. For example, Cuzzocrea et al. studied the protective effect of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(2,4,6-trimethyl-3,5-disulfonatophenyl)-porphyrinato iron (III) (FeTMPS) in a model of splanchnic artery occlusion (SAO) shock (Cuzzocrea et al., 2000). Administration of FeTMPS significantly reduced ischemia–reperfusion injury in the bowel, and reduced lipid and the production of peroxynitrite during reperfusion. Treatment with the peroxynitrite decomposition catalyst also markedly reduced the intensity and degree of *P*-selectin and ICAM-1 staining in tissue sections from SAO-shocked rats and improved survival.

As mentioned above, peroxynitrite is highly toxic to various cell types. Peroxynitrite infusion causes a reduction in myocardial contractility in isolated perfused hearts (Schulz et al., 1995) and induces an impairment of the endothelium-dependent relaxant ability (Villa et al., 1994). Our group has investigated the effects of FP15, a novel, potent, porphyrinic peroxynitrite decomposition catalyst (Szabó et al., 2002) in various animal models of disease. We have recently demonstrated the efficacy of FP15 in a large animal model of myocardial ischemia and reperfusion. Infarct size was significantly reduced (by $\approx 40\%$) in the FP15 treated group. FP15 provided a significant suppression of tyrosine nitration (a marker of peroxynitrite reactivity) in the ischemic myocardium, further confirming its mode of action (Bianchi et al., 2002).

Taken together, the above data demonstrate that peroxynitrite is an important contributor to various forms of reperfusion injury. It will be important to test the effect of potent peroxynitrite decomposition catalysts in other forms of reperfusion injury also (including stroke, renal ischemia–reperfusion, hemorrhagic shock—which is widely considered a form of whole body ischemia–reperfusion—as well as in a variety of other disease models of ischemia and reperfusion).

5. The pathophysiological relevance of peroxynitrite: inflammation

The earliest evidence demonstrating the protective effect of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(2,4,6-trimethyl-3,5-disulfonatophenyl)porphyrinato iron (III), was shown in the carrageenan-induced paw edema model, a model of acute inflammation in which peroxynitrite may play a major role (Salvemini et al., 1996). When tested in this system, the compound caused a dose-dependent reduction in swelling and lactate dehydrogenase release as well as a detectable shift to nitrate formation in paw tissue (Salvemini et al., 1998). Subsequent studies demonstrated the protective effect of the same compound in a model of experimental autoimmune encephalomyelitis, an animal model of the human disease multiple sclerosis. Mice receiving the peroxynitrite decomposition catalyst displayed less severe clinical disease, and less

inflammation and demyelination than control mice (Cross et al., 2001). In our own studies, we have found that FP15 also exerts protective effects in a wide variety of inflammation models, including murine models of endotoxic shock, collagen-induced arthritis and various experimental models of colitis (Mabley et al., 2002).

Taken together, the above data demonstrate that peroxynitrite is an important contributor to various forms of inflammation. It will be important to test the effect of potent peroxynitrite decomposition catalysts in other forms of inflammation also (including arthritis, endotoxic or septic shock—which is widely considered a systemic inflammatory disease—as well as in a variety of other models of inflammation).

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Short communication

Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention

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Abstract

Peroxynitrite is formed in biological systems when superoxide and nitric oxide are produced at near equimolar ratio. Although not a free radical by chemical nature (as it has no unpaired electron), peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration to inhibition of mitochondrial respiration. Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis with cellular energetics (ATP and NAD) serving as switch between the two modes of cell death. Peroxynitrite also damages DNA and thus triggers the activation of DNA repair systems. A DNA nick sensor enzyme, poly(ADP-ribose) polymerase-1 (PARP-1) also becomes activated upon sensing DNA breakage. Activated PARP-1 cleaves NAD^+ into nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins. Peroxynitrite-induced overactivation of PARP consumes NAD^+ and consequently ATP culminating in cell dysfunction, apoptosis or necrosis. This cellular suicide mechanism has been implicated among others in the pathomechanism of stroke, myocardial ischemia, diabetes and diabetes-associated cardiovascular dysfunction. Here, we review the cytotoxic effects (apoptosis and necrosis) of peroxynitrite focusing on the role of accelerated ADP-ribose turnover. Regulatory mechanisms of peroxynitrite-induced cytotoxicity such as antioxidant status, calcium signalling, NFκB activation, protein phosphorylation, cellular adaptation are also discussed.
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Keywords: Peroxynitrite; Cytotoxicity; Apoptosis; Necrosis; Poly(ADP-ribose) polymerase; Poly(ADP-ribose) glycohydrolase

1. Peroxynitrite production in biological systems

Nitric oxide ($NO^{\bullet}$) is a unique diffusible molecular messenger in the vascular and nervous system. $NO^{\bullet}$ is produced by a family of enzymes called nitric oxide synthetases (NOS) through enzymatic oxidation of the guanidino group of L-

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arginine (Marletta et al., 1998). This occurs in two sequential monooxygenase reactions utilizing NADPH as cosubstrate and involving the utilization of molecular oxygen (Marletta et al., 1998). Constitutive expression of two NOS isoforms is responsible for a low basal level of $NO^{\bullet}$ synthesis in neural cells (nNOS or NOS1) and endothelial cells (eNOS or NOS3). These constitutively expressed NOS isoforms require calcium for their enzymatic activity. Induction of the inducible isoform (iNOS or NOS2) by cytokines and/or bacterial products (endotoxin/LPS) has been observed in virtually all cell types tested including macrophages, dendritic cells, fibroblasts, chondrocytes, osteoclasts, astrocytes, epithelial cells and results in the production of large amounts of NO (Nathan, 1997). Mitochondria also contain a unique NO producing enzyme (mtNOS) (Ghafourifar and Richter, 1997). Although enzymatic $NO^{\bullet}$ production accounts for the bulk of $NO^{\bullet}$ formed in biological systems, alternative pathways also exist. In the skin, for example, non-enzymatic reduction of sweat nitrite has been shown to give rise to $NO^{\bullet}$ production (Weller et al., 1996).

Whereas the physiological effects (e.g. vasorelaxation, neuronal signalling) of $NO^{\bullet}$ are mostly mediated by the activation of guanylate cyclase (Arnold et al., 1977), the mechanism of pathophysiological effects is much more complex. A heavily debated feature of $NO^{\bullet}$ is its cytotoxic effect. The controversy arises from observations reporting both cytotoxic and cytoprotective effects of $NO^{\bullet}$ depending on variables of the assay systems used. In cases where $NO^{\bullet}$ was found cytotoxic, it was questioned whether $NO^{\bullet}$ directly or indirectly, through the formation of more reactive oxidative species such as peroxynitrite exerted its cytotoxic effects (Beckman and Koppenol, 1996). Peroxynitrite ($ONOO^-$) is formed when $NO^{\bullet}$ and superoxide anion react in a near diffusion-limited reaction (Beckman and Koppenol, 1996). Sources of superoxide include the mitochondrial respiratory chain where there is a constant leak of superoxide, NADPH oxidases, xanthine oxidase and autoxidation of several biomolecules such as catecholamines or myoglobin. The most powerful cellular antioxidant system protecting against the harmful effects of superoxide is embodied by superoxide dismutases (SOD) (CuZnSOD in the cytosol and MnSOD in the mitochondria). However, it was shown that $NO^{\bullet}$ efficiently competes with SOD for superoxide (Beckman and Koppenol, 1996). Joseph Beckman has therefore proposed (Beckman and Koppenol, 1996) that under conditions of increased $NO^{\bullet}$ production, $NO^{\bullet}$ can outcompete SOD for superoxide resulting in peroxynitrite ($ONOO^-$) formation.

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^-$$

As both excess $NO^{\bullet}$ or excess superoxide decreases the bioavailability of peroxynitrite, equimolar concentrations of the radicals are ideal for peroxynitrite formation (Radi et al., 2001). Peroxynitrite anion ($ONOO^-$) is in a pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH). Homolysis of ONOOH gives rise to formation of the highly reactive hydroxyl radical ($^{\bullet}OH$) mediating molecular and tissue damage associated with peroxynitrite production (Radi et al., 2001).

2. Peroxynitrite-induced apoptosis

Despite of its non-radical nature, peroxynitrite is more reactive than its parent molecules. Peroxynitrite initiates lipid peroxidation (Radi et al., 1991b), causes DNA breakage (Salgo et al., 1995) and reacts with thiols (Radi et al., 1991a). Peroxynitrite-induced protein modifications include protein oxidation (on methionine, cysteine, tryptophane or tyrosine residues) and nitration (of tyrosine or tryptophane residues). However, enzymes containing a redox active transition metal center are the prime targets of the oxidant (Beckman and Koppenol, 1996). Reactions of peroxynitrite are affected by the local pH and the microenvironment with hydrophobic membrane compartments favoring nitration and aqueous environments favoring oxidation. Moreover, carbon dioxide reacts with peroxynitrite resulting in the formation of nitroso-peroxocarbonates (Radi et al., 2001). The ubiquitous presence of CO_2 at high concentration may favor this reaction route. As nitroso-peroxocarbonates divert peroxynitrite-induced protein modifications toward nitration,

CO_2 is now considered as key determinant of peroxynitrite chemistry.

When peroxynitrite-induced cellular damage reaches a level where it cannot be handled by the repair mechanisms, cells undergo one of the basic cell death pathways, apoptosis or necrosis. Apoptosis is the 'default' death pathway characterized, among other parameters, by a compact morphology, maintenance of plasma membrane integrity, mitochondrial depolarization, secondary oxidant production, activation of caspases (*c*ysteinyl *as*partate specific prote*ases*) and oligonucleosomal DNA fragmentation (Green and Kroemer, 1998). The first report indicating that peroxynitrite can trigger apoptotic death came from Pryor's laboratory. They have detected DNA fragmentation in peroxynitrite treated thymocytes (Salgo et al., 1995). Later, activation of caspase-3, a key player in the caspase cascade has also been detected in thymocytes (Virag et al., 1998b) and HL-60 cells (Virág and Szabó, 1998c; Lin et al., 1998). These early reports have later been followed by a series of publications on the cytotoxic effects of peroxynitrite in lymphoblastoid cells (Li et al., 2002), dopaminergic SH-SY5Y cells (Saeki et al., 2000; Yamamoto et al., 2002), human aortic endothelial cells (Foresti et al., 1999), osteoblasts (Reiff et al., 2001), HaCaT keratinocytes (Szabó et al., 2001), cardiac myocytes (Arstall et al., 1999) human and rat islet cells (Hadjivassiliou et al., 1998). Prototypical apoptosis models utilize apoptosis inducers such as tumor necrosis factor or FAS ligand acting upon cell surface death receptors. Channeling the death signal from these receptors to apoptotic effector machineries is well described (Green and Kroemer, 1998). However, it is not quite clear, how peroxynitrite triggers the apoptotic machinery. Mitochondria are likely sites for peroxynitrite-induced apoptosis initiation. Mitochondria are now recognized as central organizators of apoptosis (Green and Kroemer, 1998). A characteristic sequence of events including opening of mitochondrial permeability transition pore, mitochondrial depolarization, secondary superoxide production, release of apoptotic mediators from the intermembrane space to the cytoplasm, takes place in apoptosing cells (Green and Kroemer, 1998). Some of the mitochondria-derived apoptogenic factors act as nucleases (e.g. endonuclease G), nuclease activators (e.g. apoptosis-inducing factor, cytochrome *c*), or serine proteases (e.g. Omi/HtrA2) (Fig. 1). Peroxynitrite was found to inhibit the mitochondrial respiratory chain by inactivating complexes I–III (Lizasoain et al., 1996). Furthermore, adenosine nucleotide translocator, a member of the permeability pore is also targeted by peroxynitrite (Vieira et al., 2001). The characteristic mitochondrial perturbations have also been described in peroxynitrite treated cells (Virag et al., 1998b). The role of mitochondria in peroxynitrite-induced apoptosis is also supported by findings that bcl-2, a mitochondrial antiapoptotic protein inhibits peroxynitrite-induced apoptosis (Virág and Szabó, 2000; Spear et al., 1998). The cellular energetics may become compromised by peroxynitrite also via alternative mechanisms (e.g. inactivation of creatine kinase in cardiomyocytes) which may also contribute to peroxynitrite cytotoxicity (Mihm et al., 2001a).

Recent reports from Bauer's laboratory indicate a possible role for free 3-nitrotyrosine, in peroxynitrite-induced apoptosis (Mihm et al., 2000). They found that preincubation of rat thoracic aorta segments with 3-nitrotyrosine resulted in selective, concentration-dependent impairment of acetylcholine-induced vasorelaxation indicative of endothelial dysfunction. Moreover, nitrotyrosine triggered DNA damage in the endothelial cells (Mihm et al., 2000) and proved to be neurotoxic in vivo (Mihm et al., 2001b). These data suggest that nitrotyrosine, released from proteins nitrated by peroxynitrite, is more than a benign biomarker in vivo, and may contribute to vascular endothelial dysfunction and neurotoxicity through promotion of DNA damage and/or apoptosis (Mihm et al., 2000). Nonetheless, the exact mechanism of peroxynitrite-induced apoptosis initiation remains to be investigated. The executional phase of apoptosis is carried out by caspases which are likely to be activated by mitochondria-derived apoptogenic factors in peroxynitrite treated cells (Green and Kroemer, 1998). Caspase activation has also been reported to occur during the course of peroxynitrite-induced apoptosis (Virag et al., 1998b; Virág and Szabó, 1998c; Lin et al., 1998). A detailed analysis of caspase activation has revealed that

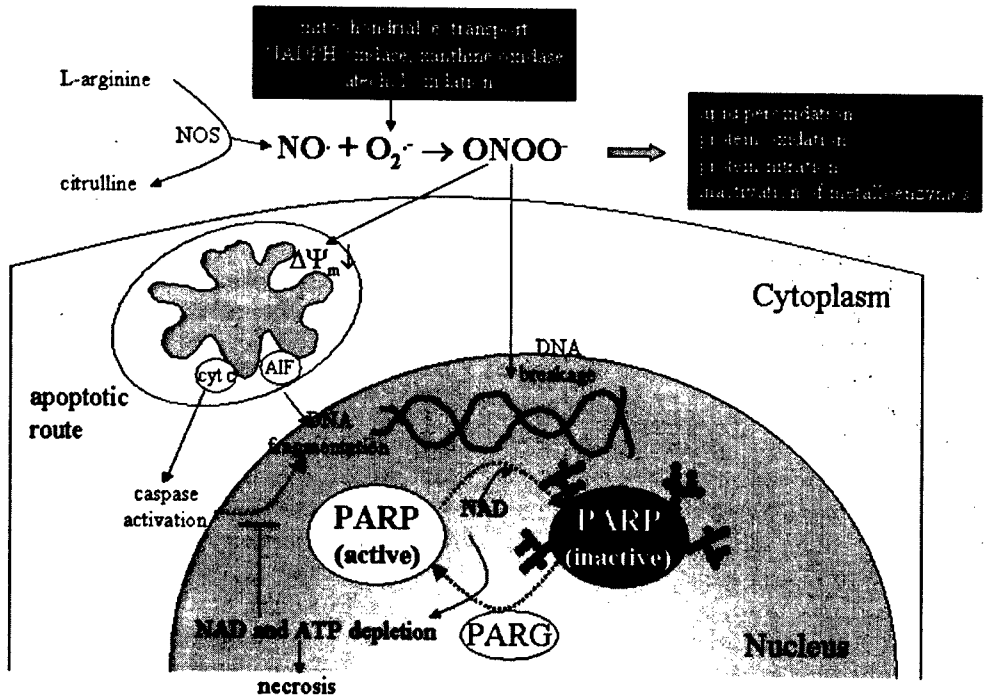

Fig. 1. Peroxynitrite-induced cytotoxic pathways. Nitric oxide and superoxide react to form peroxynitrite which damages cells via various damaging effects such as lipid peroxidation, inactivation of metalloenzymes and other proteins by oxidation and nitration. Peroxynitrite also acts on mitochondria triggering the release of proapoptotic factors such as apoptosis-inducing factor (AIF) and cytochrome *c*. These factors mediate caspase dependent and independent apoptotic death pathways. Moreover, peroxynitrite-induced DNA breakage activates PARP leading to NAD and ATP depletion and consequently to necrosis.

caspase 3-like proteases and (to a lesser extent) caspase 2, but not caspase 1 or caspase 6, are required for peroxynitrite-induced apoptosis (Zhuang and Simon, 2000).

3. Peroxynitrite-induced necrosis: role of poly(ADP-ribose) polymerase-1

Whilst low concentrations of peroxynitrite trigger apoptosis, higher concentrations of the oxidant compromise the apoptotic machinery forcing the cells to die by necrosis (Virag et al., 1998a,b; Bonfoco et al., 1995). For a long time, necrosis was thought to be a passive process resulting from the inability of the cells to cope with high degree of oxidative stress. Recently, a new paradigm has emerged identifying an active element in oxidative stress-induced necrosis. According to this concept, degree of the activation of poly(ADP-ribose) polymerase-1 (PARP-1) determines the fate of the oxidatively-injured cells (Virag and Szabo, 2002). PARP-1 is activated by DNA strand break. Activated PARP-1 catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins. The branched polymer, the size of which varies from a few to 200 ADP-ribose units, may facilitate recruitment of DNA repair enzymes to the sites of DNA injury (Virag and Szabo, 2002). In vivo the most abundantly poly-ADP-ribosylated protein is PARP-1 itself and auto-poly(ADP-ribosylation) represents

a major regulatory mechanism for PARP-1 resulting in the downregulation of the enzyme activity. The polymer is degraded by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase with the latter enzyme removing the protein proximal ADP-ribose residue (Virag and Szabo, 2002). The concerted action of PARP-1 and PARG maintains a highly accelerated ADP-ribose turnover in peroxynitrite treated cells. As a result, NAD becomes depleted in the cells leading to malfunctioning glycolysis, Krebs cycle, mitochondrial electron transport and eventually to ATP depletion (Berger et al., 1986). Moreover, shortage on ATP is exaggerated by attempts of the cells to resynthesize NAD from ATP and nicotinamide. The net result of this pathway is a dramatic drop in cellular ATP (Berger et al., 1983). As the apoptotic machinery is known to depend on ATP (Volbracht et al., 1999; Nicotera et al., 1998, 2000), apoptosis is incapacitated and necrosis takes predominance. This cellular suicide hypothesis described by Nathan Berger (Berger et al., 1983) has been applied by our group to peroxynitrite cytotoxicity. We have used mouse thymocytes (and later several other cell types, too) to validate the role of PARP-1 in peroxynitrite-induced cytotoxicity. Low concentrations (<20 μM) of peroxynitrite caused apoptotic thymocyte death characterized by phosphatidylserine exposure, caspase activation and DNA fragmentation (Virag et al., 1998b). At higher concentrations of peroxynitrite, however, PARP activation and ATP depletion occurred, apoptotic parameters (DNA fragmentation, caspase activation) declined and necrotic death occurred as indicated by the breakdown of plasma membrane integrity (Virag et al., 1998b). Inhibition of PARP-1 by 3-aminobenzamide or the absence of PARP-1 in PARP-1 deficient thymocytes resulted in dramatic protection against the loss of plasma membrane integrity (necrosis). In the same time, output of apoptotic parameters (DNA fragmentation and caspase activation) increased in cells treated with PARP inhibitors or in PARP-1 deficient cells (Virag et al., 1998b). These findings indicate that PARP-1 activation diverts the default apoptotic process toward necrosis.

An interesting new finding of our work was to establish that similarly to apoptosis, peroxynitritrite-induced, PARP-1 mediated necrotic death is also accompanied by mitochondrial alterations (collapse of mitochondrial membrane potential, overproduction of superoxide and mitochondrial membrane damage) and calcium mobilization (Virag et al., 1998a). Inhibition or the absence of PARP-1 provided remarkable protection from derailment of mitochondrial functions indicating the central role of PARP-1 in peroxynitrite-induced mitochondrial perturbation (Virag et al., 1998a).

The deterioration of cellular energetic status may play a central role in the 'cell death switch role' of PARP-1. This hypothesis is supported by findings that cellular ATP levels determine the mode of cell death (apoptosis versus necrosis) (Bonfoco et al., 1995; Leist et al., 1997; Nicotera et al., 1998, 2000). Moreover, a recent report from Swanson's laboratory supports a central role of cellular energy homeostasis in PARP-1 mediated cell death (Ying et al., 2002). In these experiments, mouse cortical astrocyte and astrocyte-neuron cocultures were treated with the DNA alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in order to activate PARP-1. Studies using the 2-deoxyglucose method confirmed that glycolytic flux was reduced by more than 90% in MNNG-treated cultures. The addition of 5 mmol/l of alpha-ketoglutarate, 5 mmol/l pyruvate, or other mitochondrial substrates to the cultures after MNNG treatment reduced cell death from approximately 70% to near basal levels (Ying et al., 2002).

4. Regulation of peroxynitrite-induced cytotoxicity

The mechanism of peroxynitrite-induced cytotoxicity is cell type dependent. The ratio of PARP dependent and PARP independent pathways varies between different cell types with thymocytes and other primary lymphoid cells representing one end of the spectrum (mostly PARP independent) and HL-60 myeloid cell line standing at the other end (no PARP dependence). Of note, out of the many cell lines tested in our laboratory, HL-60

cells were the only ones not protected by PARP inhibitors from peroxynitrite-induced cytotoxicity. The question arises, what the underlying principle behind the differential peroxynitrite sensitivity of the various cell types may be? These factors may or maynot be linked to PARP activation.

4.1. PARP dependent factors

Thymocytes represent an ideal cellular model for the analysis of PARP dependent resistance factors as in this cell type, peroxynitrite-induced cell death is mainly PARP dependent (Virag et al., 1998b). We have reported that TPEN, a zinc chelator, inhibits peroxynitrite-induced PARP activation and necrosis (Virág et al., 1999a). The mechanism of cytoprotection is not known. However, given that two zinc finger motives are responsible for DNA binding of PARP-1, TPEN may interfere with this process. Furthermore, peroxynitrite induces calcium mobilization both from intra- and from extracellular sources and intracellular calcium chelation protects from peroxynitrite-induced PARP activation and necrosis (Virag et al., 1999b). In these experiments, calcium chelators abolished peroxynitrite-induced DNA breakage indicating that they may act upstream to PARP activation. In a cell-free system, calcium chelators did not inhibit peroxynitrite induced DNA breakage (Virag et al., 1999b). These findings support the hypothesis that calcium signalling triggers secondary events leading to DNA damage and subsequently PARP activation. These secondary events may include production of secondary ROI (reactive oxygen intermediates) in the mitochondria. This novel concept implies that although peroxynitrite can directly break DNA in cell-free system, the mechanism of DNA breakage may be fundamentally different in a cellular environment. A calcium-dependent, mitochondrial production of secondary oxygen radicals has been reported in other cellular models of oxidative stress (Guidarelli et al., 2000a,c).

We have also identified purines (hypoxanthine > inosine > adenosine) as potential endogenous PARP inhibitors (Virag and Szabo, 2001). This observation may have implication for ischemia reperfusion injury where these substances reach high enough concentrations during ischemia to modulate PARP activation by ROI overproduced during the reperfusion phase. Recently, we have identified cell density signalling as a new factor regulating peroxynitrite sensitivity of HaCaT keratinocytes. We have showed that subconfluent (10–95%) cultures are more sensitive to peroxynitrite or hydrogen peroxide-induced cell death than confluent monolayers. The resistance to oxidative stress provided by high cell density involved both inhibition of caspase activation and PARP activation but not protein kinase C signalling. Our data may explain the resistance to oxidative stress of superficial, highly differentiated keratinocytes and may indicate that basal proliferative keratinocytes are possible sensitive in vivo targets of oxidative stress injury. By virtue of the epidermal calcium gradient (increasing calcium concentration in baso-superficial direction), our data also raise the possibility that calcium signalling and density-dependent signalling are interrelated.

Recently a new regulatory element of PARP activation has been identified. It has long been known that PARP-1 auto-poly-ADP-ribosylates itself leading to downregulation of enzyme activity. By removing inhibitory ADP-ribose residues from PARP-1, PARG may reactivate PARP-1 and thus may help maintain a high NAD/ADP-ribose turnover. This hypothesis has recently been tested by Swanson's group reporting that PARG inhibition by gallotannin and nobotanin B protected astrocytes and neuronal cells from oxidative stress (Ying and Swanson, 2000; Ying et al., 2001). We have confirmed these data in HaCaT keratinocytes and A549 pulmonary epithelial cells and found similar cytoprotection by gallotannin (submitted for publication).

Two studies have reported that peroxynitrite induces the expression of heat shock proteins. One of the studies has shown that the cytoprotective effect of the heat shock response is related to inhibition of PARP-1 activation (Szabo et al., 1996b). It is worthwhile that nitric oxide and peroxynitrite were found to have different effect on heat shock protein 70 expression in human monocytes with peroxynitrite inducing and nitric oxide not affecting HSP 70 expression (Adrie et

al., 2000). However, the mechanism by which heat shock inhibits PARP activation and other cytotoxic pathways remains to be elucidated.

4.2. PARP independent factors

The overall antioxidant status obviously determines the sensitivity of cells toward peroxynitrite toxicity. The importance of glutathione is supported by several observations. Increased glutathione levels, as achieved by administration of gamma-glu-cys-ethyl ester, has been shown to protect cortical synaptosomes from peroxynitrite-induced damage (Drake et al., 2002). Furthermore, depletion of cellular glutathione pools by buthionine sulfoximide sensitizes cells and animals to peroxynitrite toxicity or peroxynitrite-mediated inflammatory tissue injury, respectively (Cuzzocrea et al., 1998). In an in vivo model of myocardial ischemia, coadministration of ascorbic acid with glutathione methyl ester (GSHme) markedly enhanced the protective effects of GSHme, although ascorbic acid alone had no effect (Gao et al., 2002). The protection exerted by the combination of GSHme and ascorbic acid was significantly greater than that observed with 1 mM GSHme alone. Moreover, treatment with GSHme alone or GSHme plus ascorbic acid markedly reduced myocardial nitrotyrosine levels, suggesting that these treatments attenuated myocardial peroxynitrite formation (Gao et al., 2002). Manganese- (Szabo et al., 1996a; Ferrer-Sueta et al., 1999) or ferrous porphyrine compounds (Shimanovich and Groves, 2001; Salvemini et al., 1998) often sold as superoxide dismutase mimetics or peroxynitrite decomposition catalysts also protect from peroxynitrite. Several other antioxidants such as ebselen (Roussyn et al., 1996; Masumoto and Sies, 1996; Sata et al., 1997) or melatonin (Gilad et al., 1997; Cuzzocrea et al., 1997) as well as phytopharmacons (plant-derived antioxidants) (Choi et al., 2002a,b; Valdez et al., 2002) have also been shown to protect from the deleterious effects of peroxynitrite.

Peroxynitrite-induced apoptotic death can be prevented by classical apoptosis inhibitors such as caspase inhibitors (Virag et al., 1998b; Zhuang and Simon, 2000) or bcl-2 overexpression (Virág and Szabó, 2000; Spear et al., 1998). Although bcl-2 has been reported to provide protection against both apoptotic and necrotic stimuli, we have shown that it protected thymocytes from peroxynitrite-induced apoptotic but not against PARP-1 mediated necrotic death (Virág and Szabó, 2000).

In addition to antioxidants and direct antiapoptotic interventions other alternatives may also exist to modulate peroxynitrite-triggered cytotoxic pathways. The most intensively studied pathway is the inhibition of tyrosine kinase cascades by peroxynitrite-mediated tyrosine nitration (Beckman, 1996). Nitration of critical tyrosine residues by peroxynitrite in tyrosine kinase substrates interferes with the phosphorylation of the proteins and may inhibit downstream signalling events (Gow et al., 1996; Kong et al., 1996). Peroxynitrite has been shown to activate several signal transduction pathways. For example, peroxynitrite triggers the activation of various types of kinases, the G protein-phosphatidylinositol 3 kinase (PI3 kinase) pathway and phospholipase A2 (Klotz et al., 2000; Kaji et al., 2002; Guidarelli et al., 2000b). Many of these signalling pathways have been implicated in the regulation of cell death (Cross et al., 2000; Holmstrom and Eriksson, 2000; Sarmay, 2002). The G receptor-coupled PI3 kinase activation pathway for example was proposed to counteract peroxynitrite toxicity in primary rat astrocytes, a cell type expressing opioid receptors. Treatment of cells with morphine significantly protected astrocytes from apoptosis mediated by the peroxynitrite donor SIN-1, whereas it did not in other types of cells including C6 glioma, RAW 264.7, and HL-60 cells (Kim et al., 2001). The effects of morphine on SIN-1-induced cytotoxicity were inhibited by pretreatment with the G(i) protein inhibitor, pertussis toxin, and the PI3 kinase inhibitors, wortmannin and LY294002 (Kim et al., 2001). These results suggest that morphine may protect primary rat astrocytes from peroxynitrite-induced cytotoxicity via the signalling cascades that involve both G protein and PI3 kinase.

Maeda's group has investigated the activation of mitogen-activated protein kinase (MAP kinase) in relation to cell death induced by peroxynitrite in human neuroblastoma SH-SY5Y cells (Saeki et al., 2000). Exposure of the cells to peroxynitrite

caused transient increase in MAP kinase activity, and resulted in cell death. PD98059, a selective inhibitor of MAP kinase kinase, reduced peroxynitrite-induced cell death suggesting that activation of MAP kinase may be involved in cell death induced by peroxynitrite (Saeki et al., 2000). Furthermore, stimulation of several growth factor receptors for example by insulin like growth factor (Saeki et al., 2002), acidic fibroblast growth factor (Reiff et al., 2001), fibroblast growth factor-1 (Spear et al., 1998) or nerve growth factor (Spear et al., 1997, 1998) has also been found to modulate (in most cases to protect from) peroxynitrite toxicity. In the case of epidermal growth factor receptor (EGFR), peroxynitrite was found to crosslink the receptors in A431 epidermoid carcinoma cells resulting in dimer formation. Covalent EGFR dimerization by peroxynitrite probably involved intermolecular dityrosine cross-linking and was enhanced after receptor activation with epidermal growth factor. Furthermore, irreversibly cross-linked EGFR was more extensively tyrosine-phosphorylated compared with the monomeric form. However, exposure of A431 cells to peroxynitrite markedly reduced the kinetics of tyrosine phosphorylation of a downstream EGFR substrate, phospholipase C-gamma1. This study indicates that peroxynitrite may also interfere with tyrosine kinase pathways with a mechanism different from tyrosine nitration.

An interesting issue is the potential regulatory role of NFκB in peroxynitrite cytotoxicity. NFκB is a redox-sensitive transcription factor regulating the expression of various inflammatory mediators (Janssen-Heininger et al., 2000). The antiapoptotic effects of NFκB activation are also well documented (Aggarwal, 2000; Mattson et al., 1997), however, the outcome (death promotion or prevention) is cell type and stimulus dependent (Bours et al., 2000). The parent molecules of peroxynitrite appear to regulate NFκB activation in an opposing manner with superoxide (likely via hydrogen peroxide formation) activating and nitric oxide inhibiting NFκB activation (Schmidt et al., 1995; von Knethen et al., 1999; Marshall and Stamler, 2002). Inhibition of NFκB activation by NO-mediated nitrosylation has—at least in part—been made responsible for the proapoptotic effect of NO (Marshall and Stamler, 2002). Furthermore, immunoprecipitation studies showed that NO stabilized the NF-kappa B inhibitor, I kappa B alpha, by preventing its degradation from NF-kappa B (Peng et al., 1995). NO also increased the mRNA expression of I kappa B alpha (Peng et al., 1995). The contrasting roles of superoxide and NO on NFκB activation point toward the importance of understanding conditions regulating peroxynitrite formation. Peroxynitrite itself has also been found to activate NFκB (Matata and Galinanes, 2002). However, the role of NFκB in the regulation of peroxynitrite-induced cell death has not yet been investigated in detail. Only one study addressed the issue and found that peroxynitrite treatment did not activate NFκB in IEC-6 enterocytes but inhibition of NFκB by transfection with AdIκB, a mutated IκB functioning as a superrepressor of NFκB activation, significantly enhanced peroxynitrite-induced apoptosis in IEC-6 cells (Potoka et al., 2002).

Chronic exposure of cells to sublethal peroxynitrite concentrations (e.g. in inflammation) may also allow cells to develop adaptive responses to oxidative stress. This kind of regulation may involve upregulation of defense proteins/antioxidant enzymes. In A549 cells, peroxynitrite has been shown to trigger expression of mRNA for MnSOD, a key antioxidant enzyme (Jackson et al., 1998). However, MnSOD protein and enzyme activity were not changed. In Dr. Ischiropoulos's lab, a peroxynitrite resistant cell line has been generated by repeated exposure of the cells to sublethal concentrations of peroxynitrite. DNA chip technology will permit large-scale analysis of changes in gene expression pattern of peroxynitrite resistant cultures and may provide insight into the mechanism of cellular adaptation to oxidative stress situations.

5. Conclusions

Peroxynitrite has been implicated in the pathomechanism of various diseases. However, the lack of specific tools to unequivocally verify whether peroxynitrite is indeed produced in all of these

conditions and is responsible for at least part of the tissue injury has put peroxynitrite in the center of heated debates. Nonetheless, as evidence supporting the pathophysiological role of peroxynitrite is constantly increasing, targeting peroxynitrite-induced cytotoxic pathways is now accepted as a viable strategy to alleviate disease signs in numerous diseases. Which one of the above listed interventions or combinations of them provides the highest therapeutic benefit with the least risk remains to be seen.

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Review

Defenses against peroxynitrite: selenocompounds and flavonoids

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Abstract

The inflammatory mediator peroxynitrite, when generated in excess, may damage cells by oxidizing and nitrating cellular components. Defense against this reactive species may be at the level of prevention of the formation of peroxynitrite, at the level of interception, or at the level of repair of damage caused by peroxynitrite. Several selenocompounds serve this purpose and include selenoproteins such as glutathione peroxidase (GPx), selenoprotein P and thioredoxin reductase, or low-molecular-weight substances such as ebselen. Further, flavonoids, such as (−)-epicatechin, which occurs in green tea or cocoa as monomer or in the form of oligomers, can contribute to cellular defense against peroxynitrite.
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1. Introduction

The generation of reactive oxygen species is part of the inflammatory response and occurs at the NADPH oxidase complex expressed in phagocytic cells; upon stimulation, the enzyme generates superoxide from oxygen at the expense of NADPH. Superoxide, in turn, may dismutate, either spontaneously or catalyzed by superoxide dismutases (SOD), to form hydrogen peroxide which is then a possible source of other reactive oxygen species. Nitrogen monoxide (nitric oxide; NO) is also generated in cells involved in the inflammatory response by action of NO synthases in inflammatory immune cells (iNOS) or in the vascular endothelium (eNOS). The reaction of NO with superoxide results in the generation of peroxynitrite ($ONOO^-$), and due to the high second-order rate constant for this reaction of about 1×10^{10} M^{-1} s^{-1}, this reaction, as pointed out by Koppenol (1998), is very likely to occur even in the presence of physiological concentrations of SOD. Catalysis of superoxide disproportionation occurs with a rate constant of approximately 3×10^9 M^{-1} s^{-1} (for Cu, Zn-

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SOD). For a recent comprehensive review on peroxynitrite biochemistry, see Radi et al. (2001).

In addition to the reaction outlined above, peroxynitrite generation was also hypothesized to be feasible with NO synthases (see Alderton et al., 2001, and references therein), which may generate superoxide at the expense of NADPH in a reaction uncoupled from NO synthesis. Further, NO synthases have been described to generate nitroxyl anion (NO^-) also, which would react with molecular oxygen to form peroxynitrite (Kirsch and de Groot, 2002; Klotz and Sies, 2002; Schmidt et al., 1996).

Peroxynitrite is a strong oxidant and nitrating species, the formation of which may be beneficial in inflammatory reactions in terms of an oxidative destruction of intruding microorganisms. Higher concentrations and an uncontrolled generation of peroxynitrite, however, may result in unwanted oxidation and consecutive destruction of host cellular constituents. Peroxynitrite may oxidize and covalently modify all major types of biomolecules, including DNA (Inoue and Kawanishi, 1995; Salgo et al., 1995), proteins (Ischiropoulos and Al-Mehdi, 1995; Grune et al., 2001; Kröncke et al., 2002) and lipids (Radi et al., 1991) as well as various low-molecular-weight biomolecules. Peroxynitrite-induced modification of proteins has been connected with the development of several diseases (for review, see Greenacre and Ischiropoulos (2001)). Nitration of tyrosyl residues in proteins may interfere with signaling pathways relying on tyrosine phosphorylation/dephosphorylation and regulating cellular proliferation and apoptosis (Kong et al., 1996), and oxidation and nitration of proteins mediated by peroxynitrite may disturb the tertiary structure of proteins and, at higher degrees of damage induced, may render them inaccessible for degradation by the proteasome (Grune et al., 1998, 2001). Such proteins accumulate and tend to form insoluble aggregates, a pathogenic process inhibited by molecular chaperones in vivo (Naylor and Hartl, 2001). Aggregate formation is considered to be a major contributing factor in the pathogenesis of various neurodegenerative diseases associated with accumulation of insoluble proteins (e.g. Alzheimer's disease, Huntington's Chorea, Parkinson's disease; for review, see (Layfield et al., 2001). Peroxynitrite is thought to be involved in these disease states (Beckman et al., 1994; Torreilles et al., 1999).

Cellular and extracellular defense mechanisms against peroxynitrite are thus crucial for an attenuation of pathobiochemical processes.

2. Levels of defense against peroxynitrite

Three basic strategies serve the defense against peroxynitrite (Sies, 1993; Arteel et al., 1999a), the prevention of formation of the reactive species, interception with its damaging targets and repair of damage done (see Fig. 1). Further, signal transduction pathways affected by peroxynitrite may interact with all three above defense strategies. Signaling pathways may influence the expression of NO or superoxide-generating or -scavenging proteins (prevention), may influence the expression of peroxynitrite-scavenging proteins or proteins involved in the biosynthesis of scavenging molecules, including those involved in glutathione biosynthesis (interception), and they may affect repair mechanisms as well as general cellular survival or apoptosis (repair).

2.1. Prevention

Prevention of the exposure of cells to peroxynitrite can simply be prevention of its formation. Generation of peroxynitrite can be prevented by inhibiting the formation of nitric oxide and/or of superoxide by either inhibiting enzymatic systems responsible for the generation of these two radicals (nitric oxide synthases, xanthine oxidase, NADPH oxidase) or inhibiting their upregulation induced by inflammatory processes. Further, nitric oxide and superoxide can be scavenged prior to their generating peroxynitrite.

2.2. Interception

Low-molecular weight compounds such as carbondioxide, thiols, ascorbate, selenocompounds or synthetic metalloporphyrins as well as proteins such as certain peroxidases, hemoglobin, albumin and selenoproteins have been shown to react with

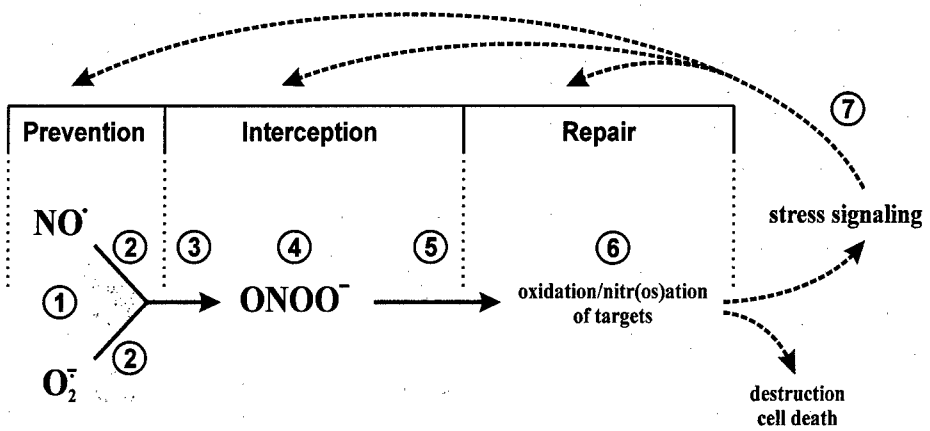

Fig. 1. Formation of, and strategies of defense against, peroxynitrite. Three categories of defense are listed: prevention, interception and repair, with several respective examples (1–7). (1) Prevention of formation of nitrogen monoxide or superoxide, e.g. by inhibition of enzymes such as NO synthases, xanthine oxidase or NADPH oxidase; (2) prevention by scavenging of either NO or superoxide, e.g. with thiols, hemoproteins or with SOD; (3) once peroxynitrite is formed, interception may be at the level of inhibiting the diffusion of peroxynitrite to reach its targets, an example being the blocking of the band 3 anion exchanger in the erythrocyte membrane (Macfadyen et al., 1999); (4) interception by scavenging peroxynitrite (e.g. thiols) or by catalyzing its decomposition (certain metalloporphyrins); (5) interception of reaction of peroxynitrite with targets, e.g. by interacting with intermediate products, as exemplified with epicatechin possibly interacting with tyrosyl radicals; (6) moderately damaged biomolecules can be repaired, e.g. by the usual DNA repair mechanisms or by selective proteolysis. (7) The signaling effects elicited by peroxynitrite may result in the expression of genes the products of which act in one of the three categories of defense (see text).

peroxynitrite (for reviews, see Arteel et al., 1999a; Koppenol, 1998; Squadrito and Pryor, 1998). Considering the approximate concentrations in vivo and the reaction rate constants of the respective compounds with peroxynitrite, one can conclude that CO_2 as well as glutathione are the most likely low-molecular-weight molecules to react with peroxynitrite (Arteel et al., 1999a). It is debatable whether the reaction with CO_2 can indeed be regarded a detoxication, because the product, nitrosoperoxycarbonate, is reactive on its own.

2.3. Repair

Repair of damage resulting from the reaction of peroxynitrite escaping the regulatory mechanisms serves the regeneration of normal cellular conditions. Correspondingly, the cellular repair systems available for damaged DNA, lipids, proteins are employed. It is part of the restitution process for the cells to actively degrade mildly oxidized or nitrated proteins (Grune et al., 1998, 2001).

Using the yeast *S. cerevisiae* as a model organism, it has been found that proteins are not only nitrated but also ubiquitinated upon exposure of cells to peroxynitrite, implying that these proteins are tagged for degradation (Buchczyk et al., 2000).

2.4. Peroxynitrite as inducer of cellular stress responses

Peroxynitrite can be recognized by cells as a stress, capable of inducing a stress response. If damage imposed by peroxynitrite exceeds the capacity of a target cell to restore a normal state, stress-responsive mechanisms lead to the induction of apoptosis (Brito et al., 1999; Lin et al., 1998; Virag et al., 1998).

Nitration of protein–tyrosine residues by peroxynitrite can prevent phosphorylation of tyrosines. Accordingly, it has been found that some signaling events are impaired by peroxynitrite due to tyrosine nitration (Brito et al., 1999; Kong et al., 1996; Mallozzi et al., 1997). However, phosphotyrosine signaling can also be induced by perox-

ynitrite, as shown for *src* tyrosine kinases in erythrocytes and synaptosomes (Mallozzi et al., 1999). In A431 epidermoid carcinoma cells, EGF receptors were shown to be a target of peroxynitrite and covalently dimerized upon treatment with consequences on downstream signaling as exemplified with PLC-γ1 (van der Vliet et al., 1998).

One of the pathways originating from receptors like the EGF receptor or the PDGF receptor is the phosphoinositide 3-kinase (PI3K)/Akt pathway. The protooncogene product c-Akt, also termed protein kinase B, has been implicated in the antiapoptotic and proliferative cellular response to growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), but also in the response to stressful stimuli such as heat shock or oxidants including hydrogen peroxide (Konishi et al., 1997; Wang et al., 2000) and nitric oxide (Datta et al., 1999). This PI3K/Akt pathway is activated in human skin fibroblasts by peroxynitrite, either added to the cells as a bolus of authentic peroxynitrite or generated in situ from SIN-1. This activation is mediated by PI3K and is initiated mainly at the level of PDGF receptors that are tyrosine-phosphorylated upon treatment (Klotz et al., 2000).

Further stress signaling events induced by exposure to peroxynitrite include the activation of pathways such as the p38 (Oh-hashi et al., 1999; Schieke et al., 1999) or the c-Jun-NH_2-terminal kinase (JNK; Go et al., 1999; Schieke et al., 1999) pathways, the induced expression of stress genes such as c-fos (Müller et al., 1997), heme oxygenase-1 (Foresti et al., 1999) or the growth arrest and DNA damage-inducible genes (Gadd) 34, 45, and 153 (Oh-hashi et al., 2001).

All of these responses can be linked to apoptosis. Interestingly, pathways considered antiapoptotic and proliferative, i.e. the ERK- and PI3K/Akt pathways, seem to be activated in parallel with some that are considered proapoptotic, i.e. the p38 and JNK pathways (Klotz et al., 2000; Schieke et al., 1999). It may be speculated that a balance between anti- and proapoptotic stimuli may be tipped, deciding about which process prevails after a stress, survival or apoptosis. For a recent review on peroxynitrite-induced signaling, see Klotz et al. (2002).

3. Protection against peroxynitrite by selenocompounds

The low-molecular-weight selenium compound ebselen reacts with peroxynitrite efficiently, exhibiting one of the highest second-order rate constants for a low-molecular-weight compound with peroxynitrite known so far (Masumoto et al., 1996; Masumoto and Sies, 1996). Ebselen and other low-molecular-weight selenocompounds such as selenomethionine are effective—usually more so than the corresponding sulfur analogs—in protecting several model compounds from being oxidized or nitrated by peroxynitrite (Briviba et al., 1996). Likewise, plasmid DNA was protected against peroxynitrite-induced single-strand breaks (Roussyn et al., 1996). Ebselen was also used to block peroxynitrite induced increase in susceptibility towards proteolytic degradation of isolated ferritin (Grune et al., 2001).

The selenoprotein glutathione peroxidase (GPx) is capable of efficiently reducing peroxynitrite and preventing oxidation and nitration of model compounds as well as nitration of proteins (Sies et al., 1997). The system of GPx plus GSH works catalytically in a manner resembling the detoxication of hydroperoxides by GPx at the expense of GSH. The selenocysteine residue at the active site of the enzyme is oxidized by peroxynitrite (or peroxynitrous acid) to the selenenic acid and reduced back to the selenol at the expense of two reducing equivalents provided by glutathione. The second-order rate constant for the reaction of reduced GPx with peroxynitrite has been reported to be $8 \times 10^6\ M^{-1}\ s^{-1}$ per tetramer (Briviba et al., 1998), which, on a selenium basis, is similar to the rate constant of $2 \times 10^6\ M^{-1}\ s^{-1}$ reported for ebselen (Masumoto and Sies, 1996). Different from what was expected from these data on the peroxynitrite reductase activity of GPx, GPx1-deficient murine hepatocytes are less rather than more susceptible to peroxynitrite-induced apoptosis than wild-type cells (Fu et al., 2001). This may be due to regulatory circuits affected by deletion of GPx1 that are not yet fully understood but result in upregulation of peroxynitrite-scavenging mechanisms.

A similar mechanism applies for selenomethionine (Assmann et al., 1998). Interestingly, methionine oxide, unlike methionine selenoxide, is not reduced by glutathione alone, but rather an enzyme is necessary for this reduction to occur, methionine sulfoxide reductase (Levine et al., 1996).

Selenoprotein P in human plasma also protects against peroxynitrite (Arteel et al., 1998), suggesting that it may serve as a protectant in human blood. The heparin binding domains of selenoprotein P enable surface coating of cellular membranes; this may serve as a protective barrier against peroxynitrite (Arteel et al., 2000a).

A third selenoprotein that has been demonstrated to be capable of assisting the reduction of peroxynitrite is thioredoxin reductase (Arteel et al., 1999b); the enzyme uses NADPH to reduce the oxidized forms of selenocysteine or ebselen. Although, transiently inactivated in human umbilical vein endothelial cells exposed to the peroxynitrite generating substance SIN-1, this very exposure to peroxynitrite results in an induced expression of thioredoxin reductase (Park et al., 2002).

Most of the selenocompounds mentioned here have been shown to protect cells or cell lysates from protein nitration (Schieke et al., 1999; Sies et al., 1997). This would also prevent cells from impairments in phosphotyrosine signaling necessary for growth and survival. As mentioned above, peroxynitrite is capable of activating p38 MAP kinases in liver epithelial cells. This activation is prevented by a preincubation of the cells with selenite, shown to increase specific GPx activity (Schieke et al., 1999). The same is true for gap junctional intercellular communication: peroxynitrite induces a loss of intercellular communication, which is not seen in cells pretreated with selenite (Sharov et al., 1999). These data exemplify that interception of peroxynitrite, e.g. by induction of a selenoprotein, makes it unnecessary for a cell to react towards an exposure to peroxynitrite by inducing stress pathways: there is no need for repair and disposal of debris.

4. Flavonoids as protectants against peroxynitrite-induced damage

Flavonoids are present in high amounts in dietary sources and can protect against the effects of peroxynitrite both by efficiently scavenging the precursors of peroxynitrite (at the level of prevention), nitrogen monoxide (van Acker et al., 1995) and superoxide (Robak and Gryglewski, 1988), and by prevention of its interaction with targets (at the level of interception; Haenen et al., 1997; Pannala et al., 1997). Regarding the interaction with peroxynitrite, much work has been done with the flavan-3-ol, (−)-epicatechin. Epicatechin and/or its oligomers are particularly abundant in certain chocolates, green tea and red wine (Scalbert and Williamson, 2000). In vitro, these polyphenols protect against peroxynitrite-induced oxidation of dihydrorhodamine 123 and the nitration of tyrosine with efficiencies similar to those of ebselen (Arteel et al., 2000b; Arteel and Sies, 1999).

The notion that flavonoids are direct scavengers of peroxynitrite has been challenged based on kinetic studies (Tibi and Koppenol, 2000). In line with this, and with respect to the reaction of peroxynitrite with tyrosine, Schroeder et al. (Schroeder et al., 2001a) proposed an interaction of epicatechin not with peroxynitrite itself but rather a reaction intermediate, the tyrosyl radical. This hypothesis is based on an efficiency of (−)-epicatechin in preventing the peroxynitrite-induced dimerization of tyrosine equal to that of preventing its nitration. The authors found that epicatechin inhibits tyrosine nitration by peroxynitrite several orders of magnitude more efficiently than it inhibits oxidation reactions by peroxynitrite (Schroeder et al., 2001b). Oxidation reactions examined were the oxidation of thiols, the oxidative inactivation of glyceraldehyde-3-phosphate dehydrogenase or of purified soybean lipoxygenase-1 and the oxidation of 2′,7′-dichlorodihydrofluorescein in cells. The apparent selectivity of (−)-epicatechin with regard to interfering with tyrosine nitration but not oxidation reactions was hypothesized to be due to interference with tyrosyl radicals rather than to a direct interaction with peroxynitrite.

Employing (−)-epicatechin to selectively inhibit tyrosine nitration by peroxynitrite did not diminish activation of ERK- and p38-MAP kinases as well as the PI3K/Akt pathway by peroxynitrite (Schroeder et al., 2001b), implying that activation is not due to, or negatively influenced by, tyrosine nitration. As p38 is regarded an oxidant-responsive kinase, these findings are in line with the selectivity of epicatechin in preventing nitration rather than oxidation reactions of peroxynitrite.

5. Conclusion

There is a requirement for defense against peroxynitrite. This may be accomplished using several strategies: prevention, interception and repair as well as stress signaling mechanisms. Two groups of compounds that exert protective effects in this respect are selenocompounds, including selenoenzymes, and flavonoids. Although it is generally assumed that dietary uptake of these substances or of precursors thereof is beneficial in terms of a general prevention, it remains to be established in how far the knowledge about the interaction of these with peroxynitrite may be exploited medically.

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The role of multidrug transporters in drug availability, metabolism and toxicity

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Abstract

Multidrug resistance is frequently observed when treating cancer patients with chemotherapeutic agents. A variety of ATP binding cassette (ABC) transporters, localized in the cell membrane, cause this phenomenon by extruding a variety of chemotherapeutic agents from the tumor cells. However, the major physiological role of the multidrug transporters is the protection of our cells and tissues against xenobiotics, and these transporters play a key role in drug availability, metabolism and toxicity. Three major groups of ABC transporters are involved in multidrug resistance: the classical *P*-glycoprotein MDR1, the multidrug resistance associated proteins (MRP1, MRP2, and probably MRP3, MRP4 and MRP5), and the ABCG2 protein, an ABC half-transporter. All these proteins were shown to catalyze an ATP-dependent active transport of chemically unrelated compounds. MDR1 (*P*-glycoprotein) and ABCG2 preferentially extrude large hydrophobic, positively charged molecules, while the members of the MRP family can extrude both hydrophobic uncharged molecules and water-soluble anionic compounds. By examining the interactions of the multidrug transporters with pharmacological and toxic agents, a prediction for the cellular and tissue distribution of these compounds can be achieved. Oral bioavailability, entering the blood–brain and blood-CSF barrier, reaching the fetus through the placenta, liver and kidney secretion, cellular entry for affecting intracellular targets, are all questions, which can be addressed by basic in vitro studies on the multidrug resistance proteins. Investigation of the substrate interactions and modulation of multidrug transporters may pave the way for predictive toxicology and pharmacogenomics. Here we show that by using in vitro assay systems it is possible to measure the interactions of multidrug transporters with various drugs and toxic agents. We focus on the characterisation of the MRP1 and MRP3 proteins, their relevance in chemoresistance of cancer and in drug metabolism and toxicity.
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Keywords: Chemotherapeutic agents; Multidrug resistance; Multidrug resistance protein; Active transport; In vitro studies

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1. Introduction

The multidrug resistance phenotype in tumors is associated with the overexpression of certain ATP binding cassette (ABC) transporters, termed MDR proteins. The *P*-glycoprotein (Pgp, MDR1, ABCB1) mediated multidrug resistance was the first discovered and probably still is the most widely observed mechanism in clinical multidrug resistance (Goldstein et al., 1992; Gottesman and Pastan, 1993). There are two other ABC transporters, which have been definitely demonstrated to participate in the multidrug resistance of tumors: the multidrug resistance protein 1 (MRP1, ABCC1; Cole et al., 1992; Deeley and Cole, 1997; Borst and Elferink, 2002), and the mitoxantrone resistance protein (MXR/BCRP, ABCG2; Bates et al., 2001). Furthermore, other human ABC proteins capable of actively transporting various compounds out of the cells, may also be players in selected cases of multidrug resistance. These include the homologues of MRP1–5. MRP2 and MRP3 seem to be key players in organic conjugate transport in various tissues, while MRP4 and MRP5, may have special functions as nucleoside transporters (Borst et al., 1997, 1999; Hipfner et al., 1999; Konig et al., 1999a; Hirohashi et al., 1999; Wijnholds et al., 2000).

All ABC proteins contain at least three characteristic peptide sequences: the Walker A and B motifs and the so-called ABC-signature sequence. Whereas the Walker motifs are present in several classes of ATP binding proteins, the presence of the signature region is diagnostic for the ABC proteins (Klein et al., 1999). According to our current notion, in addition to an MDR1-like core, MRP1, MRP2, MRP3, and MRP6 contain an additional N-terminal segment of about 280 amino acids. A major part of this region is membrane embedded with five transmembrane helices (TMD0), while a small cytoplasmic loop of about 80 amino acids (L0) connects this area to the core region (Bakos et al., 1996, 1998). Recent studies revealed a special role for the L0 region both for the transport activity and for the proper intracellular routing of the MRP1 and MRP2 proteins (Fernandez et al., 2002).

The generally accepted basic mechanism of multidrug resistance is that the MDR proteins actively expel the cytotoxic drugs from the cells, maintaining the drug level below a cell-killing threshold. Drug extrusion mediated by these primary active transporters is driven by the energy of ATP hydrolysis. The most intriguing characteristic, distinguishing the MDR proteins from other mammalian transporters is their wide substrate specificity. Unlike other, selective (classical) transport proteins, multidrug transporters recognize and handle a wide range of substrates.

The three major MDR proteins are highly promiscuous transporters, they share the ability of recognizing and translocating a large number of various, mainly hydrophobic compounds. In addition to their overlapping substrate specificity, each transporter can handle unique compounds. Pgp-MDR1 is a transporter for large hydrophobic, either uncharged or slightly positively charged compounds, while the MRP family is mostly transporting hydrophobic anionic conjugates, and also extrudes hydrophobic uncharged drugs. The MRP-related uncharged drug transport is quite an enigma, and is somehow linked to the transport or allosteric effect of cellular free reduced gluthatione (Deeley and Cole, 1997, Loe et al., 1998; Borst et al., 1999; Kool et al., 1999; Zeng et al., 1999; Kruh et al., 2001).

All multidrug transporters are localized predominantly in the plasma membrane. Pgp-MDR1 in polarized cells is localized in the apical (luminal) membrane surface, e.g. in the epithelial cells of the intestine and the proximal tubules of kidney, or in the biliary canalicular membrane of hepatocytes (Gottesman and Pastan, 1993). In contrast, MRP1 expression in polarized cells is restricted to the basolateral membrane. The expression of MRP2 is predominant in the canalicular membrane of hepatocytes, while MRP3 and MRP5 are expressed in the basolateral membranes of these cells. MRP2 is also highly expressed in the apical membranes of kidney proximal tubules (Borst et al., 1997, 1999; Hipfner et al., 1999; Konig et al., 1999a,b).

In addition to their contribution to the multidrug resistance in cancer, MDR proteins play an important physiological role in the protection of

the body against xenobiotics (foreign compounds), occurring in the environment. This function is accomplished by the active pumping of these toxic agents. The physiological function of MDR1 has been best studied in a knock out mouse model, showing that mice lacking MDR1-like transporters were hypersensitive to xenobiotics (Schinkel et al., 1997).

The physiological role of MDR proteins is also revealed by their tissue distribution. These transporters are highly expressed in important pharmacological barriers, such as the brush border membrane of intestinal cells, the biliary canalicular membrane of hepatocytes, and the luminal membrane in proximal tubulus of the kidney. MDR proteins are also present in the endothelial cell of the brain capillaries, and in the epithelial cells in the choroid plexus, both contributing to the blood–brain barrier (Borst et al., 1999; Rao et al., 1999). It is important to note that MRPs also mediate the transport of partially detoxified compounds, such as glutathione and glucuronide conjugates (see above).

An extremely important question in current pharmacological studies is whether certain drugs can cross these barriers. Since MDR transporters play a key role in these transport processes, the interaction between pharmaceuticals and MDR transporters is an essential information for drug targeting. Therefore, testing the interaction between compounds and various ABC transporters may significantly help the understanding of these phenomena.

In the ABCC (MRP) family of the MDR proteins, MRP1 and MRP3 are the closest homologs (58% similarity). Both may have an important role in numerous organs, including the liver, kidney, the blood–brain barrier, etc., in transporting anionic conjugates or other organic anions, and both proteins are localized in the basolateral membrane of epithelial cells (Deeley and Cole, 1997; Kool et al., 1999; Zeng et al., 1999; Hirohashi et al., 1999). Several reports indicate an overlapping, but non-identical transported substrate specificity for these proteins (Kruh et al., 2001). In the present paper we provide data for the interactions of MRP1 and MRP3 with a number of physiological organic compounds and pharmacological agents.

2. Materials and methods

2.1. Expression of MRPs in insect cells

Recombinant baculoviruses containing the MRP cDNAs were prepared as described in Bakos et al. (1998). Sf9 (Spodoptera frugiperda) cells were cultured and infected with a baculovirus as described in Müller et al. (1996). MRP3 cDNA was obtained from Prof. Piet Borst and inserted into a baculovirus vector as described in Bakos et al. (2000).

2.2. Membrane preparation and immunoblotting

Virus-infected Sf9 cells were harvested, their membranes isolated and stored, and the membrane protein concentrations determined, as described in Sarkadi et al. (1992). Gel-electrophoresis, and immunoblot detection was performed and protein–antibody interaction was determined using the enhanced chemoluminescence technique as described in Bakos et al. (2000).

2.3. Membrane ATPase measurements

ATPase activity was measured basically as described in Sarkadi et al. (1992), by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction. The incubation media contained 10 mM $MgCl_2$, 40 mM MOPS–Tris (pH 7.0), 50 mM KCl, 5 mM DTT, 0.1 mM EGTA, 4 mM Na-azide, 1 mM ouabain, and 4 mM ATP. Membrane ATPase activity was measured for 60 min at 37 °C in the presence of 4 mM ATP (control points), plus or minus 1 mM Na-orthovanadate (difference of the two values means the vanadate-sensitive component), and various concentrations of additional compounds, as indicated in the figures.

2.4. Multidrug transporter assays in isolated inside-out membrane vesicles

Membrane vesicles were incubated in the presence of 4 mM ATP in a buffer containing 10 mM $MgCl_2$, 40 mM MOPS–Tris (pH 7.0), and 50 mM KCl at 37 °C (Bakos et al., 1998). Aliquots of the membrane suspensions were added to excess cold transport buffer and then rapidly filtered through nitrocellulose membranes (0.25 μm pore size). After washing the filters with 10-ml ice-cold washing buffer, radioactivity associated with the filters was measured by liquid scintillation counting. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP. The figures present means values obtained in three independent experiments.

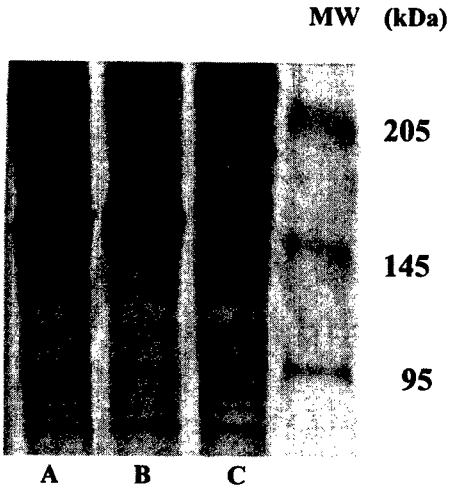

Fig. 1. Detection of MRP1 and MRP3 by SDS-polyacrylamide gel electrophoresis and Coomassie staining in isolated membranes of Sf9 cells. MW, molecular weight marker, (lane A) isolated membranes of Sf9 cells expressing MRP1; (lane B) isolated membranes of Sf9 cells expressing MRP3; (lane C) isolated membranes of Sf9 cells expressing β-galactosidase as control.

3. Results

3.1. Expression of human MRP1 and MRP3 in Sf9 cells

Fig. 1 shows a Coomassie stained blot of the isolated membranes, obtained from Sf9 cells, and separated by SDS-gel electrophoresis. The Sf9 cells were infected with the recombinant baculoviruses inducing human MRP1 or MRP3 expression. As documented, both MRP1 and MRP3 were successfully expressed in high levels (with an apparent molecular mass of ~160 kDa) in the Sf9 insect cells. Immunoblotting by specific antibodies clearly identified the respective human MRP proteins expressed (data not shown here). In this heterologous expression system these human proteins were produced in an underglycolisated form, which has been demonstrated not to have any effect on their transport functions (Gao et al., 1996; Bakos et al., 1998; van Aubel et al., 1998; Bakos et al., 2000). In the following experiments we used isolated membranes, forming inside-out membrane vesicles (Bakos et al., 1998) from these human MRP1 and MRP3 expressing Sf9 cells.

3.2. ATPase measurements

It has been shown previously that vanadate-sensitive ATPase activity of multidrug transporters closely correlates with their substrate transport activity. Transported substrates significantly stimulate this ATPase activity, whereas inhibitors block the ATPase cycle. As both the human MRP1 and MRP3 are presumed to be organic anion transporters, we examined the modulatory effects of organic anions, regarded as substrates or inhibitors, on their membrane ATPase activity.

In the present paper we provide data for the effects of GS-NEM, as a typical model glutathione conjugate, and $E_2 17\beta G$, a typical glucuronide conjugate, which may be physiologically relevant model substrates of these transporters. We have also compared the effects of the organic anion pharmacological agents, furosemide, and indomethacin, as well as previously described MRP inhibitors, MK571 and benzbromarone (BBR) on the ATPase activity of MRP1 and MRP3.

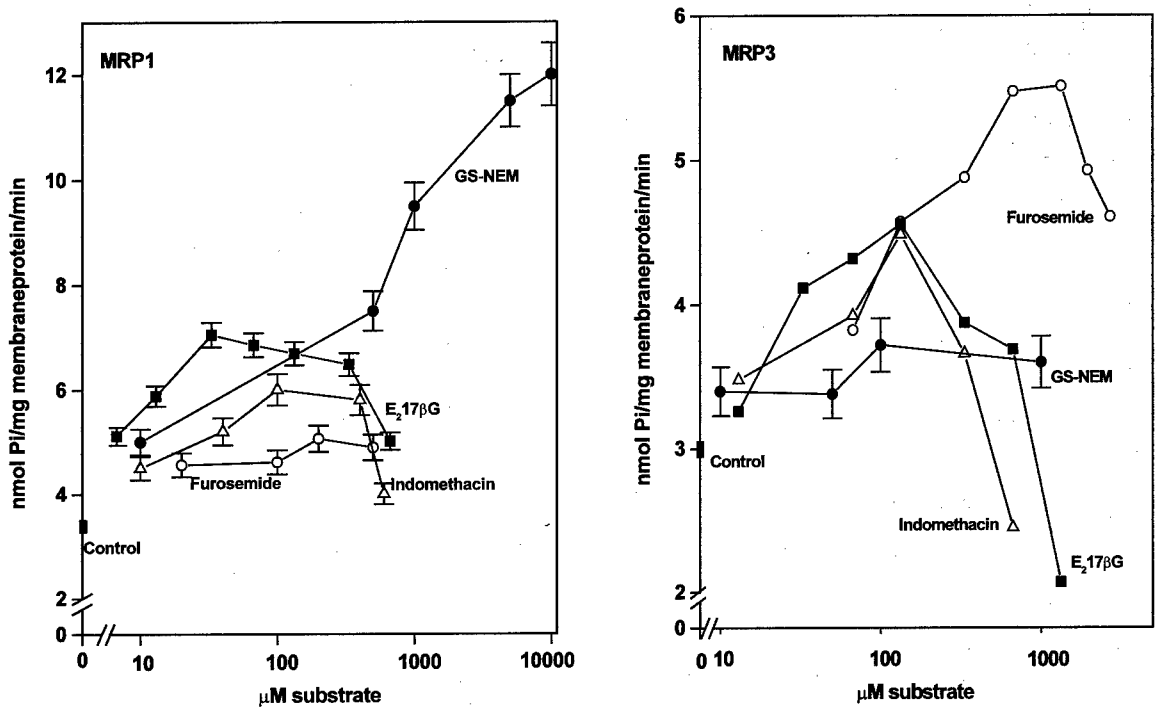

Fig. 2. Effects of $E_2$17ßG, indomethacin, furosemide and GS-NEM on vanadate-sensitive ATPase activity of MRP1- (panel A) and MRP3- (panel B) expressing Sf9 cell membranes.

Fig. 2 documents the ATPase activity measurements carried out by using human MRP1 (Panel A) or human MRP3 (Panel B) expressing Sf9 cell membranes. The ordinate shows the vanadate-sensitive fraction of the ATPase, which is reflecting the activity of the expressed MRPs. A baseline vanadate-sensitive ATPase activity (3–5 nmol/mg membrane protein/min) is mostly due to the presence of endogenous ATPases in the membrane preparation (as also found in the control, β-galactosidase expressing membranes).

As documented in Panel A, Fig. 2, in the case of MRP1 containing membranes NEM-GS was a powerful activator of the ATPase activity, although a relatively high (5–10 mM) NEM-GS concentration was required for maximum activation. $E_2$17βG and indomethacin produced only a moderate increase in the ATPase activity in MRP1-containing membranes, and no measurable effect of furosemide was observed. We have shown earlier (Bakos et al., 1998) that an additive effect of the indomethacin stimulation of MRP1–ATPase by 5 mM free GSH could be observed, but no major change in the relative activation curves occurred for any of the other compounds examined (data not shown).

As shown in Panel B, Fig. 2, in the case of MRP3 membranes NEM-GS was practically ineffective on the ATPase activity, in the wide concentration range examined. In contrast, $E_2$17βG and indomethacin produced a significant increase in the ATPase activity in these human MRP3 containing membranes, and furosemide induced the most pronounced activation of the MRP3–ATPase. It is to be noted that the relative ATPase activity levels were lower in the case of MRP3 than with MRP1, which is most probably reflecting a lower turnover rate of the former transporter protein. The ATPase activity of MRP3 was not significantly affected by the addition of free GSH.

In the following experiments we examined the effects of previously described MRP1 inhibitors, benzbromarone (BBR, an effective uricosuric

agent) and MK571 (a leukotriene receptor inhibitor) on the ATPase activity of the human MRP1 and MRP3. As shown in Fig. 3, Panel A, in MRP1 containing insect cell membranes both benzbromarone and MK571 had a well measurable inhibitory effect on the NEM-GS activated ATP hydrolysis. Both compounds produced a half-maximum inhibition at about 10 μM concentration, although in these experiments a slight activation of the MRP1–ATPase by MK 571 was seen at 0.5–2 μM.

In the case of MRP3-expressing membranes the effect of benzbromarone and MK571 was investigated in the presence of 15 μM $E_2 17\beta G$, causing a slight stimulation of the MRP3–ATPase activity (Fig. 2). Interestingly, in this case both benzbromarone (10–30 μM) and MK571 (10–50 μM) caused a significant stimulation of the MRP3–ATPase, which, in the case of benzbromarone, greatly exceeded any other ATPase stimulation caused by potential organic anion substrates (Fig. 2). These ATPase activation data suggested, that BBR and MK571 may be potential transported substrates of MRP3.

These experiments collectively indicate that presumed substrates and inhibitors of the MRP1 and MRP3 protein may have overlapping, but also entirely different effects on these proteins—inhibitors of MRP1, like BBR or MK 571, may be excellent transported substrates of MRP3. In the following experiments we have directly examined the vesicular transport of some of the organic anions by MRP1 and MRP3, as well as the effects of other potential modulatory agents.

3.3. Transport measurements in inside-out membrane vesicles

In order to compare the transport characteristics of human MRP1 and MRP3, we studied the ATP-dependent uptake of radiolabeled NEM-GS and $E_2 17\beta G$ in isolated Sf9 cell membrane vesicles. Both of these compounds have been indicated to be transported substrates for MRP1 and MRP3

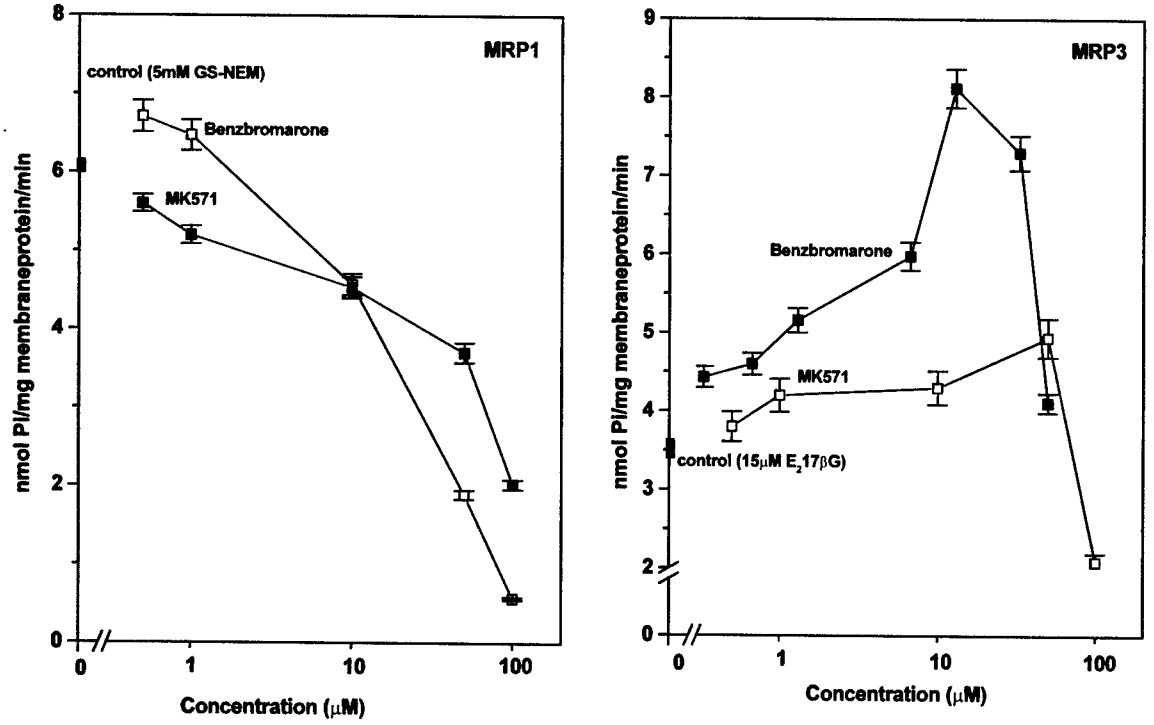

Fig. 3. Modulatory effect of MK 571 and benzbromarone on NEM-GS (5 mM, in MRP1) or $E_2 17\beta G$- (15 μM, in MRP3) stimulated, vanadate-sensitive ATPase activity in isolated Sf9 cell membranes expresssing MRP1 (panel A) or MRP3 (panel B).

(Loe et al., 1996). In these experiments we examined the ATP-dependent tracer uptake by subtracting the values obtained in the presence of AMP (which was low in all experiments presented). Also, as a control, we used vesicles obtained from Sf9 cells expressing β-galactosidase. In these latter vesicles ATP-dependent tracer uptake was negligible. In all these experiments the linear phase of the tracer uptake was determined (2 min for $E_2 17\beta G$, and 4 min for GS-NEM), and these periods were used for studying the concentration dependence of the uptake.

Fig. 4A shows the MgATP-energized NEM-GS and $E_2 17\beta G$ uptake by membrane vesicles prepared from human MRP1-expressing Sf9 cells. As documented, MRP1 performs an efficient ATP-dependent transport for NEM-GS, saturating at millimolar NEM-GS concentrations, with an approximate Km of about 300 μM (Bakos et al., 1998), and a Vmax of about 500 pmol/mg membrane protein/min). In the case of MRP1, $E_2 17\beta G$ uptake is also measurable, but this transport has a much lower Km (~ 8–10 μM), and also, a much lower Vmax (about 50 pmol/mg membrane protein/min) value.

In the case of human MRP3, the transport activity for these model substrates shows a reversed picture (Fig. 4B): $E_2 17\beta G$ is transported with a high rate, approaching 1300 pmol/mg membrane protein/min, with a Km of about 30–35 μM. In contrast, the transport rate of NEM-GS is almost unmeasurable (a maximum of 20 pmol/mg membrane protein/min), and due to this low transport activity, the Km value could not be properly estimated.

In the following experiments we have examined the modulatory effects of benzbromarone and MK571 on the substrate transport activity of MRP1 and MRP3 in isolated membrane vesicles. In these experiments we studied labeled $E_2 17\beta G$ uptake, as this compound was a well measurable, actively transported substrate for both proteins. In these experiments tracer uptake was measured at a fixed, relatively low concentration (1 μM) of $E_2 17\beta G$, so as $E_2 17\beta G$ uptake could be examined below saturating substrate concentrations.

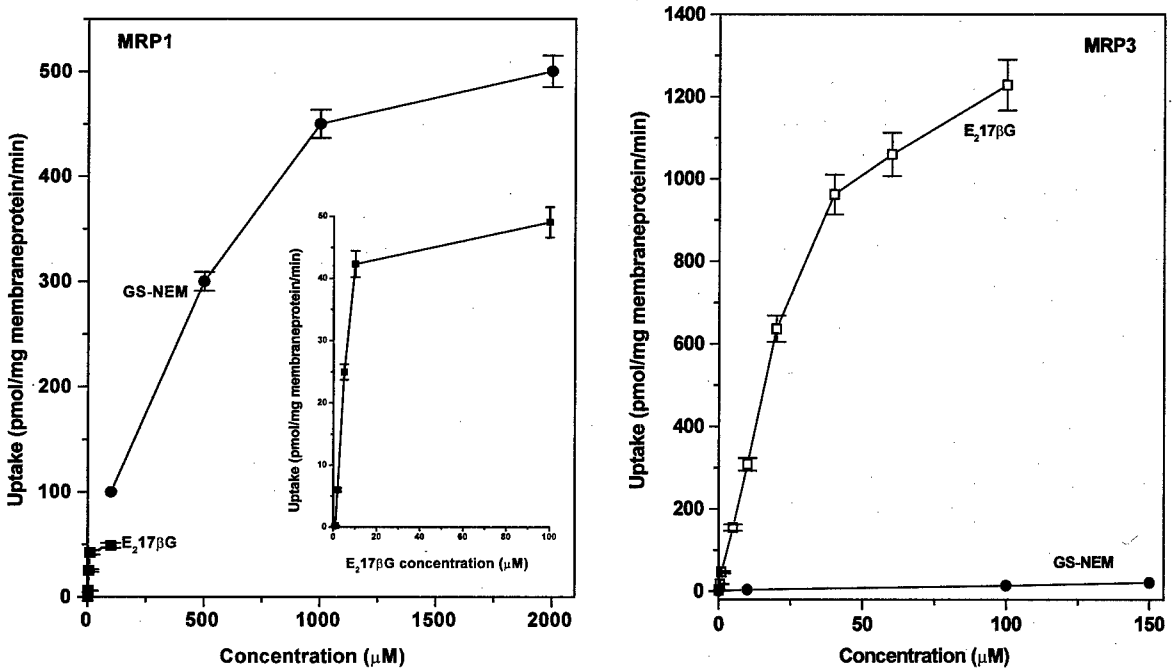

Fig. 4. ATP-dependent uptake of [^{3}H]-$E_2 17\beta G$, and [^{3}H]-GS-NEM in Sf9 cell membrane vesicles, expressing MRP1 (A) or MRP3 (B). Membrane vesicle preparations were incubated with different concentrations of labeled compound at 37 °C, for 2 min in case of $E_2 17\beta G$, and for 4 min in case of GS-NEM (Section 2).

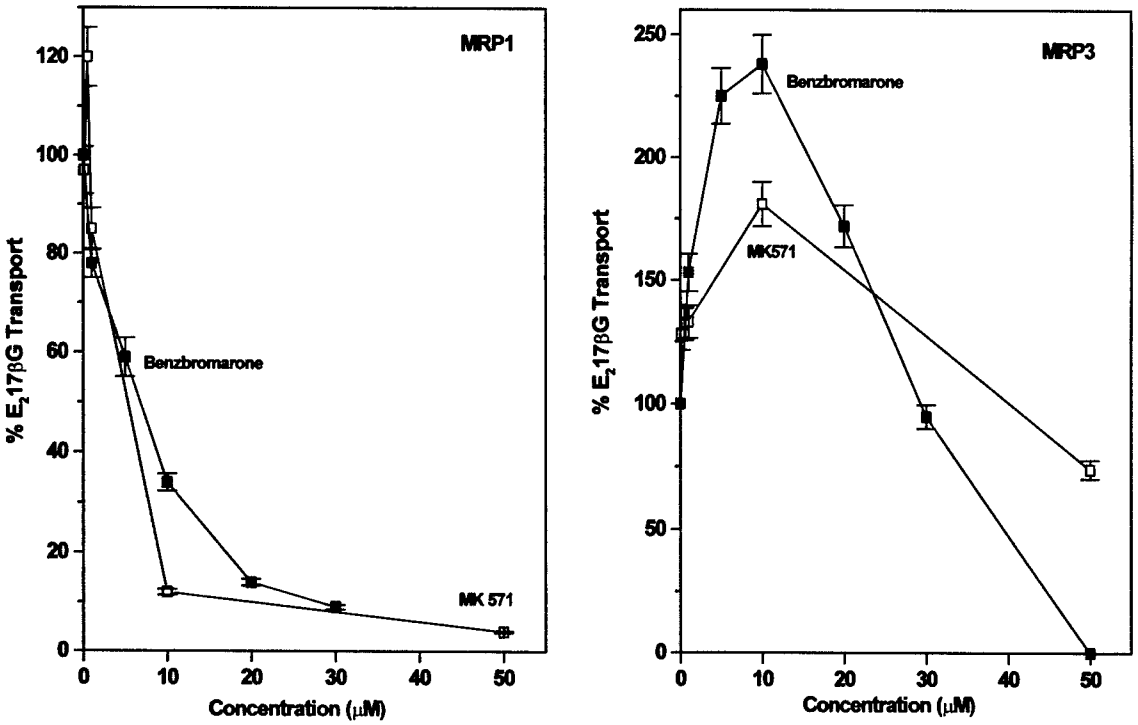

Fig. 5. Modulation of ATP-dependent [^{3}H]-$E_2$17βG transport in MRP1- (panel A) or MRP3- (panel B) expressing membrane vesicles by BBR and MK 571. Sf9 cell membrane vesicles were incubated with 1 μmol [^{3}H]-$E_2$17βG at 37 °C for 2 min. (Section 2). Transport rates are expressed as per cent of the tracer uptake measured in the absence of any additional compounds.

As shown in Fig. 5A, in MRP1 containing membranes benzbromarone strongly inhibited $E_2$17βG uptake at micromolar concentrations, and a half-maximum inhibition was caused by 5–8 μM BBR. The effect of MK 571 was somewhat different—this compound caused a slight (~20%) stimulation of $E_2$17βG uptake by MRP1 at 0.5–1 μM concentrations, while a full inhibition of the $E_2$17βG uptake occurred at 10–50 μM MK 571.

In the case of human MRP3-expressing membranes, as shown in Fig. 5B, $E_2$17βG uptake was greatly stimulated by benzbromarone in a relatively wide concentration range (1–20 μM), and this stimulation produced a 2–2.5 times activation of the $E_2$17βG uptake process. In higher concentrations, at about 50 μM, BBR caused a strong inhibition of the $E_2$17βG uptake by MRP3. In the case of MK571 a similar biphasic curve was obtained: at low concentrations (2–5 μM) MK571 stimulated $E_2$17βG uptake, while 50–100 μM this compound caused an inhibition of the $E_2$17βG transport by MRP3.

4. Discussion

The present study focussed on a comparative investigation of the in vitro ATPase and transport properties of human MRP1 and MRP3, the closest homologs of the MPR subfamily of the ABC transporters. These proteins were expressed in a Sf9 baculovirus expression system, as the heterologous expression in insect cells produces high and comparable expression levels of various human ABC proteins (Sarkadi et al., 1992; Bakos et al., 1996; Özvegy et al., 2001).

MRP1 has been described to function as a GS-X pump, preferentially transporting LTC_4 with a high affinity, but MRP1 has been also demonstrated to extrude natural product drugs, in a co-transport with free glutathione (Deeley et al., 1997,

Loe et al., 1998; Borst et al., 1999; Kool et al., 1999; Zeng et al., 1999; Kruh et al., 2001). Earlier data in the literature indicated that MRP3 has a low affinity for glutathione conjugates, but preferentially transports glucuronide- and bile salts conjugates (Loe et al., 1996; Hirohashi et al., 1999), and may also extrude organic anions and natural vinca alkaloids (Kool et al., 1999; Hirohashi et al., 1999; Zelcer et al., 2001). Recent studies suggested, that the substrate selectivity of human and rat MRP3 are different (Zeng et al., 1999; Kruh et al., 2001), thus comparative experiments for examining the transport properties of human MRP1 and MRP3 may help our understanding the characteristics of these transporters.

In the present experiments the ATPase and transport activities of human MRP1 and MRP3 were compared. We found that NEM-GS, representing GS-conjugates of toxic agents, is a good substrate for MRP1, while its transport by MRP3 is very inefficient. We also examined the uptake of tritiated LTC_4, and found the same feature for the two transporters: MRP1 is an efficient, high affinity transporter for LTC_4, while LTC_4 transport by MRP3 was practically unmeasurable (data not shown). We found that human MRP3 preferentially transports $E_2 17\beta G$, with a medium affinity and a very high capacity, while MRP1 can transport $E_2 17\beta G$ with a higher affinity both with much lower capacity. The values obtained for these parameters in the ATPase assays closely correlate with those obtained in direct transport studies.

Furosemide, an anionic diuretic, has a strong stimulatory effect on the MRP2–ATPase activity in high concentration (Bakos et al., 1998), and produced a similar effect in case of MRP3-containing vesicles, while furosemide had little effect on MRP1. Indomethacin, a non-steroid anti-inflammatory agent, showed a weak stimulatory effect on MRP3 ATPase activity at relatively high concentrations, while gave a more pronounced stimulatory effect in the case of MRP1.

Benzbromarone has been reported to be an inhibitor of both MRP1 and MRP2 (Bakos et al., 1998). In our current experiments we showed that benzbromarone significantly stimulated the MRP3–ATPase activity in a low concentration range. Moreover, BBR directly activated $E_2 17\beta G$ uptake by MRP3. MK 571, an efficient antagonist of a leukotriene receptor, inhibited both MRP1-dependent transport and ATPase activity. (Gekeler et al., 1995). This compound turned out to be a weak activator of MRP3–ATPase activity, also showing a small stimulatory effect on $E_2 17\beta G$ uptake.

These results, in accordance with the ATPase measurements suggest that both MK571 and benzbromarone can produce an allosteric activation on $E_2 17\beta G$ transport by MRP3, based on the presence of multiple binding sites on these transporters. In the absence of labeled BBR or MK571, the direct ATP-dependent vesicular transport of these compounds cannot be examined, but the combination of the ATPase and transport data indicate a co-activation and a possible co-transport for these compounds with $E_2 17\beta G$, in the case of MRP3. MRPs have already been reported to function as co-transporters for non-conjugated hydrophobic drugs and GSH. Our results suggest that there are several agents which may modify MRP3-mediated organic anion and glucuronide conjugate transport, through allosteric activation or actual co-transport mechanisms.

The present experiments also demonstrate major differences in the transport handling and inhibitor-sensitivities of human MRP1 and MRP3. These may have profound effects on liver or renal toxicity of various compounds, depending on the expression levels of these proteins. MRP1 expression has a low level in the liver, while MRP3 has been shown to be an important transporter in this organ (Konig et al., 1999a, Scheffer et al., 2002). Elevated levels of MRP3 expression have been detected in human hepatocellular carcinoma (Nies et al., 2001; Rost et al., 2001), and in Dubin–Johnson patients, when in the absence of a functional MRP2, MRP3 seems to have a compensatory transport function (Konig et al., 1999b). In this case various compounds are transported by MRP3 into the sinusoidal blood, while these metabolites are normally extruded into the bile by MRP2. MRP3 may also be up-regulated under cholestatic conditions (Donner and Keppler, 2001)

In summary our results indicate that there is a significant overlap in the transport capacity of

MRP1 and MRP3, although we also observed significant differences in their substrate interactions. Different organic anions may be differently transported by these MRPs, and common substrates or substrate analogs may have entirely different inhibitory or modulatory effects on these transporters.

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Review

Toxicogenomics: challenges and opportunities

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Abstract

Toxicogenomics describes the measurement of global gene expression changes in biological samples exposed to toxicants. This new technology promises to greatly facilitate research into toxicant mechanisms, with the possibility of assisting in the detection of compounds with the potential to cause adverse health effects earlier in the development of pharmaceutical and chemical products. In this short review, I discuss the opportunities presented by toxicogenomics, the challenges we face in the application of these tools, and the progress we have made in realising the potential of these new genomic approaches.
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Keywords: Toxicogenomics; Microarrays; Mechanistic toxicology; Predictive toxicology

1. Introduction

The publication of the draft sequence of the human genome almost 2 years ago signalled the arrival of the genomic era of the biological sciences (International Human Genome Sequencing Consortium, 2001). This newfound knowledge accelerated the development of tools that allow biological processes to be examined on a global scale. Among these tools are those that facilitate the simultaneous measurement of the expression levels of thousands of different genes, technologies known collectively as gene expression profiling (Duggan et al., 1999; Brown and Botstein, 1999). Toxicologists quickly realised the potential of these new tools to advance their discipline and a new field was born. The application of gene expression profiling to toxicology, termed toxicogenomics, presents us with opportunities to define, at unprecedented levels of detail, the molecular events that precede and accompany toxicity, promising to shed light on toxic mechanisms that are presently poorly understood (Afshari et al., 1999; Farr and Dunn, 1999; Nuwasyr et al., 1999; Pennie, 2000; Pennie et al., 2000; Orphanides et al., 2001; Gant, 2002; Ulrich and Friend, 2002). Moreover, it is hoped that gene expression changes induced upon chemical exposure will provide a means of predicting mechanisms of toxicity more rapidly.

Used in conjunction with existing tools available to the toxicologist, toxicogenomics promises significant advances in research and investigative toxicology. These advances include:

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- a more detailed appreciation of molecular mechanisms of toxicity.
- faster screens for substance toxicity.
- enhanced extrapolation between experimental animals and humans in the context of risk assessment.

In this article, I discuss the use of toxicogenomics in mechanistic and predictive toxicology. In particular, I examine how far we have come towards realising the full potential of these tools.

2. Use of toxicogenomics to predict mechanisms of toxicity

A goal of modern toxicology is to protect the human population from exposure to harmful substances by identifying compounds with the potential to cause toxicity. Most current testing strategies measure the effects of long-term chemical exposure in experimental animals. Through the identification of gene expression changes associated with chemical exposure, the hope is that toxicogenomics will facilitate the development of methods that predict the long-term effects of compounds using short-term assays. The underlying assumption is that compounds that induce toxicity through similar mechanisms will elicit comparable changes in gene expression. It is, therefore, possible that toxicant-induced expression changes will act as sensitive and specific indicators of toxic mechanism. In this way, gene expression 'fingerprints' can be identified for multiple mechanisms of toxic insult and entered into a database. The gene expression profile of a suspected toxicant can then be analysed for similarity with the expression fingerprints of known toxicants.

The predictive capacity of gene expression profiling has been demonstrated most compellingly in a clinical setting. A number of studies have reported the classification of tumour type using transcript profiling (reviewed by Clarke et al., 2001). For example, van't Veer et al. (2002) identified a gene expression 'fingerprint' capable of distinguishing metastatic and non-metastatic breast tumours. This approach has also been used successfully to predict chemical activity. The most comprehensive study of this kind involved a combination of chemical treatments and mutant strains of the yeast *Saccharomyces cerevisiae* to generate a gene expression database capable of predicting the biological effects of exogenous compounds (Hughes et al., 2000).

Two recent studies indicate that toxicogenomics can be used to predict chemical mode of action in toxicologically relevant species (Waring et al., 2001; Hamadeh et al., 2002). These reports demonstrate that the liver gene expression profiles associated with exposure of rats to different hepatotoxins segregate according to mechanisms of toxicity. Thus, it appears that the assertion that toxicogenomics has the potential to provide enhanced methods for predicting toxicity is well founded. The rodent liver is ideally suited for demonstrating proof of principle: the hepatocyte is the predominant cell type, therefore hepatotoxic chemicals will induce mechanistically linked gene expression changes in the majority of cells that make up the organ. However, many toxicants target only a small proportion of cells in an organ. A challenge for the future application of toxicogenomics in a predictive context is the identification of diagnostic gene expression changes originating from cells that represent a minority population. Nevertheless, it appears that this general approach holds much promise.

3. Toxicogenomics as a mechanistic tool

The global analysis of gene expression levels has found many diverse applications in modern biology. A particular strength of this approach as applied to toxicology is that it is holistic and, therefore, provides an unbiased view of alterations in cellular processes associated with chemical insult. In this regard, global gene expression profiling is an ideal tool for hypothesis generation in the context of mechanistic toxicology. Individual genes, or entire pathways, implicated in a mechanism of toxicity using this technology can be further evaluated using more conventional approaches.

A major challenge in the application of gene expression technologies to mechanistic toxicology is the identification of gene regulation events linked directly to the mode of toxicity under investigation. Successful application of toxicogenomics in this context requires an understanding of the link between gene expression changes and phenotype (Smith, 2001). The simultaneous measurement of changes in the expression levels of tens of thousands of genes is now becoming routine. However, the increase in the rate at which gene expression data can be generated has not been accompanied by corresponding advances in our ability to interpret them as biologically meaningful information.

Any given toxicant is likely to induce alterations in the expression levels of many different genes, and only some of these genes will play a role in the mechanism of toxicity. Appropriate experimental design can facilitate the identification of relevant gene changes. For example, the use of animal models in which pathways relevant to the mode of action have been inactivated or modified can aid the identification of gene expression changes directly linked to the molecular mechanism of a toxicant. Transgenic 'knock-out' mice resistant to the toxic effects of the compound being studied can be used to identify genes whose regulation is not directly related to the development of toxicity. Changes in gene expression seen in these knock-out mice exposed to toxicant are unlikely to be linked to the adverse effects of the compound. Therefore, any changes in gene expression that occur in a sensitive wild-type animal, but not in a resistant knock-out animal, are more likely to be directly associated with the mechanism of toxicity. While, not all gene expression changes that match this description will be directly involved in the mode of action of a toxicant, this strategy focuses attention on the most likely candidates. This approach as been used to implicate the lactoferrin protein in the mechanism of rodent non-genotoxic hepatocarcinogenesis induced by peroxisome proliferators (Hasmall et al., 2002).

Toxicant-induced gene expression changes are often difficult to interpret in isolation. Careful selection of compound dose and time of exposure and the concurrent collection of conventional toxicology data (e.g. biochemical, clinical and histopathological data) can greatly facilitate the interpretation of toxicogenomic data. A successful toxicogenomic study will, therefore, be multidisciplinary, requiring the expert skills of the toxicologist, pathologist and molecular biologist (Orphanides et al., 2001).

4. Conclusions

Toxicogenomics is an evolving science. We have witnessed many successes of the genomic sciences in other fields of biology, and these tools are now beginning to enhance our ability to understand and predict mechanisms of toxicity. It is likely that toxicogenomics, along with other global profiling tools such as proteomics (Pandey and Mann, 2000) and metabonomics (Nicholson et al., 2002), will revolutionise research and investigative toxicology, leading to a holistic appreciation of molecular responses to toxicants. However, there is still a long way to go before the full potential of toxicogenomics is realised. The sheer weight of data generated by gene expression profiling can be overwhelming. Extraction of value from this data will be facilitated by the development of toxicogenomic databases capable of being interrogated by expert and non-expert user alike. Moreover, the identification of gene expression changes of predictive value or mechanistic significance often requires the use of sophisticated computational tools, which will evolve alongside gene expression methodologies (Bassett et al., 1999). One thing we can be confident about is that the tools of the genomic era are here to stay. The toxicologist of the future may feel equally at home with a toxicogenomic data set as with a histopathology slide.

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Review

Genomic analysis of stress response genes

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Abstract

Mammalian cells respond to a wide range of external stimuli including growth factors, peptide hormones, cytokines, osmotic stress, heat shock, pharmacological agents and toxicants via multiple signalling pathways. Genome-wide transcript profiling simultaneously monitors the gene expression programs downstream of all signal transduction pathways and can identify novel molecular targets for stress-inducing signals. Our laboratory has combined transcript profiling of cytotoxic compounds with experimental systems in which signalling components are disrupted (e.g. small molecule protein kinase inhibitors) to reveal the contribution of specific signalling pathways to the transcriptional response to toxicant-induced stress. A complementary approach for elucidating the molecular mechanisms that regulate transcriptional responses to toxicants involves DNA sequence analysis of gene regulatory regions obtained via data mining of recently completed mammalian genome sequences. Together, these approaches reveal the molecular mechanisms used to finely tune alterations in gene expression, enabling cells to react in an appropriate manner to external stress-inducing stimuli.
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Keywords: Stress response; Protein kinase; Signalling pathways; Gene regulation; Transcript profiling; Microarray

1. Introduction

The exposure of a cell to external stimuli triggers complex intracellular signalling cascades that result in finely tuned alterations in gene expression, enabling the cell to react in an appropriate manner via regulation of cell growth and division, differentiation, metabolism and many other functions. Individual stimuli can activate multiple signalling pathways, including mitogen-activated protein (MAP) kinases, inositol lipid signalling, nuclear factor-κB, protein kinase C, intracellular Ca^{2+} release or signal transducers and activators of transcription that produce phosphorylation-dependent activation of transcription factors. These recruit active transcription factor complexes to upstream regulatory sequences of immediate-early (IE) genes and initiate transcription by RNA polymerase II. IE genes encode a range of proteins that include transcription factors and cytokines, and these in turn regulate secondary transcriptional responses appropriate for the particular stimulus to which a cell is exposed. Cellular

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responses to stress involve a number of defensive processes aimed at minimising cell damage and redressing the balance of homeostasis and a component of this response is mediated via the expression of IE genes. IE genes such as *c-jun* and *c-fos*, which encode components of the signal-responsive transcription factor AP-1, are rapidly and transiently induced by a wide range of stress stimuli, including heavy metals (e.g. arsenite), UV radiation, heat shock, and the stress-inducing pharmacological agent anisomycin. Although each of these stimuli induces overlapping signalling pathways and target genes, the exact profile of IE gene expression is stimulus-specific. Thus a detailed characterisation of molecular responses to stress-inducing stimuli requires the simultaneous analysis of multiple signalling pathways and the expression of their target genes.

2. Transcript profiling of stress response genes

Genome-wide transcript profiling using DNA microarray technology has the potential to simultaneously monitor the gene expression programs of all signal transduction pathways and can identify novel molecular targets for both naturally occurring and synthetic stress-inducing signals. In addition to identifying specific target genes, transcript profiling can also provide additional mechanistic information on the molecular responses a cell elicits upon exposure to stress-inducing stimuli. Kinetic studies allow the elucidation of staged transcriptional responses to external stimuli and can potentially reveal co-regulated genes. This is exemplified by the elucidation of the transcriptional program associated with the response of human fibroblasts to serum (Iyer et al., 1999) and the elucidation of gene expression signatures of immune responses (Shaffer et al., 2001). It is noteworthy that these transcript profiling experiments generated enormous data sets, the interpretation of which relied heavily on computational approaches for classifying and displaying gene expression patterns (reviewed in Young, 2000). In addition to regulating secondary transcription responses, some IE genes may also be involved in feedback regulation of signalling pathways. For example, a common gene target of activated MAP kinase signalling pathways is MAP kinase phosphatase 1 (MKP-1) that dephosphorylates (thus attenuating) MAP kinases responsible for the initial signalling event. Although transcript profiling is a powerful approach for genome-wide comparisons of the effects of external stimuli on cell behaviour and generally provides a good indication of differences in protein synthetic capability between the two mRNA populations, it is important to note that the amount of RNA accumulated at a given time after a cell responds to an external stimulus is dependent on the rates of transcription, RNA processing and export, and mRNA turnover. For example, many but not all, IE genes possess AU-rich stability elements, whose activity can also be regulated by signal transduction pathways. Thus, dissecting the molecular links between signal transduction and gene regulation will require the use of experimental systems in which the contribution of individual signalling components can be assessed.

Combining transcript profiling with animal models or cultured cell lines in which specific signalling components have been disrupted or modified is a powerful approach for identifying genes that are regulated by individual signalling pathways (Fig. 1). A variety of small molecule inhibitors allow specific signalling molecules (e.g. protein kinases) to be inhibited, thus revealing the target genes they regulate. Cells bearing targeted deletions or modifications of specific signalling molecules can also be generated by reverse genetics. These approaches facilitate the identification of gene expression changes directly linked to the molecular mechanism of a particular stress-inducing agent.

3. Genomic analysis of gene regulatory regions targeted by stress-activated signalling pathways

A complementary approach for elucidating the molecular mechanisms that regulate transcriptional responses to toxicants involves DNA sequence analysis of gene regulatory regions. Mammalian gene regulation relies on a wide range of complex gene regulatory elements that can lie

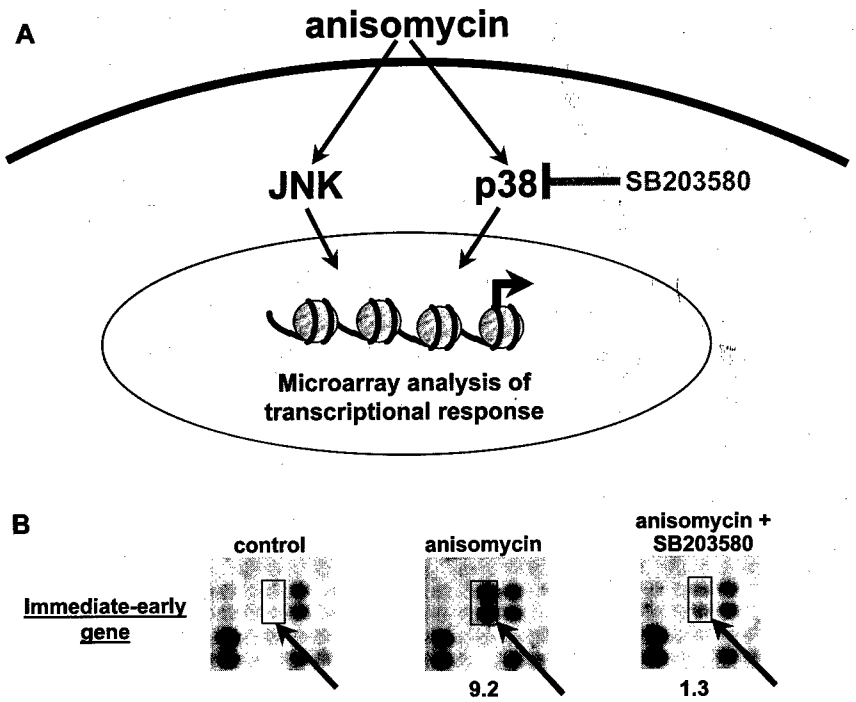

Fig. 1. Identification of genes regulated by specific signalling pathways using transcript profiling of cells treated with small molecule protein kinase inhibitors; (A) Anisomycin is a stress-inducing pharmacological agent that acts as a potent activator of the JNK and p38 MAP kinase cascades in mammalian cells. Genome-wide transcript profiling using DNA microarray technology can simultaneously monitor all the downstream gene targets induced by anisomycin; (B) Combining transcript profiling with the small molecule p38 MAP kinase inhibitor (SB203580) allows anisomycin-induced genes to be subsetted into p38-dependent and p38-independent classes. The example shows portions of cDNA microarrays after hybridisation with probes derived from mouse fibroblast mRNA. The pair of spots in a box contains duplicate cDNAs for an IE gene that is up regulated 9.3-fold by anisomycin. This transcriptional response to anisomycin is almost completely inhibited (to 1.3-fold) by the addition of SB203580. The adjacent pairs of spots represent genes that are not anisomycin-inducible.

within, close to, or at large distances from a given gene. Only a small number of mammalian promoter sequences have been experimentally characterised to date (e.g. European Promoter Database http://www.epd.isb-sib.ch/) and data mining of the recently completed human and mouse genome sequences (e.g. http://www.ncbi.nlm.nih.gov/genome/guide/) can potentially fill in the blanks by revealing the flanking DNA sequences of every gene. A variety of web-based genome sequence browsers (http://www.sciencemag.org/feature/plus/sfg/resources/res_maps.shtml) have been recently developed to facilitate the analysis of these databases. Examining the regulatory regions of genes identified through transcript profiling experiments has the potential to define subsets of genes that are directly regulated by specific transcription factors and signalling pathways (Fig. 2). The IE genes *c-fos* and *c-jun* contain different configurations of transcription factor binding sites and this presumably facilitates the fine tuning of transcriptional responses to specific extracellular stimuli. Examining the promoter regions of a wider range of IE genes, identified using transcript profiling experiments, should enhance our understanding of how this fine tuning is achieved. Although the bioinformatic tools for the prediction of mammalian promoter regions and gene regulatory elements are still evolving, it is already possible to rapidly analyse the flanking sequences of a given gene for transcriptional start sites, transcription factor binding sites (http://transfac.gbf.de/TRANSFAC/) and mRNA stability elements (http://bigarea.ar-

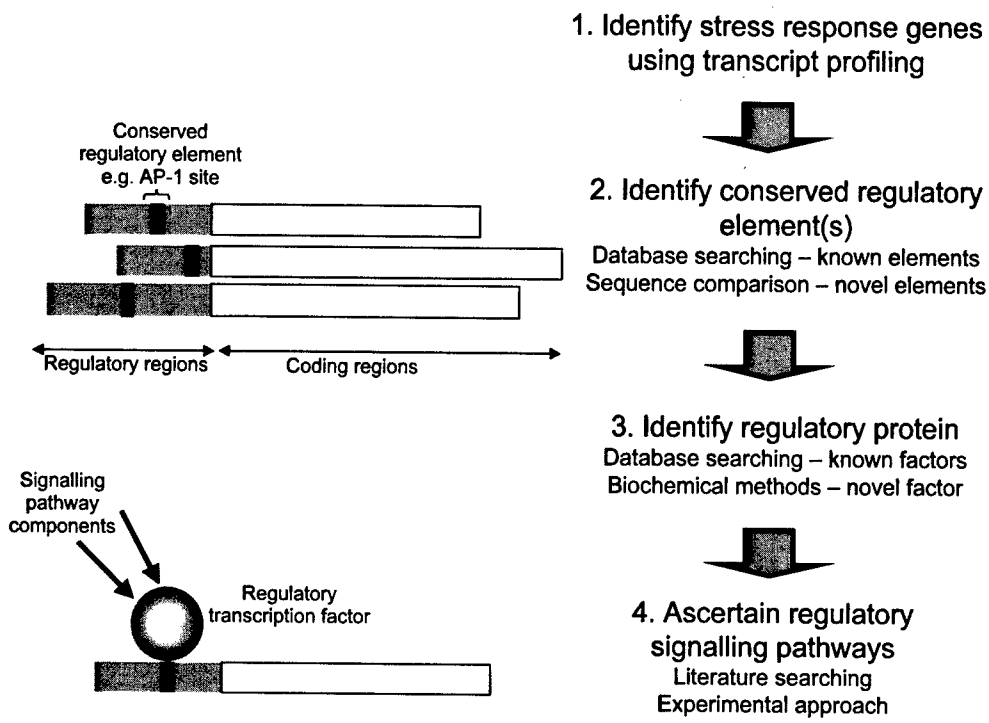

Fig. 2. Sequence analysis of gene regulatory regions as a basis for characterising the signalling pathways that regulate transcriptional responses to toxicants. Following the identification of stress response genes using transcript profiling, their regulatory regions are determined with the aid of promoter databases or genome sequence browsers. Genes co-ordinately regulated through the same pathway, and therefore by the same transcription factor(s), are predicted to contain the same DNA sequence motifs in their regulatory regions. Comparing the regulatory sequences of co-ordinately regulated genes can identify these conserved motifs. The transcription factor(s) that bind to these conserved DNA motifs to regulate gene expression can then be identified. The identity of the controlling transcription factor(s) may be sufficient to reveal the pathway of gene regulation and the signalling components involved. Where this is not known, or where the transcription factor has not been characterised previously, this may be determined by further experimentation.

ea.ba.cnr.it:8000/EmbIT/UTRHome/). Comparison of the promoter regions of co-regulated genes may reveal common gene regulatory elements and can lead to the identification of the controlling transcription factor. This genomic information can then be used to design biochemical experiments that measure directly the occupancy of candidate transcription factors on target gene regulatory elements. For example, the recruitment and modification of gene regulatory proteins bound to the promoter regions of stress response genes has been explored using the chromatin immunoprecipitation (ChIP) assay (Thomson et al., 2001 and references therein). The ChIP assay involves cross-linking transcription factors and regulatory proteins to their natural chromosomal targets in vivo followed by cell lysis, genome fragmentation and immunoselection of specific gene regulatory proteins along with the genomic DNA to which they were bound. Occupancy of specific genomic locations (e.g. stress response gene promoters) can then be measured via PCR using promoter-specific primers. This powerful assay can directly measure the integration of transcription factors and signalling pathways at the promoter of a given gene.

The sequences of many genes represented on DNA microarrays are expressed sequence tags (ESTs) that have not yet been assigned functions, because the EST represents either a novel gene or part of a putative mRNA. ESTs can now be rapidly mapped to the genome sequence, potentially revealing the intron/exon structure of the gene, flanking regulatory sequences and single nucleotide polymorphisms. Proteins encoded by

novel genes can be deduced using exon prediction algorithms such as GENSCAN (http://ccr-081.mit.edu/GENSCANM.html). Since most known ESTs are now mapped to the human genome, one can also obtain information on the likely tissue-specific expression pattern for given genes based on the tissue origin of ESTs that map to the same genomic location. There is already a wealth of information contained within the human and mouse genome databases but the number of annotations for each gene is rapidly increasing. Additional bioinformatic tools will be required to integrate information from transcript profiling data sets and their associated gene regulatory sequences and subsequent biochemical experiments will be crucial in demonstrating the functional significance of predicted gene regulatory elements.

4. Conclusions and perspectives

Genome-wide transcript profiling can simultaneously monitor the gene expression programs regulated by signal transduction pathways in response to stress-inducing signals. Combining transcript profiling with suitable experimental systems in which specific signalling components have been disrupted or inhibited is a powerful approach for identifying genes that are regulated by individual signalling pathways. The molecular mechanisms by which these genes integrate information from complex intracellular signalling cascades are being elucidated using a combination of genomic and biochemical approaches. Bioinformatic analysis of recently completed mammalian genome sequences has dramatically improved our ability to identify target gene regulatory sequences and subsequently predict which transcription factors recognise them. This genomic information is being used to design biochemical experiments that measure the occupancy of candidate transcription factors on target gene regulatory elements. It is important to note that the earliest intracellular response to an external stimulus involves post-translational modifications of signalling cascade proteins. The global responses of a cell's 'proteome' to the activation of a signalling pathway can be analysed by combining functional proteomics and mass spectrometry (Lewis et al., 2000). These data will complement genomic approaches in defining the molecular responses to stress-activated signalling pathways. A more detailed understanding of molecular mechanisms underlying transcriptional regulation of stress response genes will enhance our understanding of xenobiotic responses in mammals and should facilitate the development of compounds with favourable toxicity profiles.

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Review

Current status of developmental neurotoxicity: regulatory view

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Abstract

The need for developmental neurotoxicity testing has been recognized for decades and guidelines are available, as the USEPA guideline and the OECD draft TG 426. Regulatory testing of industrial chemicals for developmental neurotoxicity is required to some extent, especially for pesticides in the US. Until recently, however, developmental neurotoxicity testing of industrial chemicals has not been a clear regulatory requirement in EU, probably due to the lack of an accepted OECD TG. The revised EU Technical Guidance Document for Risk Assessment (EU-TGD) has now included the OECD draft TG 426 in the testing strategy for new and existing substances, and biocides. Hopefully, this will lead to an improved database for risk assessment of potential developmental neurotoxicants. However, the regulatory authorities and toxicologists will also be faced with the challenge that decisions have to be made concerning e.g. when testing should be requested, how testing should be performed, as well as evaluation of the results and the regulatory consequences. In this paper, these three issues will be discussed based on the recommendations given in the EU-TGD.
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Keywords: Developmental neurotoxicity; OECD guideline; Regulatory testing

1. Introduction

A number of chemicals are known to produce developmental neurotoxicity in humans and other species, and as such the importance of assessment of chemicals for potential developmental neurotoxicity has been recognized for decades. At a workshop in the US in 1989 the degree of qualitative and quantitative comparability between human and experimental data was assessed for chemicals where the data were available, i.e. lead, methyl mercury, selected agents of abuse, phenytoin, PCB, ethanol, and ionising radiation (Francis et al., 1990). The workshop evaluated the following functions: motor development and function, cognitive function motivational/arousal behaviour, sensory function, and social behaviour. Although a number of limitations were identified for cross-species comparability, the degree of comparability was considered remarkable. It was not possible to make definitive quantitative comparisons, but there were indications, that for some

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of the agents discussed, cognitive function appeared to be the most sensitive category. Comparisons of the administered effective doses revealed a wide range of differences among species, while comparisons using internal doses (e.g. blood or brain levels) showed a remarkable correlation (generally, 1–2 fold difference). The workshop concluded that developmental neurotoxicity should be recommended on a case-by-case basis and agreed upon a number of criteria to be used as triggers for deciding the need for testing.

A guideline for developmental neurotoxicity study was issued by USEPA (US Environmental Protection Agency) in 1991 and a revised US guideline was proposed in 1995 (USEPA 1995). During recent years a proposal for an OECD Test Guideline 426 Developmental Neurotoxicity Study has been developed based on the US guideline (OECD 1999).

The USEPA has recently required registrants to conduct developmental neurotoxicity studies for a number of neurotoxic pesticides (EEA, 2002). In EU, the SCF (Scientific Committee for Food) has recommended that appropriate experts should address this issue with the view to setting criteria to decide when developmental neurotoxicity studies in the future are necessary (EU SCF, 1998). Until recently, however, developmental neurotoxicity testing of industrial chemicals has not been a clear regulatory requirement in the EU, probably partly due to the lack of an accepted OECD TG. The revised EU Technical Guidance Document for Risk Assessment (EU-TGD) has now included the OECD draft TG 426 in the testing strategy for new and existing substances, and biocides (EU-TGD 2001). Hopefully, this will lead to an improved database for risk assessment of potential developmental neurotoxicants. However, the regulatory authorities and toxicologists will also be faced with the challenge that decisions have to be made concerning e.g. when testing should be requested, how testing should be performed, as well as evaluation of the results and the regulatory consequences. In the following, these three issues will be discussed based on the recommendations given in the EU-TGD.

2. When is developmental neurotoxicity testing requested?

There is at present no general requirement that pesticides or other chemicals be tested for developmental neurotoxicity prior to their registration and use (Claudio et al., 2000). Instead, the testing strategy in the EU-TGD and in the US relies upon a tiered approach to determine when testing be recommended.

In the EU-TGD, the key data requirements for reproductive toxicity testing for the new substances, existing substances and biocides are generally identified as: a two-generation study (OECD TG 416), and a prenatal developmental toxicity (teratogenicity) study in two species (OECD TG 414). However, these key data requirements can be modified, resulting in either a reduced testing or a need for accelerated or extended testing, depending on the regulatory program, and influenced by factors such as structural relationships with a known reproductive toxicant, the results of other toxicity studies, concerns for endocrine disruption and anticipated use and human exposure patterns.

The first specific reproductive toxicity test to be conducted should usually be the two-generation study. Ideally, this test should be initiated after the rat 90-day sub-chronic repeated dose toxicity study (if it is to be conducted) as this study can provide information necessary for selecting the appropriate dose levels for the two-generation study. Additionally, repeated dose toxicity studies can provide toxicity information of relevance to reproduction that should be taken into account when designing the two-generation study. For example, the observation of neurological effects can trigger the need for a developmental neurotoxicity evaluation.

In determining the necessity for developmental neurotoxicity testing, a weight-of-evidence approach should be used. Data from all available toxicity studies, as well as potential human exposure information should be considered. Developmental neurotoxicity testing should be conducted to further characterise neurological effects observed in other studies, and should be considered if the substance has been shown to cause neurotoxicity or structural abnormalities of

the CNS in other studies, or suspected of interfering with neurotransmission or neuroendocrine pathways (thyroid, pituitary, or circulating sex hormones) at the CNS level. For example, neuroendocrine interference at the level of the hypothalamic-pituitary axis might be inferred from changes in the levels of circulating gonadotrophins or steroid sex hormones.

The above criteria or triggers for developmental toxicity testing in the EU-TGD are rather similar to those used in the US and recently it has been questioned based on US-EPA experience whether these criteria are sufficient (Claudio et al., 2000).

When looking at the triggers it is clear that some toxicity information is needed.

For example, information on adult neurotoxicity may come from repeated dose toxicity study. However, specific neurotoxicity testing in adults may only be performed to a limited extent and for some chemicals and therefore this knowledge may not be present.

Relevant data on structural abnormalities in the CNS can be obtained only from the prenatal developmental toxicity study. This study is part of the minimum data requirements in EU-TGD, but as already mentioned the two-generation test is usually recommended as the first reproductive test. A developmental neurotoxicity study can be conducted as a separate study, or as an add-on study. In the EU-TGD, it is recommended to perform developmental neurotoxicity testing as an add-on to a two-generation study using offspring that would otherwise be discarded at weaning since this will not involve the use of additional groups of animals. Consequently, the need for inclusion of a developmental neurotoxicity evaluation has to be considered at the planning stage of a two-generation study, i.e. normally before the prenatal developmental toxicity study has been performed. Therefore, the relevant data needed for triggering developmental neurotoxicity testing may not be available.

A number of existing chemicals will probably meet the criteria for the requirement of developmental neurotoxicity testing, e.g. some pesticides, organic solvents and metals, and hopefully the testing of these chemicals for developmental toxicity will lead to a better basis for risk assessment. However, limitations in the data set for a number of chemicals indicates that the triggering schema may not be sufficient to elicit testing of all chemicals that may be developmental neurotoxicants. This is worrying as there is a regulatory need for identifying chemicals that may induce neurotoxicity during development.

3. How to test-developmental neurotoxicity studies

A standard test method is currently being developed, as the OECD draft TG 426 and this guideline is recommended for regulatory testing in the EU-TGD. Consequently, the choice of guideline will not be a great challenge for the regulators.

The evaluation consists of observations to detect gross neurological and behavioural abnormalities; the assessment of physical development, including sexual maturation, reflex ontogeny, motor activity, motor and sensory function, and learning and memory; and the evaluation of brain weights and neuropathology during postnatal development and adulthood. The overall design of the study as well as the functional end points are relevant and should ideally allow the identification of potential developmental neurotoxicants. Some functions of potential relevance for e.g. endocrine disruption such as social interaction and mating behaviour are not included in developmental neurotoxicity guidelines or any guidelines at present. Further studies are needed to evaluate the need for testing these functions in order to have a sufficient assessment of potential developmental neurotoxicity.

The functional end points rather than specific test methods are mentioned in the guideline in order to have flexibility and allow the use of the most up to date behavioural methods. Some guidance on method selection and specific examples are given in the guideline. However, the methods mentioned for some functional end points, e.g. hearing function and cognitive function, range from tests with a reasonable sensitivity to some test with a relatively low sensitivity (Hass, 2002). For example, a simple water maze is part of many testing batteries used to collect data on learning and memory functions. However, the

demands on the nervous system in this test may not be sufficient to detect subtle damages in the brain (Ulbrich and Palmer, 1996). Therefore, the flexibility in the OECD guideline may become a disadvantage if methods with a low sensitivity are chosen. Choice of appropriate testing methods is crucial for the identification of developmental toxicants and it will be a challenge for regulators to encourage the use of sufficiently sensitive methods.

4. Results of the developmental neurotoxicity study and further action

The experience of offspring especially during infancy may affect their later behaviour. For example, frequent handling of rats during infancy may alter the physiological response to stress and the behaviour in tests for emotionality and learning. In order to control for environmental experiences, the conditions under which the offspring are reared should be standardised within experiments with respect to variables such as noise level, handling and cage cleaning. The performance of the animals during the behavioural testing may also be influenced by the time of day, and the stress level of the animals. Therefore, the most reliable data are obtained in studies where control and treated animals are tested alternately and environmental conditions are standardised. Performance of developmental neurotoxicity testing according to the OECD draft guideline would normally ensure that reliable data are obtained. However, these issues have to be considered when evaluating e.g. non-guideline developmental neurotoxicity studies for regulatory purposes.

Developmental neurotoxicity can be indicated by behavioural changes or morphological changes in the brain. The severity and nature of the effect should be considered. Generally, a pattern of effects (e.g. impaired learning during several consecutive trials) is more persuasive evidence of developmental neurotoxicity than one or a few unrelated changes. The reversibility of effects should be considered, too. Irreversible effects are clearly serious, while reversible effects may be of less concern. However, it is often not possible to determine whether an effect is truly reversible. The nervous system possesses reserve capacity, which may compensate for damage, but the resulting reduction in reserve capacity should be regarded as an adverse effect. For example, if developmental neurotoxicity is observed only during some part of the lifespan then compensation should be suspected. Also, effects observed for example during the beginning of a learning task but not at the end should not be interpreted as truly reversible effects. Rather the results may indicate that the speed of learning is decreased. The above considerations are the guidance given in the EU-TGD and they briefly mention some important aspects when evaluating developmental neurotoxicity studies. However, other issues may be also important such as evaluations of gender-specific effects, statistical issues, and effects occurring at doses causing maternal toxicity. Also, the EU-TGD gives no guidance on how to evaluate the nature and severity of the effects. Normally, a study report on a guideline study includes a discussion and evaluation of the results obtained, but evaluations of developmental neurotoxicity data may sometimes differ among study directors and other experts. Therefore, regulators are generally advised to perform their own evaluation or have the study evaluated by other experts than the study directors.

Equivocal results may need to be followed up by further investigation. The most appropriate methods for further investigations should be determined on a case-by-case basis guided by the effects seen in the developmental neurotoxicity study, adult neurotoxicity studies and/or structure-activity-based predictions.

Further testing will normally not be required when the results are clearly negative. However, a second study in another species and/or examination of developmental neurotoxicity end points not covered yet may be considered when there are potential widespread exposure of women of childbearing age and indications of developmental neurotoxicity in humans (EU-TGD 2001).

No further testing will normally be required when the results of the developmental neurotoxicity study are clearly positive. Ideally, such results should lead to classification of the chemical as

being toxic to reproduction. However, for many regulators behavioural effects are a relatively new area compared to e.g. malformations and consequently the severity of behavioural effects may not be recognized. Therefore, the future regulatory use of developmental neurotoxicity data would benefit from increased knowledge among regulators.

5. Conclusions

The revised EU-TGD has now included the OECD draft TG 426 Developmental Neurotoxicity Study in the testing strategy for new and existing substances, and biocides. Hopefully, this will lead to an improved database for risk assessment of potential developmental neurotoxicants. However, the regulatory authorities and toxicologists will also be faced with the challenge that decisions have to be made concerning, e.g. when testing should be requested, how testing should be performed, as well as evaluation of the results and the regulatory consequences. The testing strategy in the EU-TGD and in the US relies upon a tiered approach to determine when testing be recommended. A number of existing chemicals will probably meet the criteria for requiring developmental neurotoxicity testing, however, limitations in the data available for a number of chemicals indicates that the triggering schema may not be sufficient to elicit testing of all chemicals that may be developmental neurotoxicants. This causes concern as there is a regulatory need for identifying chemicals that may induce neurotoxicity during development.

Choice of appropriate testing methods is crucial for the identification of developmental toxicants and the overall design of the study as well as the functional end points included in the OECD draft guideline are relevant and should ideally allow the identification of potential developmental neurotoxicants. However, the guideline allows the use of some methods with a relatively low sensitivity and it will be a challenge for regulators to encourage the use of sufficiently sensitive methods.

Developmental neurotoxicity data is a relatively new area for many regulators and they will be faced with the challenge that more data come from specific tests involving sophisticated techniques sometimes requiring specialists for interpretation. The future regulatory use would therefore benefit from a generally increased knowledge among regulators.

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Review

Current status of developmental neurotoxicity: an industry perspective

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Abstract

The chemical industry, along with the rest of society, shares the fundamental goal to protect the health and safety of children. Most of the existing testing programs for environmental chemicals primarily address the adult organism. Developmental neurotoxicity testing (DNT) studies are especially designed to address the specific risks of the developing nervous system. At time, DNT studies are not a regulatory requirement (US EPA) for all pesticides, however, these studies are recommended for and are now being required as Tier II studies for compounds which have shown evidence of neurotoxicity, endocrine modulation or in some cases developmental or reproduction toxicity. Laboratories, which are going to conduct this type of study, should have broad experience in reproduction toxicology and neurotoxicology studies. The DNT study includes a so called *'behavioral testing battery'* to examine the development of autonomic, motor, sensory, and cognitive functions, where tests are performed at different time-points to cover important developmental stages of the nervous system. The development of the structure of the nervous system is carefully examined by neuropathological and morphometric examinations at two time points to detect morphologic abnormalities and growth defects. Critical issues concerning the conduct and interpretability of DNT study data include experience in the handling and logistics of large number of animals, historical control data, lack of scientific data of certain developing processes and uncertainties concerning the choice of the best methodical approaches.
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Keywords: Developmental neurotoxicity; DNT studies; Rat animal model; Practical approaches; Critical issues; Industry perspective

1. Introduction

BASF—as the chemical industry worldwide—strongly supports and is fully committed to the 'Principle of Responsible Care and Sustainable development'. In recent years, there has been a growing discussion, whether environmental chemicals may be a special hazard for children due to the fact that children are in many ways not only little adults. The development of children can be disturbed during different developmental stages in pregnancy as well as early and late childhood. Indeed, still young adults show processes of maturation that could make them differently

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susceptible compared with adults where organ development has been fully matured. Related to their body weight, children breathe more air, drink more water, and eat more food than adults. Their different behavior patterns when compared with adults (hand-to-mouth activities, play close to the ground) can increase exposure to environmental toxicants. Some of the metabolic capacities of children are still developing, making them less able to metabolize, detoxify, and excrete exogenous substances; so, children may react less or more sensitive than adults (Dorman et al., 2001; EPA, 1998a). On the other hand there is also a large body of evidence from the use of pharmaceutical products that children need higher doses per kg body weight to achieve the desired effects of the drug (Küttler, 2002). The potential effects of chemicals on the intra-uterine development is investigated for drugs, pesticides and partly industrial chemicals in prenatal toxicity studies in rats and rabbits. Moreover, the pre-, peri- and postnatal development for the above-mentioned chemicals is also investigated in one or two generation studies with continuous administration of the test substance. Although there has been no evidence that the current use of chemicals has resulted in any harm to the neurological development of children, to more fully address the potential hazard of a chemical to children's brain development, the developmental neurotoxicity testing study (DNT study), as required by the US EPA was introduced to the BASF Experimental Toxicology Laboratories, as an experimental tool for testing a chemical compound's potential of developmental neurotoxicity.

2. The scientific background

The developing brain is not unprotected. In utero, the *placental barrier* plays critical roles in maintaining and protecting the developing fetus by maintaining an appropriate hormonal environment and nutrient transfer. During lactation, the *blood–milk barrier* protects the nursing offspring. For example, high molecular-weight or hydrophilic compounds cross these membranes very poorly when at all. Conversely, low molecular-weight and lipophilic chemical compounds are fully capable of crossing the placental and blood–milk barriers (Dorman et al., 2001). So, as these 'external' barriers do not completely protect the developing brain, some important neurological differences between the immature, developing brain and the mature, adult brain have to be considered more closely. These neurological differences correlate with the 'specific risk' or 'different vulnerability' of the developing brain of the unborn, newborn and young children in respect to the kind of neurotoxic impacts when compared with the adults.

One of the specific features of the central nervous system is the blood–brain barrier (BBB) that represents an important protective system in the adult brain, as it actively isolates the brain from the blood (Aschner, 1998). As long the critical process of angiogenesis in the growing brain is progressing, the BBB is still immature and functionally not fully competent. This causes a different neuropathology in the case of a neurotoxic impact. Experimentally, the different outcome of lesions in the adult versus the developing brain is well-documented for cadmium poisoning (Gabbiani et al., 1967a,b; Reuhl, 1988). When treated between postnatal days (PD) 4 and 20, rat pups developed a hydrocephalus internus and severe diffuse hemorrhages in the cerebral white matter and the cerebellum, associated with extensive neuronal and glial necrosis. Young adults treated on PD 30 showed only a hemorrhagic necrosis at the trigeminal and spinal sensory ganglia, a location where no BBB exists. Heavy metals in general and lead and mercury in particular belong to the most critical group of developmental neurotoxicants with a different outcome of structural and functional neurotoxicity during the different stages of nervous system development and the adult stage (Verity, 1997).

A second neurological difference is important to consider as well. The 'time window' of a neurotoxic impact on the developing nervous system is critical for the kind of lesion as brain nuclei and structures are only generated during predetermined, narrow time windows. So, *timing of exposure* with a developmental neurotoxicant plays a key role for the kind of lesion and its structural and functional consequences whereas in

the adult nervous system the *timing* of exposure plays no role for the neurotoxic outcome. Bayer et al. (1993) reported the different neuropathology of X-irradiation from a series of experiments in rats as a consequence of such a different timing of exposure. Antimitotic agents, like methylazoxymethanol (MAM) are further examples where a different timing of exposure during pregnancy led to different neuropathology lesions in rat experiments. For example, a single ip dose of approximately 30 mg/kg MAM caused a hypoplasia of the cortical mantle most severely when treatment was on gestational day (GD) 15 (Fischer et al., 1972). Newborn pups treated on PD 1 showed a 31% decrease in the cerebellum weight with a reduced number of granule cells (Kabat et al., 1985). In these animals the cortex cerebri was of normal size.

A third neurological difference has to be addressed in addition. The important developmental processes of *neuronal differentiation*, *axiogenesis* and *synaptogenesis* may be influenced by xenobiotics as well. For example, treatment with receptor-acting agents during that critical period of brain maturation may influence the process of neuronal differentiation and may cause an irreversible 'imprinting' of receptor densities whereas in adults, receptor-mediated actions have reversible character ('pharmacological effects'). The sexual differentiation of the brain is a good example in this respect. In rats, the critical period of the sexual differentiation is between PD 7 and 9. Testosterone propionate injections into the female neonate for 9 days after birth changes the perinatal differentiation of sexual dimorphic nuclei and lead to androgenized females. Those females are characterized by a male sexual behavior pattern and a size of the medial preoptic nucleus, one of the sexual dimorphic nuclei, typical for males (Diaz et al., 1995; Maeda et al., 2000).

These neurological differences are the key to understand that a variety of environmental conditions and factors may harm the brain development in children, which show not necessarily neurotoxic effects on the adult brain as well. Such additional factors may be virus infections of the pregnant woman (e.g. rubella, measles, influenza), hormonal influence (androgens, estrogens) or hormonal deficiencies (iodine, hypothyroidism). Prenatal and lactational mal- or undernutrition during the rapid growth period of the brain is critical for the immature brain with possible long-lasting functional and structural consequences (Wiggins, 1982; Levitsky and Strupp, 1995).

Beside the well-documented developmental neurotoxicants lead, mercury, ethanol and the PCBs, only a little information exists to what extent pesticides and environmental chemicals in use are a true risk for the children's brain development. Epidemiological studies may add only limited information. Too many confounding factors, as maternal smoking and drinking, drug abuse and socio-economic factors (life-style, hygiene standards, poverty), and the state of infection rates may make it impossible to come to a reliable assessment on the etiologic meaning of environmental chemicals and pesticides to which the population (via food residues) or certain subgroups (farm worker families) were exposed in addition. At least the appropriate experimental testing and animal model may give some more information concerning the neurotoxic potential of chemicals and their possible impact on children. This may help to eventually clarify the question of an impact of environmental chemicals on children's health.

3. What is already done and where we are going

Considering the core toxicology data set required by governmental regulatory agencies for new chemical compounds, a broad variety of endpoints must be tested. This includes tests on mutagenicity, acute and dermal toxicity studies, chronic toxicity and carcinogenicity studies, acute and sub chronic neurotoxicity studies (at 'neurotoxicity screening battery'), and reproduction toxicology studies (prenatal toxicity, one- and multi-generation studies). Many of these studies are performed with different species, both rodents and nonrodent, and are designed to collect clinical, biochemical, and clinical pathology and pathomorphology data. All these data taken together are used for a risk assessment for humans and are relevant for adults as well as in many regards also

for children, especially those studies involving in utero exposure (prenatal toxicity, and generation studies) and exposure during postnatal development (generation studies). Especially pesticides and drugs belong to the chemicals where a very broad database already exists. It was long accepted by all regulatory agencies that these data are sufficient to assess the risk for the human population, for adults as well as children. A more sophisticated approach was only triggered when indeed some neurotoxic effects in adult neurotoxicity studies (ANT-studies) or teratogenic, especially neuroteratogenic effects (e.g. brain malformations) were found. For these cases, a new study type was developed in the beginning of the 1990s, designed and discussed by academic, regulatory and industrial scientists which let to the first DNT guideline (US EPA OPPTS Developmental Neurotoxicity Testing Guideline § 83-6) which was finalized in 1991. In the meantime, a revised version was finalized and published in 1998 (EPA, 1998b). The new OECD Revised Draft Guideline 426 (OECD, 1999) principally follows the same approach. Until 1998, only a total of 12 DNT studies were submitted to the OPPTS of the US EPA for evaluation and to support registration for use of pesticides and toxic substances, among them organophosphates, carbamates, and solvents (Makris et al., 1998). At that time the children health policy of the US EPA required DNT studies only on the basis of the chemical database (weight-of-evidence) as a second tier toxicology study.

The US Food Quality Protection Act (FQPA) and the Safe Drinking Water Act Amendments of 1996 which focused on children as susceptible population, required a much more extensive evaluation of potential risks to children (Fenner-Crisp, 1998). In that sequence, an EPA toxicology-working group was established which recommended the inclusion of a developmental neurotoxicity study in the minimum core toxicology data set for all chemical food-use pesticides (EPA, 1998c). On 10 September 1999, the US EPA issued a Data Call In (DCI) that requires DNT studies for all registered organophosphate (OP) insecticides. In addition, DNT studies are highly recommended and required as a Tier II test for all other pesticides showing evidence of neurotoxicity, endocrine modulation or in some cases developmental or reproduction toxicity. The DNT study results help determine the level and need of an additional FQPA safety factor.

3.1. The animal model used—advantages and disadvantages

Rats (Wistar or Sprague–Dawley strains) are the experimental animals of choice for performing DNT studies. They belong with mice to the most commonly used test animals in behavioral teratology studies. The extensive background information present concerning normal behavioral and neural development made them the favored species in screening studies on toxin-induced injuries during brain development (Meyer, 1998).

In principle, the development of the nervous system of all mammals follows the same schedule and is comparable with the brain development in humans. Many papers and literature reviews confirm the comparability of developmental neurotoxic effects gained in the laboratory rat with those reported in humans (Stanton and Spear, 1990; Schardein, 1998; Kaufmann, 2000). But there are some differences to be considered carefully when planning such experiments. First, in relation to the time of birth, the period of the 'brain growth spurt' (period of enhanced brain growth) is differently positioned. In humans, as well as in guinea pigs and dogs, this period is almost completed before birth, in rats this period lasts far into the postnatal developmental period (around PD 10). So, a possible in utero exposure in humans via the placental barrier has to be compared with a lactational exposure after birth in rats via the blood–milk barrier. For pesticides, recommendations were developed which include the proof of the presence of the test compound or its metabolites in the milk or the use of alternative exposure routes (direct pup dosing) to get data that postnatal exposure levels in rats compare with in utero exposure scenarios of humans. For testing of pharmaceuticals for use in children, the use of dogs or mini pigs in a different study design seem to be more appropriate to cover this important period of brain development. Rats are as well not suitable test animals testing toxicant effects of

higher cognitive functions (Meyer, 1998). In summary, apart from these limitations, the DNT studies with rats are recognized as good predictors for a compound's potential of developmental neurotoxicity risk in humans (Kimmel, 1998).

3.2. The 'classical' DNT approach for pesticides

DNT studies performed in industry follow in general the requirements of existing testing guidelines (EPA, 1998b; OECD, 1999) and further recommendations given by the agencies. The full scope of examinations include different subsets of rat pups which are used for observations in an open arena ('open field observation' with a thorough documentation of many parameters including impacts on *autonomic functions* as shown in Table 1), in automated devices for measuring the development of motor activity (impact on *motor function*?) and the auditory startle response (impact on *sensory* function?). Separate subsets of pups are used for testing the learning and memory (impact on *complex* ['cognitive'] functions?) after weaning and as young adults. At least, two different subsets are taken for neuropathology investigations including brain weight measurements, examinations by macroscopy, light microscopy, and morphometry of selected major brain areas (impact on the *development of structure?*). The assignment of subsets and the days of testing as it is done in the BASF Experimental Toxicology and Ecology Laboratory are listed in Table 2.

Table 1
Observation in an open area ('open field observation')

		Score/comments
1	Behavior in handling	
2	Fur	
3	Skin	
4	Posture, body tone	
5	Salivation	
6	Respiration	
7	Activity/arousal level	
8	Tremors	
9	Twitches	
10	Convulsions	
11	Abnormal movements (bizarre, stereotypic)	
12	Gait abnormalities	
13	Lacrimation	
14	Palpebral closure	
15	Exophthalmus	
16	Assessment of the feces discharged during the study (appearance/consistency)	
17	Assessment of the urine discharged during the study (quantity, stain)	
18	Pupil size	

Checklist with parameters assessed and recorded for all scheduled animals. Findings are scored or described in detail.

In difference to the finalized OPPTS DNT Guideline from 1998, we follow the recommendations to dose dams up to weaning (PD 21) and use ten instead of six animals per test group for neuropathology and morphometry. Instead of selecting PD 11, PD 22 is taken for the first subset for neuropathology, but the brains of one subset sacrificed on PD 11 are kept in fixation fluid as well. These brains will be only investigated when further effects trigger a better recognition of structural changes at that time-point (e.g. characterizing effects of impacts on the external germinal layer of the cerebellum).

As seen in the Table 2, a variety of subsets are needed to conduct the diverse tests. Around 1000 pups have to be handled in a DNT study and at least 140-mated dams are used to produce enough pups from different litters available to the tests. A large number (640) of pups are kept for at least 3 weeks up to weaning, and 240 pups kept up to the young adult stage around PD 60–68. Together with the prenatal period, the in-life period of a DNT study lasts at least 3 months. That means many technician-hours per day, which continues after sacrifice with the histotechnical processing of the nervous system, including perfusion fixation and preparation of homologous sections, which are mandatory for any following morphometry. Sophisticated neuropathological examinations and morphometric measurements of selected brain regions of at least 80 and up to 160 test animals from two time-points (PD 22 and 62) follow. This scope of neuroclinical and neuropathological examinations makes a DNT study challenging from the logistic point of view, very laborious and costly.

Table 2
Assignment of pups to different subsets

Subset	Number of pups selected per sex per group	Day of examination	Study examinations
I	10	PD 11	Storage of the brain in fixative fluid
II	10	PD 22	Perfusion fixation, brain weights and neuropathology
III	10	PD 24 and 60	Auditory startle test
		PD 60 (+/−2)	Perfusion fixation, brain weights and neuropathology
IV	10	PD 4, 11, 21, 35, 45 and 60	Open field observation (OFO)
		PD 13, 17, 21 and 60 (+/−2)	Motor activity (MA)
V	10	PD 21 (+/−2)	Learning and memory test (L/M)
VI	10	PD 60 (+/−2)	Learning and memory test (L/M)

One subset consists of 80 pups, ten males and ten females per group: one male or one female out of 20 litters.

3.3. The 'special' DNT approach for organophosphates and carbamates

Decreases in the plasma acetyl cholinesterase (AchE) activity are a commonly accepted biomarker for exposure to organophosphate and carbamate pesticides (Tilson, 2000). Cholinesterase (ChE) inhibition data may, therefore, characterize the adequacy of postnatal dosing in DNT studies and are useful to compare the sensitivity of young and adult organisms. In addition to the DNT protocol discussed above, the DCI of the US EPA for DNT studies for the organophosphate pesticides includes a set of more investigations addressing this biomarker and support risk assessment for specific sub-populations and exposure scenarios (EPA, 2001). Exposures to pregnant and lactating women may be met by providing data, which include assessment of dams at the end of gestation (GD 20) and at the end of lactation (PD 21). Examinations on GD 20 cover exposures to fetuses and provide baseline measures for comparison with lactational exposure measures in pups on PD 4, the first time-point taken to cover exposures to nursing infants. The PD 4 ChE values may represent some carryover of prenatal exposure via blood (passing the placental barrier) and postnatal exposure via the milk (passing the blood–milk barrier). The exposure risk of nursing infants is addressed furthermore by taking values on PD 21 at the end of lactation, a time-point less likely influenced by prenatal exposure. These examinations may be all incorporated in a main DNT protocol by including a satellite group of dams for the GD 20 measurements in dams and fetuses (see Tables 3 and 4).

As recommended in the guidance on ChE measures in DNT and related studies (EPA, 2001), laboratories that conduct this kind of DNT studies should give data for the adequacy of postnatal dosing (e.g. milk concentrations of the test substance, along with milk and food consumption data in pups) or alternatively by evaluation of the toxicity data, e.g. survival, body weight, clinical signs and ChE inhibition. In the absence of signs of toxicity or ChE inhibition in the pups during one or more critical phases of neurological development, the adequacy of the DNT study as a whole may be called into question (EPA, 2001).

The testing of age-dependent differences in response to acute or repeated exposures of an organophosphate pesticide is performed in separate 'Comparative Sensitivity Studies'. Pups (both sexes) are tested after acute exposure on PD 11 (early–mid lactation) and 21 (late lactation) and as adults on PD 60 or older at the time of peak effect of ChE inhibition, which has to be determined in a range finding study. The repeated exposure testing of pups (both sexes) has to be started not later than on PD 11 and lasts to PD 21, preferably the same time-point when the acute measures are carried out. In adults, the same treatment regimen and exposure duration will be used and the day of sacrifice should be comparable to what was used

Table 3
Assignment of pups to different subsets-additional subsets for DCI organophosphorus compounds

Subset	Number of pups selected per sex per group	Day of examination	Study examinations
VII	10	PD 4[a]	Cholinesterase measurements (ChE)
VIII	10	PD 21	Cholinesterase measurements (ChE)
Dams	10	PD 21	Cholinesterase measurements (ChE)

Main study.
[a] Culled pups may be taken.

after acute exposure. All pups are directly dosed by gavage.

Direct pup dosing by gavage is a challenging effort for experimental laboratories. Pup gavaging should be done only by well-trained technicians to prevent the risk of a high loss of pups. Practical considerations associated with direct dosing of pups are summarized by Dorman et al. (2001): the logistical problems of a direct pup dosing of a large number of pups can be significant, there are limitations in the volume of solution that can be administered repeatedly to young animals, and direct pup exposure might injure or stress young animals which could lead to behavioral and functional effects not associated with exposure to the chemical.

4. Critical issues concerning DNT studies

Some of the newer regulatory requirements, particularly in the area of developmental neurotoxicity, have been introduced by extrapolation from basic biology without developmental toxicological validation. Hence the area is increasingly burdened by accumulation of data from studies which have the potential to be useful but which have no clear precedents for its interpretation (Ray et al., 2002). Coordinated, inter-laboratory validation efforts are, therefore, urgently needed to improve interpretability of data gained in DNT studies. For example, the US STP ad hoc Committee on Pathology Evaluation for DNT recognized the ILSI neuropathology review (Garman et al., 2001) as careful summation of the strengths and weaknesses of the various morphological approaches in DNT studies that provides a number of suggestions for the appropriate use of certain techniques. But a clear recommendation of the use of an optimal set of definitive techniques in DNT studies is missing. This emphasizes the relative uncertainty in the scientific community and lack of data regarding the best practical approaches in DNT studies. Secondly, a thorough understanding of indirect (via maternal toxicity) versus direct neurotoxic impacts on the development of the nervous system is still missing. The need and usefulness of more pharmacokinetic and pharmacodynamic data for assessment of developmental neurotoxicity is addressed by Dorman et al. (2001) and research needs are identified by the ECETOC LRI Health Effect teams (Ray et al., 2002) for developmental toxicokinetics. The basic parameters affecting toxicokinetics in the developing animal or child are still poorly understood (e.g. timing of maturation of xenobiotic metabolic enzymes).

These examples may illustrate the range of uncertainties still connected with the adequate conduct of DNT studies and interpretation of

Table 4
Gestational exposure group (GD6–20)

	Number of dams/fetuses selected per sex per group	Day of examination	Study examinations
Dams	8	GD 20	Cholinesterase measurements (ChE)
Fetuses	8	GD 20	Cholinesterase measurements (ChE)

collected data. Efforts have to be undertaken for more validation and setting appropriate standards in DNT studies to increase interpretability and comparability of DNT study data.

5. Conclusions

The pesticides sector has been subject to increasingly demanding regulatory requirements. One of these additional regulatory requirements includes the conduct of DNT studies to address the potential hazard to children as sub-population. DNT studies are a new experimental tool to detect a possible potential of a chemical compound to act on or to interfere with the development of the nervous system. The EPA Health Effects Test Guidelines OPPTS 870.6300 Developmental Neurotoxicity Study and the OECD Draft Guideline 426 from 1999 mainly guides the conduct of these studies. Two practical approaches are described, which are currently in use or introduced to our experimental laboratories. The high number of test animals used, the wide scope of clinical, neurobehavioral, and neuropathological examinations performed, make the conduct of this study type logistically a challenge, very laborious and costly in general and in special for ChE-inhibiting insecticides. Considerable industry resources are mobilized to collect data in DNT studies according to regulatory requirements for pesticides. However, some critical issues concerning DNT studies exist, among them a lack of scientific data concerning developmental toxicokinetics in the developing body and the nervous system and the need to increase the interpretability of DNT data. This will probably give more information what the best methodologies are and may lead to more standardized approaches.

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Short communication

The impact of intrauterine exposure versus postnatal environment in neurodevelopmental toxicity: long-term neurobehavioral studies in children at risk for developmental disorders

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Abstract

Various investigators have shown that enriched environment may positively affect the early brain development of experimental animals. Environment was also shown to positively affect the development of young children born to mothers of low socio-economic class (low SES). It is unknown, however, to what extent can an enriched environment improve the developmental outcome of children born with slight brain damage. We studied the development of preschool and early school age children born to heroin dependent parents raised at home or adopted in comparison to children suffering only from environmental deprivation (low parental SES) and to controls. They were examined by several professionals, using standard, age appropriate, neurological and psychological tests. Similar evaluations were performed on a group of early school age children born to mothers with pregestational or with gestational diabetes and on a group of children born prematurely, with a birth weight of less than 1500 g using various developmental tests. Young children born to heroin dependent mothers and fathers raised at home and children of low SES had, in comparison to controls, lower intellectual skills and a higher rate of inattention. This persisted at school age, too. Children born to heroin dependent mothers adopted at a young age and hence being raised in a good environment had normal intellectual function but a high rate of inattention and behavioral problems. We also examined the school age children for possible presence of ADHD and found a high rate of ADHD among all children born to heroin dependent parents including those adopted, as well as in the children with low parental SES. Similar findings regarding the strong positive influence of an enriched environment were observed in children born to diabetic mothers, where the intellectual abilities of the children were directly related with parental education. The cognitive abilities of the children born prematurely were also strongly associated with parental education and not with the degree of perinatal complications. In conclusion, in all groups of children at high risk for developmental problems was found that the environment has a strong influence on their intellectual abilities but not on motor skills or attention span. A good environment (high parental SES) may significantly improve the outcome.
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1. Introduction

One of the first investigators stating that the developing brain may be affected by the environment was Charles Darwin who attributed the larger brains of wild animals in comparison to the domesticated ones to the effects of wild life with more stimulation and experience. Later, Hebb (1949) introduced the concept of 'use-dependent plasticity of the brain'. This initiated various studies, amongst them the study by the group led by Rosenzweig who have demonstrated an increase in cortical brain weight, in dendritic branching and of acetylcholinesterase activity in rats subjected to enriched environments in comparison to rats reared in deprived environments (Rosenzweig and Bennett, 1996). Similar results were observed in experiments with other animals. Post-mortem examinations of the human brain have revealed a similar effect of the environment on brain development in man. For example, Jacobs et al. (1993) in right handed men and women a positive correlation between education and dendritic branching in Wernicke's center located in the temporal lobe and responsible for the processing of language (Jacobs et al., 1993).

The environment the child is raised in seems to be one of the most important factors that determine its developmental outcome. In children born small for gestational age (SGA) the parental socioeconomic status (SES) influences the development, especially during the early years of life, with children in families from lower SES failing to show developmental recovery. In fact, the relative impact of the clinical and biological factors of these children seems to be overshadowed by the 'Family' factors (Escalone, 1982; Teberg et al., 1988). A similar phenomenon was repeatedly described in very low birth weight infants, where the major factors affecting cognitive development of the children were the home environment and the neurological score (Weisglas-Kuperus et al., 1993).

1.1. Heroin in pregnancy

It is generally accepted that children born to heroin dependent mothers have no increase in the rate of congenital anomalies. However, heroin use during pregnancy is associated with low birth weight, small head circumference, and increased neonatal and perinatal mortality (Chasnoff, 1988; Little et al., 1991). Withdrawal symptoms may also develop in 40–80% of the newborns; a high incidence of sudden infant death was also described (Kandall and Gaines, 1991; Little et al., 1990).

Developmental delay, as well as behavioral problems were often encountered in children born to drug-dependent mothers using heroin or methadone during pregnancy, (Johnson et al., 1984; Sumner et al., 1993). Some investigators have demonstrated an improvement of the developmental scores in these children with the advancement of age, but others did not (Van Baar, 1990). In most studies the number of children investigated was relatively small, and the age difference among the studied children was wide. A high proportion of children suffered from behavioral abnormalities such as inattention, hyperactivity, aggressiveness, and lack of social inhibition (Olofsson et al., 1983; Ornoy et al., 1996).

Several studies have found an association between ADHD and substance abuse. The prevalence of substance abuse is therefore much higher among persons with ADHD, and ADHD was found among 35% of cocaine abusers (Martin et al., 1994; Schubiner et al., 1995).

It is therefore logical to presume that one of the major factors affecting the development of children born to heroin dependent mothers is the influence by the surrounding environment they are raised in. In many studies on the development of children born to drug-dependent parents, the control children are not matched by SES and therefore the possible effects of the environment are not appropriately addressed. As heroin is not considered to be teratogenic, we hypothesized that the developmental abnormalities observed in the children of heroin dependent mothers may be influenced by postnatal environmental factors related to the children's poor home environment, rather than the in-utero exposure. This can be tested in children born to drug-dependent fathers as they were not exposed in-utero to heroin, or in children born to drug-dependent mothers but

adopted at a very young age as they were raised in 'optimal environments'.

1.2. Diabetes in pregnancy

Various studies have shown an increase in the rate of congenital anomalies among offspring of mothers with pregestational diabetes. Other studies have addressed the question of possible brain damage induced by diabetic metabolic factors during the second half of pregnancy.

Stehbens et al. (1977) examined children born to diabetic mothers at 1, 3 and 5 years of age. The SGA children born to diabetic mothers had lower cognitive scores in comparison to controls. Similarly, Petersen et al. (1988) found that SGA children of diabetic mothers had lower verbal performance at 5 years, but the children that did not suffer in-utero from growth retardation were normal. Cummins and Norrish (1980) did not find differences in cognitive scores of children born to diabetic mothers at 4.25–13.5 years of age as compared to controls, and Person and Gentz (1984) found no differences on these measures at 5 years of age. Rizzo et al. (1991) did not find developmental delay in children born to diabetic mothers or to mothers with gestational diabetes, but found an inverse correlation between maternal blood β hydroxybutyrate levels and scores on IQ tests for these children. In a later study Rizzo et al. (1995) evaluated psychomotor development of children at ages 6–9 years and found a significant negative correlation between maternal second and third trimester β hydroxybutyrate level and performance on the Bruininks–Oseretzki test that measures fine and gross motor abilities.

It is possible that children born to diabetic mothers suffer from slight neurological damage that does not necessarily affect their scores in IQ tests. In recent studies on the developmental outcome of children born to diabetic mothers we found normal cognitive performance of these children at school but slight motor impairment. Their abilities were inversely correlated with the degree of control of maternal diabetes (Ornoy et al., 1998, 1999).

1.3. Premature infants

Many studies have shown that children born prematurely, especially those born with very low birth weight (< 1500 g), suffer from a variety of neurological and developmental disorders, in addition to the well known medical complications of prematurity (Ella et al., 1992; Yu, 2000).

In all the groups of children described above, many of the infants are born with slight brain damage and have a high risk to develop long-term developmental problems. It is reasonable to presume that the environment will influence the development of these children. However, studies addressing this specific issue in man, as well as in experimental animals, seem to be scanty.

We had the opportunity to study the developmental outcome of preschool and early school age children born to heroin dependent mothers (and hence may have slight brain damage) that were either raised at home or sent for adoption at a very young age. This enabled us to 'isolate' the prenatal effects of heroin on the neurobehavioral development from the postnatal possible impact of environmental deprivation, which is so common in families of drug addicts. Comparison groups were composed of children born to heroin dependent fathers, children with severe environmental deprivation and a group of normal, age matched children. In addition we studied the development of early school age children born to diabetic mothers and of children born prematurely, with a birth weight of 1500 g or less, and correlated the findings with parental SES.

2. Methods

2.1. Preschool age children

In the first phase of the study on the effects of heroin we studied children at 0.5–6 years of age. The first group consisted of 93 children born to *heroin-addicted mothers*, of which 48 were *adopted*. These children were either adopted immediately after birth or first placed for several months in foster homes and then adopted. Forty-five children were raised by their biological mother with

the help of other members of the 'extended' family, usually the grandparents. There were 85 children born to *heroin dependent fathers* of whom 83 were raised by their biological parents; none of the mothers in this group was heroin dependent. We also examined 50 children who were born to families of *low SES* and suffered from environmental deprivation and neglect. This included inadequate feeding and handling, emotional deprivation but no physical abuse. To be included in the study the children had to have an IQ or DQ above 70, and be without significant neurological impairment. Additionally, 87 children from kindergartens and nurseries in Jerusalem and surroundings, served as *normal controls*.

2.2. Early school age children

We also examined 160 children, 6–12 years of age, from 5 groups as described above. There were 30–34 children in each group. All children studied in regular schools; about half of them were also examined by us at preschool age.

2.3. Evaluation of the children

In all children the SES was evaluated according to parental profession and/or education on a scale of 1–5, one being the highest SES status (Ornoy et al., 1996, 1998, 1999, 2001). We examined all siblings in the family of either study or control groups if they were of appropriate age. To the best of our knowledge, none of the control children were born to drug addicted parents. Only children born post-32 weeks of gestation were included in the study.

2.3.1. Children born to heroin dependent parents

The following evaluations were carried out in preschool aged children (Ornoy et al., 1996):

1) A comprehensive medical and neurological examination.
2) A psychological examination using the Bayley or McCarthy's developmental scales.
3) The child's behavior in relation to attention span, level of activity, and behavior was recorded by both the physician and the psychologist and was stated in the medical and psychological records independently.

2.3.1.1. The school age children had the following examinations (Ornoy et al., 2001).

1) A medical and neurological evaluation, using the Touwen and Prechtl neurological examination for the child with minor neurological dysfunction. Abnormal neurological findings on this test were scored, and a total score of 10 or more was considered abnormal—i.e. pointing to some neurological impairment.
2) A psychological examination using WISC-R and Bender Gestalt tests.
3) Evaluation of reading and arithmetic skills using standard tests.
4) The Pollack Taper test. This test is designed to assess attention deficits. The child is asked to repeat a specific sequence of light blinks and auditory taps presented by the tester. The number, sequence and duration of these stimuli is adapted to the child's age. Children with attention deficit tend to obtain lower scores than children with normal attention span.
5) The Conners abbreviated parent–teacher's questionnaire for the study of hyperactivity and inattention was administered to the parents. The higher the score is, the more hyperactivity and attention problems (ADHD) the child has.

2.3.2. Children born to diabetic mothers

We compared the development of 57 early school age children born to mothers with pregestational diabetes and of 32 children born to mothers with gestational diabetes to 57 age matched control children using a number of developmental tests as used for the children born to heroin dependent parents. Only children born after 32 weeks of gestation participated in the study. In addition to the tests outlined above, we used for these children the following tests (Ornoy et al., 1998, 1999):

1) Bruininks–Oseretzky motor development test. This test examines fine and gross motor development of children aged 4.5–14.5 years.
2) Southern California Integration test, for the evaluation of children's sensory functioning. This test includes three sub tests: manual form perception (MFP), finger identification (FI) and localization of tactile stimuli (LTS).

2.3.3. Premature infants with a birth weight than 1501 g

We studied the developmental outcome of 50 early school age children born prematurely (< 33 weeks of pregnancy) and with a birth weight of less than 1501 g in comparison to 50 matched controls, and related the developmental outcome to a variety of factors including parental SES. We used the same tests described above for the other groups of children and in addition the Good-enough–Haris IQ scores (Ella et al., 1992).

2.4. Statistical analysis

The results are given as mean ± S.D. Significance of differences between groups were analyzed by the two tail t-test, χ^2 and multiple analysis of variance using SAS statistical computer program. P values of 0.05 or less were considered to be significant. For comparison of the groups on the Touwen and Prechtl neurological examinations we used the Wilcoxon Matched-pair signed-ranks test. Pearson correlation was calculated between the metabolic findings of the diabetic mothers and the scores on the neurodevelopmental tests of their children. The correlations were calculated for each trimester of pregnancy and then for the entire period of pregnancy.

3. Results

3.1. Children born to heroin dependent parents

As expected, a significantly lower SES was found in the heroin dependent parents and among the parents of children with environmental deprivation in comparison to the control group. A lower birth weight and a shorter gestation were recorded in the children born to heroin dependent mothers and fathers when compared to the other groups. There was no difference in the physical parameters at examination among the different groups of children.

3.1.1. Preschool age children

The results of the psychological evaluation are given in Table 1. The group of children born to heroin dependent mothers raised at home had lower Bayley or McCarthy developmental scales when compared to normal controls. However, the scores of the children born to heroin dependent mothers raised by adopting families were not different from the control children (Table 1). These low scores were comparable to those of children born to heroin dependent fathers which were all, except two, raised at home. The lowest scores, even significantly lower than that of children born to heroin dependent mothers, were found among the children who suffered from severe environmental deprivation (Table 1). We also evaluated the number of young children over 2 years of age that exhibited behavioral disorders: hyperactivity, inattention, impulsivity and aggression and found that more than half of children born to heroin dependent mothers had behavioral disorders. Behavioral disorders were also found in children born to heroin dependent mother adopted at a young age, in children of heroin dependent fathers and in children who suffered from severe environmental deprivation, but to a lesser extent. Only 5% of normal controls had these symptoms. Among the children with behavioral disorders, over 2/3 were males, as expected in ADHD (results not shown).

The medical examinations of the children born to heroin dependent mothers did not show any specific abnormalities at examination in comparison to the control children regarding head circumference, height and weight, as well as physical findings.

The results of the psychometric evaluation are shown in Table 2. There were lower scores on the various cognitive tests and in reading and arithmetic skills in the children born to drug-dependent parents raised at home, and in those with environmental deprivation. However, the children born to

Table 1
Average scores on Bayley and McCarthy Scales in young children born to heroin dependent mothers from low SES and environmental deprivation

	Addicted mothers, children raised at home		Addicted mothers, adopted children		Environmental deprivation		Control normal	
Bayley	MDI	PDI	MDI	PDI	MDI	PDI	MDI	PDI
	96.4±9.7[a]	97.5±12.0	108.8±17.7	97.0±14.1	87.3±9.85*,**	84.4±12.9*	112.0±14.9	100.9±14.5
McCarthy	GCI	Mot	GCI	Mot	GCI	Mot	GCI	Mot
	95.9±10.3***	47.9±9.1****	111.1±13.0	56.1±3.9	88.0±11.4***	46.5±5.7	108.4±12.2	51.0±7.5

MDI, mental developmental index; PDI, psychomotor developmental index; GCI, general cognitive index; Mot, motor achievement scores. The children were raised at home or adopted. The control group consisted of normal children. In each group there were at least 45 children.

[a] Mean±S.D.

* Significantly lower than MDI, adopted children an controls, $P = 0.04$.

** Significantly lower than addicted mothers raised at home, $P = 0.04$.

*** Significantly lower than GCI, adopted children and controls, $P = 0.03$.

**** Significantly lower than Mot, adopted children, $P = 0.02$.

heroin dependent mothers and adopted at a young age functioned, in most parameters, normally (Table 2).

The results of the Conner's parental questionnaire and of the Pollack Taper audio–visual test are shown in Table 3. The average failure score on the parental Conner's questionnaire was significantly higher in the children born to drug-dependent mothers whether raised at home or adopted. However, high scores were also found in the children born to heroin dependent fathers and in children with environmental deprivation (Table 3). If the cutting point for ADHD was set as 21, than 54% of children born to heroin dependent mothers and raised at home had ADHD, none of the control children had ADHD and 21–24% of the children from the other groups had ADHD.

The results of the Pollack Taper test show lower scores in the children born to heroin dependent mothers and fathers raised at home but the differences with controls were not statistically significant (Table 3).

3.2. Children born to diabetic mothers

3.2.1. Cognitive and neurological development

Table 4 shows the WISC-R and Bender tests as well as the motor assessment for the children born to diabetic mothers and the control. No differences were found between the children born to diabetic

Table 2
Results of psychometric evaluations of school age children born to drug-dependent parents raised at home in comparison to controls and adopted

Group	WISC-R verbal	WISC-R performance	Bender	Reading	Arithmetics
Drug-dependent fathers	11.33±2.73[a],*	11.45±2.63**	−1.19±1.64**,*	48.53±9.64**,*	34.62±28.57**,*
Drug-dependent mothers, at home	9.98±2.21**,*	10.17±2.31**,*	−1.26±1.63**,*	49.73±12.65**	29.60±27.14**,*
Low SES	10.42±2.70**	10.83±2.50**	−1.07±1.58**,*	52.65±11.12	30.09±24.65**,*
Controls average SES	12.15±2.99	13.23±2.20	−0.10±1.30	60.58±13.37	54.96±29.05
Adopted	11.70±2.68	11.32±2.45**	−0.46±1.36	57.06±17.50	43.87±21.57**

[a] Mean±S.D.

* Significantly lower than adopted, $P < 0.05$.

** Significantly lower than controls, $P < 0.05$.

Table 3
Results of the parent's Conners questionnaire and of the Pollack Taper tests in school age children

Conners (average score)	% of children with ADHD (with Conners above 21)	Pollack (total score)	
Drug-dependent fathers	16.06±8.94[a,*]	24	29.77±7.07
Drug-dependent mothers, home	20.07±13.50*,**	54	29.25±10.07
Controls, low SES	12.62±11.77*	21	33.86±5.36
Controls, average SES	5.13±6.16	0	33.21±7.66
Adoption	14.22±10.36*	21	33.08±6.45

There were 30–34 children in each group.
[a] Mean±S.D.
* Significantly higher than controls $P < 0.05$.
** Significantly higher than adopted $P < 0.05$.

mothers and the control group in the WISC-R scores, but the Bender scores of the children born to mothers with gestational diabetes were lower than controls (Table 4). The motor assessments of the children born to mothers with gestational or pregestational diabetes showed significantly lower scores on the Bruininks–Oseretzky fine and gross motor scores as compared to controls (Table 4). The differences between the controls and 'diabetic' groups were higher in the younger, 5–8 years old group children than in the older, 9–12 years old children (results not shown). No differences between the children of both diabetic groups and the control group were observed in any of the 3 subtests of the Southern California Integration test (MFP, FI and LTS) that were designed to reflect sensory-motor functioning. Children born to mothers with pregestational diabetes, but not with gestational diabetes, had a significantly higher number of soft neurological signs in the Touwen and Prechtl examination. A higher score is indicative of a larger number of soft neurological deficiencies, implying more children with attention deficit and/or hyperactivity (Ornoy et al., 1998, 1999, 2001).

There was a marked difference on the Pollack Taper test between the control group children and the children born to mothers with pregestational or gestational diabetes, the average score of these children being lower than in controls (results not shown).

Children born to mothers with pregestational diabetes obtained higher scores on the Conners abbreviated parent–teacher's questionnaire in comparison to controls, indicating more hyperactivity and inattention. However, the differences in

Table 4
Comparison of cognitive scores on WISC-R and on Bender Gestalt test and of motor abilities on Bruininks–Oseretzky test of control children and those born to diabetic mothers or mothers with gestational diabetes

Test	Control	Diabetic mothers	Mothers with gestational diabetes
IQ	118.5±11[a]	117.7±12	113.5±14.3
Verbal	114.4±12	112.4±12	108.0±11.5
Performance	119.7±11.5	120.4±19	116.0±16.0
Bender (%)	48.6±26.5	48.0±24	32.0±27.0
Bruininks total	138±21	129±20*	121±27*
Bruininks gross motor	60.8±12	57±11*	57±15*
Bruininks fine motor	62.5±9	58±10*	49±11*

[a] Mean±S.D.
* Significantly lower than controls.

the average scores were not statistically significant. When the number of children having 15 or more failure points was compared among the groups, it was significantly higher in both research group children in comparison to controls.

3.2.2. Correlation between neurodevelopmental assessment and severity of pregestational diabetes

A negative correlation was found between the percent of HbA1c and the scores on the Bender Gestalt test, the total motor scores on the Bruininks–Oseretzki test and the results of the Pollack Taper test. This indicates that sensory-motor functions of children born to diabetic mothers and their attention span tend to be lower with higher glycosylated hemoglobin levels. A high percent of HbA1c indicates more severe diabetes. A similar negative correlation was found between positive urinary acetone and the motor ability of the children: the higher the acetonuria, the lower were the total motor scores on the Bruininks test. No correlation was found between the medical status (i.e., hypoglycemia, increased or decreased birth weight) of the newborn infants and outcome of any of the associated variables in the children born to women with gestational or with pregestational diabetes. However, a positive correlation was found between the cognitive scores of the children born to diabetic mothers and maternal education, implying the positive effect of the environment on the intellectual outcome of these children.

Table 5
Results of WISC-R and Goodenough–Haris IQ scores in 50 VLBW infants (BW ≤ 1500 g) in relation to parental education

	Parental education		
	Low	Middle	High
	(0–11 y)	(11–14 y)	(> 14 y)
Goodenough			
Controls	109.0±14.8[a]	110.4±19.7	108.9±10.6
Prematures	100.1±17.6	102.2±27.3	118.1±24.2
WISC-R			
Full scale IQ			
Controls	114.4±7.0	118.6±10	118.5±8.4
Prematures	96.4±9.9*	104.0±20*	113.6±11.6

A correlation with parental education is found in both tests.
[a] Mean±S.D.
* Significantly lower than controls.

3.3. Premature infants

Table 5 shows the results of the cognitive evaluation of the children in relation to parental education. While there is no significant difference in the cognitive scores among the 3 groups of control children, the cognitive scores of the children born to the mothers with 0–11 years of schooling are the lowest, and the cognitive scores of children born to mothers with over 14 years of schooling is similar to that of the controls. This was found in spite of the fact that the birth weight of the premature infants among these 3 groups was similar, implying that improved environment affects the intellectual outcome of children with slight brain damage more than it does in children with a normal brain. No correlation was found between perinatal complications of the infants and their intellectual abilities.

4. Discussion

Children of heroin dependent mothers, if born without significant neurological damage, seem to have a normal developmental potential in spite of the fact that they have been exposed in-utero to heroin, methadone, and in many cases to a variety of psychoactive drugs. The developmental outcome of the children born to heroin dependent mothers seemed to be influenced mainly by the environment, as those raised in adopting families had normal cognitive development. Moreover, children not exposed in-utero to opiates but raised in 'neglecting' and 'abusing' environments function even less well than the children born to heroin dependent mothers.

The environment in which the young child is raised has may have a major influence on the postnatal development of his brain (Brazelton, 1986; Levine et al., 1983; Ornoy, 1986). This is clear when evaluating the results of studies where either premature, SGA, handicapped children, or even normal children with environmental depriva-

tion were exposed to early intervention programs (Brazelton, 1986; Farran, 1990). Moreover, the parental SES seems to be especially important in children who were born with slightly damaged CNS or had suffered in-utero from developmental deprivation such as offspring of diabetic mothers (Ornoy et al., 1998, 1999).

Families that are allowed in Israel to adopt children are known to have a relatively high SES. The higher the SES of the parents, the better are the chances for their children to have normal behavior, socialization and cognitive function (Levine et al., 1983). It is therefore not surprising that the adopted children born to heroin dependent mothers functioned normally.

Sowder and Burt (1980) found that children born to heroin dependent fathers were at high risk for early school behavioral and learning problems. Similarly, children born to drug-free parents but of a similar underprivileged environment and low SES were also at risk for early school problems, but to a lesser extent. These results are similar to our findings in school age children. Herjanic et al. (1979) found slow mental development in 44% of children born to heroin dependent fathers. By age 12, conduct disorders and behavioral problems were common among these children. Behavioral problems and ADD–ADHD was also described in the offspring of cocaine using mothers (Sumner et al., 1993).

In our study, a very high proportion of preschool and early school age children born to heroin dependent mothers and raised at home had diagnostic features of ADHD. It may be postulated that this may have resulted mainly from the damage to the fetal brain caused by heroin. However, the supportive evidence for this from our study is not conclusive since in the children born to heroin dependent fathers the incidence of hyperactivity and inattention was also very high.

A high incidence of ADHD was also found among children born to diabetic mothers, but to a lower degree than in children born to heroin dependent parents or in children with severe environmental deprivation. This high rate of ADHD is probably attributed to both genetic and prenatal as well as postnatal environmental factors, as supported by the literature (Stevenson, 1992; Rappaport-Cohen et al., 1998). Moreover, a close association between ADHD during childhood and substance abuse in adulthood was repeatedly demonstrated, mainly for alcohol and cocaine (Martin et al., 1994).

We found that the cognitive ability of the preschool age children who suffered from severe environmental deprivation were lower than that of children born to drug-dependent parents raised at home (Ornoy et al., 1996). In the school age children, the cognitive ability of the children with environmental deprivation was better than that of the children born to heroin dependent mothers raised at home, although the differences were not statistically significant. This is explained by the fact that we have in Israel many 'enrichment' programs for children of lower SES. Mothers of children with environmental deprivation tend to enroll their children in such programs more often than heroin dependent mothers, and hence their children's intellectual abilities improve.

It is also possible that the high incidence of inattention, hyperactivity, and behavioral disorders found among the children in our study is related to a high rate of ADHD among their parents, who were therefore prone to substance abuse more than the general population. When using specific questionnaires to assess for maternal ADHD, we indeed found a very high rate of ADHD among drug-dependent mothers, which was in high correlation to the rate of ADHD among their children.

Early school age children born to mothers with pregestational diabetes or to mothers with gestational diabetes had more soft neurological signs, and lower gross and fine motor achievements than pair matched control children born to non-diabetic mothers. Soft neurological signs may be a sign of mild, non-specific brain damage (Ornoy et al., 1998; Smith, 1992). Negative correlations were found between several parameters related to the degree of maternal glycemic control and some of the neurodevelopmental tests of children born to mothers with pregestational diabetes. However, the cognitive function of these children mainly correlated with parental SES.

Lambert and Sandoval (1980) found a high prevalence of pre and perinatal complications in children suffering from ADHD, including more health problems in their mothers, when compared to control (normal) children. Intellectual ability in children with ADHD is usually within the normal range (Rappaport-Cohen et al., 1998), which is in accordance with the findings of the present study: variability in muscle tone (hypertonicity or hypotonicity) may cause delayed or abnormal motor development (Smith, 1992). Children born to diabetic mothers may be able to compensate for slight motor impairment, and their daily function may be normal. However, when coping with complex motor tasks, they may have difficulty in performing adequately. These motor abilities are apparently not modified by parental SES.

It is accepted (Little et al., 1990, 1991; Ornoy, 1986) that various maternal factors (such as exposure to high levels of opiates) may induce delay in the intrauterine maturation of the fetal brain. With age and with the provision of an appropriate environment, postnatal brain maturation may become normal. It is possible that the diabetic metabolic derangement during pregnancy delays brain maturation and therefore fine neurological functions are impaired to a larger extent at a young age, as found by us. Advancement in age will enable functional recovery, providing the environment the child is raised in is optimal for growth and development.

In conclusion we studied the developmental outcome of preschool age and early school age children born to heroin dependent parents in comparison to appropriate controls. The children of heroin dependent parents suffered form a high rate of hyperactivity, inattention, and behavioral problems. The children born to diabetic mothers or born prematurely had the same problems but to a lesser extent. The cognitive developmental and learning abilities of the children in our studies were influenced to a large extent by their environment, emphasizing the need to improve the biological homes and environment of children born to heroin dependent parents or belonging to other 'high risk' groups.

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Short communication

Overview of testing methods used in inhalation toxicity: from facts to artifacts

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Abstract

For smaller rodent species, homogenous in size and growth, small head or nose-only chambers are commonly used up to subchronic exposure durations, whereas larger whole-body exposure chambers are used for long-term exposures or exposure paradigms exceeding the normal working day. The advantages and disadvantages of each different technique have already been identified and published in detail. It is often believed best that whole-body inhalation chambers simulate potential human exposure to environmental chemicals or pesticides and this serves as a justification for preferring this mode of exposure. However, real-life exposure conditions of humans cannot be readily duplicated. A comparable mode of exposure may be employed rather than duplicating both the exposure regimens and atmospheres similar to those present in real-world settings. Especially in inhalation studies with complex mixtures, in which atmosphere generation is difficult to control, non-homogenous exposure atmospheres and artifacts are more likely to occur in larger whole-body chambers than in the smaller nose-only inhalation chambers. Inhalation studies with complex mixtures not only face all the challenges of traditional inhalation toxicity testing, but also they are frequently subject to artifacts not readily detected. Thus, a disproportionation of volatile and particulate constituents might occur in inhalation chambers depend on selected technical features, i.e., whether a dynamic or (quasi)static mode of exposure is chosen. Inappropriate timing of the sampling of biological specimens may lead to the underestimation of effects, especially in whole-body exposed animals.
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Keywords: Disproportionation of atmosphere; Sampling artifacts; Timing of tissue collection; Mode of exposure

1. Introduction

Inhalation studies using laboratory animals are carried out under well-controlled conditions to assess the toxicity of aerosols, gases, and vapors or mixtures thereof. Methodologies to generate atmospheres and to expose animals depend on the study objective, exposure regimen and whether the exposure is to low or high concentrations. The degree of complexity increases when complex mixtures of formulated products, produced by end-user devices, have to be tested. Today, most inhalation facilities use dynamic exposure systems where the airflow and introduction of agents into

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the system are continuous. A dynamic inhalation exposure system with a suitable online computer control system is desirable to monitor the inhalation chamber atmospheres with respect to aerosol and/or vapor concentrations, particle size, air flow rates, temperature, humidity, stability and reproducibility. Real-time and direct reading monitoring devices (e.g., aerosol photometer or microbalance systems for particulates and a total hydrocarbon or infrared analyzers for volatile materials) may be useful to demonstrate that temporally stable exposure conditions prevailed and that the time required to reach the inhalation chamber equilibrium concentration is negligible in relation to the total duration of exposure. Most importantly, for compounds causing a contact-site type of toxicity which characterized by a concentration-dependent anterior-to-posterior gradation in lesion intensity and severity along the respiratory tract such piece of equipment identifies inadvertent short-term high-level excursions that might jeopardize the outcome and interpretation of study, if not readily identified, especially for these types of compounds. Moreover, it should be noticed that the continuous monitoring of a test atmosphere is an integral measurement of all dynamic parameters of the system and hence provides an indirect, however, integrative means to control all relevant, dynamic inhalation parameters. For the inhalation testing of compounds using concentrations high enough to reduce the partial pressure of oxygen or by testing products that produce smoke by fumigation, combustion or pyrolysis, the measurement of oxygen, carbon monoxide and carbon dioxide and other important combustion products needs to be included. As a rule, measurements should be performed by samples taken from the vicinity of the breathing zone of the exposed animals.

'Nominal concentrations' reflect the mass of test substance introduced into the inhalation system relative to the total volume of air available for dispersion. Many factors—including wall loss, losses on the skin and fur of animals, for the whole-body mode of exposure, sedimentation and impaction especially of larger particulates, chemical reactivity—cause the 'analytical' or 'actual' concentration to be substantially less than the nominal concentration. Therefore, the concentration present in the vicinity of the breathing zone should always be measured in an appropriate manner rather than reporting the nominal concentration alone. Commonly, actual concentrations are based on samples taken in the breathing zone and are reported in terms of the tested article. Whenever, this is a formulation, the analytical concentration must be reported for the total formulation, and not just for the active compound(s). More complex formulations that contain substances from low to highly volatile ingredients have to be sampled from the atmosphere in a way that both the aerosol and the vapor phase are sampled with equal collection efficiency. When using different instruments for atmosphere characterization an equipment-specific, size-selective sampling of atmosphere must be excluded. In this context, it must be borne in mind that in test atmospheres containing both volatile or gaseous substances and particulates the partial enrichment of atmospheres with the more volatile constituent(s) can readily occur. The partitioning (or adsorption) of more volatile agents with particulates needs to be identified and understood since particulates may function as shuttle for gases otherwise deposited and retained within the extrathoracic airways, i.e., the anterior-to-posterior gradation in lesion intensity and severity along the respiratory tract may be shifted to more posterior site as a result of this interaction.

Nominal concentrations, while useful to assess the consistency of a test, however, are of limited, if any, value for hazard identification, because laboratory-specific ramifications make it difficult to predict the concentrations actually present in exposure atmospheres. Accordingly, to make inhalation studies comparable and reproducible, they call for an elaborate and costly characterization of exposure atmospheres in regard to the concentrations of aerosol, aerosol size and vapor equilibrium concentrations, if applicable. For a more general description of the methodologies used in inhalation studies, comprehensive reviews are published (Phalen, 1984; Tillery et al., 1976; Kennedy, 1989; Cheng and Moss, 1989).

Other variables are related to the efficiency of the delivery of dose which may be contingent upon

external factors, such as whole-body, nose-only modes or other types of exposures. Also test animal specific factors have to be envisaged, because, especially in rodents, reflex reactions that are considered to be of a protective nature, may limit contact to a variety of potentially hazardous chemicals. They may range from mechanisms using the own fur as a filter for irritant particulates, or through nerves, when stimulated, result in an appreciable decrease of respiratory ventilation, heart rate, blood pressure, and body temperature. The objective of this paper is to consider some of the critical issues that may affect the delivery of dose or the modulation of effects caused by species-specific mode of exposures or by physiological response.

2. Test design and species differences

Whenever possible one should use nose-only rather than whole-body modes of exposure in inhalation studies that are designed to reduce dosing from non-respiratory routes, except for large animal number, chronic inhalation studies or study types that require virtual continuous exposure. The nose-only mode of exposure uses several technical modifications such as mixed flow, past-flow, and directed-flow types (Pauluhn and Mohr, 2000; Moss and Asgharian, 1994) that utilizing restraining tubes characteristics depicted in Fig. 1. Past- and directed-flow exposure principles minimize the re-breathing of atmospheres. Stress may be placed on animals when using hermetically sealed tubes from which urine and feces cannot escape or the rats' thermoregulation via the tail is compromised. By using a positive flow between exposure tubes and inhalation chamber exposure atmospheres cannot be diluted by bias airflows via non-sealed restraining tubes. To date, for many types of test compounds the nose-only mode of exposure is considered superior to whole-body exposure due to the reasons detailed already in Section 1. Further advantages are that for the purpose of dosimetry, the measurement of the respiratory minute volume using volume displacement nose-only plethysmographs (Fig. 1D) can be employed during exposure. Also larger laboratory animals, such as dogs, can be exposed utilizing exactly the same exposure technology and regimen. This renders it easier to identify species-specific susceptibilities. In spite of this, even when using an identical exposure paradigm (Table 1), the effective dosage, defined as the extend of bronchoalveolar lavage cells displaying increased levels of incorporated surfactant phospholipids evoked by the cationic amphiphilic drug (CAD) which is lower in dogs when compared with rats (Fig. 2). The two CAD-salts examined (Table 1) were of markedly different solubility in water. The bis-hydrochloride (CAD–HCl) was highly soluble in water whilst the mono-sulfate (CAD–SO_4) was poorly soluble in water ($\approx$ 0.1%). Both salts demonstrated a very poor bioavailablity via the gastrointestinal tract. Despite the markedly different solubility, both the toxicological side effect of concern (alveolar phospholipidosis) and the dose recovered in BAL-cells were identical. The minute ventilation per kg bw of normally breathing dogs is 2–3 times lower than rats. This relationship is mirrored in Fig. 2.

For agents known to be sequestered within the lung, the dosage of the dry powder aerosol of CAD recovered from BAL reflects that amount of drug bound to lung tissue more closely than plasma levels (data not shown). Therefore, plasma levels may be a poor predictor for that occurring in lung parenchyma. Collectively, this comparative assessment demonstrates that the dose of drug penetrating the alveolar region of either species appears to be governed by differences in ventilation rather than differences in the anatomy and branching patterns of airways or species-specific breathing patterns.

3. Test design, specific and non-specific effects

There is a great deal of controversy as to whether the restraint caused by the nose-only mode of exposure may interfere with the outcome of inhalation studies. The potential extent of immobilization distress may include factors dependent on the specific design of the restraining tube rather than this mode of exposure per se. Major factors appear to be whether restraining

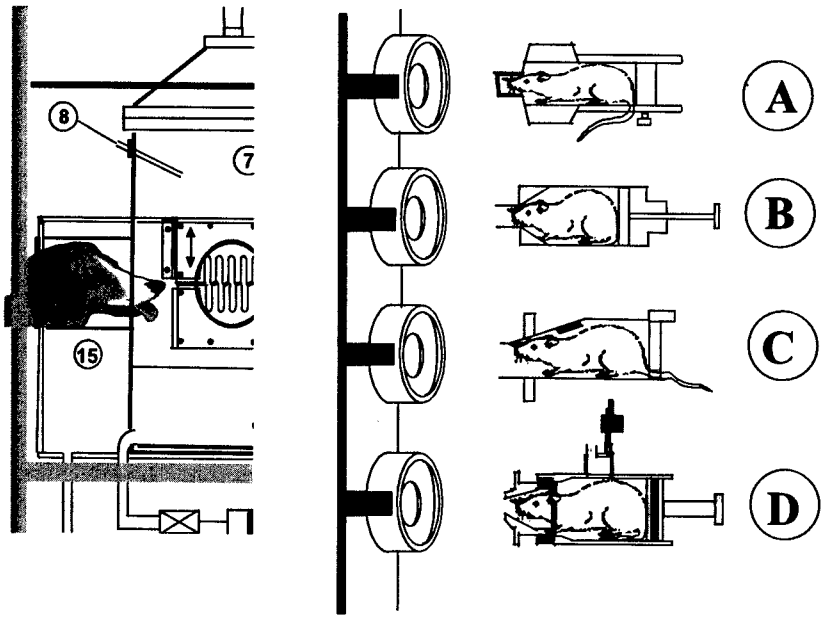

Fig. 1. Head-/nose-only mode of exposure of dogs or rats. Left panel: inhalation chamber for simultaneous head-only exposure of four dogs in canvas slings (7), sampling port for analytical characterization of test atmosphere (8), negative pressure jacket around exposure chamber (15). Dogs are accessible during exposure for blood sampling and physiological measurements. Right panel: tube-type animal holders for nose-only exposure of small laboratory rodents. (A) Standard tube used in directed-flow nose-only exposure chambers. Note that this tube allows thermoregulation via the tail and excreta do not accumulate in the restrainer during the course of exposure. (B) Sealed nose-only exposure tube. (C) Cannon-type exposure tube for flow-past nose-only inhalation chambers. (D) Volume displacement plethysmograph with attached differential pressure transducer for respiratory measurements during nose-only exposure.

Table 1
Results of the characterization of exposure atmospheres

Parameter	Low	Intermediate	High
Rat—CAD-HCl/28 × 1 h			
Concentration (mg/m^3 air)	0.28	2.0	14.6
MMAD (μm)	0.68	0.63	0.68
GSD	1.37	1.19	2.76
Daily dose (μg/kg/day)	17	120	876
Cumulative dose (μg/kg)	476	3360	24 528
Dog—CAD-SO_4/28 × 1 h			
Concentration (mg/m^3 air)	0.28	1.76	13.2
MMAD (μm)	1.4	1.3	2.2
GSD	1.6	1.5	2.3
Daily dose (μg/kg/day)	5.9	40.0	277.2
Cumulative dose (μg/kg)	165	1120	7762

Sampling was made in the vicinity of the breathing zone of animals. All concentrations reflect the concentration of the base of a CAD. Concentrations and doses are converted to the free base; MMAD, mass median aerodynamic diameter, GSD, geometric S.D.

tubes are hermetically sealed or partially open. The often reported stress in nose-only exposed rodents has led to recommendations that for specialized study types, such as reproduction studies, whole-body exposure should be given preference to nose-only. Conversely, by using directed-flow nose-only exposure tubes (Fig. 1A), the respective reproductive endpoints of rats exposed daily to conditioned air or a commonly used vehicle (polyethylene glycol 400) from gestational days 6–15 (6 h/day) are not significantly different from rats dosed by gavage (Table 2). This means restraint-specific stress effects could not be ascertained. This finding is further substantiated by physiological measurements in rats, demonstrating that the least stress occurs when using positive pressure nose-only chamber systems and restraining tubes that are not sealed so that rats can to thermoregulate via the tail. Inhalation exposure to upper respiratory tract sensory irritants, e.g., is known to evoke in

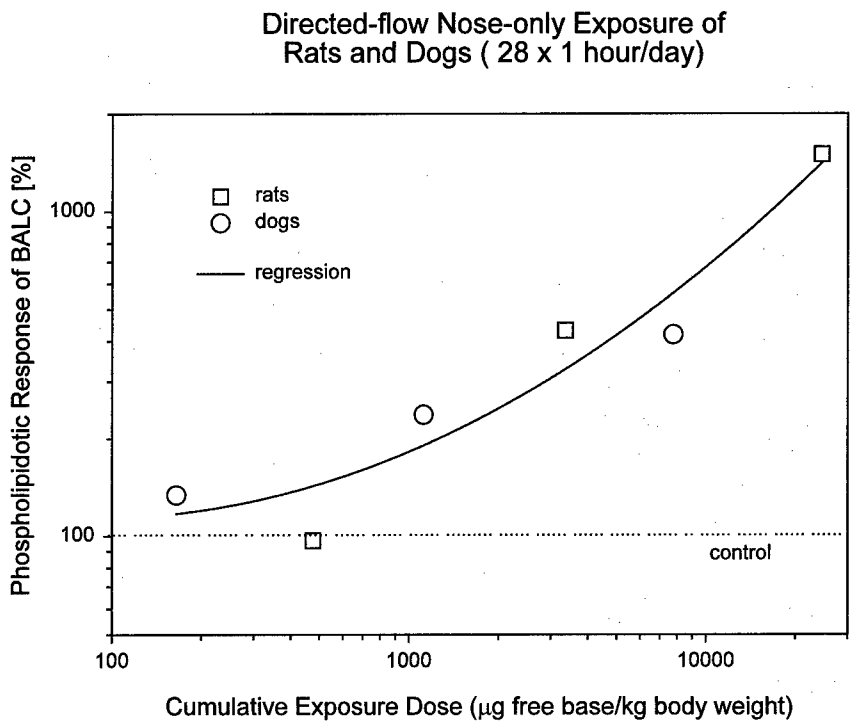

Fig. 2. Synopsis of intracellular concentrations of phospholipids in bronchoalveolar lavage cells (BALC) from rats and dogs exposed in a directed-flow nose-only inhalation chamber. Changes are illustrated relative to air exposed controls (= 100%). Exposure was to a dry powder aerosol of a CAD, daily for 1 h/day on 28 consecutive days. Squares: rat 28 × 1-h/day; circles: dog 28 × 1-h/day. Exposure concentrations represent the means over the duration of study and were converted to the respective free base.

Table 2
Comparison of reproductive indices of Wistar rats exposed nose-only (chamber see Fig. 1, nose-only restrainer see Fig. 1A) to conditioned, dry air or to a polyethylene glycol 400 (PEG) aerosol daily for 6 h/day or receiving PEG or water by gavage

Reproductive index	Gavage		Inhalation	
	PEG	Water	Air	PEG-aerosol
Number of rats/group	22	22	21	22
Corpora lutea	14.8±2.41	14.4±1.74	14.3±1.39	14.2±1.62
Implantations	10.9±3.41	11.8±2.60	12.3±2.44	12.8±2.09
Preimplantation losses	3.9±3.82	2.6±2.30	2.0±2.39	1.4±1.33
Live fetuses	9.7±4.04	10.5±2.76	11.6±2.71	12.0±1.76
% of implantations	87.8±19.81	89.7±13.15	92.8±9.69	94.1±6.05
Litter mean (% males)	51.4±16.47	53.4±15.92	46.6±12.41	52.5±14.39
Litter mean (% females)	48.6±16.47	46.6±15.92	53.4±12.41	47.5±14.39
Resorptions % of implantations	1.2±2.0	1.3±1.70	0.8±0.83	0.8±0.85
Number of rats	13	14	11	12
Mean placental weight	0.67±0.160	0.62±0.058	0.61±0.060	0.60±0.057
Weight of live fetuses	3.51±0.251	3.48±0.221	3.41±0.205	3.50±0.232

Exposure/administration was from gestational days 6 through 15. Cesarean section was on day 20. Data represent the number per female with viable fetuses±S.D.

rodents a remarkable decrease in body temperature, possibly via reflex stimulation of local receptors in this region of the tract. Measurements made in nose-only exposed rats using an intra-abdominal probe and telemetry revealed the control group body temperatures in the normal range (Fig. 3a) whilst in rats exposed to volatile sensory irritant (isocyanate) evidence of a concentration-dependent hypothermia was observed during exposure durations as short as 2 h exposure (Fig. 3b). In the absence of irritant stimuli the rats' normal temperatures are attained rapidly, i.e., with 1 h after cessation of exposure. In rodents exposed by inhalation to compounds eliciting upper respiratory tract irritation commonly a concentration-dependent hypothermia occurs. This type of physiological response is specific to the inhalation route of exposure and seen as a result of local stimulation of the trigeminal nerve.

In this context, it is important to recall that in rodents, reflex reactions are considered to be of a protective nature, i.e. to limit contact to a variety of potentially hazardous chemicals. Some of the uncertainty regarding the nature and magnitude of the pulmonary toxicity observed in rodents following exposure to respiratory tract irritants may be attributable to a modulatory influence exerted by extrapulmonary factors. For example, it is well known that in small laboratory rodents basic functional parameters such as heart rate, blood pressure, and core temperature are extremely labile and highly sensitive to a number of external stimuli such as upper respiratory tract sensory irritation (Watkinson and Gordon, 1993; Watkinson et al., 1993). Seemingly routine experimental procedures and fluctuations in ambient temperatures can produce striking changes in these parameters, independent of any treatment effects (Jaeger and Gearhart, 1982). In context with inhalation toxicity studies, it should be borne in mind that basic indices of cardiovascular and thermoregulatory function may be affected by rodent-specific response to respiratory tract irritants. Such a response may be capable of significantly modifying the results of toxicological studies; thus, moderate decrease of these physiological parameters is known to be associated with an attenuation or potentiation of toxicity (Costa et al., 1992).

Despite the apparent 'temporary' nature of the response of rats and mice to irritant exposure, these secondary effects are important to observe for a number of reasons. First, the induced decreases appear to be a primary component of a more general response by the rodent to toxic insult. Second, the magnitude of changes in thermoregulatory function may be potentiated or attenuated by a number of experimental conditions or stresses which may differ from one laboratory to another. Thus, initial experimental conditions may play an important role in the final toxic outcome, thereby compromising the ability to compare results across-species and studies in which these experimental factors are neither monitored nor controlled. Third, it is as yet unclear whether this physiological response to xenobiotic agents is unique to rodents or if it also occurs in larger mammals and humans. However, it is quite possible that humans have a greater thermal inertia due to the larger body mass and therefore do not exhibit this response to any measurable degree. Another issue that renders the extrapolation across species particularly complex is when inhalation studies utilize neonatal rodents, especially when they are in the transition from poikilothermy to homeothermy. In this context, poikilothermy is defined as the pattern of thermoregulation of a species exhibiting a large variability of core temperature as a proportional function of ambient temperature. The antonym is homeothermy, a pattern of temperature regulation in a tachymetabolic species (high level of basal metabolism) in which the cyclic variation in core temperature, either nycthemeral or seasonally, is maintained within arbitrarily defined limits ($\pm$ 2 °C) despite much larger variations in ambient temperature (Bligh and Johnson, 1973). Especially in neonatal mice their large surface area:body mass ratio and relatively small size plays a significant role in facilitating heat loss. This makes immature neonatal mice thermally more labile (Serdarevich and Fewell, 1999) when compared to adult rodents (Watkinson et al., 1993; Watkinson and Gordon, 1993). In contrast, larger species have lower metabolic rates and rely more on their

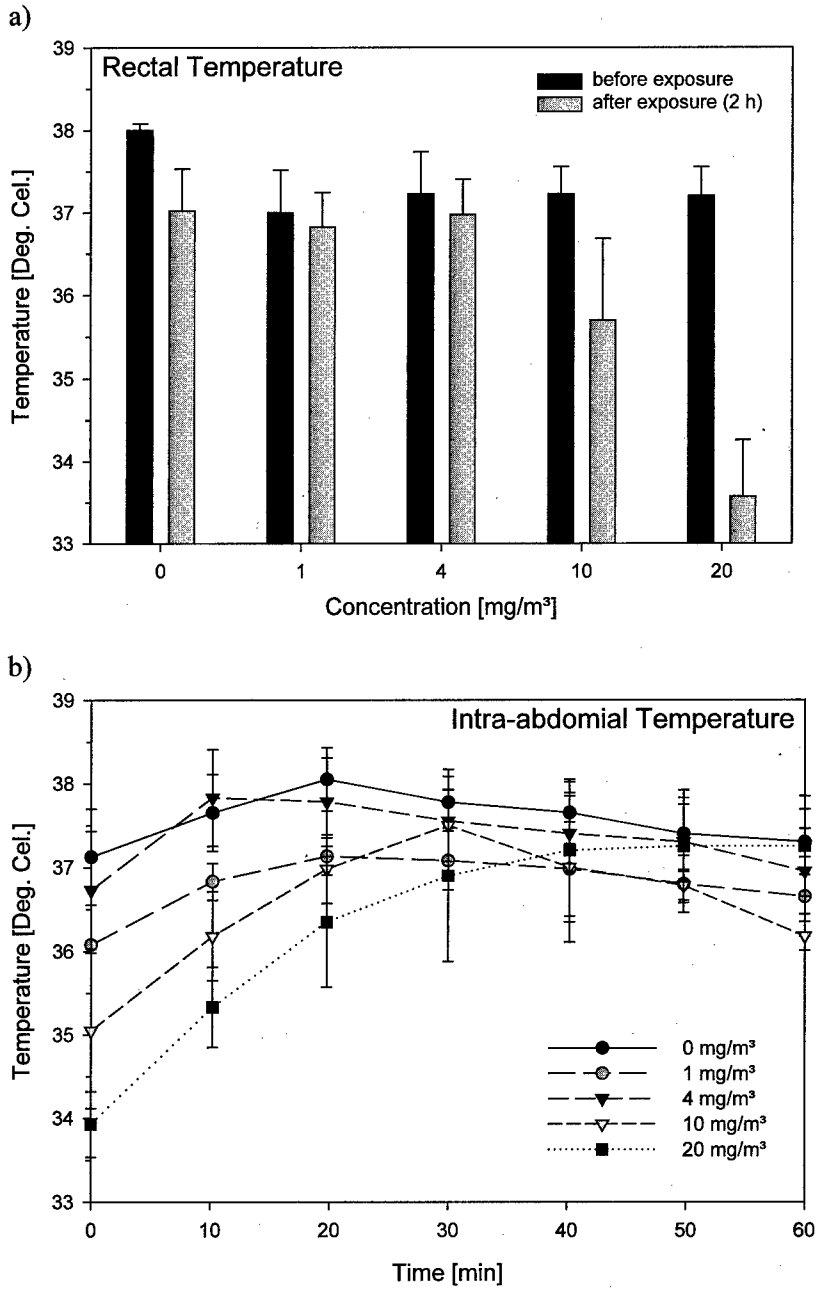

Fig. 3. Body temperature before and after a 2 h nose-only exposure to an upper respiratory tract sensory irritant (phenyl isocyanate) in rats ($n = 4$). (a) Rectal temperatures measured by a rectal probe prior to and after cessation of exposure. (b) Intra-abdominal temperature measured by telemetry starting shortly after exposure up to 1-h postexposure (means ± S.D.).

mass and insulation to regulate a constant body temperature. Because of this relationship, one would expect that a given decrease in metabolic rate, as could occur following toxic insult, would result in greater hypothermia in species with a smaller body mass. One general finds that in those instances where hypothermia is the principal thermoregulatory effect, smaller and especially

poikilothermic species exhibit larger drops in body temperature. Thus, with respect to thermoregulation and surface area:body mass ratio, results obtained in poikilothermic cannot be compared to any equivalent human situation.

4. Artifacts related to the timing of tissue specimens

Although useful for specialized study types, the whole-body exposure mode might be associated with specific artifacts when not specifically taken into consideration at the outset of study. Major disadvantages of this mode of exposure are that losses onto chamber surfaces especially when atmospheres are generated at elevated temperatures. When using such combustion principles of generating test atmospheres a severe mismatch of particulates (condensates) and gases can occur especially in whole-body chambers (Fig. 4, upper panel). The particle size of combustion atmospheres is both dependent on the concentration as well as the time available for coagulation (Fig. 4, lower panel). With respect to the gaseous constituents (carbon monoxide), an inhalation chamber steady-state could not be attained within an exposure period of 4 h whilst for particulates it was attained in approximately 1 h. After extinguishing the source, CO and particulates disappeared from the chamber with elimination half-times of 360 and 60 min, respectively. Such spatial uniformity of airborne materials readily occurring in whole-body chambers as a result of adsorption of particulates onto chamber surfaces, including the hair-coat of exposed animals, does not occur in nose-only systems (Achmadi and Pauluhn, 1998).

As illustrated in Fig. 4, larger whole-body chambers require a longer time period between the cessation of atmosphere generation and the ability to collect samples for specialized examinations, e.g., blood sampling for analysis of methemoglobin (MetHb) and carboxyhemoglobin (COHb) concentrations or cholinesterase activity (e.g., carbamates). Previous work by Kim and Carlson (1986) has shown that following inhalation exposure of rats to aniline the half-life of methemoglobin was as short as 75 min. Thus, with regard to the quantification of methemoglobinemia, this rapid recovery of hemoglobin might pose logistical challenges especially in inhalation studies. MetHb levels determined immediately after cessation of exposure demonstrated unequivocal effects whilst the more commonly applied procedures failed to display a concentration-dependent formation of MetHb (Fig. 5) (Pauluhn and Mohr, 2001). The same applies for other compounds, such as COHb, which elimination half-time is approximately 12.4 min in rats (Fig. 5). These examples demonstrate that possible shortcomings

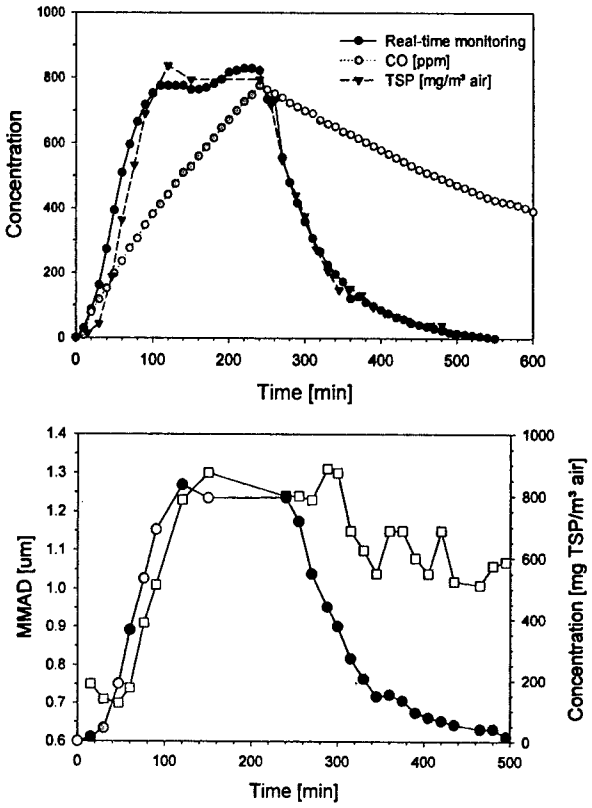

Fig. 4. Whole-body exposure in 2 m^3 inhalation chambers to combustion products generated within the chamber (for details see Achmadi and Pauluhn, 1998). The combustion device was switched-on for 4 h and then switched-off whilst the exposure atmosphere was monitored for the remaining time period of 6 h. Upper panel: airborne concentrations of particulates (total suspended particulate matter, TSP) and carbon monoxide (CO), lower panel: particle size (mass median aerodynamic diameter, MMAD) as a function of concentration and time. Mass concentrations were monitoring continuously (real-time aerosol photometer), measured by a laser Velocimeter (TSI APS 3300) or filter analysis.

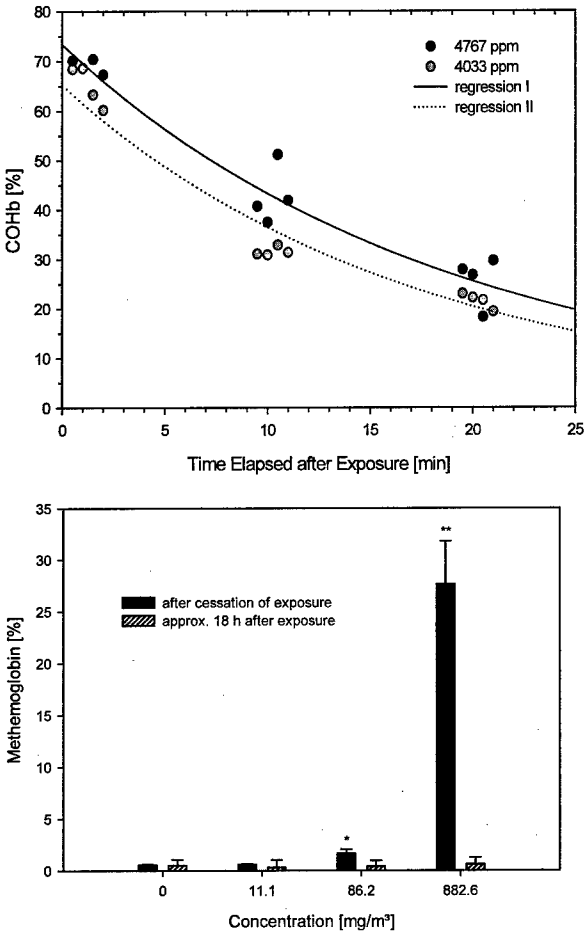

Fig. 5. Impact of timing of blood collection after cessation of exposure. Upper panel: elimination of carbon monoxide (CO) in spontaneously breathing rats after cessation of a 30-min exposure to CO. Lower panel: methemoglobin formation in rats exposed to various concentrations of phenetidine; blood was sampled either after cessation of the 6 h exposure period of the following day (18 h after cessation of exposure).

in study design and timing of collection of blood relative to the end of exposure must be envisaged for certain agents. Thus, commonly employed procedures may result in false negative results.

5. Artifacts related to the generation and characterization of atmospheres

Low to moderately volatile liquids may contain volatile constituents that may either be artificially enriched in exposure atmospheres by stripping of the liquid or by the procedures used to optimize of particle size. Thus, minute impurities in the test compound may become a major constituent in the test atmosphere. Powders may contain low concentrations of particulates small enough to penetrate the lower respiratory tract. The specific procedures used in inhalation toxicology commonly favor the dispersion of finer particulates whereas the larger ones often constituting the major product are entrapped by, e.g., pre-separators, elutriators or cyclones. This means, the physical and chemical composition of test atmospheres might differ appreciably from the actual compound tested. Such disproportionation needs to be identified and assessed appropriately. Especially for mixtures, whether related to a 'virtual mixture' of the same agent but existing of a wide range of particle sizes or a 'mixture of different agents' it is difficult to judge at the outset of study from which constituent the inhalation hazard of most concern actually originates. Therefore, it requires a careful balance of analytically and toxicologically relevant endpoints to identify such potential artifacts. Moreover, this example demonstrates further, that for complex atmospheres the generation of which is difficult to control, a challenging characterization of atmosphere is mandatory in order to verify the actual concentration of volatile and particulate materials in the vicinity of the breathing zone of the test animals. To what extent this particular exposure atmosphere resembles that observed under real-life exposure can only be demonstrated by elaborate analytical characterization of the respective atmospheres rather than by comparison of nominal settings.

These considerations suggest that laboratory-specific 'nominal settings' may be decisive for the outcome of an inhalation study and cannot be used for hazard identification without careful appreciation of all facets of the test model. As alluded to above, difficulties may also arise with substances (solids or liquids) with moderate vapor pressure because animals are exposed to a mixed exposure atmosphere consisting of an equilibrium of aerosol and vapor phase of the same agent. The generation of vapors or solid aerosols may not

necessarily mean that the test atmosphere consists solely of one phase or another. High concentration atmospheres, particularly when generated at higher temperatures, may form supersaturated vapors that will form condensation aerosols under specific circumstances. On the other hand, solid powders which have a substantial tendency to sublimate or evaporate may again challenge the analytical characterization of atmospheres because one phase can analytically be omitted at the expense of the other phase. For instance, cyanuric chloride, when tested as a dust, is present in the inhalation chamber in an equilibrium of vapor and aerosol. Analytical procedures that focus on particulate material (dust) only would lead to greatly erroneous data and misclassification of toxicity since the collection efficiency of the major phase (vapor) approaches zero (Pauluhn and Mohr, 2000).

Sampling of test atmospheres is subject to significant bias when collection efficiencies of different sampling devices are poorly defined. Sampling ports should be designed in such a way that potential sampling errors as a result of anisokinetic sampling or size-selective sampling are minimized. Aerosol sampling implies that all those particles should be sampled which are present in the inhalation chamber than restricting it to a size fraction likely to be deposited at a specific location within the respiratory tract of any test species. Extraction of a representative sample of an aerosol is subject to error due to the influence of inertial, diffusional, gravitational, thermal, or electrical forces as well as to the heterogeneity of dispersion aerosols. Errors due to anisokinetic conditions become greater as particle size, density, and air velocity increase. They are minor, however, for unit-density matter smaller than about 5 μm in diameter under all circumstances.

6. Conclusions

Several animal species have traditionally been used in inhalation studies. The limitations involved with each need to be identified and accounted by the respective study design. For the purpose of comparison, similarity of procedures commonly applied in humans will usually be considered as an advantage, though bioassay specific constraints have to be closely observed. These include the adjustment of time points of tissue collection as well as using exposure regimens close to those relevant for humans. With respect to laboratory rodents, species-specific responses, often protective in nature, need to be identified and not to be confounded with early toxic effects. When generating complex exposure atmospheres under specialized laboratory conditions, the composition of test atmospheres may not necessarily resemble that at the workplace or in conditions of interest. The optimization or maximization of respirability of aerosol atmospheres in laboratory settings may identify potential hazards not likely to occur under real-world use of the respective product. Test compounds, when airborne, may change their characteristics, and specialized analytical procedures are required for their identification, especially in a particle–vapor phase equilibrium. The extraction of particles from moving streams may result in anisokinetic sampling errors. If the main stream is flowing at a different velocity and orientation than that maintained at the orifice of a probe, then greater-than-actual or more often less-than-actual concentrations can be found, especially with larger particles (which represent the fraction of particles containing the highest mass). Exposed animals breathe in a cyclical manner, and thus their 'collection efficiency' may be different from that of the probe. Accordingly, the results of inhalation studies need to be analyzed and interpreted with caution and in relation to the effects observed.

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Review

Toxic responses of the lung to inhaled pollutants: benefits and limitations of lung-disease models☆

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Abstract

The widely accepted notion that certain individuals are more susceptible to air pollutants than others has been revitalized by recent epidemiology that strongly suggests that the elderly, particularly those with underlying cardiopulmonary diseases (e.g. chronic obstructive pulmonary disease (COPD), infection), and children with asthma are more susceptible to the adverse outcomes associated with ambient particulate matter (PM). Pulmonary toxicologists have adopted 'susceptibility' as an issue that can be approached experimentally and have begun to develop as well as study more relevant animal models. These models may have specific genetic traits or cardiopulmonary impairments analogous to human diseases. The goal is to identify potential susceptibility characteristics and elucidate whether responsiveness is due to impair compensation or some unique mechanisms. Several rodent models have been used with PM: pulmonary vasculitis, bronchitis, COPD, allergic asthma, infectious lung diseases, systemic hypertension, and congestive heart disease. Transgenic and knockout mice are of growing interest but have seen limited use in air pollutants studies, with primary interest being directed to specific mechanistic questions. No model should be used without careful consideration of its strengths and limitations. However, when interpreted in the context of field and epidemiology findings, they may reveal generic susceptibility attributes or useful biomarkers.
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Keywords: Air pollution; Health effects; Cardiopulmonary impairments; Animal models

1. Introduction

Animal toxicology has traditionally had its strength in the control of variables that frequently confound the outcomes of human and population studies, or otherwise increase data variability that impair statistical acumen. One aspect of this control is apparent in the refinement in modern day test animal genetics and husbandry. Great efforts and expense have been invested over the

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last 30 years to develop and standardize inbred strains of laboratory rodents, deriving them by caesarean section, and rearing them in pristine, infection-free environments with standardized diets. This emphasis has provided substantial control of host variables, and indeed, is at the core of most testing imperatives as well as exploratory research. Standardization is also central to the paradigms of risk assessment, but with the caveat that studies should focus on the 'most sensitive species or strain' as the model for evaluation. In practice, however, there is usually insufficient knowledge to judge the 'appropriateness' of a given animal model for study. When coupled with mandated protection for so-called susceptibility, most risk assessments must ultimately rely on the application of uncertainty factors.

Despite the quest for standardization in toxicologic assessments, research addressing clinical questions involving disease pathogenesis or its mitigation have been explored in various respiratory disease models. Among these are a number of models with lung fibrosis, emphysema, or allergic asthma. The current emphasis is on transgenic or knockout mice, which can be genetically crafted to test the roles of specific gene related products or mechanistic questions. However, aside from some recent inquiries into the genetics underlying responsiveness to selected toxic inhalants (e.g. ozone—Kleeberger et al., 2000), disease or susceptibility models have not been widely used in inhalation toxicology. When they have been used, usually they are included as 'accepted or relevant' models of disease with little or no characterization or appreciation of their appropriateness with regard to disability or chronologic disease state, per se. Actually, it is not surprising that results of these toxicology studies generally are unremarkable with minimal differences in response found between most disease models and healthy animals.

The concept of 'susceptibility' is undergoing a revitalization within the field of inhalation toxicology. Perspectives have begun to mature with more attention to both generic and specific attributes underlying the 'state of susceptibility' and how any given model may relate to the analogous human clinical disease. The renewed interest largely has been fueled by recent epidemiological studies investigating the health effects of ambient air pollutants, notably particulate matter (PM). These reports have and continue to draw attention to the statistical weight of evidence for effects in presumptive susceptible subpopulations, including the aged, especially those with underlying cardiopulmonary diseases (e.g. chronic obstructive pulmonary disease (COPD); cardiovascular disease) as well as in children with asthma (Dockery et al., 1993; Burnett et al., 1995; Morris et al., 1995; Peters et al., 1997). That these effects on health appear at concentrations thought to be safe based on prior assessments, the associations have been provocative. Questions continue to arise regarding the 'biological plausibility' of these effects at such low concentrations.

Thus, toxicologists have been challenged to explore this issue empirically in a rigorous, hypothesis-based fashion. To do so with rapidly developing cellular and molecular tools seems appropriate, but most existing animal models are less than adequate. Given the goal to explore biological phenomena that occur in a tiny fraction of the human population (i.e. groups that appear to be frail or responsive), it becomes highly problematic to discern effects within the limits of a laboratory study utilizing relatively few and especially healthy test animals. Then, the task of the animal toxicologist is to develop and implement research strategies that can elucidate host attributes that underlie susceptibility that can be applied to humans (Fig. 1). Moreover, it is clear

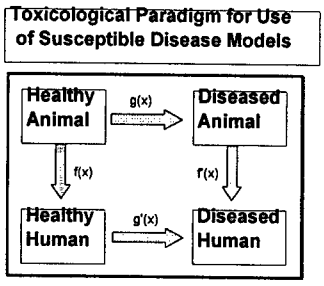

Fig. 1. Toxicological paradigm showing the hypothetical extrapolation of susceptibility disease models to the humans. The functions, $f(x)$, $f'(x)$, $g(x)$ and $g'(x)$ indicate algebraic functions related to the extrapolation models that link animal-derived data to those of the human with and without disease.

that the development or refinement of appropriate models will be invaluable in predictive risk assessments if their utility in standardized protocols for safety testing can be achieved.

In the last few years, there has been an increasing number of studies employing animal models of pulmonary and cardiovascular diseases in air pollution toxicology, and likewise there is more effort to improve and develop new models. The utility and limitations of many common cardiopulmonary models, especially in air pollution-related investigations, recently have been reviewed along with a proposed criteria for model selection (Kodavanti et al., 1998; Kodavanti and Costa, 1999). Other publications have also attempted to address these issues both generically (Brain et al., 1988) as well as specific to respiratory disease (Slauson and Hahn, 1980; Snider, 1992).

Briefly, the following generic questions were derived from the criteria we felt should be addressed when selecting an animal model of susceptibility for an air pollution study.

(1) *Does the animal model fit the question being asked*? In other words, does the hypothesis address a specific susceptibility feature under a specific air pollution challenge, which might require accurate knowledge or assessment of the existent disability at the time of challenge? Or, is the question more one of altered disease pathogenesis in which one is assessing the state of health at two points in time and the focus is more one of altered slope of deterioration? The approach and selection of a model, as well as its characterization would differ between these goals.

(2) *Does the model have human clinical relevancy*? Factors to consider would include: (1) pathogenic analogy to the human disease; (2) homology or coherence of end points or outcomes between the human clinical assessment and that for the animal; and (3) pulmonary, cardiac and systemic interactions as may relate to any human disease of chronic deterioration (i.e. general or holistic health status).

(3) *Is the disease being modeled in the animal persistent or does it progress, recover, or invoke compensatory mechanisms not common to the human*? Rodents, especially less than 14 weeks of age, have the ability to regenerate lung tissue, either as new lung or as hyperinflated lung, to ensure adequate gas exchange surface area (Costa et al., 1983). Thus, animal models given sufficient time may recover not only by repair processes but also by replacement compensatory processes, which obviously could have impact in studies extended in duration.

The goal of this paper is to provide a perspective on the utilization of animal models of cardiopulmonary disease. Emphasis is placed on recent advances regarding attributes of host susceptibility gleaned from the use of such animal models in studies of PM. Also discussed are some advances in the use of uni- or poly-genic models and how these can be bridged with experimental manipulation to sort out of the complexities of host susceptibility. Clearly, the better understanding of the disease entity being modeled and the model itself will assure maximum utility and interpretability.

2. Animal susceptibility models in air pollution toxicology

The very concept of 'host susceptibility' is complex. It involves aspects of dosimetry, innate sensitivity of organs and tissues, as well as alternate mechanisms or modes of action (reviewed by Brain et al., 1988; Kodavanti and Costa, 1999). Clearly, any host factor that can alter dosimetry of an inhalant to the lung (e.g. breathing pattern changes, narrowed airways etc.) may result in a shift in the dose–response curve, perhaps even the slope depending on the cellular target and its role in the response. Increased deposition in the airways of an asthmatic may initiate a response that for dose reasons alone would not affect a healthy person. Alternatively, a genetic polymorphism may create a unique status of susceptibility for an individual based on a genetic predisposition in metabolism, for example see Fig. 2. Likewise, gender also provides an innate attribute that may alter responsiveness, as lung size and ventilation differs by sex. Add to this list: age, nutrition, and the presence/degree of pre-existent disease and one finds that susceptibility is almost individually

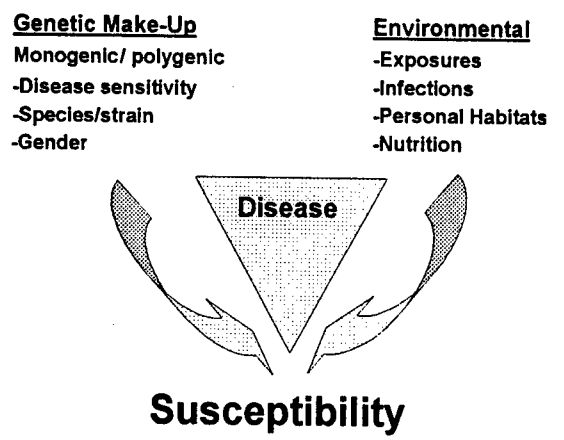

Fig. 2. Genetic and environmental factors that interact to define individual susceptibility, either to disease or to an environmental challenge.

unique (Kodavanti et al., 1998; Kodavanti and Costa, 1999). Lastly, these determinants may in fact interact with one another to alter overall susceptibility—no one trait is determinant.

The recent interest in PM-related susceptibility has stimulated the use of a number of rodent models (mostly in the rat) of pre-existent pulmonary or cardiac disease in air pollution toxicology. These models are being exploited in an attempt to investigate the presumptive risk factors that have been suggested by the epidemiology. The models are: pulmonary vasculitis, bronchitis, COPD, allergic asthma, infectious lung diseases, systemic hypertension, and cardiovascular disease (Gilmour and Selgrade, 1993; Clarke et al., 1999; Gavett et al., 1999; Kodavanti et al., 1999, 2000a,b; Wellenius et al., 2002). Studies in mice have largely been limited more to strain differences in response to specific air pollutants (Ohtsuka et al., 2000; Kleeberger et al., 2000) and selective use of knockouts and transgenics for mechanistic determinations (Ghio et al., 2000; Yoshida et al., 1999; Gavett et al., 1999). The challenge of all studies involving animal models, however, includes establishing their relevance as analogues of actual human disease states or pathogenesis, detecting a 'signal above the noise' associated with disease, and pin-pointing the appropriate severity status of disease in the animal.

3. Human chronic obstructive pulmonary disease

Clinically, human COPD is characterized by chronic airflow obstruction arising from one or more of the following: destruction of small airways and septal infrastructure, loss of alveolar wall structures (emphysema), and mucus plugging because of hypersecretion. The presence of inflammation is considered critical to the chronic progression of the disease (Scientific Committee, 1999). Not surprisingly, the major risk factor for COPD (also a risk factor for cardiovascular disease) is chronic cigarette smoking (Surgeon General, 1984). Mechanistically, lung damage and remodeling in COPD is thought to be due to an imbalance between pulmonary proteases and antiproteases released by inflammatory cells. Because the pathogenesis of COPD (emphysema, bronchial mucus hypersecretion, inflammation) is complex and involves both the bronchial and parenchymal compartments, it has been difficult to experimentally induce this disease in laboratory animals. This has slowed the development and testing of effective therapeutic targets (Shapiro, 2000), and not surprisingly, it has restricted air pollution studies to animal models that generally fall short of the human paradigm.

4. Rodent COPD models for air pollution studies

Much of our knowledge of the protease–antiprotease hypothesis has come from laboratory rodent models. A single intratracheal instillation of elastase will induce 'emphysema' in laboratory animals, characterized by rapid neutrophil recruitment/inflammation, release of fragments of extracellular matrix into the air spaces, and destruction of alveolar walls (Snider, 1992). With resolution of the initial acute inflammation, there is considerable alveolar remodeling of damaged alveolar structures with loss of compliance and airflow obstruction analogous to observations in humans with advanced emphysema. It is worth noting, however, that only a small percentage (10–20%) of chronic smokers develop COPD and this process often take decades; hence the development of an

'acute' model of this disease entity is likely to be problematic.

Emphysema and mucus hypersecretion are both critical pathological features of COPD, but relatively little effort has been invested in developing the bronchitis feature in laboratory rodents. Although elastase instillation is associated with acute mucus hypersecretion during the initial phase of injury in rodent models, it is reversible with resolution of inflammation over ensuing days (Hayes and Christensen, 1978). Cigarette smoke itself in rats and mice has yielded inconsistent results regarding chronic mucus hypersecretion and inflammation. On the other hand, long-term, high-level exposures to the gaseous irritant, sulfur dioxide (SO_2), has become a widely used model of chronic bronchitis. The exposure regimen is 200–700 ppm for 4–8 weeks, which has been associated with damage to ciliated epithelial cells, neutrophilic inflammation, mucus hypersecretion, and mucus cell metaplasia (Shore et al., 1995; Snider, 1992). Unfortunately, the bronchial mucus hypersecretion in rodents caused by SO_2 persists for only a few days after the termination of exposure; likewise, the acute neutrophilic inflammation seen following SO_2 exposure is almost entirely reversed within a week following termination of exposure (Kodavanti et al., 2000c), with virtually no effect on alveolar structures.

Nevertheless, this SO_2-induced rat model of bronchitis has been used in concentrated ambient PM (CAPS) studies where both inflammation and heightened alveolar permeability followed acute CAPS (Clarke et al., 1999; Kodavanti et al., 2000a). The increased acute responsiveness of the model is variable and may well be associated the variability of the lesion or perhaps with altered particle deposition due to mucus in the airways. Interestingly, studies have shown that overall more particles deposit in the bronchitic as compared to the healthy lung and that there are deposition 'hot spots' (Sweeney et al., 1995).

Since emphysema and bronchitis typically coexist in COPD, we have attempted to combine these two experimental manipulations in the rat, whereby elastase-induced emphysema was induced prior to SO_2 exposure to overlay mucus hypersecretion on parenchymal destruction (Kodavanti et al., 2000c). The goal was to render the rats more susceptible to the SO_2-induced bronchitis and inflammation by prior induction of emphysema, and hopefully achieve more persistent inflammation and mucus hypersecretion. However, neither emphysemic Sprague–Dawley nor Brown Norway rats exhibited increased sensitivity to SO_2 or persistent bronchitis. Furthermore, exposure to a toxic combustion source PM (residual oil fly ash) showed no increase in inflammation or lung permeability relative to healthy controls. Interestingly, this is contrary to what we observed in PM exposed bronchitis-only rats (Kodavanti et al., 2000a). It could be argued that the emphysema may have counteracted the effect of bronchitis. Indeed, altered respiratory pattern in emphysematous rodents has been shown to result in less particle deposition in the lung compared to that of healthy animals (Sweeney et al., 1987; Mauderly et al., 1990). This pattern of dosimetry is contrary to what has been observed in COPD patients exposed to particle aerosols (Kim and Kong, 1997). Thus, although these models have more features of COPD, some critical factor(s) remain inadequate which limits their apparent relevance to the human disease. These studies place further emphasis on how genetic predisposition (in contrast to a temporary disease phenotype) may impact predisposition to disease or air pollutant health effects.

As noted earlier, the role of genetic predisposition to environmental disease has been gaining interest as new genetically-manipulated monogenic and polygenic models are developed (Shapiro, 2000). Based on the spectrum of risk factors attributable to COPD (Koyama and Geddes, 1997; Repine et al., 1997; Barnes, 2000) we introduced the use of the spontaneously hypertensive (SH) rat with known underlying oxidative stress, systemic inflammation, increased fibrinogen levels, and borderline-pulmonary hypertension, into the experimental COPD paradigm. We hypothesized that these combined risk factors may render SH rats more susceptible to experimental disease with persistent airway inflammation and alveolar destruction. Our preliminary studies show that SH rats are indeed susceptible to more persistent inflammation during elastase-induced emphysema and SO_2-induced bronchitis than

Sprague–Dawley rats (Kodavanti et al., unpublished observations). Related studies are in progress as we also attempt to characterize the sensitivity of the SH rat to long-term cigarette smoke exposure (Smith et al., 2001).

Mouse models, on the other hand, provide some features of COPD, such as loss of ciliated epithelial cells, airway inflammation, emphysema, and restructuring of small airways. However, mucus production is still relatively deficient in these rodents even following cigarette smoke exposure. A recent review by Shapiro (2000) describes how different mouse models with inherited deficiencies in elastin processing have provided insight on emphysema. Macrophage accumulation and release of metalloproteinases-12, for example, have been shown to be important in cigarette smoke-induced emphysema (Hautamaki et al., 1997). However, emphysema is likely the product of a constellation of factors which involve injury, inflammation, degeneration of tissues, and remodeling to one degree or another.

5. Human cardiovascular disease

The intimate hemodynamic and neurohumoral relationships of the lungs and heart make it important to consider the cardiac consequences of pulmonary insults, and vice versa. Cardiac disease involves complex etiologies and represents the number one cause of mortality worldwide (Wilson, 1997). With age, the risk of heart disease increases, often insidiously. Its pathogenesis often involves blood hypercoagulability, atherosclerosis, cardiac hypertrophy due to hypertension, ischemic damage to cardiac tissue, and myocardial infarction, which complicates to myopathy and compensatory hypertrophy. Low ambient PM levels have been associated with mortality/morbidity in humans with ischemic and cardiac vascular diseases (Dockery et al., 1993; Burnett et al., 1995; Morris et al., 1995; Schwartz and Morris, 1995).

A variety of animal models have attributes of cardiac disease and the complex pathobiology of humans on course to congestive heart failure. Genetically and experimentally-induced animal models of cardiovascular disease have been described in many reviews (Ganten and de Jong, 1994; Elsner and Riegger, 1995; Johns et al., 1996; Doggrell and Brown, 1998; Kodavanti et al., 1998). The following section describes their limited use in air pollution studies.

6. Rodent cardiovascular disease models in air pollution studies

Hartroft et al. (1976) were the first to demonstrate that rats with experimentally induced chronic cardiovascular disease were more sensitive to a combined atmosphere of PM and oxidant gases. Rats that were maintained on a thrombogenic diet or had systemic hypertension, suffered greater mortality and morbidity relative to healthy rats. In a rat model, genetically susceptible to salt-induced hypertension, O_3-exacerbated pulmonary vascular leakage and mortality were found to be linked to underlying cardiovascular disease (Drew et al., 1983; Costa et al., 1985). These genetically-predisposed Dahl-S rats (salt sensitive hypertension) were found to be more sensitive to O_3 when compared to the Dahl-R (salt insensitive) rats regardless of salt in the diet or degree of hypertension. Hence, there appeared to be a genetic basis for the pollutant sensitivity that was genotypically segregated with, but distinct from, the phenotype of salt-induced hypertension.

Using the polygenic SH rat model for human cardiovascular disease, we have initiated the investigation of possible cardiovascular mechanisms of increased susceptibility to pollutants (Kodavanti et al., 2000b). The SH rat is the most extensively used animal model of human cardiovascular disease as its pathogenesis, progression, and failure mechanisms have been shown to corroborate human disease and involve polygenic traits (Committee on Care and Use of Spontaneously Hypertensive (SHR) Rats, 1976; Schmid-Schonbein et al., 1991; Suzuki et al., 1995; Harjai, 1999).

Our purpose in using the SH rat in PM health effects is to investigate how pulmonary injury mediates cardiovascular effects in the presence or absence of cardiovascular disease. In characterizing the model, we have shown that the SH rat

exhibits a borderline lung inflammation as well as a number of associated systemic indicators of heightened oxidative stress (Kodavanti et al., 2000b). These common risk factors are found in human cardiovascular patients, including higher plasma fibrinogen levels, blood neutrophil counts and decreased antioxidant levels (Committee on Care and Use of Spontaneously Hypertensive (SHR) Rats, 1976; Schmid-Schonbein et al., 1991; Suzuki et al., 1995; Harjai, 1999). These very same changes have also been associated with COPD as discussed above (Jousilahti et al., 1999; Barnes, 2000). When exposed to metal-rich toxic particles either via intratracheal instillation or inhalation, SH rats have exhibited increased pulmonary injury and leakage, but more importantly, they failed to increase glutathione levels in the lung lining fluid. In addition, the PM-induced injury persisted for a longer period of time when compared to normotensive WKY rats (Kodavanti et al., 2000b). Together these findings suggested an impaired compensatory ability associated with the underlying disease. Also, at modest levels of inhaled PM, the acute pulmonary injury was associated with further increases in plasma fibrinogen in SH rats above already heightened baseline levels. This pattern was not found in the WKY rats suggesting that plasma fibrinogen along with concurrent oxidative stress may be associated with hemodynamic changes that contribute to increased susceptibility (Kodavanti et al., unpublished). Thus, the SH rat and similarly predisposed polygenic models are relevant in exploring cardiopulmonary interactions and genetic susceptibility. Other polygenic rat models also exist with susceptibilities to a variety of cardiopulmonary impairments (Ganten and de Jong, 1994; Elsner and Riegger, 1995; Johns et al., 1996; Doggrell and Brown, 1998; Kodavanti et al., 1998) which could further our understanding of the role of genetic versus environmental factors in susceptibility. The use of high throughput gene expression analysis, and identification of genetic polymorphisms in understanding of temporal biological networking would facilitate the identification and understanding of susceptibility traits.

7. Conclusions

Susceptibilities related to the development of chronic respiratory and cardiovascular disease in humans are the result of complex interaction of life styles, exposures and genetic polymorphisms. Similarly, responses to an air pollutant challenge likely involve a network of cascading events initiated when the pollutant interacts with the airway surface and its structural components. What determines the extent of the response at the pulmonary as well as at the systemic level may well reflect the genetic and/or health status of the individual exposed. It has become increasingly evident that the respiratory tract does not act alone in response to air contaminants, but physiologically shares environmental stresses. To address the complexities of host attributes associated with chronic cardiovascular and pulmonary disease and how these host factors modify susceptibility to inhaled toxicants require rigorous and careful approach in characterizing, selecting and developing appropriate animal disease models. It is expected that these technologies will help to elucidate the underlying biology of susceptibility to allow the impact of the environment, day to day life styles as well as pollution, to be evaluated.

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Review

Novel approaches for studying pulmonary toxicity in vitro

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Abstract

The in vitro study of adverse cellular effects induced by inhaled pollutants poses a special problem due to the difficulties of exposing cultured cells of the respiratory tract directly to test atmospheres that can include complex gaseous and particulate mixtures. In general, there is no widely accepted in vitro exposure system. However, in vitro methods offer the unique possibility for use of human cells, developed and validated cell culture and exposure device (CULTEX[1]) using the principle of the air/liquid exposure technique. Cells of the respiratory tract are grown on porous membranes in transwell inserts. After removal of the medium, the cells can be treated on their superficial surfaces with the test atmosphere, and at the same time they are supplied with nutrients through the membrane below. In comparison with other experimental approaches, the goal of our studies is to analyze the biological effects of test atmospheres under environmental conditions, i.e. without humidifying the atmosphere or adding additional CO_2. The system used is small and flexible enough independent of a cultivation chamber and thus offers the opportunity for onsite study of indoor and outdoor atmospheres in the field. The efficacy of the exposure device has already been demonstrated in the analysis of dose-dependent cytotoxic and genotoxic effects of exposure of epithelial lung cells to complex mixtures such as native diesel exhaust and side-stream smoke.
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Keywords: Air/liquid interface; In vitro exposure; Airborne pollutants

1. Introduction

The analysis of effects of inhalable substances by using cultivated cells of the target organ (i.e. lung) requires the introduction of new in vitro techniques in order to achieve adequate cultivation and exposure. The experimental procedure must allow exposure of the cells to chemically and physically characterized native atmospheres and must enable determination of cellular reactions. The use of target cells from human airway epithelium reduces the need for animal studies and obviously facilitates the extrapolation of test results to the human situation. The most effective experimental approach is based on the principle of the air/liquid exposure technique where the cells are cultivated on porous membranes. Nutrients are supplied basely through the porous membranes,

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[1] CULTEX: Patent No. DE 19801763; PCT/EP99/00295.

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leaving the superficial (apical) surfaces exposed for pollutant exposure (Rasmussen, 1984; Voisin et al., 1977a,b). For air/liquid exposures, the common experimental set-up uses standard or specially designed cultivation/exposure chambers (Gabrielson et al., 1994; Lang et al., 1998). The test atmospheres are heated and humidified to 90–100% rH at 37 °C. The cultures are placed on microporous membranes inside the chamber. Most of the studies using such procedures that have been reported in the literature were on the effects of model gases such as ozone. The test gas in these studies was passed through the exposure chamber above the cells by using compressed air, or the exposure chamber was statically filled once with the gas mixture.

In our studies, we set out to establish an in vitro exposure device to study the biological effects of native, environmentally relevant complex atmospheres without any modification of the gas phase. The system we developed also provides the flexibility to work independent of a cultivation chamber and thus offers the possibility for onsite investigation of indoor and outdoor atmospheres.

A description of the system we developed for exposing adherent growing cells in transwell cultures follows.

2. Material and methods

2.1. Culture module

The module is constructed entirely of autoclavable glass housing three vessels for transwells (Fig. 1). The vessels are within the inner space of the module and their temperature is determined by regulated flow of temperature-controlled water from an external water bath. The module is filled with heated water up to the level of a glass outlet tube and this stabilizes the level of water.

Nutrient medium is directed to the three transwell vessels via a glass tube system. Each module has a second outflow nozzle that can be used to obtain samples of medium for biological analysis during the experiment, e.g. for cell secretory products.

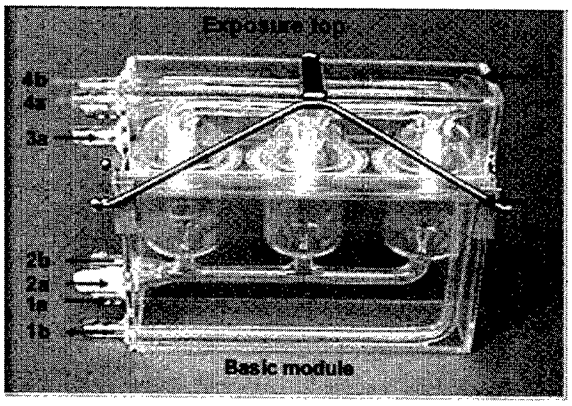

Fig. 1. Culture and exposure module. Basic module: (1a) Water inlet for the module; (1b) Water outlet of the module; (1c) Water overflow tube; (2a) Medium in- and out-let nozzle; (2b) Optional connection for separate removal of conditioned medium during the experiment. Exposure top: (3a) Inlet nozzle for heated water; (3b) Water outlet; (3c) Water overflow tube; (4a) Inlet nozzle for the test atmosphere; (4b) Outlet nozzle for the test atmosphere.

Individual modules can be run in parallel, thus creating a sequential and flexible system to meet specific experimental requirements. By connecting each module to different levels of an exposure dilution mechanism, the system enables the simultaneous testing of different concentrations of a test atmosphere (for dose–response effects) using equivalent cell populations. Since there are three transwell inserts within each module, three replicates are available for each concentration.

The test atmosphere is drawn over the top of the transwell chambers in the upper portion (exposure top) of the module via a glass tube system and Teflon tubes using negative pressure. This provides well-balanced distribution of the test atmosphere and prevents retrograde flow (Ritter et al., 2001). Thus, each cell culture can be individually exposed to the test atmosphere. The test gas/mixture is evacuated from the system via a Teflon outlet connected to another glass tube system. The gas/mixture distribution is calibrated by adjusting the diameter of the outlets. In several trial runs, the exposure system was calibrated to provide a stable and homogenous distribution of the test atmosphere with a variation of less than 5%. The exposure top of the module that provides for exposure of the transwells can also be heated to

prevent condensation. Heating is performed by the same water bath/pump system that heats the lower portion of the module containing nutrient medium (referred to earlier). The upper and lower portions of the module are connected by specially designed silicone O-rings and are held together by two metal springs (Fig. 1).

In our current tests, samples of the test atmosphere are conducted through the exposure device (25 ml/module/min) by means of a vacuum pump with flow controls. The complete exposure device consists of up to 4 culture modules driven in parallel, a vacuum pump, flow controllers and a water bath with a thermostat and pump unit.

In the experiments with diesel exhaust fumes, this experimental set-up was integrated into a mobile container, and placed in the control room of the engine test rig, next to the online monitoring system.

2.2. Exposure conditions

Cultured cells were exposed either to native gaseous compounds (Ritter et al., 2001), side-stream smoke (Aufderheide et al., 2001) or automobile exhaust fumes (Knebel et al., 2002) (Fig. 2). Therefore, the test atmospheres had to be generated individually as described in detail previously (Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001). Briefly, the model gases nitrogen dioxide and ozone were used as an example of native gaseous compounds. Nitrogen dioxide concentrations were obtained by diluting a stock of 100 ppm NO_2 in synthetic air from a gas flask with synthetic air using mass flow controllers (Tylan, Germany) for different flow ranges in a gas flow system. Ozone was generated in situ by photolysis of synthetic air using a PenRay lamp (Oriel Sarl, Paris) and was also diluted with synthetic air. Samples from these various gas concentrations were passed continuously through the cell exposure device.

Side-stream smoke was generated using a smoking machine (Teague et al., 1994), which enabled the mechanical smoking of a standard reference cigarette K1R4F (Tobacco and Health Research Institute, University of Kentucky, Lexington, USA) according to ISO guidelines. Smoke from the burning end of the cigarette was collected and transferred to a conditioning chamber, where a constant smoke concentration was achieved. The smoke atmosphere was then directed into a low-

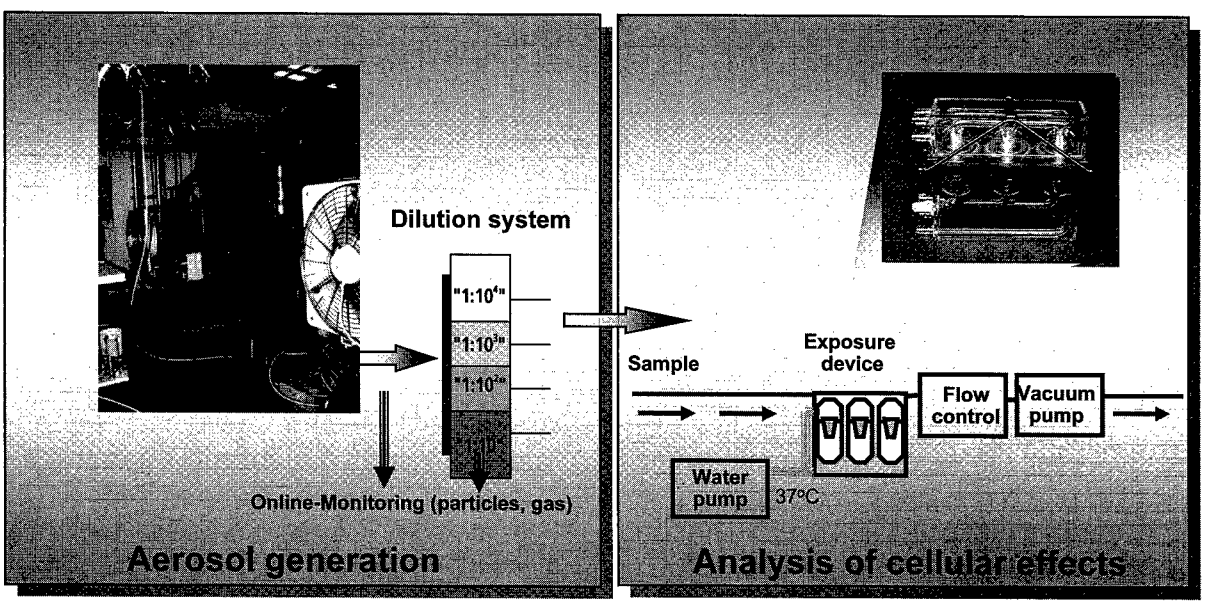

Fig. 2. Conceptual outline for the exposure of cultivated cells to different test compounds.

flow multistage aerosol mixing system where it passed through four dilution chambers, each with a constant dilution rate (factor 10). The diluted smoke atmosphere could be bled off the system at any of the four chambers and sucked through the cell exposure device.

For the generation of motor exhaust fumes, a conventional 75 horsepower, 6-cylinder diesel engine (Volkswagen, Wolfsburg) on a motor test rig (Schenck, type W320) was used without any exhaust after-treatment (e.g. oxidation catalyst). Motor emissions were generated under idling speed conditions. The motor exhaust fumes were then directed into a dilution tunnel. At the exit, the exhaust fumes were diluted further in one or two steps (dilution rate 1:100 or 1:10) using a commercially available dilution system (type VKL, Pallas) before being sucked through the cell exposure device.

Samples from each exposure situation were passed continuously through the cell exposure device using an experimental set-up as indicated in Fig. 2. Neither the temperature nor the humidity of the test atmospheres (model gases, side-stream smoke, automobile exhaust fumes) was modified, and they were not supplemented with O_2 or CO_2 when directed over the cell monolayer. Depending on the exposure situation, the analysis of several atmospheric compounds as well as particle concentrations was performed by online monitoring parallel to the cell exposure (for details see: Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001).

2.3. Cell lines and culture conditions

For the exposure experiments, human bronchial epithelial cells (Brockmeyer et al., 1990; Emura et al., 1990, 1995; Ochiai et al., 1991; Ochiai, 1992) were grown in transwell inserts on a porous membrane with defined pore size (0.4 μm) and density sufficient to produce a humidified microclimate above the cells as described previously (Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001) and also allow their nutrification from the basal side of the membrane. The test atmosphere was drawn through the exposure unit from the dilution system by negative pressure (25 ml/min/module). Under these conditions, cells could be exposed to clean air for up to 2 h without altering their status (vitality) in comparison to control cultures maintained under air/liquid conditions but without gas flow.

2.4. Endpoint measurements

The cleavage of the tetrazolium dye (WST-1) (Boehringer, Mannheim, Germany) to formazan by mitochondrial dehydrogenases was measured. Cells were incubated with a dilution of the dye according to the protocol developed by the manufacturer. After incubation, formazan production was quantified by absorption reading using a microtiter plate reader (Spectramax 340PC, Molecular Devices, Ismaning, Germany) at the wave lengths 450–630 nm.

The *number of viable cells* was determined directly after tetrazolium salt analysis of the same cells using an electronic cell counter (CASY, Schärfe Systems, Reutlingen, Germany) after trypsinisation and dilution of the cell suspension (Knebel et al., 1998). This method is appropriate for an automated, quantitative discrimination of living and dead cells (Winkelmeier et al., 1993).

The *protein content* of the cells was quantified using a commercial kit according to the protocol of the manufacturer (DC Protein Assay, Biorad, München, Germany).

The *ATP/ADP ratio* of exposed cells was analyzed using reversed phase HPLC with UV detection at 259 nm following extraction with trichloroacetic acid (Ritter et al., 1999).

Intracellular glutathione of cells was determined using a modified Tietze recycling assay after extraction with meta-phosphoric acid (Ritter et al., 1999).

The intracellular redox ratio of oxidised and reduced glutathione (*GSSG/GSH*) was quantified using extraction with meta-phosphoric acid, derivatisation using 2,4-dinitrofluorobenzene and detection of the *N*-2,4-dinitrophenyl derivatives by UV at 355 nm after HPLC (Ritter et al., 2001).

3. Results and discussion

In a series of studies, the effectiveness and practicality of the CULTEX exposure modules were analyzed for two gases (ozone and nitrogen dioxide; Ritter et al., 2001) and two different complex atmospheres (native diesel exhaust and side-stream cigarette smoke; Aufderheide et al., 2001; Knebel et al., 2002) according to the experimental strategy described earlier (Fig. 2). Direct exposure of the cells to nitrogen dioxide and ozone at the air/liquid interface resulted in a dose-dependent decrease in the viability of the cells (Ritter et al., 2001). The nature and depth of the liquid layer covering cultured cells is a critical determinant of gas-induced effects because it can modify effects through both physical and chemical mechanisms. In our experiments, the liquid layer on the surface of the cells that was established by equilibrium between evaporation above the cells and diffusion of the medium through the membrane was thin enough to interpose no significant reactive barrier between the cells and the gas.

After demonstrating the efficiency of the exposure device for single gaseous compounds, the system was used to analyze the effects of complex mixtures. The first model atmosphere was diluted side-stream cigarette smoke, to which the human bronchial epithelial cells were exposed for 60 min at a flow rate of 25 ml/module. Firstly, the smoke was collected in an 'Age Dilution Chamber' (ADC) and then serially diluted twice by a factor of 10 using synthetic air (D1 to D2). The smokes of the different dilution steps were sucked through the system and the cultures were analyzed for cell number, metabolic activity, glutathione and ATP content (3). The smoke from the ADC induced a strong cytotoxic effect. Cell number decreased to about 20% of the air/liquid control. Dilution of test atmospheres (D1) resulted in values comparable to the control cultures. These data could also be confirmed by measurements of the metabolic activity. Further dilution of the smoke (1:100) showed no significant changes in these parameters. Due to the high concentrations of reactive species in cigarette smoke, intracellular glutathione content was measured as a sensitive marker of effect (Fig. 3). Corresponding to the strong cytotoxic effect induced by the smoke from the ADC, the total glutathione content decreased significantly. By diluting the smoke, the glutathione and ATP content in the cells increased progressively above that of the control cells from the first dilution

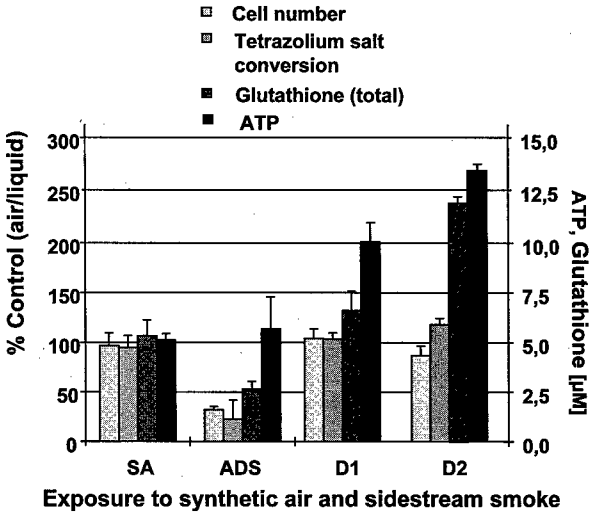

Fig. 3. Exposure of HFBE cells to synthetic air (SA) and different dilution of non-filtered side-stream smoke of the research cigarette K1R4F. ADC: ageing and dilution chamber; D1, dilution 1:10; D2, dilution 1:100.

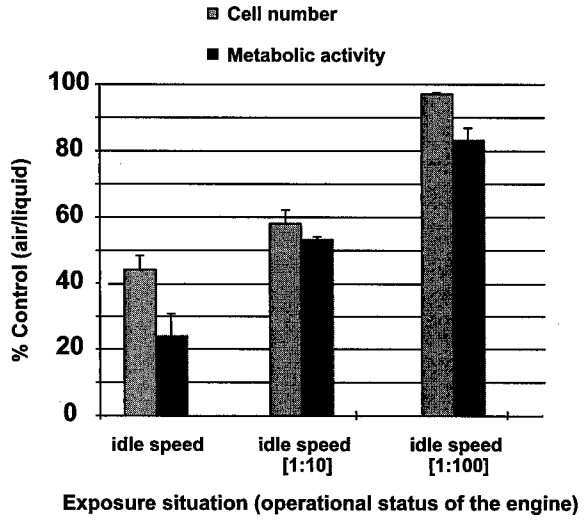

Fig. 4. Exposure of HFBE cells to diluted diesel engine exhaust under the engine operating conditions idle speed.

chamber D1 to the second one D2. There was a clear dose–response between the glutathione content and the smoke concentration.

In another study, diesel exhaust fumes generated by a conventional 75 horsepower, 6-cylinder diesel engine on a motor rig were directed into a dilution tunnel. At the exit, the exhaust was collected, diluted and connected to the sampling device accompanied by a monitoring of the test atmosphere (HC without CH_4, CO, NOx, NO_2, NO, CH_4, particles). Under these conditions, cultures were exposed undiluted and diluted (1:10, 1:100) to emissions for 60 min and analyzed for cell number and tetrazolium salt conversion (Fig. 4). Undiluted emissions induced a significant decrease in cell number (up to 50%), and a reduction of 30% in tetrazolium cleavage. The exposure to diluted exhaust fumes (1:10, 1:100) from the dilution tunnel resulted in an increase in cell viability compared to controls. In comparison to the air/liquid control cultures, 97% of living cells were found after an exposure to 1:100 diluted diesel emissions resulted in 97% of living cells compared to 100% in controls, and the tetrazolium conversion reached 83% compared to 100% in controls.

These results therefore confirmed the sensitivity of the experimental approach for evaluating substance- and dose-dependent effects of gases and complex mixtures.

4. Conclusions

These experiments demonstrated the capability of the new exposure system for testing complex atmospheres and obtaining dose-dependent effects in cultured cells. It is particularly important to note that this specialized in vitro technique enables the study of adverse health effects of inhalable compounds directly on the human target cells of the respiratory tract. The fundamental component of the exposure system is the air/liquid culture technique that allows the cultivation of target cells on porous membranes. This guarantees nutrification of the cells from below while permitting their surface exposure directly to the test atmosphere. The separation of the medium and test atmosphere by the membrane also minimizes the possibility of interactions between them. Because of the portability of the exposure system the system is suitable for the study of actual indoor and outdoor atmospheres at remote sites.

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Review

Approaches to induce and elicit respiratory allergy: impact of route and intensity of exposure

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Abstract

Although a number of test protocols have been developed to predict respiratory allergenic potential, none of these are widely applied or fully accepted. However, given the serious health problems caused by respiratory allergy and the ever-increasing stream of new chemicals into workplaces, early identification of chemical respiratory allergens is important. Inhalation exposure as well as skin application have been used in predictive tests to induce respiratory tract sensitisation. While there are good indications in laboratory animals and humans that skin exposure can act as a route for respiratory tract sensitisation and vice versa, less is known about the effect of the route on the type of allergy evoked and on dose–response relationships. Although, the responses were in general more vigorous after dermal sensitisation than after inhalation sensitisation, the nature of the immune responses seemed to be qualitatively comparable. As to the intensity of exposure, dose or concentration–response relationships have been observed both during respiratory sensitisation and challenge, suggesting that assessment of safe exposure levels is feasible. Finally, a correct distinction between respiratory allergens and non-sensitising airway irritants is needed for effective risk assessment and management.
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Keywords: Respiratory allergy; Route and intensity of exposure; Airway irritation

1. Introduction

Occupational asthma resulting from respiratory sensitisation by both high molecular weight and low molecular weight (LMW) compounds is a disorder of great health concern because of the high morbidity, the frequently poor reversibility upon withdrawal from exposure, and the sometimes life-threatening reactions (Briatico-Vangosa et al., 1994). The disease is characterized by episodic airway obstruction, airways inflammation and non-specific airways hyperresponsiveness to a variety of stimuli, including histaminergic and cholinergic agents (Sheffer, 1991; Anonymous, 1992).

Over the past years, asthma at the workplace has proved to be a difficult and controversial subject for regulatory authorities. Adoption of a clear definition has been hampered by a poor

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understanding of the mechanisms of asthma and confusion about terminology. Given the serious health problems caused by respiratory allergy and the ever-increasing stream of new chemicals into workplaces, the early identification of chemical respiratory allergens is vital. A number of animal test protocols have been published to detect respiratory allergy (see for reviews Briatico-Vangosa et al., 1994; Pauluhn et al., 1999) although none of these are widely applied or fully accepted yet. There are two routes of sensitisation: via the skin or respiratory tract. The challenge is mostly by inhalation exposure to investigate inhalation challenge-induced respiratory reactions (e.g. Karol, 1988).

Epidemiological studies suggest that respiratory irritation aggravates allergic responses, most probably because of the induction of non-specific airway inflammation. Respiratory irritants may also provoke allergy-like symptoms in susceptible individuals (Samet, 1995). Moreover, most (if not all) LMW chemical allergens also have irritating properties. The airway irritation potential of LMW allergens may in fact hamper the interpretation of allergic responses. In screening it is therefore important to know the irritating potential of LMW respiratory allergens.

In the present paper we discuss test protocols to induce and elicit respiratory tract allergy with emphasis on the impact of the route of exposure and on dose–response relationships. Also the role of airway irritation will be discussed.

2. Test protocols to detect respiratory allergy

In guinea pigs, respiratory sensitisation to LMW chemicals has been achieved via single or repeated inhalation exposures, and via intradermal or subcutaneous application. Several LMW chemicals tested positive. These were (amongst others): p-tolyl isocyanate, hexylisocyanate, toluene diisocyanate (TDI), diphenylmethane 4,4′-diisocyanate (MDI), hexamethylene diisocyanate (HMDI), trimellitic anhydride (TMA), and phthalic anhydride (PA). See papers of Karol et al., 1979; Karol, 1980, 1983; Cibulas et al., 1986; DeCeaurriz et al., 1987; Botham et al., 1988; Karol and Thorne, 1988; Pauluhn and Eben, 1991; Sarlo and Clark, 1992; Sarlo et al., 1994; Pauluhn, 1997). The guinea pig models that examine the potential of chemicals to induce respiratory allergy have the advantage of being a functional endpoint test; i.e. respiratory symptoms can be studied. In addition, like the human lungs, lungs of guinea pigs are a major shock organ for anaphylactic responses to allergens (Sarlo and Ritz, 1997). However, the tests are time-consuming, costly, and may require the use of hapten-protein conjugates which hampers comparison to the human situation. Furthermore, unlike in humans, in the majority of the studies the onset of respiratory responses was immediate with reactions occurring either during or shortly after the challenge period. Only in very rare instances were delayed-onset or dual responses reported.

The IgE test (Dearman et al., 1992a) is based on the finding that chemicals which have the potential to cause respiratory allergy in man, such as TMA, PA, TDI, MDI, and HMDI can provoke significantly elevated serum levels of total and chemical-specific IgE in mice. Conversely, skin allergens that frequently lack the potential to induce respiratory allergy in man, such as 2,4-dinitrochlorobenzene (DNCB), dicyclohexylmethane-4,4-diisocyanate and oxazolone, failed to do so. The different potential to induce IgE is thought to be the consequence of a selective Th2 and Th1 cell stimulation (Dearman and Kimber, 1991, 1992; Dearman et al., 1991, 1992a,b; Hilton et al., 1995).

The mouse IgE test is cost-effective but is not a functional endpoint test, i.e. it is not known whether increases of total serum IgE are indicative of actual induction of respiratory allergic symptoms. We, therefore, extended the IgE test with an inhalation challenge to examine whether increases of total serum IgE levels can be associated with manifest functional and morphological changes of the airways after inhalation challenge. Using a 2-times topical application of TMA followed by airway challenge of Brown Norway (BN) rats, we found specific airway reactions during and 24 h after challenge, increased lung weights, increased total and specific IgE levels, histopathological airway changes, and non-specific airways hyperresponsiveness. Inhalation challenge of TMA-sensi-

tised BN rats also induced type III/IV allergic inflammatory reactions in larynx and lungs. These latter changes were also observed in low IgE-responding Wistar rats. Moreover, delayed type (type IV) mediated reactions were observed in the larynx of Wistar rats after dermal sensitisation and inhalation challenge with the skin allergen DNCB. It is, therefore, reasonable to suppose that chemicals preferentially inducing skin sensitisation may also represent a risk of respiratory allergy when exposed by inhalation. Examination of the ability of such chemicals to attain sufficiently high concentrations by evaporation or its use (e.g. spraying) should, therefore, be a starting point to decide whether or not testing for respiratory sensitisation is necessary (Arts et al., 1998; Arts, 2001).

Sensitisation can also be measured directly by measurement of cytokine profiles. Increases in cytokine levels, induced by chemical sensitisers, can be determined using different assays such as ELISA (Ryan et al., 1998; Dearman and Kimber, 1999), RT-PCR (Ryan et al., 1998) or a multiprobe ribonuclease protection assay (Plitnick et al., 2002). Changes in cytokine profiles are generally measured in mice upon flank application of the chemicals on days 0 and 5, followed by ear application on days 10, 11 and 12. At various times following ear exposure mice are sacrificed, draining lymph nodes removed, and one of the different assays applied. Different cytokine profiles are being induced by either respiratory or skin allergens.

3. Impact of route of exposure during sensitisation

As indicated above, besides sensitisation by inhalation, the dermal route—intradermal injection or topical application—has often been used in predictive tests to induce respiratory tract sensitisation. The efficacy of topical application for sensitisation with LMW chemicals in both rats and mice suggests that skin exposure can be a significant risk factor in respiratory allergy in man. There is indeed evidence in man that dermal exposure to some chemical respiratory allergens may induce immune responses of the type necessary to cause pulmonary sensitisation (Karol, 1986; Nemery and Lenaerts, 1993; HSE, 2000). Moreover, occupational exposure of man to LMW chemicals via the skin may be considerable, such as found in auto body shop workers exposed to isocyanates despite protective clothing (Liu et al., 2000). In animal models, the advantage of sensitisation of the respiratory tract via the skin is that inflammation of the airways prior to challenge is avoided. Such an inflammation could complicate the interpretation of the response upon challenge (Holt and Sedgwick, 1987; Briatico-Vangosa et al., 1994; Kimber et al., 1996; Pauluhn et al., 1999). Moreover, challenge with atmospheres containing the hapten or appropriate chemical-protein conjugates very often failed to induce respiratory reactions. However, the guinea pigs sensitised via single or repeated inhalation exposures were immunologically sensitised as shown by the development of antigen-specific homocytropic antibodies (Karol et al., 1980; Botham et al., 1988; Pauluhn and Eben, 1991; Sarlo et al., 1994; Pauluhn, 1997). This may indicate that development of a specific immunological unresponsiveness or tolerance had occurred (Holt and Sedgwick, 1987), or that down regulation had taken place (Dearman and Botham, 1990).

While there are good indications in laboratory animals and humans that skin exposure can act as a route for respiratory tract sensitisation and vice versa, less is known about the effect of the route on the nature of induced immune responses. For instance, from the results of a study in mice by Hilton et al. (1995) it can be concluded that topical contact may require 3–30-times higher doses for induction of IgE than overall sensitisation/immunisation (viz. proliferation of immune cells in draining lymph nodes) requires. This implies that via the skin higher doses are needed for antibody (IgE-) mediated than for cell-mediated allergic reactions. In risk assessment terms, local high doses of allergen as occur in spilling/splashing on the skin may thus represent a high risk in the development of antibody (IgE-) mediated respiratory allergy.

What about sensitisation by inhalation? At first sight, it appears even more difficult to induce cytophilic antibodies via the inhalatory route than via the skin. Guinea pigs intradermally sensitised

with TMA demonstrated much higher antigen-specific IgG1 and more vigorous immediate-onset reactions upon inhalation challenge than animals sensitised by inhalation (Pauluhn and Eben, 1991). Also, exposure of BN rats to TMA by both the topical and inhalation route resulted in the stimulation of specific IgG and IgE antibody (Warbrick et al., 2002). A subsequent inhalation challenge resulted in specific pulmonary functional and inflammatory reactions (Pauluhn et al., 2002), although responses were considerably more vigorous after dermal exposure. Upon closer look, the more vigorous reaction after dermal exposure may be due to the higher total antigenic dose delivered by the dermal route (see below). Nevertheless, the results of the studies of Pauluhn and Eben (1991) and Warbrick et al. (2002) show that the nature of immune responses with respect to antibody profile provoked by dermal application of TMA was qualitatively comparable with that stimulated by inhalation exposure to the same chemical.

4. Impact of intensity of exposure during sensitisation

As indicated above, the lesser vigour of the immune responses following inhalation exposure compared to topical administration (Pauluhn et al., 2002; Warbrick et al., 2002) may be related to the lower total antigenic dose which is delivered by the inhalation route. It was calculated that in the study of Pauluhn et al. (2002) BN rats of the high concentration group had received about a 7-times lower total dose on a per kilogram body weight basis than dermally treated rats. In the study of Warbrick et al. (2002) this difference amounted to almost 1000-times. Nonetheless, the topical dose of TMA required for sensitisation was extraordinarily high in the context of risk assessment. As to dose–response (or concentration–response) relationships, there was some suggestion of a dose–response effect. An increased number of BN rats displayed elevated total serum IgE levels after 5 daily 1 h exposures to 5 mg/m^3 compared with groups that had received 1 or 3 daily 1 h exposures (Warbrick et al., 2002). Exposure of BN rats to either 25 or 120 mg/m^3 TMA 3 h/day for 5 days revealed a concentration-related increase in the respiratory response, and in lung-associated lymph node weights following challenge at a concentration of 23 mg/m^3 TMA (Pauluhn et al., 2002).

Dose–response relationships in cytokine levels (IL-4, IL-5, IL-10, IL-13) were also obtained using TMA. However, this was only observed when the TMA concentration was varied during the ear exposures. Variation in concentration during flank application did not result in dose–response relationships; variation in concentration during both exposures resulted in an initial increase at lower doses followed by a subsequent decrease at higher doses (Plitnick et al., 2002).

Topical exposure of mice to TDI, MDI, HDI, IPDI and TMA on days 0 and 7 caused a significant dose-related increase in serum IgE-concentration measured 14 days after the initiation of exposure (Hilton et al., 1995; Potter and Wederbrand, 1995). Also in the study of Potter and Wederbrand (1995), this way of application was compared with that of multiple applications of various concentrations of TDI, i.e. 15-times over a 3-week period, or 30-times over a 6-week period. Total serum IgE antibody levels were determined 7 days following final administration. The TDI threshold concentration required for IgE antibody induction increased with the number of TDI applications. In contrast, multiple applications with high TDI concentrations, increased total IgE antibody production when TDI was administered in 15 or 30 doses rather than in 2 doses (Potter and Wederbrand, 1995). It is unclear whether this apparent stimulation of IgE antibody production was due to more effective immunogen presentation when administered in multiple doses or if there was some down regulation of IgE antibody production when TDI was administered in two relatively high doses (Potter and Wederbrand, 1995). In BN rats, topical application of TDI in the range of 1–6%, followed by a range of 0.5–3% upon the second topical application (total amounts applied 3.75, 11.25, 18.75, and 22.5 mg, respectively) was not sufficient to increase mean total serum IgE levels. However, a same total amount of 22.5 mg TDI given in more applications did result in increased total IgE levels (unpublished results).

Blaikie et al. (1995) investigated dose–response relationships of TMA, PHA, and MDI in a single intradermal injection model in the guinea pig with subsequent inhalation challenge at a fixed concentration as an endpoint. Guinea pigs were sensitised on day 1; sensitisation was assessed on day 19 by serological analysis of serum antigen-specific antibodies. There was a sensitisation dose-related increase in specific antibody levels for all three compounds but no clear dose–response relationship between sensitising dose and pulmonary reactions upon inhalation challenge.

In another study, guinea pigs were exposed to various TDI concentrations (0.12–10 ppm) by inhalation for 3 h a day on 5 consecutive days. TDI-specific antibodies were measured from day 22 onwards. There was a sensitisation dose-related increase in the number of responding animals (Karol, 1983). Not only exposure concentration but also the duration of exposure seemed to be important for establishment of antibody response, rather than total exposure. No production of TDI-specific antibody was noted after 70 days of exposure (6 h/day, 5 days/week) to a low TDI concentration of 0.02 ppm (total exposure of 8.67 ppm). In contrast, exposure to 0.25 ppm for 3 h/day for 5 days (total exposure of 3.75 ppm) resulted in the production of antibodies (Karol, 1980, 1983). Pulmonary reactions upon challenge with 1% TDI-guinea pig serum albumin aerosol, measured as an increase in respiratory rate, were not detected in animals sensitised to 0.12 ppm TDI but were present in guinea pigs exposed to TDI concentrations of 0.36 ppm and higher (Karol, 1983).

5. Impact of intensity of exposure during challenge

In a few animal studies, functional pulmonary reactions were found in sensitised animals following inhalation challenge but these reactions were not clearly challenge concentration-dependently related. In a study by Botham et al. (1989), guinea pigs were sensitised by a single intradermal injection at a fixed dose of TMA on day 1. On day 19, sera from all TMA-injected guinea pigs contained TMA-specific antibodies. Animals were challenged with TMA-GPSA conjugate or free TMA for 15 min on day 22. The TMA-protein conjugate elicited reactions in less than half of the sensitised animals at the concentrations tested, and no concentration–response relationship was observed between the challenge concentration and the number of animals responding or the severity of pulmonary responses. In contrast, for free TMA the number of responding animals seemed to be concentration-dependently related to the challenge concentration but even at the high concentration of free TMA, pulmonary reactions were not observed in all sensitised guinea pigs. In a similar study, TMA, MDI, PHA, TDI, but not DNCB, induced high serum levels of specific antibodies following sensitisation. Upon inhalation challenge, respiratory reactions were observed, but there was no relationship between the inhalation challenge concentration and pulmonary responsiveness for TMA, MDI and PHA. For TDI, there were no pulmonary reactions detectable at inhalation challenge. As expected, no specific pulmonary reactions were observed for the skin allergen DNCB (Blaikie et al., 1995).

A concentration-dependence of functional respiratory reactions was absent also in our study using topically TMA-sensitised BN rats. A significant decrease in respiratory rate during TMA challenge, followed by an increase in breathing rate with a reduced tidal volume 24 h after challenge, were observed at all levels tested. In contrast, a challenge concentration-dependent histopathological response to TMA challenge was observed in the larynx and lungs, i.e. the severity of the granulomatous inflammation was concentration-dependent whereas pulmonary haemorrhages were seen at the highest challenge concentration only (Arts et al., 1998). Such a concentration-dependency has also been found in TMA-exposed humans (Zeiss and Patterson, 1993; Bernstein et al., 1997). However, using lower challenge concentrations, a linear log concentration–response relationship was obtained and a concentration was found below which functional respiratory reactions did not occur (Fig. 1). Also, non-specific hyperreactivity, histopathological changes, and increased lung weights were absent in sensitised animals at this concen-

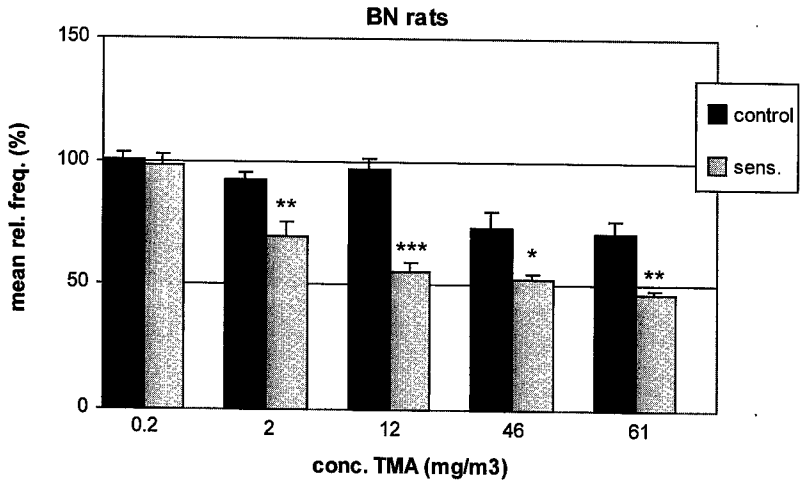

Fig. 1. Mean relative changes in respiratory frequency during inhalation exposure to different concentrations of TMA. Groups of 6 BN rats received 50% TMA (w/v) in a 4:1 (v/v) mixture of acetone and olive oil (AOO) on the shaved flanks on day 0 and 25% TMA (w/v) in AOO on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Three weeks after the first sensitisation animals were challenged by inhalation exposure to one of various concentrations of TMA for 7 min. Breathing frequency was measured each min for 20 s, starting prior to the actual challenge (means of 10 pre-challenge values). During challenge respiration was monitored continuously (means of 14 vales). Data have been expressed as means ± SEM for a 30-min pre-challenge and a 7-min challenge period. Statistics: ANOVA/t-test on differences in exposure/pre-exposure values between control and test group at each concentration; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

tration. These results indicate that challenges should be performed at a certain minimum exposure level to allow investigation of the reactions described above.

6. Airway irritation

As to the respiratory tract, irritants can influence the occurrence, severity, duration and type of allergic reactions in man (Venables and Chan-Yeung, 1997) or can cause asthma-like reactions and inflammation of the airways in the absence of prior exposure (Chan-Yeung and Malo, 1995). In addition, it has been observed in animals that changes in breathing parameters may also be caused by the irritant properties of the allergen, making distinction of irritant-induced alterations from specific allergen-induced alterations not easy (Karol, 1991; Briatico-Vangosa et al., 1994; Pauluhn et al., 1999). We, therefore, examined whether irritant and allergic effects of TMA on the airways could be discriminated by comparing TMA-sensitised with non-sensitised BN rats. TMA aerosols induced reversible alterations of respiratory cycle timing, typical of pulmonary irritation, in naive (non-sensitised) BN rats, resulting in changes in both breathing pattern and frequency (Arts et al., 2001). In TMA-sensitised rats, in contrast, a breathing pattern typical of sensitisation (irregularly lengthened pauses between a varying number of breaths) could be distinguished from the irritant pattern (Fig. 2). Several studies have reported that challenge concentrations exceeding the irritant threshold concentration are required to elicit functional allergic airway responses (Tao et al., 1991; Obata et al., 1992; Pauluhn and Mohr, 1994). However, in the case of TMA, challenge concentrations below the irritant threshold appeared capable to cause an early asthmatic reaction (Fig. 1).

As to the role of irritation and irritant-induced inflammation, we observed that, by examination of breathing pattern and histopathological changes, that exposure to 300 ppm SO_2, 2.5 h/day, for 12 days in total between sensitisation and challenge, offered some protection to TMA challenge in TMA-sensitised BN rats. The deviations from controls in breathing frequency during and 24 h after challenge were smaller in the TMA-

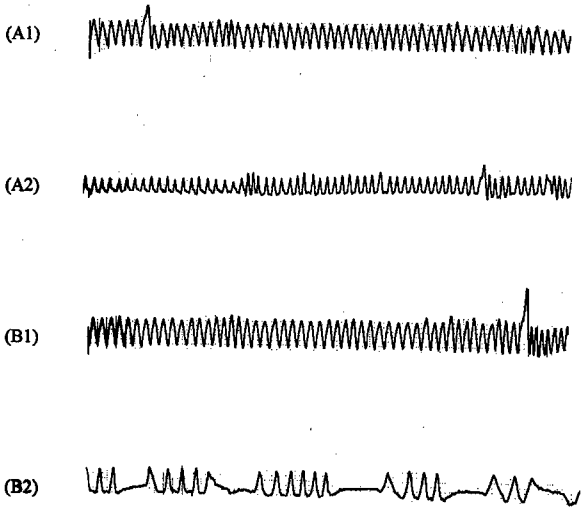

Fig. 2. Representative breathing patterns of BN rats before and during exposure to TMA. (A1) non-sensitised rat, pre-exposure; (A2) non-sensitised rat, during challenge; (B1) sensitised rat, pre-exposure; (B2) sensitised rat, during challenge.

Table 1
Summary of histopathological changes in the larynx of BN rats exposed to SO_2 alone or in combination with TMA

Sensitisation	−	+	−	+
SO_2 exposure	−	−	+	+
Number of animals examined	6	5	6	6
Epithelial ulceration				
Very slight				1
Slight	2			2
Severe	1	5		
Mixed inflammatory cell infiltration				
Slight	1			2
Moderate		2		
Squamous metaplasia and hyperplasia				
Slight			5	
Severe				6

Groups of 6 BN rats each received 50% TMA (w/v) on the shaved flanks on day 0 and 25% TMA (w/v) on the dorsum of both ears seven days later. Controls received vehicle (AOO) only. Between days 8 and 29 animals were exposed to 300 ppm SO_2 2.5 h/day for 12 days in total. Controls received clean air. On day 30 all animals were challenged by inhalation to 30–40 mg/m^3 TMA. Animals were sacrificed and airways were removed for examination 24 h after challenge.

sensitised and -challenged rats that also received SO_2 exposure when compared to similarly treated animals that did not receive SO_2. Also, there was an increase in severity of the hyperplasia of the metaplastic squamous epithelium and a decrease in ulceration and inflammation in the larynx in several animals sensitised to TMA and pre-exposed to SO_2. Replacement of sensitive epithelium by less sensitive epithelium due to the 12-day exposure to SO_2 offered an explanation for the protection. However, in a few animals the protection appeared incomplete, resulting in increased irritation effects by the allergen itself (Table 1).

7. Discussion and conclusions

Predictive tests for respiratory allergy should preferably provide information on potential and potency. Current methods, however, are usually designed to assess whether a substance has the potential to cause sensitisation (hazard identification) but not to determine concentration–response relationships and threshold levels of a particular allergen (hazard characterisation). E.g. several test methods for respiratory allergy use the skin as the induction route, or chemical-protein conjugates are used during inhalation challenge. The determination of dose–response profiles and no-effect-levels, and consequently, extrapolation from high test dose versus low dose in occupational situations is therefore hampered. In the context of hazard identification, it is supposed that the measurement of total serum IgE in high IgE-responding rats or mice provides an appropriate means to assess respiratory sensitisation potential of LMW chemicals. Testing of several concentrations in combination with multiple applications may give additional information on sensitisation potency (hazard characterisation). A subsequent inhalation challenge of the sensitised animals may provide the opportunity to identify concentrations below which adverse effects are unlikely to occur. In many instances, the exposure concentration to set off asthmatic symptoms is lower than the concentration necessary to sensitise a worker and much lower than the concentration necessary to provoke an irritation reaction (Sandler, 2000). Therefore, it may be particularly important to

consider threshold levels for elicitation when e.g. health-based occupational exposure limits are recommended.

At present, extensive attempts to correlate animal and human dose–response studies are lacking. The available data indicate that a variety of factors, both genetically and environmentally determined, may influence the development of allergy. Consequently, test results will largely depend on the specific animal strain used and there is hardly any evidence that relative potency in guinea pigs, rats or mice is similar to that in humans. Standardized and validated methods are thus needed to predict the sensitisation potency and dose–response relationships of chemical respiratory allergens (Karol et al., 1993). Although toxicologists are still miles away from recommending health-based occupational exposure levels for respiratory allergens based on animal studies, the presence of dose–response relationships and no-observed-adverse-effect levels in sensitised and challenged animals suggests that assessment of safe exposure levels is feasible. Further, the correct distinction between respiratory allergens and non-sensitising airway irritants may facilitate effective risk assessment and management. That is: 'true respiratory allergens', which may be life-threatening already at very low concentrations, could be distinguished from the large bulk of airway irritants whose effects are usually reversible following short time exposure. The role of irritation and irritant-induced inflammation in respiratory allergy needs to be studied further.

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Review

Inhalation toxicity of mineral particles: critical appraisal of endpoints and study design

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Abstract

Many of the mineral particles that are of concern in regard to lung toxicity are poorly soluble particles (PSPs). They include biopersistent mineral fibers and dusts containing crystalline silica. The preparation of well-defined test particles of respirable size range and their characterization are an essential step that may require more time and effort than the toxicity study itself. For toxicity studies with mineral particles, an investigation of the toxicokinetics is recommended. Such an investigation will yield information that will help to interpret the results if dust overload conditions occur. For mineral particles such as crystalline silica and mineral fibers, an important endpoint is their potential carcinogenicity. The following parameters are important for the design of chronic toxicity studies, and for the prediction of severe chronic effects: lung retention of inhaled materials for assessing the accumulation of particles, persistent inflammation in lungs, persistent proliferation of epithelial lung cells, progressive fibrogenicity, and genotoxicity in the lung cells. These endpoints should indicate whether the materials investigated are of concern in the health effects on exposed humans, and in the effects of the mineral particles for which chronic studies may be required. In addition, this paper focuses on the effects of PSPs combined with fibers, and on the strategies for investigating the potential carcinogenicity of quartz-containing dusts.
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Keywords: Poorly soluble particles; Mineral fibers; Crystalline silica; Carcinogenicity; Combined effects

1. Introduction

This paper addresses specific problems that may arise when conducting inhalation toxicity studies on mineral particles. Mineral particles that are of occupational relevance include mineral fibers, quartz and quartz-containing dusts, and metal oxides such as TiO_2. In Germany, registered diseases caused by occupational exposures to mineral dusts are rated high. These diseases include tumors after asbestos exposure and lung fibrosis after quartz exposure.

The toxicity patterns of mineral particles differ in many respects from those of gases. One important aspect is that poorly soluble particles (PSPs) do not act in a molecular form but act as a

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whole particle. Specific aspects of these particles include:

- aerodynamic diameter, which determines the deposition probability in the respiratory tract
- varying size distributions
- biosolubility, which governs the potential of an accumulation of particles in lungs after long-term exposure
- specific effects of dust overloading in lungs
- shape factors (e.g. fibers)
- surface properties (e.g. quartz particles)
- specific surface (ultrafine particles)
- intrinsic toxicity induced by surface properties

A specific characteristic of many mineral particles is the low solubility of the dust components involving potential accumulation in lungs. In some cases, it is difficult to define the physical and chemical parameters that are important for the characterization of test materials, e.g. of quartz. Among these parameters there are those which may be indicative of chronic toxicity or carcinogenicity (Fubini et al., 1995).

Several aspects of particle toxicity will be discussed in the present paper, e.g. the question of which assays beside biosolubility should be used to characterize potential health risks caused by the particles.

2. Specific endpoints

2.1. Lung retention of mineral particles

Lung retention of particles can be measured by various methods: (1) Analysis of inhaled mineral particles obtained after ashing of the lungs of experimental animals. The analysis of the particles can be made chemically or by scanning electron microscopy. The latter method is usually used for the analysis of mineral fibers, because, in addition to the number of fibers, the fiber length and fiber diameter are important for the toxic effects. (2) Measurement of the macrophage-mediated clearance by radiolabeled, low-solubility particles after extremely low-level short-time exposure. This provides a measure for the integrity of the macrophage clearance mechanism (Bellmann et al., 1991, 2001).

Measurements of the lung retention of inhaled mineral particles are essential for the interpretation of the results. After exposure of rats to various PSPs at high dose levels, a progressive prolongation of pulmonary dust retention was observed (Bolton et al., 1983; Muhle et al., 1988; Bellmann et al., 1991). This phenomenon is called 'dust overloading of lungs' and is defined by an overwhelming of the normal lung clearance processes (Morrow, 1988; Morrow et al., 1991). The reason for this effect is related to the design of the toxicity studies in which relatively high concentrations are commonly used, compared to those of occupationally exposed humans. In various workshops, the aspect of toxicity of particles with low solubility has been discussed (Miller, 2000; Oberdörster, 2002).

Lung retention is very important for the evaluation of numerous studies, because PSPs have shown to induce lung tumors only at high retention values. These dust-overload tumors are thought to be an artefact when experimental animals are exposed at very high concentrations (ILSI Risk Science Institute Workshop, 2000). However, the scientific evidence that there is no relevance to highly exposed humans, e.g. in coal miners or dust exposure in quarries, is not totally clear (Mauderly, 1994). It might be that lung tumors thought to be caused by exposure to crystalline silica may be enhanced by an exposure to other PSPs (Pott and Roller, 2000).

2.2. Inflammation

According to Driscoll et al. (1995, 1996) a persistent inflammatory reaction and a sustained influx of neutrophils into the lung, releasing reactive oxygen species, can induce mutagenic effects in epithelial lung cells. This hypothesis has initiated many further studies to investigate whether the carcinogenic effects of PSPs can be attributed to inflammation. Biological endpoints include mutations in the HPRT gene of isolated alveolar cells in ex vivo assays, changes in markers of cellular injury and inflammation in bronchoalveolar lavage fluid, expression of mRNA for

chemokines, and detection of oxidative DNA damage (Johnston et al., 2000).

2.3. Carcinogenicity

The question of carcinogenicity induced by PSPs at high concentrations has been discussed in various other papers (Mauderly, 1997; Mossmann, 2000; Nikula, 2000; Donaldson and Lang Tran, 2002; Driscoll et al., 2002; Schins, 2002). An open question remains, however, whether rats show a greater sensitivity to PSPs, compared to humans. A possible explanation is that compared to other species in the rat lung there is a more proinflammatory environment, which leads to oxidative stress and subsequent epithelial cell response (ILSI Risk Science Institute Workshop, 2000).

3. Strategies for toxicity testing of poorly soluble particles and fibers

Particles considered in this paper are solid carcinogens such as asbestos and crystalline silica. The question arises how materials such as man-made vitreous fibers and occupationally generated dust, which contain a fraction of crystalline silica, should be tested with regard to their toxicity?

3.1. Specific aspects of fibers

Numerous chronic inhalation studies have been performed on the potential carcinogenicity of man-made vitreous fibers (Davis et al., 1984; Hesterberg et al., 1993; Mast et al., 1995a,b; McConnell et al., 1994, 1995). However, these studies focused on carcinogenicity only. Other endpoints that would give information on the mode of action of the fibers, such as pulmonary inflammation and proliferation of alveolar epithelium, were not analyzed. Therefore, the Environmental Protection Agency of the United States of America (Vu et al., 1996) postulated to conduct a subchronic toxicity study to investigate these parameters and to find the doses relevant for subsequent carcinogenicity studies. Furthermore, in the European Union biopersistence has been introduced into the protocol 'Subchronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats' (European Commission, 1999).

Greim et al. (2000) suggested the following principles for a testing strategy to determine the occupational limit values for fibers: Identification of a non-observed effect level in experimental animals will be made at the steady-state of fiber uptake and elimination. The evaluation should be based on early inflammatory responses associated with changes in bronchoalveolar lavage, cell proliferation in lung tissue, and histopathology. Further, a basic understanding of the underlying genotoxic mechanisms will be required to differentiate between primary and secondary mechanisms and their dose dependence. However, it remains to be demonstrated whether the results obtained by this approach lead to a sensitive testing strategy sufficient for identification of safe limit values. The applicability of the concept could be validated by comparing the chronic inhalation experiments in rats with amphibol asbestos, or with epidemiological studies. If these results lead to comparable data, the fibers with unknown carcinogenic potential could be classified.

3.2. Combination effects of fibers and granular particles

The question on combination of exposure to mineral particles is important because human exposures are usually combined exposures to mixtures of fibers and particulate material. In animal experiments, the aim is generally to use one preparation of fibers only, i.e., without particles. Various papers report that mixed fibrous and non-fibrous dust exposures lead to an aggravation of effects (Davis et al., 1991; Bellmann et al., 2001). Davis investigated the effects of the exposure to both amosite and chrysotile and compared these effects with similar fiber doses, to which particles of either quartz or titanium dioxide had been added. Both fiber-particulate exposures caused more fibrosis and pulmonary tumors than the two asbestos samples alone. With quartz, which was shown to be both fibrogenic and carcinogenic to rats (Dagle et al., 1986; Holland et

al., 1986; Muhle et al., 1989, 1995; Spiethoff et al., 1992), the effect may have been additive. The increased pathogenicity of asbestos–TiO_2 mixtures is most likely due to a factor such as the modification of the clearance rate of the asbestos fibers (Davis, 1996).

Similar interpretations are possible in studies with refractory ceramic fibers. In one of the studies, type RCF1, a mixture of fibers and particles, was used (Mast et al., 1995a,b; Brown et al., 2000). In later studies, differences between a relatively pure fiber preparation (RCF1a) and a mixture of fibers and particles (RCF1) were investigated (Brown et al., 2000; Bellmann et al., 2001). The objective of the latter studies was to compare lung retention and biological effects of the two RCF samples. RCF1 contained 25% (wt.%) and RCF1a contained only 2% granular dust. Female Wistar rats were exposed 6 h/day for 3 weeks to either RCF1a or RCF1 fiber aerosol of approximately 125 fibers (>20 μm long)/ml. The results showed that the particle fraction of the RCF1 sample enhanced significantly the adverse effects seen in this 3-week study (Bellmann et al., 2001). Alveolar clearance of tracer particles was retarded after RCF1 exposure (1200 days clearance half time) compared to the exposure to RCF1a (80 days clearance half time).

3.3. *Specific aspects of exposure to quartz dust*

Various national and international organizations, e.g. the International Agency for Research of Cancer (IARC), have classified crystalline silica dust, inhaled from occupational sources as a human carcinogen. There are various caveats in this classification. IARC stated that 'carcinogenicity was not detected in all industrial circumstances studied. Carcinogenicity may be dependent on inherent characteristics of the crystalline silica or on external factors affecting its biological activity or distribution of its polymorphs'. Further, it is being discussed whether there is a threshold dose below which crystalline silica is not necessarily a carcinogen.

Four chronic inhalation studies have shown the induction of lung cancer in rats (Dagle et al., 1986; Holland et al., 1986; Muhle et al., 1989, 1995; Spiethoff et al., 1992).

Mechanistic animal studies with low soluble particles have shown that the sequence of effects in lungs after dust exposure is inflammation, fibrosis, and then cancer (ILSI Risk Science Institute Workshop, 2000). Today, it cannot be decided whether quartz is a primary carcinogen or whether it acts indirectly, e.g., by indirect pathways like reactive oxygen species.

For an investigation of dusts containing quartz, specific strategies have to be applied: (1) Identification of active and less active quartz subspecies on the basis of available toxicity data, and preparation of a sufficient amount of test materials for toxicity investigations. This may amount up to a few kilograms of test material preparation for one chronic inhalation study. (2) Characterization of the samples in regard to their physical and chemical properties. It is yet not clear which surface characteristics are responsible for the toxicity (Fubini et al., 1995).

For many of the quartz-containing materials to be assessed, information on in vitro tests is available. It is difficult to evaluate how important these results are in regard to the evaluation of carcinogenicity. Before performing chronic inhalation studies with all these materials, it is recommended to begin by performing subchronic tests as a basis for further testing (see above). On the basis of these tests, the predictive value for ranking fibrogenicity and carcinogenicity has to be evaluated.

The role of inflammatory reactions in the carcinogenicity of quartz has been investigated by Johnston et al. (2000). In a subchronic inhalation study, rats were exposed to amorphous and crystalline silica. The mutagenic response was investigated, and it was found that genotoxic effects in alveolar epithelial cells only appeared after exposure to crystalline but not to amorphous silica, despite a high degree of inflammatory cell response after subchronic exposure to both types of silica. The authors concluded that, in addition to an inflammatory response, particle biopersistence, solubility, and direct or indirect epithelial cell cytotoxicity might be key factors in the

induction of either mutagenic events or target cell death.

4. Conclusion

The role of persistent inflammation and reactive oxygen species in the carcinogenicity of solid particles needs further investigation. Antioxidant defense mechanisms of the respiratory tract could influence significantly the responses to particles. For species-to-species extrapolation, information of the antioxidant system may be important. Future study designs should also include relevant endpoints.

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Issues of dosimetry in inhalation toxicity

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Abstract

A major issue of inhalation toxicity is that of dose. An inhaled dose is more difficult to determine than the dose from other routes of administration. Via oral or parenteral routes, a discrete amount of test substance is given in a bolus. In inhalation toxicology, the delivered dose depends on the exposure concentration and duration, particle size, and associated changes in breathing patterns. Over the past few decades, the concept of dose as applied to toxicological studies has changed considerably. Initially, 'dose' simply meant the concentration in the atmosphere in inhalation studies (or the amount ingested or instilled into the gastrointestinal tract in oral dosing studies) times the duration of study. The extrapolation from one route to another is subject to tremendous errors, and caution is advised when doing so. Default values are, therefore, not recommended and conversion factors must be calculated for each individual situation, making appropriate assumptions about body weight, minute volume, percentage deposition, retention and absorption, also taking into account pulmonary and extrapulmonary pathomechanisms as well as the exposure regimen used in the bioassay and that of actual interest. Also with systemically acting agents caution is advised to perform simple route-to-route extrapolations in the absence of a detailed pharmacokinetic understanding and knowledge of the critical toxophoric moieties.
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Keywords: Route-to-route extrapolation; Rodent inhalation studies; Non-rodent inhalation studies; Portal-of-entry effects

1. Introduction

Throughout the world regulatory agencies have focused their emphasis on promoting workplace safety. To achieve this objective, inhalation studies are a basic prerequisite. Despite of this, many of the required toxicology studies mirror requirements that address continuous dosing through the oral route. Thus, many of the toxicology studies available are not designed with worker, applicator or end-user risk assessment in mind. For instance, worker exposure to agrochemical pesticides tends to be intermittent in nature, and mainly via the dermal route. However, specific use patterns are associated with exposure via the inhalation route (Krieger and Ross, 1993; Ross et al., 2001). With respect to pesticides used in residential areas in order to control and extinguish pests, non-occupational low-level, long-term exposure via the inhalation route may be of concern. Because of the regulatory focus on dietary expo-

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sure, risk assessment for occupational settings is often driven by toxicological data generated in laboratory animals with a disparate route, frequency, duration and magnitude of exposure. Yet, almost all of the required toxicity studies utilize the oral exposure route for dosing. By default, the concept of aggregating fractional exposure doses into one total body burden is given preference to assessing route-specific no-observed-adverse-effect levels (NOAELs) or for inhalation studies no-observed-adverse-effect concentrations (NOAECs). Clearly, the differences in routes, associated with intermittent exposure regimens, result in dissimilar toxicokinetics and substance-specific toxicodynamics, raising questions about the accuracy of the commonly applied procedures. In spite of the great deal of uncertainty involved in this process, these data are used in assessing the critical body burden without taking into account the exposure metric required to elicit a given toxicological effect. Examples of exposure metric considerations include not only focusing on estimates of route-specific aggregate exposures of the active agent during the time period of interest (a NOAEL may be based on concentration-dependent portal-of-entry, local responses or total body burden related systemic effects) they have also to address potential effects elicited by the inert ingredients commonly present in end-user products. When tested in the laboratory the dose metrics may change from high-concentration exposure scenarios, where the test agent is present as an aerosol, to low-level exposure environments, where exposure is commonly to the vapor phase. In addition to exposure to the active agent per se, workers exposed to these substances may inhale carrier substances, emulsifiers and stabilizing agents, solvents or propellants. Opposite to studies utilizing non-inhalation routes of exposure, following mechanical aerosolization (dispersion) of pesticide formulations the relative composition of the formulation may change dramatically when airborne because of difference in transfer efficiencies of volatile and non-volatile constituents. This paper will consider some of the essential features of portal-of-entry related damage and the potential pitfalls involved in dosimetry and route-to-route extrapolations.

2. Principle structures of the respiratory tract

When an organ system is as complex as the respiratory tract, it is convenient to simplify it by forming conceptual anatomic units or 'compartments'. Conventionally one may think in terms of three major compartments, which divide the respiratory tract into regions based upon anatomical features, upon particle deposition and clearance phenomena that occur within the tract and are specific to each compartment. The regions are called the nasopharyngeal (NP), the tracheobronchial (TB), and the pulmonary (P). The NP compartment begins at the anterior nares and includes the respiratory airway down to the level of the larynx. The deposition of inhaled substances does not occur uniformly along all airways. Specific patterns of enhanced local deposition within the respiratory tract are important in determining dosage, since the latter depends on the surface density of deposition. Nonuniformity implies that the initial dose delivered to specific sites may be greater than that occurring if a uniform density of surface deposit is assumed. This is especially important for inhaled particles that affect the tissue on direct contact, such as irritants. For large particles (> 5 μm aerodynamic equivalent diameter in humans), the predominant deposition mechanism in the extrathoracic region (head airways region) is inertial impaction. The mechanisms affecting the transport and deposition of gases involve convection, diffusion, absorption, dissolution, and chemical reactions. In this region, deposited and relatively soluble material is rapidly cleared into the blood, while for poorly soluble agents physical clearance by mucociliary transport to the throat for subsequent swallowing is predominant. The effective removal of insoluble particles may require 1–2 days. The nasal passages of small laboratory rodents are highly tortuous and are lined with four distinct nasal epithelial populations in most animal species. This includes squamous, transitional, and pseudostratified respiratory epithelium in the anterior aspect of the main nasal chamber; and olfactory epithelium, which is metabolically the most active epithelium in this region, located in the dorsal aspect of the nasal cavity.

After chemical exposure, reflex mechanisms may be invoked in order to protect an individual from inhaling excessive concentrations of the respective chemical. When inhaled through the nose, chemicals capable of stimulating the trigeminal nerve receptor will evoke a burning sensation of the nasal passages. Currently, a clear distinction cannot be made between 'nuisance' and mere perception or awareness of exposure and related somatic health effects such as headache. The onset of the response, that is, the decrease in breathing frequency in small laboratory rodents, is usually observed within a few minutes and is characterized by a stereotypic bradypneic pause during the expiratory phase of respiration (Alarie, 1966, 1973, 1981). As illustrated in Fig. 1, in bioassays using rodents a sustained decrease in ventilation may reduce the inhaled dose appreciably. Chemosensory effects of stimulation can either be irritative or odorous. Stimulation of the olfactory nerve and the trigeminal nerve results in sensation of smell, whereas stimulation of the trigeminal nerve gives rise to chemical irritation or intranasal chemesthesis, which is the activation of the trigeminal, glossopharyngeal, or vagal nerves via chemical stimulation. Historically, there have been attempts to segregate chemicals into categories of pure olfactory and pure trigeminal stimulants, although it is now conceded there are very few chemicals that fall into either category exclusively. Because most agents, at sufficient concentrations, elicit both olfactory and trigeminal activation, it is important to understand the normative function and interactions of these two systems, as well as the clinical and experimental methods used to assess and quantify odor and sensory irritation that can result from exposure to airborne chemicals or pesticides (Kendal-Reed, 2001; Feron et al., 2001; Meldrum, 2001). Animal models utilizing this endpoint were established to determine to relative potency of pyrethroid aerosols to elicit paresthesias (Pauluhn, 1998; Pauluhn and Machemer, 1998).

In experimental animals it has been shown that metal ions commonly impermeable to the blood–brain barrier may pass the brain via olfactory receptor neurons from the nasal lumen through the cribriform plate to the olfactory bulb. Some metal ions (e.g. Mn, Zn) known to occur in organometallic fungicides or airborne particulates in ambient air can cross synapses in the olfactory bulb and migrate via secondary olfactory neurons to distant nuclei of the brain. After inhalation uptake of a metal-containing solutions, transport of the metal via olfactory axons can occur rapidly, within hours or a few days (e.g. Mn), or slowly over days or weeks (e.g. Ni). The olfactory bulb tends to accumulate certain metals with greater avidity than other regions of the brain. The molecular mechanisms responsible for metal translocation in olfactory neurons and deposition in the olfactory bulb are unclear, but complexation by metal-binding molecules may be involved (Sunderman, 2001).

The TB region begins at the larynx and includes the trachea and the ciliated bronchial airways down to and including the terminal bronchioles. A relatively small fraction of all sizes of particles, which pass through the NP region, will deposit in the TB region. The mechanisms of inertial impaction at bifurcations, sedimentation, and for small particles, Brownian diffusion cause TB deposition. Interception can be an important deposition mechanism for fibrous dusts. During mouth breathing of aerosols the benefits of the collection of larger particles in the nose are lost and these larger particles tend to deposit in the TB region with high efficiency. An important characteristic of the TB region is that this region is both ciliated and equipped with mucus secreting elements so that clearance of deposited poorly soluble particles occurs rapidly by mucociliary action to the throat for swallowing. Again, relatively soluble material may rapidly enter the blood circulation. The rate of mucus movement is slowest in the finer airways and increases toward the trachea. Since particles depositing in the TB tree are probably distributed differently with respect to size, with smaller particles tending to deposit deeper in the lung, one expects larger particles to clear more quickly.

The third compartment, the P or pulmonary region, includes the functional gas exchange sites of the lung. The most prominent structure in this region is the alveolus. Each alveolus in the lung parenchyma opens directly into an alveolar duct or sac. Alveoli and alveolar ducts arising from a

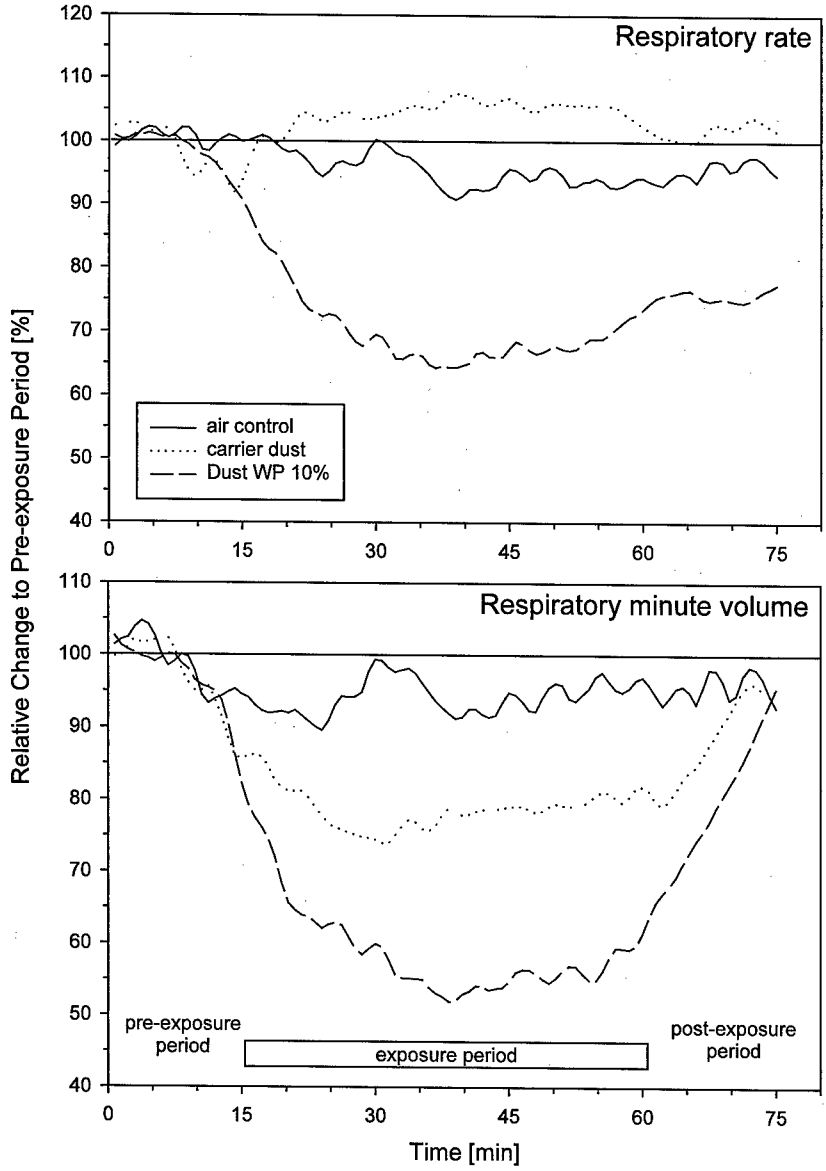

Fig. 1. Recording of respiratory rate and minute volume of rats nose-only exposed in volume displacement plethysmographs. For exposure a directed-flow nose-only inhalation chamber was used. Measurements were made in four rats simultaneously using the following sequence: pre-exposure to air, exposure to either air (control), approximately 70 mg/m^3 of carrier dust or dust containing 10% of a Type II pyrethroid (WP 10%), post-exposure to air. Pre-exposure data are normalized to 100% and effects evoked by the test substance are expressed as relative change to the pre-exposure period.

single conducting airway constitute a pulmonary acinus. A thin tissue barrier-consisting of type I and type II alveolar cells which represent approximately 25% of all the cells in the alveolar septum-provides an extremely efficient means of gas transfer over a large surface area (Fig. 2). Type I cells cover a large surface area of the lung parenchyma (ca. 90% of the alveolar surface). In regard to number, there are more pneumocyte Type II cells than Type I (67:33%). Preferential damage to type I cells by various agents may be explained by the fact that this cell type constitutes

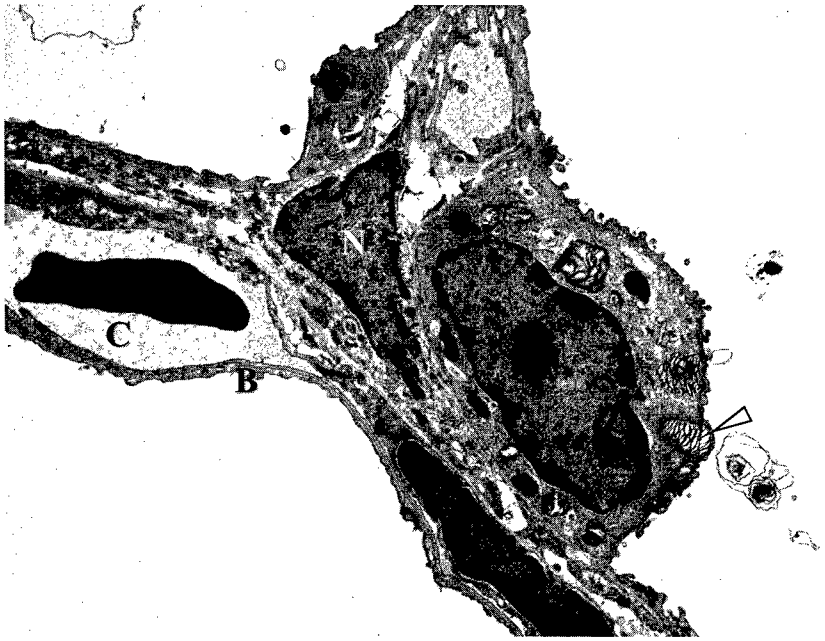

Fig. 2. Ultrastructural photograph of the septal region of a rat lung. The alveolus is separated from the blood capillary (C), which contains an erythrocyte, by a thin margin of the Type I cell. The air–blood barrier constitutes a three barrier system, that is the capillary endothelium, the basal membrane and the Type I pneumocyte (B). Note that the thickening of the interstitial space is confined to the top of the capillary (the 'service side') while the total alveolar-capillary membrane remains thin on the bottom side (the 'active side'). The type II cell can be seen containing lamellar bodies (arrowhead), the storage form of the pulmonary surfactant synthesized in this cell. N, nucleus.

a vulnerable target because of its large surface area in relation to cell mass. Type II cells are cuboidal, show abundant perinuclear cytoplasm, maintain an ion gradient within the lining fluids so that extravasated fluid is efficiently removed from the alveoli, are metabolically active and produce surfactant. In the case of damage to the type I epithelium, they may undergo mitotic division and replace damaged cells. The integrity of the delicate alveolar septa is maintained in large measure by a network of mesenchymal interstitial cell populations that produce collagen and elastin fibers. Surface tension at the air/water interface produces forces that tend to reduce the area of the interface leading eventually to a collapse of alveoli. It also reduces the pressure gradient between the vascular system (high hydrostatic pressure) and alveolus (subatmospheric pressure), thus preventing extravasation of plasma into the alveolus (Nieman, 1985; Bhalla, 1999; Vesterberg et al., 2001). In regard to the inhalation toxicity of respirable aerosols containing surface active substances they may potentially damage the blood–air barrier through a physical mode of action leading to an increased extravasation of plasma into the alveoli that can not occur by non-inhalation routes. When damaged excessively, alveolar flooding, i.e. lung edema occurs.

3. Pitfalls of route-to-route extrapolation

The route, duration and frequency of human exposure to a substance during normal use (and, as appropriate, reasonably foreseeable misuse) need to be taken into account when evaluating the data for hazard identification: hazards which may not be expressed under one exposure scenario but may become apparent under another. When data are lacking for a relevant route of human exposure, the possibility of using route-to-route extrapolation may be considered but, in general, route-to-

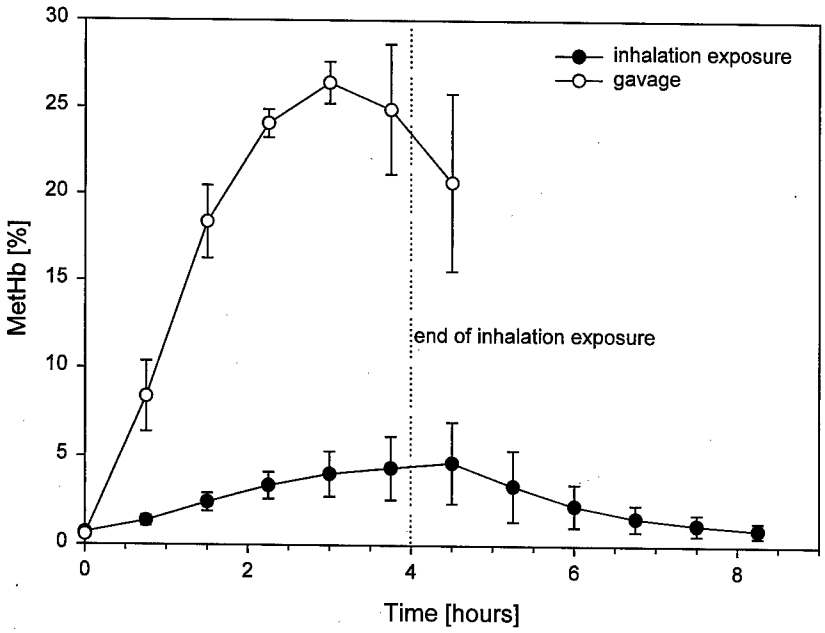

Fig. 3. Time-course of methemoglobin (MetHb) formation in beagle dogs head-only exposed to the volatilized atmosphere of aniline for 4-h. The mean exposure concentration was 174 mg/m^3 air. After complete recovery, the dogs received an equivalent of the calculated dose (15 mg/kg bw) by gavage. Blood was collected repeatedly in 45-min intervals commencing with the onset of exposure. Data represent mean ± S.D. ($n = 4$).

route extrapolation is thought to be a poor substitute for toxicity data obtained using the appropriate route of exposure. However, some pragmatic approaches to calculating an approximate NOAEL (or NOAEC in terms of inhalation toxicology) by extrapolation have been used, when specific data are not readily available, to facilitate decision taking with regard to the potential need to ensure control of exposure, or to obtain further data, for a particular route of exposure. The methods described below are for extrapolating from oral toxicity data since this is the route most often used for repeated dose toxicity studies in animals. A number of publications are available which provide guidance on route-to-route extrapolation (Gerrity and Henry, 1990; Andersen et al., 1992; Pepelko and Withey, 1985).

In the case of systemically acting substances, there are possibilities for extrapolation from one exposure route to another. However, it requires an understanding of the toxicokinetic and toxicodynamic principles involved. Empirical correlations are often used to evaluate the relationship of dose and response with little appreciation of the detailed biological interactions of the test agent. The main obstacles are differences in the degrees and rates of absorption by different exposure routes and differences in biotransformation, particularly in the case of first-pass metabolism occurring in the gastrointestinal tract or liver. For example, when such extrapolations are attempted for aniline this process may be prone to marked errors because aniline belongs to that group of substances known to be activated by a first-pass gastrointenstinal and hepatic metabolism. The activation of aniline and the ensuing methemoglobin (MetHb) formation and reduction has been described to be a multistep process (Kiese, 1974; Akintowa, 2000). Likewise it can be assumed that both the kind of dosing regimen as well as the rate of dose-delivery have major impact on the magnitude of the MetHb levels produced. The time-course of MetHb formation in beagle dogs head-only exposed to a volatilized atmosphere of aniline for 4-h demonstrates that the ostensibly equal oral dosage (15 mg/kg by gavage) elicits five times

higher MetHb levels as compared with inhalation (exposure to an actual breathing zone concentration of 174 mg/m^3, duration of exposure 4 h (Fig. 3). When using the default respiratory minute volume of dogs of 0.36 l/kg-min the total exposure dosage (0.36 l/kg-min × 240 min × 0.174 mg/l = 15 mg/kg) mirrors exactly that administered by gavage (Pauluhn, 2002). Thus, for agents known to be bioactivated by a hepatic first-pass metabolism, the conversion of findings obtained from oral dosing to inhalation exposure concentrations is subject to overestimate dramatically the magnitude of MetHb formation likely to occur following inhalation exposure. As to whether the fivefold lower potency by inhalation is solely related to the hepatic first-pass bioactivation, the rate of delivery or also to a less than 100% retention of the inhaled vapor within the respiratory tract remains to be elucidated.

Moreover, in contrast to studies where the uptake is by the gastrointestinal route, the extent of pH- and passage time-dependent degradation of modification of the test agent may be decisive for the outcome of study. Thus, the formation of new toxicophoretic entities and portal-of-entry specific types of toxicities, including site-specific, often rate-dependent compensatory mechanisms need to be envisaged before attempting such extrapolations. Furthermore, for such a local modes of action the dose to the target tissue at first contact will be determined by the kinetics of formation and elimination or scavenging of the toxicophoretic moiety rather than systemic bioavailability of the parent substance. For instance, the uptake of metal ions via the GI-tract is, to a great extent, homeostatically controlled whereas in the alveolar region metal ions may prompt a series of responses specific to this region. Accordingly, toxic species formed in the gastrointestinal tract may not necessarily be formed following inhalation. Upon inhalation, such primary responses are commonly restricted to the pulmonary region, ranging from the mere influx of alveolar macrophages (removal of poorly-soluble particulates) up to the local induction of scavenger proteins, such as metallothionein (MT), and the anti-protease or antioxidant systems. These examples demonstrate that for substances having any local mode of action at the portal-of-entry, that is within the respiratory or gastrointestinal tract, extrapolations from one route to another will often be misleading with respect to effective dose and probably even the nature of the effect. The calculation of 'systemic total body burden' based on such localized responses occurring at the initial contact may lead to erroneous conclusions.

One of the commonest problems in route-to-route extrapolation relates to the assumption that equal doses via the inhalation and oral routes may be regarded to be equitoxic. This approach is challenged using data from both acute and subchronic studies of pesticides utilizing either route of exposure. To make comparison of the oral and inhalation data possible, inhalation data are converted to inhalation dosages by using a default respiratory minute volume per kg bw of 0.75 l/min kg bw (Mauderly, 1986) {inhalation dose = exposure concentration (mg/m^3) × respiratory minute volume per kg bw × exposure duration}.

Based on data of organophosphates known to inhibit actylcholinesterase a comparison of oral LD_{50} data and calculated inhalation LD_{50} data was based on actually available LC_{50} values (Storm et al., 2000). For equal toxic potency, the ratio of oral LD_{50}/inhalation LD_{50} (calculated based on the inhalation LC_{50}) is equal to 1. The comparison made in Fig. 4 suggests that substantial deviations from expectation occur, in spite of the quite similar toxic principle of the organophosphate pesticides. At the ends of lower and higher toxic potencies (see Fig. 4), the ratios were smaller and higher, respectively, indicating that the active substance is either more toxic by the oral route than by inhalation for very toxic organophosphates or more toxic by inhalation than by oral administration for the less toxic organophosphates. Notionally, the more toxic ones appear to act more rapidly so that maximum cholinergic toxicity is more pronounced using a bolus administration when compared with the longer dosing intervals used in inhalation studies.

The NOAECs from repeated exposure guideline inhalation studies with pesticide aerosols (exposure 6 h/day on 5 days/week for 3 or 4 weeks) are converted to inhalation dosages as described above and compared with the NOAELs from subacute

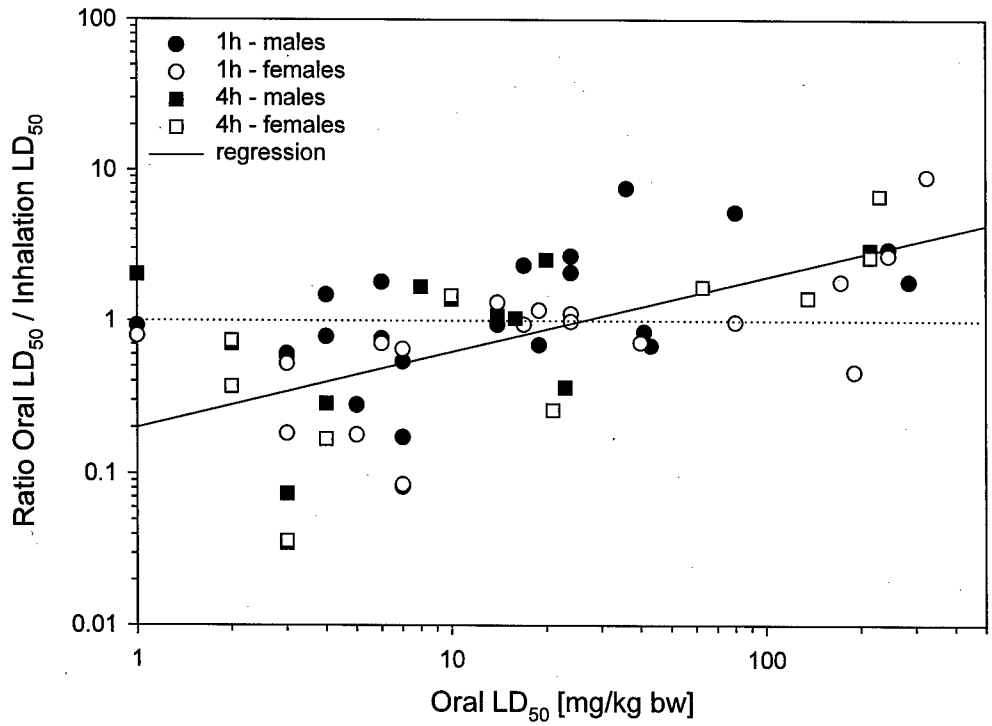

Fig. 4. Comparison of acute lethal toxic potency data of organophosphate pesticides in rats following a single 1 or 4-h inhalation exposure (LC_{50}) or oral administration (LD_{50}) (LD_{50} and LC_{50} data were from Storm et al. (2000). To make comparisons possible, exposure concentrations were converted to inhalation LD_{50} dosages.

or, if not available, subchronic oral studies (4-week feeding or gavage: 71%, $\geq$13-week dietary studies: 29%; $n = 42$) with the same pesticide. As depicted in Fig. 5, the overwhelming fraction of the ratios of NOAELs from oral and inhalation studies with various types of pesticides are in a range of 1–10 and $\geq$10 when the most critical endpoint (lowest (NOAEC) was based on systemic and lung-specific local effects, respectively. In spite the fact that 29% of the NOAELs stemmed from oral studies of appreciably longer duration than the 3 or 4 weeks inhalation studies compared with, in many cases, the prediction of inhalation NOAELs based on oral studies is biased to underestimate conspicuously the toxic potency of inhaled substances. From the data summarized in Fig. 5 one may deduce that the level of uncertainty involved in the process of risk characterization is significantly reduced in the presence of actual inhalation data.

4. Conclusions

The conversion from oral routes to ostensibly equipotent inhalation exposure concentrations is subject to both marked overestimation and underestimation in the absence of the following toxicokinetic data, whether a substance has local irritant properties at the portal-of-entry, whether it is degraded to new toxicophoretic entities within the gastrointestinal tract, whether it is bioactivated or detoxified either within the gastrointestinal tract or in the hepatic first-pass metabolism, and whether the critical mode of action is total-dose or dose-rate dependent. Extrapolations from one dosing regimen, e.g. oral administration of bulk doses to workplace-like exposure regimens, are not reliable without consideration of these aspects. Default values for the conversion from oral to inhalation are contingent upon many mechanistic and substance-related factors, which may invali-

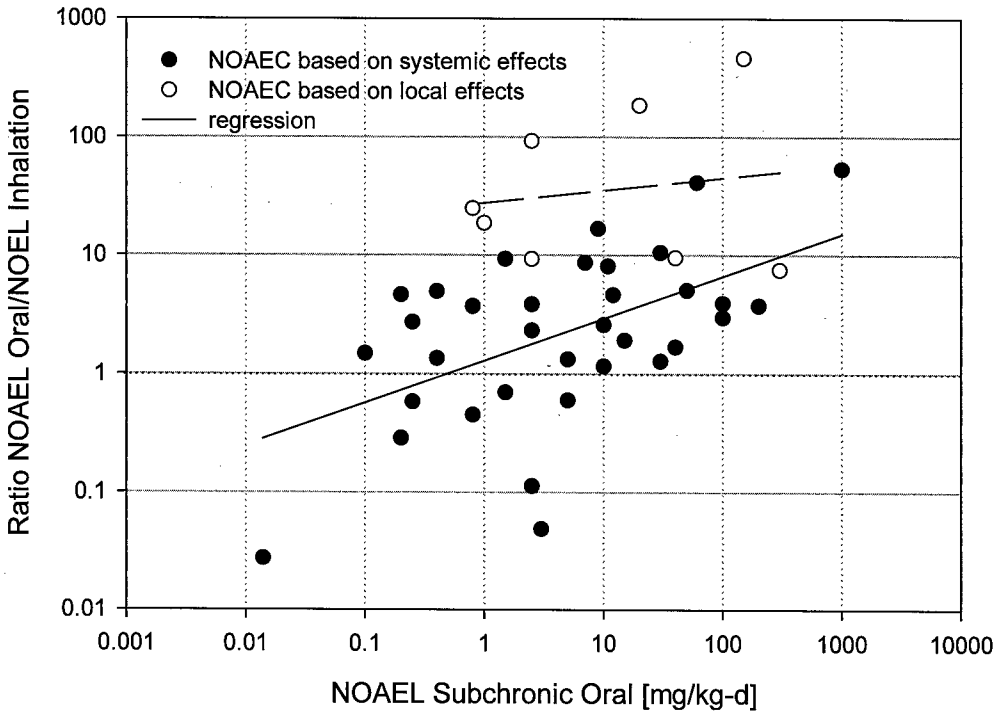

Fig. 5. Comparison of NOAELs of various types of pesticides in rats from subacute inhalation and subacute/subchronic oral (dietary or gavage) studies in rats. To make comparisons possible, NOAECs from inhalation studies were converted to inhalation dosages (NOAELs).

date the use of simple default assumptions. Biologically based modeling, which takes into account specific mechanistic steps governing tissue disposition and toxic action may lead to better predictions. Accordingly, the choice of an appropriate measure of 'dose' in inhalation toxicity studies must be defined by the nature of the pathogenesis process, i.e. defined according to the mechanism of action for the effect under consideration. Acute inhalation studies (LC_{50} per se) appear to be a poor predictor of the NOAEC following repeated inhalation exposure with the exception of agents exhibiting a low chronicity index and being highly toxic, e.g. were the limiting factor of toxicity can be related to a single target or is solely dependent on a local irritant threshold concentration. The evolution of an integrated chemical–biological concept of dose will continue as the sophistication in examining toxicological problems is enhanced by improved mechanistic understanding of a variety of toxic phenomena at the molecular, cellular, animal model and human level.

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Review

Upper airway irritation, odor perception and health risk due to airborne chemicals

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Abstract

Chemosensory irritation associated with the manufacture and use of volatile materials has been a public and employee health concern for many years. Because odor properties can often be detected at much lower concentrations than those capable of eliciting upper respiratory tract irritation, confusion between odor and irritation coupled with variability in odor sensitivity and response can produce significant obstacles for evaluating the potential for adverse effects or annoyance from worker and community exposures. Although rigorous research methods have been developed to accurately quantify chemosensory irritation in human evaluations, several important considerations should be included in the design and interpretation of such studies. Specifically, research studies evaluating chemosensory irritation from volatile materials should be capable of (1) distinguishing between the annoyance or concern elicited by odor sensation and that elicited by true sensory irritation, (2) evaluating exposure-related factors that affect odor or irritancy responses, and (3) separating true adverse health effects from those mediated via psychosocial factors. Objective measures of upper respiratory tract irritation onset obtained in conjunction with subjective reports can lend valuable input to the decision process for determining occupational exposure limits. Subjective reports of irritation at low levels that *cannot* be reconciled with objective measures should prompt a careful investigation into the other factors (e.g. cognitive or emotional) that may be modulating the sensory response. Distinguishing between the exposure that elicits local effects of sensory irritation in the upper respiratory tract and the exposure that elicits self-reports of irritation is a key component in establishing occupational exposure limits that are protective of exposed workers.
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Keywords: Odor perception; Sensory irritation; Airborne chemicals; Perceived health risk

1. Introduction

In the weeks and months following September, 11, 2001, air quality in lower Manhattan was a source of continuing concern among residents and workers, as pervasive odors wafted through the streets and into buildings, often producing symptoms of upper respiratory irritation and leading to strong perceptions of health risk. From the perspective of toxicologists and those charged with monitoring public health, concerns about risks faced by the population living or working

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in lower Manhattan were alleviated by the evidence from continuous monitoring of relatively low concentrations of toxic pollutants. However, even as health professionals and government agencies assured workers and residents that exposures to chemicals and particles were below concentrations that might pose health risks, workers exposed to a complex mixture of acid fumes, dusts, smoke and chemicals were reporting high levels of upper respiratory irritation, while residents and office workers in lower Manhattan were complaining of burning eyes, sore throats and 'World Trade Center' cough. In order to understand the discrepancy between the toxicological data and the symptom reports, it is important to recognize that odorous and irritant airborne chemicals may not be present at toxic levels but can nonetheless elicit physical symptoms (e.g. headaches, burning eyes, throat), stress and concern about the safety of the ambient environment. This paper will provide an overview of issues faced by toxicologists when evaluating the sensory effects of a chemical, namely the perception of upper airway irritancy, odor and health risk, by focusing on the following four areas: (1) What is the relationship between odor and irritation of a volatile chemical? (2) How can the human sensitivity and response to odors and irritants be evaluated? (3) What subject-based factors can influence an individual's perception of odor and irritation from a chemical exposure? and (4) Using these methods, how can experimental data be used to guide the development of occupational exposure limits?

2. How can we distinguish between odor and intranasal irritation?

2.1. Intra-nasal and upper respiratory sensations: odor and irritation

Sensations of odor and upper airway irritation are often experienced as a unitary phenomenon, principally because most airborne chemicals have the potential to activate multiple separate, yet interrelated, sensory pathways in the upper respiratory airways: the olfactory nerve, which gives rise to sensations of odor, and the trigeminal, glossopharyngeal or vagal nerves which give rise to temporary burning, stinging, tingling or painful sensations in the eyes and upper airways (Alarie, 1966). Intra-nasal irritation from airborne chemicals is almost always experienced in combination with odor perception, except in rare circumstances, e.g., following stimulation with carbon dioxide or among individuals who are anosmic (lack a functional sense of smell).

Chemical stimulation of the trigeminal nerve (known as *chemesthesis*) often combines with stimulation of the olfactory nerve to produce sensations that form an overall perception of a chemical. Because sensory irritation of the upper airways is a widely cited health effect in indoor air and occupational environments (US Department of Health and Human Services, 1994), and almost any airborne chemical at a sufficiently high concentration evokes both odor and chemesthetic sensations, there is a great need for reliable techniques capable of determining the concentration at which an airborne chemical elicits chemesthetic sensations and a greater understanding of how to interpret the data generated by those techniques.

2.2. Relationship between odor, irritation and perceived health risk

To illustrate the nature of the relationship between odor, chemesthetic perception and perceived health risk from an airborne chemical, sensory scientists use techniques based on the science of psychophysics. Psychophysics is the study of the relationship between the physical stimulus (i.e. the chemical) and the psychological experience it produces (e.g. the intensity of an odor, the degree to which two odors are different). A common way to illustrate the chemosensory response to an airborne chemical across a concentration range is to plot the psychophysical functions that relate stimulus concentration to an observer's rating of perceived intensity for both the odor and the irritancy of a chemical.

Data obtained from psychophysical scaling studies (in which various chemical concentrations are presented and the volunteer is asked to rate

intensity using a standardized scale) have shown that the exponent of the psychophysical function relating concentration to intensity for most odorants falls well below 1, implying a compression of sensation magnitude over stimulus magnitude. However, there can be considerable variation in the slope of the psychophysical function for different odorants, ranging from as little as 0.10 to more than 1.0. Apart from differences in solubility or deposition in the airways, at least some of this variation arises because, at low concentrations, most chemicals will stimulate only the olfactory system, while at higher concentrations those same chemicals will stimulate olfactory and trigeminal sensations. Data from a study by Green et al. (1996) evaluating two scaling methods for two different chemosensory stimuli are presented to illustrate this distinction. In this study, the psychophysical function for intensity of phenylethyl alcohol (Fig. 1), which does not elicit chemosensory irritation in vapor phase, is quite shallow, with a slope of approximately 0.3 across the lower concentrations and a slope of zero (implying an inability to resolve differences in concentration) across the higher ones. In contrast, intensity data for acetic acid, which is a trigeminal as well as an olfactory stimulant at the higher concentrations, were well described by a single power function with a slope of approximately 0.7. At the lower concentrations, acetic acid produced only odor sensation, but as concentration increased, the addition of trigeminal irritation to the odor sensation resulted in an increase in perceived intensity that was easily discriminable across the concentrations tested.

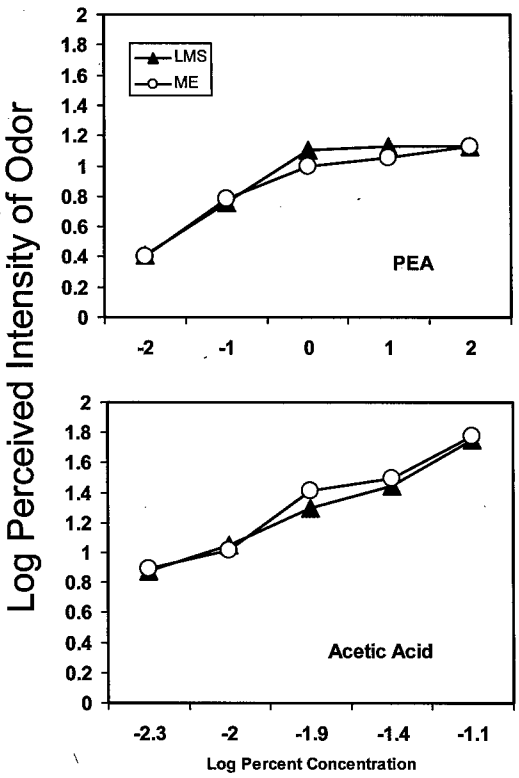

Fig. 1. The psychophysical functions for two odorants: acetic acid, which stimulates both the olfactory and trigeminal system (leading to a combined percept of odor and irritation), and phenyl ethyl alcohol (rose smell) which in vapor phase only stimulates olfactory sensations, from Green et al. (1996).

While the physical range between the acetic acid concentration necessary to stimulate olfaction and the concentration necessary to stimulate trigeminal or other upper respiratory irritant sensations is fairly wide, this may not hold true for all chemicals or classes. For some chemicals possessing a high odor threshold (e.g. methanol) or a low irritation threshold (e.g. formaldehyde) the range between odor detection and irritation onset will be much smaller. However, for the majority of chemicals for which odor and irritation thresholds have been established, odor sensation provides an adequate warning for the onset of irritation (Cometto-Muñiz and Cain, 1995).

With data from a number of studies that have established reliable estimates of odor and irritation thresholds for a variety of chemicals, how can we explain subjective reports of irritation from exposure to those chemicals at relatively low concentrations? At intermediate concentrations, i.e., those that lie above the odor detection threshold but well below the irritant threshold, reports of perceived irritation most likely signify *odor intolerance or annoyance* and are often accompanied by concerns about *perceived toxicity*. These intolerance or annoyance reactions do not represent direct effects of chemical exposure, but rather a psychological or 'stress-based' response to an airborne chemical (Fig. 2). As the concentration

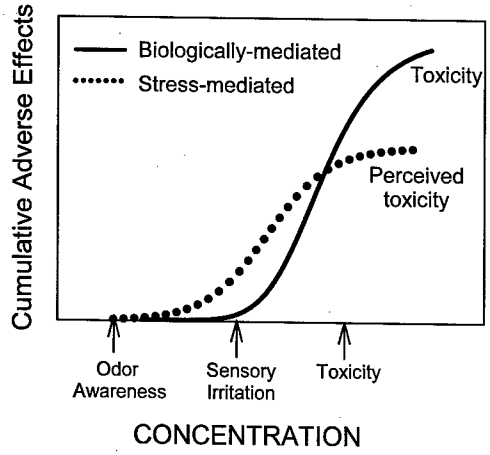

Fig. 2. Hypothetical functions illustrating the cumulative adverse symptom response to a chemical possessing both odor, irritant and toxicity properties. The dotted line represents the 'stress-based' responses that are indirectly elicited by chemical exposure, largely through the awareness of odor, while the solid line represents the "biologically-based' responses that are a function of exposure duration and concentration.

of a volatile chemical increases and the threshold for trigeminal (or other irritant) sensation is exceeded, the observed effects or symptoms are 'biologically-based' and can be directly tied to concentration or duration of exposure. However, the majority of adverse reactions to low or moderate concentrations of airborne chemicals appear to be more closely related to 'stress-based' effects following the perception of an odor than to 'biologically-based effects' following the onset of sensory irritation or toxicity (Dalton et al., 1997a).

3. How can odor and sensory irritation be measured in humans?

Although animal models have been used widely in assessing the irritative properties of airborne chemicals, most rodents are obligate nose breathers whose nasal mucosa and passages differ considerably from those of humans. Hence, the ability to measure responses to airborne chemicals in the species of interest, humans, is a great advantage. Nevertheless, there are significant limitations to studying human response as well. Although it may appear to be a straightforward exercise to expose individuals to a volatile chemical and ask them to report on the level of irritation they experience, the potential for confusion between olfactory and irritant modalities has produced extreme variability in direct scaling of upper airway irritant sensations by individuals with intact olfactory and trigeminal systems. This is exacerbated when chemicals present in the air at levels that stimulate only odor sensation can prompt exposed individuals, such as workers, to report 'irritation', even if the perception is largely mediated through the psychological discomfort incurred by smelling the odor of an unfamiliar or unpleasant chemical, or even from misattributions of unrelated symptoms that just happen to coincide with chemical exposure. Not surprisingly, considerable interest has centered on developing methods of assessing irritancy that either do not rely solely on self-report or that measure or otherwise control for the variance that is produced by the perception of odor and other factors.

Two approaches have been taken to address this problem. The first embodies the use, validation and continued development of a number of objective assays for evaluating irritant potency across a wide range of chemicals, as described in the paper by Hummel et al. (this special volume). The second approach involves the design of studies to identify the mechanisms of sensory irritation and *perceived* irritation, with a focus on systematically studying variables that may impact on the perception of irritancy in humans.

3.1. Psychophysical measures of chemosensory irritation

Two psychophysical methods have proven to be quite valuable in ongoing efforts to relate the subject's perception of irritation to the physical concentration of the chemical. Sensory scaling, a technique that has been in use for decades, relies upon the relation between systematic changes in physical concentration and corresponding changes in the subjects' report of perceived irritation intensity. For scaling sensory stimuli in other domains (e.g. vision, audition), many different types of intensity scales have been developed, of

which most can be adapted for use in the scaling of sensory irritation, including category scales, ratio scales (Stevens and Galanter, 1957) and more recent hybrids of these two types, known as category-ratio scales (Green et al., 1993, 1996). Although a detailed description of the differences between scaling methods are beyond the scope of this review, one common feature among scaling methods that is often a cause for concern is the inherent subjectivity of the response. Self-report, scalar ratings of irritation intensity reflect an integrated response to a volatile chemical that is comprised of (1) the sensory and physiological signals that the chemical elicits and (2) the interpretation of those signals as influenced by experience, expectations, personality factors and other psychosocial or situational variables. For this very reason, however, scalar ratings can provide quite valuable information in the investigation of chemical irritancy as they are often most analogous to and associated with the subjective reports of irritation and symptoms that occur in the workplace. Moreover, progress has been made in refining the utility of self-report information. For example, asking exposed individuals to provide both an affective rating (e.g. perceived annoyance or bother) in addition to the more typical sensory ones (e.g. irritation quality or intensity) can usefully partition the response to the volatile stimulus into its sensory and affective components, e.g., (Dalton et al., 2000). Such a partition has been found to increase associations between the sensory component of the response and more objective assays of sensory irritation, such as the lateralization threshold or other techniques described below.

The second group of psychophysical methods that are used to distinguish odor and irritation accomplish this by measuring absolute thresholds for irritation. Historically, irritation thresholds were estimated by measuring nasal *detection* thresholds to odorants in *normosmics*—subjects with a normal sense of smell—and comparing them with detection thresholds obtained from *anosmics*—individuals without a functional sense of smell (Cometto-Muñiz and Cain, 1990, 1991). The concentration at which a normosmic can detect a chemical is assumed to correspond to the odor threshold, whereas the concentration at which an anosmic can detect it is assumed to correspond to the nasal irritation threshold. Detection thresholds collected using this technique for a diverse set of chemicals has revealed that odor thresholds are typically orders of magnitude below irritation thresholds. However, the degree to which anosmic detection thresholds are equivalent to irritation thresholds in normal individuals has been questioned, as evidence from electrophysiological and anatomical studies has emerged to suggest that chemesthetic perception is diminished among individuals who lack a functional sense of smell (Dunnegan, 1993; Hummel et al., 1996).

For this reason, a psychophysical method that allows one to independently obtain thresholds for odor and irritation in individuals with normal olfactory ability has been of considerable value (Cometto-Muñiz and Cain, 1998; Wysocki et al., 1997). This technique, called lateralization, relies upon the fact that irritants, but not pure olfactory stimuli, can be localized in the nasal mucosa. Thus, a chemical vapor (at a concentration above odor threshold) presented to one nostril and clean air to the other nostril can elicit a smell, but cannot be localized to the stimulated nostril, as long as the concentration does not exceed the threshold for activating nasal-trigeminal receptors (the functional definition of sensory irritation). In contrast, when chemical concentrations exceed the threshold for trigeminal activation, the subject can reliably discriminate or lateralize which nostril has been stimulated. By varying the concentrations presented to the subject in a stepwise fashion throughout the test, one can determine the concentration at which the individual can reliably identify the stimulated nostril, which is taken to represent the nasal irritancy threshold. One important advantage of this method is that, in combination with an odor threshold test, it provides objective measures of both odor and intra-nasal irritation. Because sensations of irritation in the eyes can also be localized, the lateralization technique has also been adapted to obtain ocular thresholds for chemical vapor irritation that can then be compared with nasal thresholds.

3.2. Physiological and functional measures of chemosensory irritation

The human response to a chemosensory irritant can also be evaluated by examining the degree to which airborne chemicals produce local signs of irritation in the nasal or ocular mucosa, such as those used in the study by Emmen et al. (this special volume). Functional changes such as irritation-induced congestion can be evaluated using rhinomanometry or acoustic rhinometry to measure the pressure changes or volume changes in the nasal passages (Kesavanathan et al., 1996). In addition, the defensive response of the ocular or nasal mucosa to a chemical irritant can be evaluated by measuring the volume of secretion (mucus or tear production) or the presence of inflammatory mediators in that secretion (Douwes et al., 2000).

However, focusing attention only on the endpoints directly related to chemosensory irritation may overlook much of the 'stress-based' symptomatology that can be elicited in response to the presence of an environmental odor. Thus, methods for evaluating the degree to which an individual is 'aroused' in the presence of an airborne chemical stimulus form an important part of a program to identify the global response to chemosensory stimuli. For example, measures of heart rate and respiratory behavior, such as volume, rate and end-tidal CO_2 can provide important information about the individual's autonomic state as it may be influenced by their level of reactivity to a chemical stimulus. In addition, obtaining neuroendocrine measures of stress, such as salivary cortisol, in conjunction with other measures can also be useful in determining the psychological or 'stress-based' component of the adverse response to a chemical stimulus.

4. What factors can influence odor, irritant and health risk perception?

Individuals can vary in their sensitivity or response to odors and irritants just as they vary in their sensitivity and response to many sensory stimuli. Variation in the sensitivity and response to the chemosensory properties of an airborne chemical can arise from two types of factors: (1) variables that alter an individual's sensitivity, such as genetics, age, gender, disease or exposure history and (2) variables that influence the way an individual processes chemosensory information. Because each of these sources of variation can alter an individuals' response, it is critical that these sources of variation are well identified and understood when designing studies or interpreting the outcome of research.

4.1. Expectation and beliefs

Although symptoms and somatic sensations from airborne chemical exposures are determined, in part, by physiological activity, they can also be influenced by general beliefs or models about exposure risks. Thus, one important source of influence on an individual's response to a chemical is their accumulated knowledge or 'mental model' of exposure effects. Such a schema can guide the interpretation of everyday odor experiences (Reiser et al., 1985) and provide focus to the symptoms a person monitors and ultimately perceives, e.g., (Leventhal et al., 1980). For example, Shusterman (2001) surveyed a group of individuals living near hazardous waste sites about the frequency with which they perceived odors and symptoms and found that symptom rates were significantly higher among those individuals who were 'worried' about the source of the odors. These results strongly suggested that people can be cued to monitor and report symptoms that are primed by their cognitive or emotional state.

Evidence in support of these field studies comes from a series of laboratory studies that explicitly manipulated the perceived risk from chemical exposure. In those studies, individuals who were informed that the chemical to which they were being exposed (either butanol or methyl salicylate–wintergreen oil) was an industrial solvent reported significantly more odor, irritation and health symptoms from exposure than did individuals who were exposed to the exact same chemical but who were told that it was a natural extract (Dalton, 1999) (Fig. 3). Moreover, the frequency of spontaneous (Dalton, 1996) and

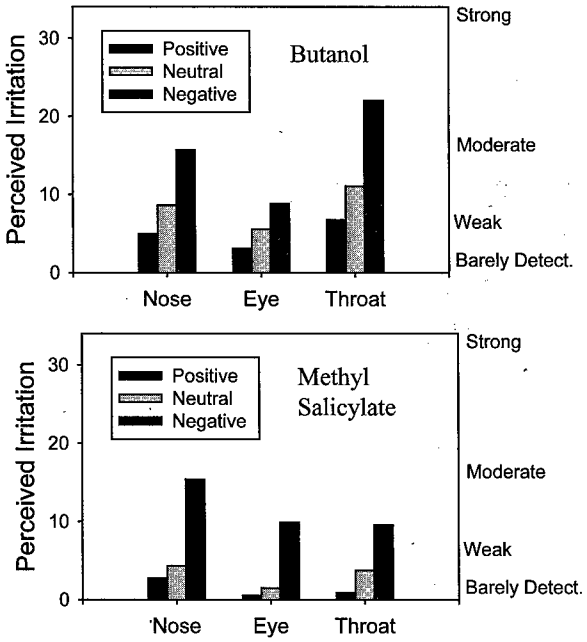

Fig. 3. Average intensity ratings of the perceived irritation from a 20-min chamber exposure to butanol or methyl salicylate (wintergreen) for participants given a positive or negative bias about the nature of the odorants. Intensity ratings were made using the Labeled Magnitude Scale, from Dalton

surveyed (Dalton et al., 1997a,b) symptom reports varied significantly with perceived odor intensity, suggesting that symptom perception was correlated with, or perhaps triggered by, the awareness of an odor.

4.2. Bias and chemosensory response

The role of response bias has emerged as a critical variable in studies of sensory irritation, particularly where odors are also present. Numerous studies of irritancy from airborne chemicals have contrasted responses during exposure to the chemical with responses made during exposures to clean air. However, for most people, non-odorized air signals the absence of a volatile chemical. For this reason individuals may adopt very different criteria for reporting irritation in the presence of an odor than in the presence of relatively odorless air. For example, subjects who were successively exposed to clean air and the chemical PEA (which is used as a positive control for odor because it has an odor, but is not an irritant) reported significantly more irritation in the presence of PEA than in the presence of clean air. As revealed in the study by Emmen et al. (this special volume) measuring bias and sensitivity in studies that use positive odor controls can shed considerable illumination on the factors that govern the reports of irritancy in the workplace.

4.3. Psychosocial factors

Reports of sensory irritation in the workplace may also be significantly influenced by the reactions of co-workers and other bystanders. A study that employed a confederate subject, (e.g. an actor who was trained to follow a carefully scripted set of verbal or behavioral responses to the ambient odor in the chamber) showed that the perception of chemical odor and irritancy can be significantly altered by the reactions of other people (Dalton et al., 1999). In these studies, the confederate (who presented him or herself as a research participant to the real subject) delivered either a set of positive, negative or neutral comments/behaviors about the ambient odor. The measure of this effect was on the responses of the real subjects in each of the three conditions. Subjects who were exposed to a solvent odor while being given negative cues were much more likely to report irritation and health symptoms (70%) than were individuals who were given positive cues (12%) or neutral cues (34%).

4.4. Personality variables

A significant amount of the variation in irritant and symptom perception in normal, healthy individuals can be attributed to differences in personality orientations. In general, positive affective orientations appear to lower individuals' expectancies of becoming ill, while negative orientations appear to heighten those same expectancies. Negative affectivity (NA) (Tellegen, 1985; Watson and Clark, 1984) is a personality dimension that reflects stable and pervasive differences in emotional processing, negative mood and self-concept. Individuals who are high in NA are more likely to experience distress in the absence of overt

stressors, exhibit hyper-vigilance in scanning their environment, interpret ambiguous stimuli in a negative manner, and report more subjective health complaints (Watson and Pennebaker, 1989). These tendencies may allow environmental stimuli, such as odors, to trigger detection of baseline levels of physiological activity that would otherwise go unnoticed (Moyle, 1995; Wickramasekera, 1995).

Smeets, Maute and Dalton (unpublished data) conducted a study aimed at understanding the relationship between NA and responses to odor exposure. Healthy, normal volunteers who scored at the low and high extremes on the NA items of the Positive and Negative Affectivity Scale (PANAS) were exposed to isopropyl alcohol at 400 ppm for 30 min on each of two visits during which a variety of subjective (e.g. reported symptoms) and objective (e.g. eye redness) adverse effects were measured. High NA individuals gave significantly higher ratings of ocular irritation both at baseline and following exposure than did low NA individuals; moreover, ratings of ocular irritation on their second visit were significantly higher at baseline than on visit 1. No such differences were observed for the low NA individuals (Fig. 4). Despite reports of high levels of eye irritation among the high NA group, no objective signs of ocular irritation (i.e. exposure-related differences in conjunctival hyperemia (redness)) were observed.

5. Advantages to studying chemosensory responses in human vs. animals

Although animal models of sensory irritation have been widely used to evaluate the irritant potency for many chemicals, differences in breathing patterns and the anatomy and biochemistry of the upper respiratory tract in humans and rodents necessitates sometimes significant adjustments to values derived from animal studies in order to set human exposure guidelines. Thus, the availability of safe, non-invasive assays to directly measure odor and irritant responses in the species of interest, *humans*, can both simplify and improve accuracy in the process of developing appropriate occupational exposure guidelines.

It is also important to acknowledge, however, that factors that produce variation and complicate the measurement of human sensory responses in experimental situations (such as non-specific responses to odors and generalized tendencies to report irritation) are likely to be critical components of people's reactions to irritants in occupa-

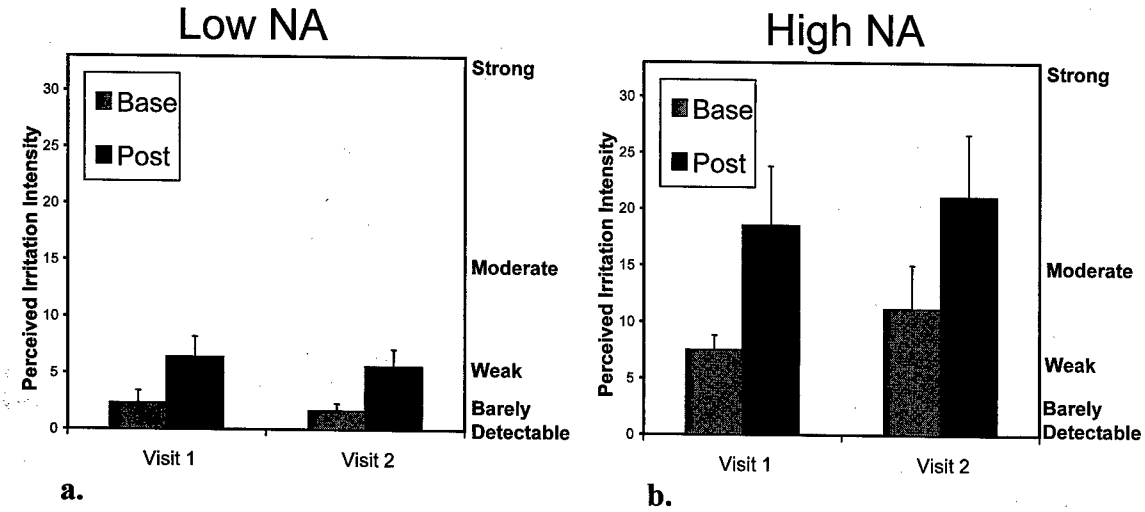

Fig. 4. Reported ocular irritation following two, 30-min exposures to 400 ppm isopropanol for individuals scoring high and low in NA. Although there was no objective evidence of ocular irritation, the high NA group rated more sensory irritation at baseline and following exposure than did the low NA group.

tional environments as well. Fortunately, the general nature of this problem for measuring human responses in other domains has prompted researchers in cognitive and sensory psychology to develop methods that separate and quantify measures of generalized response tendencies (bias) separately from measures of perceptual sensitivity. Thus, rather than adopting a strict focus on designing studies that eliminate the influence of bias, a more comprehensive strategy may be to explicitly study and quantify the degree and types of bias that influence subject reports of sensory irritation and identify experimental analogues of real-world conditions under which biases are likely to modify reports of irritation following chemical exposure.

6. Discussion and conclusions

Why is it important to differentiate between stress-mediated and biologically-mediated symptoms and adverse responses to airborne chemicals? One primary reason is that the sources of variation in the population response to perceived (stress-mediated) and actual (biologically-mediated) toxicity are quite different, even if the origin of the adverse responses are indistinguishable at first glance. For example, the variation in responsiveness due to sensitivity factors (e.g. age or gender) is likely to be far smaller than the variation in reported symptoms or stress-mediated responses that can occur because of differences in beliefs or attitudes about the possible consequences of exposure to a chemical in the environment. Moreover, strategies that are capable of mitigating responses to perceived risk, such as information communication and education about the chemicals in the environment, are likely to be ineffective mediators of biologically-based effects such as sensory irritation, headaches, nausea, etc. Thus, one important reason for attempting to differentiate the origin of the adverse response is to facilitate the development of effective response strategies to a worker or community population.

Methods to evaluate chemosensory response to odors and irritants can be of significant utility in the development of exposure guidelines for occupational (and residential) environments. This paper has attempted to provide an overview of such methods and to describe some of the factors underlying the lack of concordance between exposure concentration, objective signs of exposure-related symptoms and adverse reports that are frequently observed in occupational environments. Well-controlled, objective and subjective assays of human response to chemosensation can be used to (1) distinguish thresholds for odor, annoyance and sensory irritation, (2) measure irritant potency and inflammatory response and determine a NOAEL for irritation, and (3) determine the basis (stress or biologically-mediated) for any observed health effects in the exposed population.

Objective measures of irritation onset obtained in conjunction with subjective responses can lend valuable input to the decision process for determining occupational exposure limits. Objective measures of irritation that cannot be reconciled with subjective reports occurring at much lower levels of exposure should prompt a careful investigation into the other factors (e.g. cognitive or emotional) that may modulate the sensory responses. For example, anxiety or worry over the consequences of exposure, and other workplace stress can elevate symptom reports and irritation and may not be ameliorated by a reduction in the exposure level if the odor of the chemical is still detectable. Combination of data from studies that evaluate chemosensory responses of a chemical with other toxicological data can form a solid basis for policies and guidelines to protect chemically-exposed workers.

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Review

Human volunteer study with PGME: eye irritation during vapour exposure

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Abstract

The objective of this study was to establish the possible occurrence of eye irritation and subjective symptoms in human volunteers exposed to propylene glycol monomethyl ether (PGME) vapour at concentrations of 0, 100 and 150 ppm. Testing was conducted in 12 healthy male volunteers using a repeated measures design. Each subject was exposed for 2.5 h to each of the three exposure conditions that were spaced 7 days apart. The exposure sequences were counterbalanced and the exposure to the test substance and the effect measurements were conducted in a double-blind fashion. During all exposure sessions, 20 ppm diethyl ether was used as a 'masking agent' for vapour exposure. Measurements of pre- and post exposure eye redness, corneal thickness, tear film break-up time, conjunctival epithelial damage, blinking frequency, and subjective ratings on discomfort were used to evaluate the possible irritating effects of PGME. The results indicated no significant treatment effects for any of the objective parameters. Results of the subjective ratings indicated very slight effects on the eyes in the 150 ppm PGME condition only. No significant effects of treatment were found for the remaining questions concerning the perceived intensity of the smell in the room, the (un)pleasantness of the smell, the perceived effects on the skin, effects on the throat, shivering, muscle aching, and intestinal cramps. In conclusion, the results of the present study indicated minimal subjective eye effects at 150 ppm only, and no impact on the objective measures of eye irritation at either of the two exposure levels. It was concluded that the no adverse effect concentration for eye irritation due to PGME vapour was at least 150 ppm.
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Keywords: Eye irritation; Subjective symptoms; Propylene glycol monomethyl ether; Human volunteer study

1. Introduction

Stewart et al. (1970) determined the potential irritating and anaesthetizing properties of propylene glycol monomethyl ether (PGME) (1-methoxy-2-propanol; CAS 107-98-2) at vapour concentrations of 50–2000 ppm. When six subjects entered the exposure chamber containing 100 ppm PGME, four of them immediately described the odour as too strong to be tolerated. Odour tolerance developed within 25 min, and all subjects judged the odour as mild in intensity. After one

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hour of exposure to 100 ppm, one subject perceived mild eye irritation. After 2 h, two of the six were complaining of slight eye irritation. None of the subjects complained of nose or throat irritation. During 3.5 h of exposure, there was no decrement in visual acuity, coordination and neurological responses. Furthermore clinical laboratory outcomes after the vapour exposure were normal and similar to pre-exposure values.

Stewart et al. (1970) concluded that symptoms like eye, nose or throat irritation became objectionable prior to the first signs of central nervous system impairment, which occurred at 1000 ppm in one of two subjects. The reliability of these findings is unclear given the small number of subjects, non-double-blind study design and no use of objective measurements for eye irritation.

In this report, the results of a human study examining the irritating effects of PGME are presented. The objective of this study was to establish the possible occurrence of eye irritation and subjective symptoms in human volunteers exposed to PGME vapour at concentrations of 100 ppm (the 8-h maximal allowable concentration level (MAC)) and 150 ppm. The methods were based on those of Iregren et al. (1993), who described the use of a number of objective endpoints for quantification of irritation effects after experimental exposure. The degree of success of the masking agent was also examined.

2. Materials and methods

2.1. Study design

Testing was conducted with 12 subjects using a within-subjects, Latin square design. Subjects complying with all study selection criteria were exposed to three conditions: 0 ppm PGME (masking only; see Section 2.2), 100 ppm PGME (+ masking) and 150 ppm PGME (+masking), respectively. The test sessions for each subject were spaced 7 days apart. Each subject was exposed for 2.5 h to each of the three exposure conditions. The sequence of exposure conditions was balanced as follows: (1) four subjects receiving the 0 ppm (placebo) condition first, the 150 ppm condition second and the 100 ppm condition third (i.e. 0–150–100). (2) The other four subjects receiving the 100 ppm condition first, the 0 ppm condition second and the 150 ppm condition third (100–0–150). (3) The last four subjects receiving the 150 ppm condition first, the 100 ppm condition second and the 0 ppm condition third (150–100–0). Assignment to treatment sequence was random. The exposure to the test substance and the effect measurements were conducted in a double-blind fashion, i.e. neither the subject nor the investigator/assistant was aware of treatment condition.

2.2. Characterization of the test substance and masking agent

The test substance used in the study was a sample of commercial propylene glycol monomethyl ether (PGME), supplied by Dow Europe S.A. PGME (1-methoxy-2-propanol; CAS Reg. No. 107-98-2) is a colourless liquid with a sweet ether-like smell. Since PGME has a distinct odour, exposed subjects are not blinded with respect to a control condition (i.e. no PGME present). For this reason, the masking agent diethyl ether was used in the present study. Diethyl ether was chosen as it possesses similar odour characteristics to PGME and the odour threshold of this compound lies far below the MAC-level of diethyl ether (400 ppm = 1200 mg/m^3). Diethyl ether (DEE; CAS Reg. No. 60-29-7; obtained from Labscan, Dublin, Ireland; a colourless liquid with the well-known ether smell) was simultaneously generated with PGME at a target level of 20 ppm.

2.3. Subjects

2.3.1. Population and selection

Twenty-two male subjects were recruited from a pool of persons living in the vicinity of Zeist who were known to be willing to volunteer as subjects for participation in human studies. For inclusion in the study, each subject had to meet the following criteria: (a) age between 20 and 50 years; (b) health as determined by medical and laboratory examinations, including lung function measurements; (c) ophthalmologic measurements in

normal range; (d) native language Dutch; and (e) written informed consent. The exclusion criteria were: (a) a history of medical/surgical disease (that may significantly affect the study outcome); (b) a history of alcohol, amphetamine, cocaine, barbiturate or other drug abuse; (c) participation in a clinical study within 3 months before the start of the present study; (d) present use of psychopharmacological medication (including medication for sleeping disorders); (e) use of more than 28 alcoholic beverages a week; (f) evidence of lung, liver or kidney dysfunction; (g) employed by TNO or first or second removed relative employed by TNO; (h) claustrophobia; (i) wear of contact lenses or spectacles; (j) smoking.

The principles of informed consent in the current revision of the Declaration of Helsinki (Hong Kong, 1989) were implemented in this study.

Prior to and after the admission into the study, a check of the subject's general health was performed. The pre-study screening involved: (1) filling out a health questionnaire; (2) measurement of body weight (in indoor clothing, without shoes), height and blood pressure; (3) an interview with the deputy medical investigator; (4) examination of the eyes including measurement of corneal thickness, tear film break-up time (BUT), and conjunctival epithelial damage. The ophthalmologic measurements were performed as a selection criterium for inclusion (baseline values). Further, (5) lung function measurement; and (6) blood and urine collection. The following laboratory tests were carried out: serum chemistry (fasted) (ALP, ALAT, ASAT, γ-GT, total bilirubin, creatinine, glucose, total protein, sodium, potassium), and dipstick urinalysis (protein, glucose and sediment if indicated).

The screening of subjects took place within 2 weeks prior to the beginning of the experimental phase of the study. Twelve subjects were selected according to the criteria given above.

At the post-study check-out, serum chemistry (ALP, ALAT, ASAT, γ-GT, total bilirubin, creatinine, total protein, sodium, potassium) was carried out and a medical check-out interview was held.

2.3.2. Inclusion and allocation

On the first experimental day, the medical investigator checked the results of the Daily Findings Questionnaire and determined whether the subjects fulfilled the criteria for participation. An entry number was allocated to those included in order of their arrival at the research centre.

Prior to the experimental phase of the study, the following treatment codes were defined: A (representing exposure sequence 100–0–150), B (representing exposure sequence 0–150–100) and C (representing exposure sequence 150–100–0). The entry numbers 01-04 were allocated to treatment code A, the entry numbers 05-08 were allocated to code B, and the entry numbers 09-12 were allocated to code C.

The treatment code was blinded for the subjects, sponsor, monitor, medical investigator, deputy medical investigator and personnel involved in the conduct of the study, but not for the inhalation technician.

2.4. Generation of test atmosphere

The exposure took place in an exposure chamber with a volume of 13.6 m^3. Within the chamber temperature and humidity were controlled. The air in the exposure chamber was refreshed at a rate of about 40 m^3/h. The test atmosphere was generated by pumping at an adjustable rate a precisely prepared mixture of propylene glycol monomethyl ether (PGME) and diethyl ether (DEE in liquid form) to an evaporation unit, which was placed in the air supply of the exposure chamber. The evaporation unit was operated at a sufficiently high temperature to allow instant evaporation. The generated concentrations of PGME and DEE were measured by infrared absorption in a MIRAN apparatus (Foxboro, USA) at 3.5 μm and a path length of 11.25 m (see below).

The MIRAN was calibrated with tedlar air bags filled with metered amounts of non-absorbing nitrogen gas and were injected through a septum with the necessary amounts of PGME. Six concentrations of PGME were used, viz.: 0, 50, 100, 125, 150 and 175 ppm. The calibration curve:

$$\text{MIRANreading(mV)} = -0.5 + 4.406592*[\text{PGME(ppm)}] - 0.002953*[\text{PGME(ppm)}]^2$$

could be fitted almost perfectly ($R = 0.99996$).

PGME concentrations were read at 15-min time intervals and also recorded continuously with a chart recorder. The 15-min intervals were averaged over an experimental session by first converting MIRAN readings to PGME concentrations by using a tangent approximation at the target concentration value. The infrared absorption spectra of DEE and PGME were not sufficiently different to allow estimation of PGME concentrations without interference from DEE with the MIRAN. Therefore, the sensitivity of the MIRAN for DEE was established separately. It appeared that 20 ppm DEE had the same absorbance as 13 ppm PGME. Target 'concentrations', therefore, were 13, 113 and 163 ppm for exposure to 20 ppm DEE alone, 100 ppm PGME combined with 20 ppm DEE and 150 ppm PGME combined with 20 ppm DEE, respectively.

2.5. Study parameters

Eye irritation was measured using selected ophthalmologic tests, i.e. eye redness, corneal thickness, tear film break-up time (BUT), conjunctival epithelial damage (CED) and the blinking frequency. Subjective symptoms were assessed by means of a questionnaire. In addition, at the end of the third session subjects were asked to indicate during which test session they believed to have been exposed to PGME (100 and 150 ppm).

2.5.1. Eye redness

Close-up slides of the eyes of the subjects were made with a camera attached to a close-up lens with a ring-light flash (Nikon F401s, Medical NIKKOR 120 mm f/4 IF). Both eyes were photographed prior to and immediately after the termination of exposure. For the evaluation of results, the slides were projected in pairs (pre- and post-exposure) on a screen and the difference in redness of the eye prior and after exposure were independently judged by three different scorers without knowing the background of the slides (pre- or post-exposure) or the exposure concentration. The post-exposure eye observations were characterized as either more, equally, or less red than the pre-exposure eye. In case of a discrepancy between the outcome of the three scorers, the opinion of the ophthalmology consultant was asked and considered decisive.

For standardization in the development of the slides, a standard colour card provided by the photographic service, was photographed at least once per film. In addition, all films (Kodak Ektachrome 64 from the same emulsion batch) were developed simultaneously.

2.5.2. Corneal thickness

Prior to and within 1 h after termination of exposure, the corneal thickness of both eyes of each subject was accurately measured at the corneal apex using the Depth Measuring Attachment no. II for the Haag-Streit slit-lamp microscope (Slit-lamp 900 CN, Haag-Streit AG, Liebefeld-Bern, Switzerland), and expressed in the instrument units. Baseline corneal thickness was also measured about 2 weeks prior to the exposure sessions.

2.5.3. Tear film break-up time (BUT)

The precorneal tear forms a stable and continuous covering. It consists of three layers, viz. an outermost lipid layer, an underlying aqueous layer and an innermost mucin layer. The latter, produced by conjunctival goblet cells, is spread over the cornea by the eyelids, adsorbs onto the corneal surface and thus forms a hydrophilic surface for the aqueous layer. If the periodic resurfacing of the film by blinking is interfered with, the precorneal film will eventually break up, i.e. dry spots will develop causing areas of discontinuity of the tear film. BUT can be defined as the interval between a complete blink and the appearance of the first randomly distributed dry spot (Lemp and Hamill, 1973).

A total of 10 μl of fluorescein sodium BP 2% w/v (Minims, Smith & Nephew, Romford, UK) was instilled into the conjunctival sac of the eye with a micro pipette (Nichiryo, model 8100, Tokyo,

Japan). The subject was instructed to blink a few times and then to keep the eyes open. A stopwatch was started immediately after the last blink and the whole precorneal film was searched from side to side using a cobalt filtered-light beam from the slit lamp. Time was measured until the first rupture in the precorneal film was observed, or for a maximum duration of 30 s (sometimes less due to difficulties in delaying blinking). Measurements were made three times on each eye within 1 h after termination of exposure and after the corneal thickness measurement. The maximum BUT for each eye was used for evaluation. Baseline-level of BUT was measured about 2 weeks prior to the exposure sessions.

2.5.4. Conjunctival epithelial damage (CED)

Conjunctival epithelial damage was visualized by instilling 10 μl of 1% lissamine green in the conjunctival sac of both eyes. The subject was instructed to blink freely, and after ca. 1 min, the conjunctivae were inspected with the slit lamp in white light. If a minimum of ten stained cells were seen they were counted. Isolated and widely dispersed single stained cells were not counted. The numbers of stained dots in each eye were summed for evaluation. These determinations were made within 1 h after termination of exposure and after the measurement of BUT. A baseline value was measured about 2 weeks prior to the exposure sessions.

2.5.5. Blinking frequency

Blinking frequencies were measured by making videotape recordings of the subject's face (without informing the subject that blinking was recorded) and counting the number of blinks for 1 min during playback. Recordings of the subject's face were made during a 5-min period in the exposure chamber prior to the start of the exposure (baseline level), and during minutes 0–15 and 30–35 after the start of the exposure. Blinking frequencies were counted for 1 min during minutes 2–3 of the baseline recording and during minutes 2–3, 7–8, 12–13 and 32–33 after the start of exposure. The blinking frequencies were judged independently by two different scorers. The mean score was used in this study.

2.5.6. Subjective rating on general irritation

A questionnaire (I) designed to assess subjective reactions was administered three times during each exposure session. At 15, 30 and 120 min during the exposures, the subjects were asked to rate the perceived intensity of the smell in the room, the (un)pleasantness of the smell, the perceived effects on the eyes, skin, nose and throat. In addition, the subjects were asked to indicate several symptoms (shivering, muscle aching and intestinal cramps) on a 7-point scale ranging from 1 (not at all) to 7 (extremely). The subjects were not able to consult their previous answers. Most of the questionnaire items were obtained from Iregren et al. (1993).

2.5.7. Exposure perception

At the end of the third exposure session, subjects were asked to indicate during which test session they believed to have been exposed to PGME (100 and 150 ppm). In addition, they were asked to rate how certain they were of their answer (Questionnaire II).

2.6. Study procedures

The schedule of assessments on each experimental test day is given below:

Scheduled clock

Time	Activity
08:00	Arrival at TNO Daily findings Instruction about the program of the test day Breakfast Ophthalmologic measurements: – eye redness – blinking frequency – corneal thickness
08:30	Start of vapour generation
09:00	Start of exposure
09:05	Blinking frequency
09:10	Blinking frequency

Scheduled clock *(Continued)*

Time	Activity
09:15	Blinking frequency
	Questionnaire I
09:30	Blinking frequency
	Questionnaire I
11:15	Questionnaire I
11:30	End of exposure
11:35	Eye redness
< 12:30	Corneal thickness
	Tear film break-up time
	Conjunctival epithelial damage
12:45	Check well being
	Questionnaire II[A]
	Medical check-out[A]
13:00	Departure from TNO

[A] Only at the end of the third test day.

2.7. Statistical evaluation

The outcome parameters were evaluated using Friedman's Test and repeated-measures analysis of variance (ANOVA). Analyses were conducted using the BMDP statistical software package (Dixon, 1990). The Newman–Keuls procedure was used for evaluating pairwise comparisons among means (Kirk, 1982). For all statistical tests $P < 0.05$ was considered significant.

2.8. Criteria and clinical relevance for eye irritation

The following criteria for assessment of the eye irritation were applied: (1) a corneal swelling of ≤ 5% was considered normal, > 5% was considered abnormal. (2) A BUT below 5 s was considered abnormal, 5–10 s was considered borderline, and > 10 s was considered normal. (3) For conjunctival damage the number of stained dots per eye determined its significance, viz. > 50 dots was considered abnormal, 10–50 dots was considered borderline, and < 10 dots was considered normal. (4) An increase of the blinking frequency with 50% or more from the baseline frequency was considered abnormal.

3. Results

3.1. Demographics

The mean age of the study subjects was 23 years and ranged from 20 to 26 years. The mean body weight was 77.0 kg (range: 66.6–95.4). The mean height was 181.8 cm.

3.2. Safety and adverse events

At the end of each test session, the well-being of the subject was checked by the deputy medical investigator. None of the 15 registered adverse events (AEs) were judged to be treatment-related per se. For eight AEs, the relation to treatment was judged 'unlikely' (i.e. a relation is not likely, but not impossible): fatigue (2 ×), common cold (2 ×), dry lips (1 ×), thirst (1 ×), and ALAT (2 ×). For seven AEs, the relation to treatment was judged 'possible' (i.e. a relation is not likely, but may exist): lethargy (2 ×), thinking abnormal (slow) (2 ×), dizziness (1 ×), headache (1 ×) and nausea (1 ×). All subjects were considered healthy at the end of the experimental phase of the study as judged by the deputy medical investigator.

3.3. Exposure

Mean exposure concentrations were calculated by averaging the 15-min interval values over the period during which subjects were exposed. The mean concentrations for the three 100 ppm PGME-sessions were respectively 97.2 ppm (S.D. = 9.5), 98.8 ppm (S.D. = 6.4) and 103.4 ppm (S.D. = 7.6). The mean time-weighted concentrations for the three 150 ppm PGME-sessions were respectively 150.6 ppm (S.D. = 12.2), 151.3 ppm (S.D. = 11.6) and 153.5 ppm (S.D. = 9.8).

Mean time-weighted DEE ('masking') concentrations during the 0 ppm PGME (control) sessions were respectively 19.5 ppm (S.D. = 2.0), 19.9 ppm (S.D. = 1.6) and 20.9 ppm (S.D. = 1.2). During the 100 ppm PGME-sessions DEE concentrations were respectively 19.4 ppm (S.D. = 1.9), 19.8 ppm (S.D. = 1.3) and 20.7 ppm (S.D. = 1.5). During the 150 ppm PGME-sessions DEE concentra-

tions were respectively 20.1 ppm (S.D. = 1.6), 20.2 ppm (S.D. = 1.6) and 20.5 ppm (S.D. = 1.3).

Mean temperatures in the exposure chamber ranged from 22.3 to 23.1 °C. Relative humidity ranged from 53 to 56%.

3.4. Study parameters

3.4.1. Eye redness

Data grouped by exposure condition are presented in Table 1. Using Friedman's two-way analysis of variance, no significant differences between exposure conditions were observed. The Friedman statistics for the analysis of the left and right eye recordings were 2.54 ($P = 0.28$) and 2.63 ($P = 0.27$), respectively.

3.4.2. Corneal thickness

Data grouped by exposure condition are presented in Table 2. Repeated-measures analysis of variance (ANOVA) revealed no significant (treatment) differences both for the left and right eye measurements ($F = 0.25$, $P = 0.78$; $F = 0.08$, $P = 0.93$). There were no significant interactions between treatment and the order of exposure ($F = 0.62$, $P = 0.65$; $F = 0.39$, $P = 0.81$).

3.4.3. Tear film break-up time (BUT)

Data grouped by exposure condition are presented in Table 3. On several occasions during baseline and experimental determination of BUT, several subjects experienced difficulties in delaying blinking for a maximum period of 30 s. The baseline BUT of ten out of the 12 subjects were within the normal range (BUT > 10 s), while borderline BUTs (5–10 s) were recorded in subject no. 07 (left eye 7 s) and in subject no. 06 (left eye 10 s, right eye 9 s). After each of the three exposure conditions, no abnormal BUTs (< 5 s) were recorded in any of the 12 subjects.

ANOVA's on the BUT-scores for the left and right eye, indicated no effect of treatment (Left eye: $F = 0.75$, $P = 0.49$; Right eye: $F = 0.61$; $P = 0.55$). There were no significant interactions between treatment and the order of exposure ($F = 0.75$, $P = 0.57$; $F = 0.15$, $P = 0.96$).

Table 1
Eye redness (ratings*) vs exposure conditions

Score	0 ppm		100 ppm		150 ppm	
	Left	Right	Left	Right	Left	Right
1*	6	9	4	4	3	8
2*	4	0	2	0	1	0
3*	2	3	6	8	8	4

1* Pre-exposure more red than post-exposure; 2* Post-exposure more red than pre-exposure; 3* No difference between pre- and post-exposure eye redness.

3.4.4. Conjunctival epithelial damage (CED)

Data grouped by exposure condition are presented in Table 4. The baseline CED was borderline (i.e. 10–50 dots) in only one subject (no. 10: left eye, 12 stained dots). Five subjects did not show any stained dots. The remaining six subjects showed limited staining (up to 4 dots). After each of the three exposure conditions, no abnormal CED (> 50 dots) were recorded in any of the 12 subjects.

ANOVA's on the CED-scores for the left and right eye, indicated no effect of treatment (Left eye: $F = 0.09$, $P = 0.91$; Right eye: $F = 0.52$; $P = 0.60$). There were no significant interactions between treatment and the order of exposure ($F = 0.63$, $P = 0.64$; $F = 0.69$, $P = 0.61$).

3.4.5. Blinking frequency (BF)

Data grouped by exposure condition, are presented in Table 5. Individual BF varied considerably between the 12 subjects prior and during the three exposure conditions (1–59 blinks/min). Per subject the BFs varied to a much lesser extent, with exception of one volunteer (no. 07) showing high pre-exposure BFs and much lower BFs during the 100 and 150 ppm exposure sessions.

ANOVA on the BF-score indicated no effect of treatment ($F = 0.04$, $P = 0.99$). There was no significant interaction between treatment and the order of exposure ($F = 1.84$; $P = 0.16$).

3.4.6. Subjective rating on general irritation

Data grouped by exposure condition are presented in Table 6. Repeated-measures analysis of variance (ANOVA) revealed statistically significant effects on the eyes ($F = 3.70$; $P = 0.045$) and

Table 2
Corneal thickness [instrument units] vS exposure conditions

Score	0 ppm		100 ppm		150 ppm	
	Left	Right	Left	Right	Left	Right
Pre	82.8	83.0	82.7	82.8	82.4	82.8
Post	82.5	82.6	82.3	82.3	82.3	82.3
Swelling %	−0.4	−0.5	−0.5	−0.7	−0.2	−0.6

Baseline value left eye: 81.1; baseline value right eye: 81.6.

Table 3
Mean BUT[a] (s) vs exposure conditions

	Baseline	0 ppm	100 ppm	150 ppm
Left eye	22 (3)	22 (3)	21 (3)	25 (2)
Right eye	23 (2)	22 (3)	20 (3)	22 (2)

[a] The maximum BUT recorded for each eye was averaged over the 12 subjects; ( ) S.E.M.

Table 4
Conjunctival epithelial damage[a] vs exposure conditions

	Baseline	0 ppm	100 ppm	150 ppm
Left eye	2 (1)	4 (2)	4 (2)	3 (2)
Right eye	1 (0)	4 (2)	4 (2)	3 (1)

[a] Summed number of stained dots (nasal and temporal) averaged over 12 subjects; ( ) S.E.M.

nose ($F = 4.06$; $P = 0.035$). According to the Newman–Keuls procedure, the scores on the question 'do you feel effects on your eyes' were rated higher during the 150 ppm condition (mean = 1.92) than during the 0 ppm (mean = 1.31) condition. The scores on the question 'do you feel effects on your nose' were both rated higher during the 150 ppm condition (mean = 2.00) and 100 ppm condition (mean = 1.81) than during the 0 ppm (mean = 1.06) condition. In general, these mean scores are very low, since the subjects were asked to indicate the possible symptoms on a 7-point scale ranging from 1 (not at all) to 7 (extremely).

With respect to the remaining questions, no significant effects for treatment were found: the (un)pleasantness of the smell ($F = 0.35$, $P = 0.71$), the perceived intensity of the smell in the room ($F = 0.75$; $P = 0.49$), the perceived effects on the

Table 6
Subjective ratings [scale: 1(not at all)–7(extremely)] vs exposure conditions

Questionnaire item	0 ppm Rating[a]	100 ppm Rating[a]	150 ppm Rating[a]
(Un)pleasantness of smell	3.2 (0.2)	3.0 (0.2)	3.1 (0.2)
Smell intensity	3.5 (0.4)	3.6 (0.3)	4.0 (0.3)
Effects on the eyes	1.3 (0.1)	1.4 (0.2)	1.9 (0.3)
Effects on the skin	1.1 (0.0)	1.1 (0.1)	1.1 (0.1)
Effects on the nose	1.1 (0.1)	1.8 (0.3)	2.0 (0.4)
Effects on the throat	1.2 (0.1)	1.4 (0.2)	1.6 (0.4)
Shivering	1.0 (0.0)	1.0 (0.0)	1.1 (0.1)
Muscle aching	1.0 (0.0)	1.1 (0.1)	1.1 (0.1)
Intestinal cramps	1.0 (0.0)	1.1 (0.0)	1.0 (0.0)

[a] Mean of ratings at 15, 30 and 120 min during exposure; ( ) S.E.M.

Table 5
Blinking frequency (number of eye blinks/min) vs exposure conditions

	0 ppm	100 ppm	150 ppm
Pre-exposure (12[a])	17.1 (8.3) [5.0–31.0]	16.2 (9.7) [5.5–34.5]	17.6 (10.6) [6.0–40.0]
During exposure (48[a])	17.1 (10.4) [6.5–35.5]	17.3 (10.5) [5.5–34.1]	17.3 (13.3) [3.1–49.4]

( ) S.D.; [ ] range.
[a] Number of observations.

skin ($F = 0.41$, $P = 0.67$), effects on the throat ($F = 1.02$, $P = 0.38$), shivering ($F = 2.10$, $P = 0.15$), muscle aching ($F = 0.54$; $P = 0.59$), and intestinal cramps ($F = 0.17$, $P = 0.85$). There were no significant interactions between treatment and the order of exposure with P values > 0.05.

3.4.7. Exposure perception

Results of the questionnaire designed to obtain information regarding exposure perception during the test days indicated that eight subjects correctly identified the (two) PGME exposure conditions. Four subjects (06, 07, 11, 12) reported having been exposed to PGME while, in fact, they were exposed to the placebo (diethyl ether) condition.

Only one of the eight subjects who correctly identified the PGME exposure conditions, reported to be 'very sure' (score of 7 on a scale of 1–7) of his perception. In contrast, two out of the eight subjects were 'not at all sure' (score 1) of their exposure impression. One of the four subjects who did not identify the PGME exposure conditions, was nevertheless 'very sure' that he was exposed. The other subjects reported to be less certain of their exposure impression.

4. Discussion and conclusions

The objective of the present study was to establish objectively the possible occurrence of eye irritation and subjective symptoms in human volunteers exposed to propylene glycol monomethyl ether (PGME) vapour at concentrations of 0 (control condition), 100, and 150 ppm. Measurements of eye redness, corneal thickness, tear film break-up time, conjunctival epithelial damage, blinking frequency, and subjective ratings were used to evaluate the possible effects of PGME.

Testing was conducted in 12 healthy male volunteers using a repeated (within subjects) measures design. This was selected based on the superior statistical power which can be achieved in studies in which subjects serve as their own control compared to that of study designs with independent groups (Maxwell and Delaney, 1990).

During all exposure sessions, 20 ppm diethyl ether, which possesses similar odour characteristics to PGME, was used as a masking agent. Results of the questionnaire items concerning the (un)pleasantness and strength of smell indicated no significant differences between the exposure conditions. On a scale ranging from 1 (very unpleasant) to 7 (very pleasant) the mean ratings for the 0, 100 and 150 ppm PGME conditions were respectively 3.2, 3.0 and 3.1. With respect to the ratings of the odour strength (1 = very strong; 7 = not at all strong), mean values for the exposure conditions were respectively 3.5, 3.6 and 4.0. Thus, it can be concluded that the subjects perceived a similar overall odour in the control condition (20 ppm DEE) and in both PGME exposure conditions.

Furthermore, based on the results of the questionnaire designed to obtain information regarding the correctness and certainty of the subjects' exposure perception, it was concluded that diethyl ether was successful in creating doubt for 11 out of 12 subjects comparable to the true exposure condition and provided an effective masking of the exposure condition of this experiment.

In order to quantify irritative effects of exposure to various substances, the use of rating scales as well as objective measures are recommended (Iregren et al., 1993). For the objective measurement of irritation to the eyes, Iregren and coworkers suggested the application of techniques for the evaluation of eye redness, corneal thickness, tear film break-up time, conjunctival epithelial damage, and blinking frequency.

Photographic methods to record changes in eye redness during exposure to n-decane (Kjaergaard et al., 1989), n-butyl acetate (Iregren et al., 1993) and tobacco dust (Kjaergaard and Pedersen, 1989) have been reported. The mechanism of a possible increase in eye-redness may be chemical irritation to the mucous membranes in the eye. In the present study, the eye redness of two of the 12 subjects in the 100 ppm condition was more intense post-exposure than pre-exposure. This was also the case for one subject in the 150 ppm condition. However, in the 0 ppm (control) con-

dition the eye redness of four subjects was more intense post-exposure than pre-exposure. In addition, it was observed that in the 0, 100 and 150 ppm conditions the eye redness of respectively nine, four and eight subjects was more intense pre-exposure than post-exposure. Statistical analyses found no significant differences in (increased) eye redness between exposure conditions.

Measurement of lipid layer thickness on the cornea has been used in a study of exposure to n-butyl acetate (Iregren et al., 1993). In the present study, on an individual level no change or only a slight increase or decrease of the corneal thickness (up to $\pm 2.4\%$) was observed but no statistically significant treatment effects were found.

Tear film break-up time (BUT), which is dependent on the mucin layer in the eye, has been used in studies of exposure to n-decane (Kjaergaard et al., 1989), n-butyl acetate (Iregren et al., 1993), indoor climate problems (Franck, 1986), and exposure to man-made fibres (Stockholm et al., 1982). In this study, statistical analyses on the BUT-scores for the left and right eye did not indicate an effect of treatment. The magnitude of BUT in the present study (mean: 22–25 s) was comparable to that reported in other investigations (e.g. Lemp and Hamill, 1973; Kjaergaard et al., 1989; Iregren et al., 1993; Franck, 1986).

Measurements of conjunctival epithelial damage (CED) has been used in studies of exposure to n-butyl acetate (Iregren et al., 1993), man-made fibres (Stockholm et al., 1982), and indoor climate problems (Franck, 1986). The baseline CED was borderline (i.e. 12 dots) in only one subject. Five subjects did not show any stained dots. The remaining six subjects showed limited staining (up to 4 dots). After each of the three exposure conditions (0, 100 and 150 ppm), no abnormal CED (>50 dots) were recorded in any of the 12 subjects. Borderline CED (10–50 dots) was recorded in respectively four, three and two subjects. The other subjects showed no or limited staining (up to 8 dots). Statistical analyses on the CED-scores for the left and right eye did not indicate an effect of treatment.

The effect of PGME vapour on the blinking reflex, mediated by the trigeminal cranial nerve endings on the epithelium of the eye, was measured by making videotape recordings of the subjects' face and counting the number of blinks during playback. This method was successfully used by Iregren et al. (1993) in an exposure study with n-butyl acetate. In the present study, individual blinking frequencies (BFs) varied considerably between the 12 subjects prior to and during the three exposure conditions (1–59 blinks/min). Per subject the BFs varied to a lesser extent. Statistical analyses indicated that there was no effect of PGME vapour on blinking frequency.

In the present study no significant treatment effects for any of the objective parameters were found. With respect to the subjective ratings, the answers indicated some very slight effects on the eyes and nose. Eye effects were reported in the 150 ppm PGME condition. The response to the question 'do you feel effects on the eyes' were rated higher during the 150 ppm condition (mean = 1.92) than during the 0 ppm condition (mean = 1.31). In general, these means are very low, since the subjects were asked to indicate the possible effects on a 7-point scale ranging from 1 (not at all) to 7 (extremely). The response to the question 'do you feel effects on the nose' were both rated higher during the 150 ppm conditions (mean = 2.00) and 100 ppm condition (mean = 1.81) than during the 0 ppm condition (mean = 1.06). It is likely that the reported effects on the nose were directly influenced by reporting of the strength of smell for some subjects, reflecting the design of the question ('do you feel *effects* on the nose?'). This view is supported by an additional analysis of covariance using the reported smell-strength as covariate, when no significant effect on the nose was apparent ($F = 3.28$; $P = 0.063$). It is therefore argued that the perceived effects are related to smell, rather than irritation. With respect to the remaining questionnaire items, no significant effects for treatment were found.

In conclusion, the results indicated minimal subjective eye effects at 150 ppm only, but no impact on objective measures of eye irritation at either of the two exposure levels. It was, therefore, concluded that 150 ppm PGME vapour was not irritating to the eyes in human volunteers.

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Review

Physiological and psychological approaches to chemosensory effects of solvents

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Abstract

Workplace related standard settings for solvents are based in a remarkable extent on information about sensory irritations. However, data from controlled human exposure studies are seldom available. Therefore, the aim of this study was to present the association of self-reported symptoms and physiological processes leading to sensory irritations. Three series of laboratory experiments each with 24 young male subjects were performed. Ethyl benzene (EB), 2-butanone (methyl ethyl ketone or MEK), isopropyl alcohol (IPA), 1-octanol (OCT), and 2-ethylhexanol (EHEX) were investigated in low and high concentrations. Ratings for sensory irritations (eyes and nose), olfactory symptoms, and annoyance were assessed repeatedly before, during and after the 4-h-exposures. The anterior active rhinomanometry (AAR) was employed measuring the nasal flow. The nasal lavage was used for the analysis of the neuropeptide substance P as indicator of nasal chemosensory irritations. Goodness-of-fit was calculated for non-linear regression analyses by fitting the sine function on the data of the ratings given during the 4-h-exposure. In general, ratings for annoyance and odor symptoms were fitted on a higher level than those for sensory irritations. However, a high fit could be shown for nasal irritations due to EHEX. In these experiments, a significant reduction of the nasal flow and a significant increase of substance P could be proved.
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1. Introduction

Recent reviews (Dick and Ahlers, 1998; Edling and Lundberg, 2000) revealed the significance of sensory irritations for the regulation of hazardous chemicals. Accordingly, up to 40% of the solvents at workplaces are regulated by the responsible

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national agencies to avoid sensory irritation. In contrast to this strong impact a precise definition of sensory irritation as well as standardized methods for their assessment are lacking. Based on the extensive work done by Cain and Cometto-Muñiz (e.g. Cometto-Muñiz and Cain, 1990, 1995, 1998; Cometto-Muñiz et al., 1998) there is evidence that humans detect chemicals, and especially volatile organic compounds (VOCs), via the olfactory nerve and those parts of the trigeminal nerve that innervate the nasal, ocular, and anterior oral mucosae (Cometto-Muñiz et al., 1998). Stimulation of the latter system is usually called sensory irritation, while odors are perceived via olfaction. For many substances humans are not able distinguish between odor and irritation (Feron et al., 2001). Nevertheless, this distinction is important for the evaluation of adverse health effects associated with the chemoreception of chemicals. Whereas odors are considered as a sensory aspect of annoyance, irritation may in some cases reach the significance of a clinical sign (Cometto-Muñiz et al., 1998). Moreover, Paustenbach et al. (1997) suggested that avoiding sensory irritations caused by acute exposure to formaldehyde reduces the possibility that it causes carcinogenic effects.

Due to the interaction of the two chemosensory systems subjective ratings should point very precisely on the different sensations associated with olfactory and trigeminal stimulation. The symptom-based questionnaire 'acute symptoms' from the neurobehavioral test battery Swedish Performance Evaluation System (SPES) is the only differentiated method available in standardized test batteries used in occupational neurotoxicology (Iregren, 1998). However, the initial SPES version included only a small number of chemosensory symptoms (e.g. running nose, watering eyes). By combining questions from validated questionnaires (Franck and Skov, 1991) with questions on sensations clearly associated with trigeminal stimulation (e.g. itching, burning; Hummel, 2000) the current SPES version (Seeber et al., 2002) asks very precisely about olfactory symptoms, nasal irritations, and eye irritations. However, self-reported chemosensory symptoms should be supported by objective methods assessing irritations of the eyes and the nose. Biochemical indicators of inflammatory processes might be an objective source of information about the irritative potency of a substance. For example, occupational exposures to fuel-oil ash (Woodin et al., 1998) and environmental exposures in newly painted buildings (Wieslander et al., 1999) can influence the content of different cells (e.g. neutrophils, eosinophils, epithelial cells) as well as the cellular mediators (e.g. IL-8, ECP, MPO, lysozyme, albumine) in the nasal fluid. In addition, stimulation of peripheral c-fiber neurons of the trigeminal nerve might initiate neurogenic inflammation indicated by the release of neuropeptides (e.g. substance P) into the nasal fluid (Bascom et al., 1997). Swelling of the intranasal mucosa, associated with inflammatory processes (Meggs, 1993), might reduce the nasal air flow after exposure to irritants. Anterior active rhinomanometry (AAR) has been used to evaluate swelling of the mucosa in the nasal cavity after controlled exposure to chemicals (Lundqvist et al., 1992).

However, there is a lack of controlled exposure studies in humans investigating associations between sophisticated subjective methods and physiological measurements of sensory irritations. Therefore, the aim of the present study is the investigation of associations between psychological variables assessing chemosensory effects on one hand, and physiological markers of sensory irritations on the other hand. Especially the simulation of workplace conditions is intended which includes several hours of exposure and changing levels of exposure.

2. Methods

The experiments have been conducted in the exposure laboratory of the Leibniz Research Center for Working Environment and Human Factors. It is a secluded room built of glass and stainless steel with spatial dimensions of $4.80 \times 2.65 \times 2.27$ m (≈ 29 cbm). Inside the lab there are four PC workplaces with 15 in color computer monitors and response panels for neurobehavioral testing, and four subjects can be exposed simultaneously.

From an adjacent room a climate control unit provided the lab with tempered and humidified air. During exposures the relative humidity ranged between about 35 and 40%, temperature varied from 23 to 25 °C. The average air exchange rate during the experiments was approximately 10.5 times per h.

By means of an ultrasonic vaporizer or a heater defined amounts of the solvents investigated can be added to the airflow. As a function of the exposure duration (t) the respective solvent concentration (c) can be described by the following equation: $c = A_0 + a \sin(2\pi t/60 + 1.5)$, A_0 being the average solvent concentration and a being the difference from the average to the intended maximum concentration (i.e. short-term exposure limit; STEL). According to the regulation of STEL exposures in Germany, the temporal parameters cycle duration and phasing were set to 60 min and 1.5. The session started with a concentration maximum and lasted for four complete cycles. Air samples were taken every 2.5 min and analyzed by gas chromatography using flame ionization detection (GC-FID).

2.1. Substances

In experiments the organic solvents ethyl benzene (EB), 2-butanone (methyl ethyl ketone, MEK), isopropyl alcohol (IPA), 1-octanol (OCT), and 2-ethylhexanol (EHEX) were used. Experiment I investigated low and high concentrations of EB and MEK. Experiment II examined low and high concentrations of IPA and OCT. In both experiments, the concentrations of low exposure conditions were kept constant and corresponds on average to the respective odor threshold of the substances. The high concentrations match the current German exposure limits (MAK, maximum workplace concentration). Experiment III used EHEX in three concentrations (low, moderate, and high), all below the current German MAK (50 ppm). The conditions are described in Table 1.

The subjects were exposed according to a crossover design with repeated exposures and permutation of the respective conditions. Thus, in the experiments I and II each subject completed four exposure sessions, and in the experiment III three sessions. An interval of at least 2 days between successive sessions was strictly adhered to. Embedded in 1/2-day sessions of approximately 6 h, the net exposure time in the lab was 4 h. Four subjects were exposed simultaneously. Before and after these exposure periods various physiological measurements were performed. Prior to the experiments, the subjects passed a 1-day training to become familiar with all requirements.

2.2. Subjects

After a medical examination each experiment consisted of 24 male participants. Most of them were students from the University of Dortmund. The mean age of the subjects was 26.04 years (S.D. = 4.58 years) in experiment I, 25.83 years (S.D. = 4.26 years) in experiment II, and 23.96 years (S.D. = 3.71 years) in experiment III. Each participant signed a declaration of consent to the conditions of the experiment. The experiments were accepted by the responsible ethic commission.

2.3. Ratings of chemosensory exposure effects

Three dimensions of well-being, tenseness (relaxed vs. stressed), tiredness (awake vs. tired), and annoyance (not annoying vs. very annoying) were assessed by a validated scaling procedure (Seeber et al., 1997). Together with seven-point visual rating scales the keywords of the dimensions were presented on the computer screen. Ratings were given by means of a numeric pad on a response panel (range: 1–7).

Acute health symptoms were assessed with an extended version of the test 'Acute Symptoms' from the SPES (Iregren, 1998). The initial SPES version was extended with 12 symptoms related to the olfactory system, to nasal irritations, and eye irritations (Seeber et al., 2002). Altogether, 29 symptoms were successively presented on the computer screen, and their severity was evaluated by ratings ranging from 0 (not at all) to 5 (very, very much) also given by means of a numeric response panel. Symptoms of similar type (e.g. stink, unpleasant odor) were grouped and their

Table 1
Average, minimum and maximum concentrations of the 11 exposure conditions used in the three experiments

Experiment	Substance	Condition	Concentration (ppm)		
			Mean	Minimum	Maximum
I	EB	Low	10.28	8.61	11.14
		High	98.30	8.10	188.20
	MEK	Low	9.58	8.23	9.89
		High	189.40	9.49	379.43
II	IPA	Low	34.90	33.20	35.50
		High	189.90	37.00	356.80
	OCT	Low	0.105	0.096	0.113
		High	6.40	0.40	12.50
III	EHEX	Low	1.53	1.39	1.58
		Moderate	10.63	1.23	20.20
		High	21.88	1.76	42.07

respective means were calculated. These symptom groups were: pre-narcotic (4 symptoms), olfactory (4), symptoms of bad taste (3), respiratory (3), nasal irritations (5), eye irritations (7), and other irritations (3).

Since the present study focuses on chemosensory exposure effects annoyance, nasal irritations, eye irritations and, a combined score of nasal and eye irritations (sensory irritations) were analyzed. Ratings of well-being and acute symptoms were given 50 min before exposure onset (baseline), nine times during the exposure (1, 26, 59, 85, 129, 145, 173, 199, and 232 min after exposure onset), and 52 min after exposure offset. The assessment of the ratings during the exposure period corresponded to the minima and maxima of the changing concentration.

2.4. Anterior active rhinomanometry (AAR)

During all experiments AAR was performed before and after the exposures. AAR is a method for quantitative measure of nasal airway resistance. Nasal airflow and transnasal pressure gradient between the nostrils and the epipharynx are measured simultaneously with a computer-based system (Atmos Inc., Lenzkirch, Germany). The flow volume is measured by ring diaphragm spiroceptor, which is adapted to the test person by a half-mask. For anterior pressure measurement one of two different adapters was connected to the nostril according to the size of the individual nostril and without any deformation of the nasal lobule. Flow volume and pressure gradient were calculated from respiratory cycles recorded over 30 s by the computer-based system. To reduce artifacts the data were checked by the so-called CAR (computer aided rhinomanometry) from Bachert and Feldmeth (1988). AAR was performed immediately before and after the exposure period.

2.5. Biochemical parameters from nasal secretion (nasal lavage)

In experiment III, nasal lavages were performed 30 min before and immediately after the exposures. Therefore, a sterile pipette (NUNC™) filled with 10 ml of phosphate-buffered-saline (PBS; prewarmed) was adapted successively to the left and right nasal cavity and 5 ml of the PBS was inserted into each cavity. The PBS was left in the nose for 10 s and subsequently subjects let the lavage fluid flow into funneled 15 ml PP-test tubes without sniffing. The volume of the lavage fluid recovered after the nasal lavage was recorded. Immediately after the lavage the samples were stored on ice and within the next 30 min frozen at −80 °C. Analysis of substance P was performed by means of ELISA kits from Cayman Chemical (Ann Arbor, MI, USA; local dealer: IBL-Ham-

burg, Nr. CM59211) with a detection level of 7.0 pg/ml.

2.6. Statistics

Non-linear regression fitting the sine functions $[f(t) = A_0 + a \sin(2\pi t/60 + 1.5)]$ on both, the 96 air samples measured during each exposure session and the nine ratings obtained during these periods, were performed. All regression analyses estimated the two descriptive parameters A_0 and a and the adjusted explained variance (R^2) was calculated to express the goodness-of-fit.

Exposure-related effects of the physiological measurements were analyzed non-parametrical with Wilcoxon-Tests. Two comparisons were calculated: (a) pre- and post-measurements of the physiological methods were compared to estimate the strength of the exposure effect within the experimental condition, and (b) standardized difference scores (pre- minus post-measurement) of the dependent variables were used to compare the different concentrations within the experiments.

The associations between the physiological indicators of sensory irritations and the ratings were expressed as Spearmans rank correlation coefficients calculated for the standardized difference scores. The standardized difference scores of the subjective ratings were calculated by subtracting the pre-exposure score from the mean of the nine ratings obtained during the exposure period. All analyses were performed with SPSS 10.0 (1999).

3. Results

The analyses of the air samples taken during the high and moderate exposures of the three experiments revealed adjusted R^2 ranging from 0.85 to 0.96. The values were $R^2 = 0.96$ (EB_{high}), $R^2 = 0.93$ (MEK_{high}), $R^2 = 0.91$ (IPA_{high}), $R^2 = 0.85$ (OCT_{high}), $R^2 = 0.96$ ($EHEX_{moderate}$), and $R^2 = 0.96$ ($EHEX_{high}$). Thus, the intended lapses of concentrations could be confirmed with high accuracy. Low exposures were realized on a constant level without necessity for adjusted R^2.

3.1. Exposure-related effects—rating scales

Using the same temporal parameters (cycle duration: 60 min and phasing: 1.5), the non-linear regression analyses revealed that chemosensory symptoms and annoyance were differently affected by the different substances. Table 2 shows the estimated parameters A_0, a, and the explained variance R^2 for the high and moderate exposure conditions of the experiments.

The average level of the ratings, expressed in the parameter A_0, differed markedly between the different symptom scores. The ratings of sensory irritations (range 0–5) amounted only to one-third of the ratings obtained for olfactory symptoms. This ratio was lower during the EHEX exposures. The level of annoyance ratings (range 1–7) was close or even above the scale mean of 3.5. The highest annoyance was reported during the experiment investigating EB and 2-butanone (MEK). IPA yielded the weakest ratings for all subjective variables.

Regarding the variation of the ratings across the nine assessments, expressed in the parameter a, Table 2 shows nearly no variation for the sensory irritations during the high exposures to EB, 2-butanone, IPA and OCT. In contrast, the two EHEX exposures provoked variations of these symptoms, especially during the high exposure. Similarly, the goodness-of-fit (R^2) for sensory irritations is above 0.50 only for these two conditions. The time-course of the sensory irritations is describable with a sine function similar to the lapse of concentrations of EHEX. Sixty-seven and 80% of the variance of the ratings for sensory irritations during the moderate and high EHEX exposures are explained by the non-linear regression analyses. Considering the weak variation of the sensory irritations during the other experimental exposures, obviously the sine function could not be fitted to the small variability of the perceived sensory irritations.

With respect to the other chemosensory exposure effects, olfactory symptoms and annoyance, the R^2 values for the investigated substances were higher and nearly comparable to each other. With the exception of the olfactory symptoms during OCT exposure the R^2 values indicated that all

Table 2
Parameters and goodness-of-fit estimated by fitting the sine function on the data of the chemosensory ratings obtained during the high/moderate exposures of the three experiments

Exposures	Sensory irritations			Olfactory symptoms			Annoyance		
	A_0	a	R^2	A_0	a	R^2	A_0	a	R^2
EB_{high}	0.71	0.04	0.05	2.42	0.60	0.65	3.93	0.54	0.66
MEK_{high}	0.53	0.08	0.32	2.00	0.98	0.89	3.58	0.94	0.92
IPA_{high}	0.38	0.03	0.19	1.02	0.31	0.57	2.44	0.22	0.68
OCT_{high}	0.53	0.06	0.23	1.75	0.17	0.21	2.99	0.21	0.64
$EHEX_{moderate}$	0.69	0.19	0.67	1.64	0.51	0.73	3.04	0.53	0.73
$EHEX_{high}$	0.99	0.49	0.80	1.87	0.87	0.86	3.23	1.04	0.86
Mean	0.64	0.15	0.38	1.78	0.57	0.65	3.20	0.58	0.75

ratings showed time-courses comparable to the oscillations of the changing concentrations.

Since EHEX showed R^2 values above 0.50 for sensory irritations, the two subordinated aspects nasal and eye irritations were analyzed separately. Fig. 1 shows the ratings of these symptoms across the 240 min of the moderate EHEX exposure and the fitted sine functions.

The reported nasal irritations varied in accordance to the sine function while the eye irritations showed a deviating pattern (Fig. 1). The 95%-confidence interval (95%-CI) plotted in Fig. 1 obviously shows the different residuals of the two symptom areas. While all means observed for the nasal irritations are embedded into small 95%-CI's this intervals are much larger for eye irritations. This difference in goodness-of-fit can be described by the resulting $R^2 = 0.92$ for nasal irritations and $R^2 = 0.17$ for eye irritations caused by the 10 ppm EHEX exposure. The correspond-

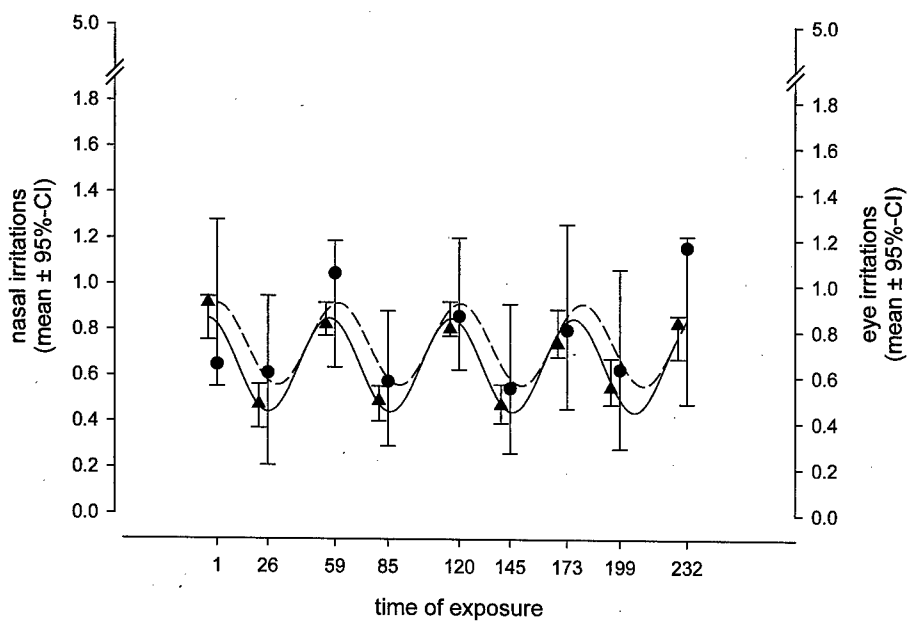

Fig. 1. Time-courses of nasal (▲, solid line) and eye irritations (●, dashed line) during changing exposures to the moderate EHEX concentration and the two sine function fitted on the data.

ing values for the high exposure (20 ppm) to EHEX were $R^2 = 0.91$ for nasal irritations, and $R^2 = 0.54$ for eye irritations.

3.2. Exposure-related effects—physiological measurement

Table 3 shows the pre- and post-exposure values of the AAR. The medians revealed that the average nasal flow decreased in nearly all exposures. The level of significance could be achieved for three exposures: IPA_{low}, OCT_{high}, and $EHEX_{high}$. The results on IPA do not fit exactly into the expected tendency ($P < 0.05$ for the low condition indicates stronger effects than $P = 0.09$ for the high condition). High exposures to OCT and EHEX caused the expected reduction of nasal flow. Low exposures showed a lower statistical significance for the differences analyzed. According to comparison (b) the standardized difference scores of the high and the low exposure concentrations were compared. Higher difference scores at higher exposures indicate dose-dependency. This relation was only significant for the two concentrations of OCT (−5.05 vs. −16.28%; $z = -2.06$, $P = 0.04$).

The results of the pre–post data for the nasal lavage carried out in experiment III are given in Fig. 2. The increase of substance P concentrations during the exposure is stronger for the moderate and high exposures than for the low one. The Wilcoxon-Test revealed significance ($z = -2.61$, $P = 0.01$) for the mean difference of 4.82 pg/ml obtained during high exposure. Comparing the standardized difference scores of the three concentrations, the analyses revealed a significant difference only between the low and the high exposure conditions (1.51 vs. 38.79%; $z = -2.20$, $P = 0.03$).

3.3. Associations between ratings and physiological variables

Spearman rank correlation between the standardized difference scores for the anterior active rhinometry and the 'baseline-corrected' ratings were calculated for all experiments with high exposure conditions. It resulted in a matrix of 24 correlation coefficients (six experiments × four dependent variables indicating the psychological reactions). The majority of coefficients was insignificant. Only two significant associations between flow changes and subjective ratings could be proved. During the high IPA exposure an increase of annoyance ratings and of reported eye irritations was correlated with a decrease of nasal air flow ($n = 22$, $\rho = -0.42$ and -0.45, respectively, $P < 0.05$).

For substance P an analogous approach was possible only for the experiments with ethylhex-

Table 3
Median of the flow-values (150 Pa) obtained before and after the eleven exposures of the experiments and P-values of the respective Wilcoxon-tests

Experiment	Substance	Condition	Flow-value (ml/s)		
			Pre-exposure	Post-exposure	P-value
I	EB	Low	636	656	0.22
		High	788	666	0.22
	MEK	Low	728	678	0.33
		High	692	732	0.18
II	IPA	Low	768	596	0.01
		High	676	628	0.09
	OCT	Low	690	644	0.19
		High	710	602	0.01
III	EHEX	Low	716	620	0.06
		Moderate	694	560	0.09
		High	660	540	0.01

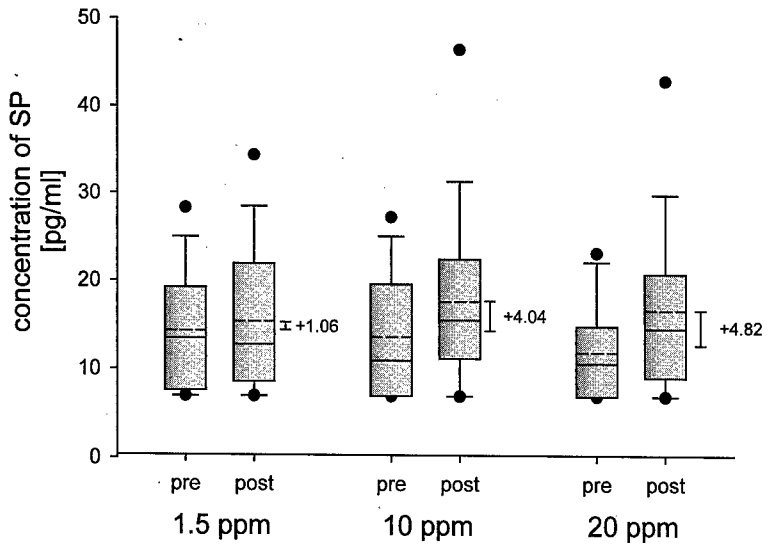

Fig. 2. Boxplot (showing 5th, 10th, 25th, median, mean, 75th, 90th, 95th percentile) of the concentration of substance P in nasal fluid before and after exposures to EHEX.

anol. Out of 12 rank correlations (three concentrations × four dependent variables) only for the low level exposure of 1.5 ppm the association between substance P and the nasal irritation reached significance ($n = 24$, $\rho = 0.45$, $P < 0.05$).

Thus, on the individual level, neither for the nasal flow nor for the neuropeptide substance P, remarkable and clear reproducible associations to the psychological variables could be detected.

4. Discussion

4.1. Odor and annoyance

The study revealed that the ratings of annoyance and partly those of the olfactory symptoms reflect sufficiently the time-course of simulated concentrations exploiting the respective STELs of the solvents. Thus, in a dynamic exposure approach, only the psychological indicators of olfactory stimulation showed sequences of dose–response relationships. This observation did not apply to OCT. Exposure to this long-chain alcohol yielded a level of olfactory symptoms that is comparable to those of the 2-ethylhexanol exposures. However, due to its weak variation across time, the goodness-of-fit is insufficient. This substance-related difference might be related to the low odor threshold of 0.1 ppm (Ruth, 1986) of OCT. The slope of its psychophysical function indicates a very intense odor (Patte et al., 1975), and thus, changing concentrations might not affect the perceived intensity.

The annoyance ratings, which aside from sensory stimulation also covered an affective component (Dalton et al., 2000) showed satisfactory results in the non-linear regression analyses. Subjects reported high levels of annoyance during exposures to EB and 2-butanone (MEK). The American and the German TLV committees consider the avoidance of annoyance as an important goal for the limit setting (DFG, 2001; ACGIH, 2001). Therefore, these results might be beneficial for 'limit setting'. The annoyance levels of the other substances were below the scale mean of the rating scale. Generally, the evaluation of annoyance ratings with respect to possible adverse effects remains problematical and further research is needed.

4.2. Sensory irritations and subjective effects

Both the level and the intra-session variation of provoked sensory irritations were low in the first experiments. Especially the IPA exposure yielded

nearly no temporal variation of these ratings. Thus, in accordance with a recent study (Smeets et al., 2002), exposure to IPA up to 400 ppm did not provoke sensory symptoms, and thus, the German MAK value is sufficient to protect workers from this adverse exposure effect. Furthermore, the results obtained for EB and 2-butanone yielded no evidence of acute exposures to TLV concentrations provoking sensory irritations on a subjective level.

Since the average concentration of 6.4 ppm for OCT was very low (12.8% of American WEEL of 50 ppm) and the results showed ratings of sensory irritations as high as those given during TLV exposure to 2-butanone, this occupational exposure limit seemed doubtful.

The results of the third experiment indicated a strong irritative potency of EHEX. Airborne concentrations of 20 and 40% of the current German MAK value (50 ppm) were able to elicit stronger reports of sensory irritation than the other substances investigated in this study. Additionally, the non-linear regression analyses yielded evidence that these ratings showed a strong dose–response relationship.

4.3. Sensory irritations–objective effects

The experiments I and II revealed no effects of the investigated substances on the nasal flow, except for OCT. There was a reduction of the nasal flow in nearly all experiments, indicating that some climate factor of the exposure lab might influence the measurements. The effect of a stronger flow increment of the high concentration observed for OCT has to be evaluated carefully. As reported in the results, the same subjects showed comparable flow increments during exposures to 35 ppm of IPA. This result is in contrast to the possible dose–response relationship when the results for OCT were evaluated individually.

The results of experiment III are to some degree in accordance with a dose–response relationship. The reduction of nasal flow increases with higher concentrations of EHEX. However, statically the dose-dependency could not be confirmed. Regarding the amount of reduction, the value of the high 2-ethyhexanol exposure did not differ markedly from the other exposure scenarios.

Generally, the application of such rhinological methods in experimental exposure studies is problematic. The results of recent studies have shown no exposure-related effects in these techniques for acute solvent exposures (Smeets et al., 2002; Wiesmüller et al., 2002). Thus, the effect of the inhaled solvent on the nasal mucosa might be too small or the duration of the exposure might be insufficient. Therefore, not the functional level but the biochemical level should be studied more carefully.

The results of experiment III supported this suggestion. At the biochemical level, stronger effects were produced by higher concentrations of EHEX. Nevertheless, since this study is one of the rare studies using biochemical markers of irritations after solvent exposure, the evaluation of this effect is difficult. Nasal provocation with hypotonic saline (2.7, 5.4, 10.8 and 21.6% NaCl) causes a dose-dependent increase of substance P (Baraniuk et al., 1999). Also the challenge with bradykinin in allergic individuals shows a dose-dependent increase of substance P release (Baumgarten et al., 1997). However, these studies did not report the difference between the conditions numerically, and thus, a comparison to our results is impossible. Furthermore, the substances investigated are chemically unrelated to the solvents investigated in our study.

Thus, the result of experiment III has to be replicated, and within such a study additional, standardized provocation should be incorporated to 'benchmark' the results of the solvent exposures.

4.4. Association of subjective and objective effects

Hardly any associations between subjective and objective methods assessing chemosensory effects were revealed in our experiments. These negative results might be associated with differences regarding the temporal resolution of the methods. Subjective ratings were assessed nearly continuously, and even small variations of the solvent concentrations were reflected by the time-course of the ratings. Thus, a fine-tuned psychological

measurement was correlated with a method based on two rough assessments. Furthermore, the subjects were perhaps not aware of functional and biochemical changes in the nasal cavity, and their rating behavior might depend on other information coming from physiological sources. Thus, objective measurements of the reactions of the eyes (e.g. eye blinks), probably possessing higher awareness, might be more capable to show an association between objective and subjective exposure effects.

4.5. Concluding remarks

In this study convergent validity between the subjective and objective markers of chemosensory stimulation could not be achieved on the individual level. However, the results in the EHEX experiments show that specific psychological reactions—self-reported nasal irritations—can be observed in relation to physiological reactions proved by specific methods which indicate different processes of chemosensory irritations. The results might contribute to improve the risk assessments for OCT and EHEX.

Acknowledgements

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Effects of olfactory function, age, and gender on trigeminally mediated sensations: a study based on the lateralization of chemosensory stimuli

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Abstract

The present investigation aimed to compare trigeminal nasal function of anosmic and hyposmic patients to healthy controls. Further, we aimed to study effects of age and gender on trigeminally mediated sensations following intranasal chemosensory stimulation. Participants were 35 patients with olfactory dysfunction ($n = 13$: functional anosmia; $n = 22$: hyposmia; age 28–69 years, mean age 56 years). Their results were compared with 17 normosmic subjects (28–82 years, mean 52 years). To analyze effects of age and gender in healthy subjects, an additional 24 healthy subjects were included (19–27 years; mean 24 years). Olfactory function was assessed using the 'Sniffin' Sticks' test kit (butanol odor threshold, odor discrimination, odor identification). The subjects' ability to lateralize odors was investigated for benzaldehyde and eucalyptol. Patients with olfactory dysfunction had lower scores in the lateralization task than controls ($P < 0.001$) indicating decreased trigeminal sensitivity. Among anosmic patients scores were not different in relation to different causes of olfactory dysfunction ($P > 0.29$). There was a weak, but significant, correlation between localization of eucalyptol and duration of olfactory dysfunction ($P = 0.017$). When investigating normosmic subjects only, no gender-related difference was apparent for lateralization scores. However, older subjects had lower scores than younger ones ($P < 0.01$). Results of partial correlational analyses controlling for age suggested a relation between the trigeminal and the olfactory systems. In conclusion, results of the present study indicate that patients with olfactory dysfunction have lower trigeminal sensitivity compared with normosmic controls. This seems to be independent of the cause of olfactory loss. The deficit appears to improve with duration of the olfactory dysfunction, possibly indicating adaptive mechanisms. Further, the data suggest an age-related decrease of intranasal trigeminal sensitivity in healthy subjects. Analyses additionally indicate a correlation between olfactory and trigeminal sensitivity.
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Keywords: Nociception; Chemosensory; Olfaction; Irritation; Multisensory integration

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1. Introduction

Most odorants produce not only olfactory but also trigeminally mediated sensations (Elsberg et al., 1935; Fröhlich, 1851; von Skramlik, 1926). Numerous reports indicate to the role of the trigeminal intranasal chemosensory systems in the perception of odors. For example, it has been shown that at certain concentrations trigeminal activation may suppress olfactory mediated sensations (Cain and Murphy, 1980). On the other hand, research also indicates that olfactory activation increases sensitivity to trigeminally mediated stimuli (Kobal and Hummel, 1988a; Livermore et al., 1992). This has been shown on an electrophysiological level where patients with anosmia exhibit smaller response amplitudes of the EEG-derived event-related potential to trigeminal stimuli (Hummel et al., 1996). On a behavioral level, respiratory reflexes to intranasal trigeminal stimuli have been reported to be significantly faster and stronger in normosmic subjects compared with anosmic subjects (Kendall-Reed and Walker, 1998). Clinical observations (Kobal and Hummel, 1988b; Van Toller, 1999) indicate that olfactory loss is frequently accompanied by a decreased responsiveness to trigeminal stimulation (compare Gudziol et al., 2001).

However, these changes of trigeminal sensitivity in relation to olfactory dysfunction are still a matter of debate (e.g. Cometto-Muniz et al., 1998). Thus, the investigation aimed to compare trigeminal nasal function of anosmic and hyposmic patients to healthy controls. Further, we aimed to study effects of age and gender on trigeminally mediated sensations following intranasal chemosensory stimulation.

2. Materials and methods

The study was performed according to the Declaration of Helsinki (WMA, 1997) on guidelines for biomedical research involving human subjects. All subjects/patients provided written consent after they were thoroughly acquainted with all details of the investigation. All testing was performed in well ventilated rooms.

2.1. Study participants

Participants were 35 patients with olfactory dysfunction (13 patients with functional anosmia; 22 hyposmic patients; age range 28–69 years, mean age 56 years). In 18 patients olfactory dysfunction followed infections of the upper respiratory tract, in two patients it was caused by sino-nasal disease, and in eight patients it was due to head trauma; an additional seven patients had idiopathic olfactory dysfunction. Diagnosis was based on extensive investigations performed at the Smell and Taste Clinic of the Department of Otorhinolaryngology, University of Dresden Medical School, which included a detailed history, state-of-the-art ENT-examinations, and imaging of the head. The patients' results were compared with 17 normosmic subjects (age range 28–82 years, mean age 52 years). Dysosmic patients and normosmic controls did not differ significantly in terms of age ($t = 0.78$, $P = 0.44$) or gender (Mann–Whitney test: $Z = 1.30$, $P = 0.19$). In order to analyze effects of age and gender in healthy subjects, an additional 24 young, healthy subjects were studied (age range 19–27 years; mean age 24 years; eight women, 16 men).

2.2. Assessment of trigeminal sensitivity

Trigeminal sensitivity was assessed using an experimental design described previously (e.g. Berg et al., 1998; Kobal et al., 1989; Roscher et al., 1996) where trigeminal activation is quantified by the subjects'/patients' ability to lateralize stimuli presented to either the left or the right nostril. Based on previous studies benzaldehyde and eucalyptol were used for the odor localization paradigm (Berg et al., 1998; Doty et al., 1978). The two odorants were presented to either one nostril in a high density polyethylene squeeze bottle (total volume 250 ml) filled with 30 ml of the odorant; at the same time an identical bottle filled with 30 ml of odorless propylene glycol was presented to the contralateral nostril. The bottles had a pop-up spout that was placed into either nostril. A puff of approximately 15 ml air was delivered by pressing the two bottles at the same time by means of a hand-held squeezing device.

The subjects held onto the spouts to prevent movements, which might accompany squeezing of the bottles; movement of the spouts might produce mechanical irritation which, in turn, might interfere with the subject's ability to localize the odor. A total of 40 stimuli were applied to the blindfolded subjects/patients at an interstimulus interval of approximately 30 s; stimulation of the left or right nostril followed a pseudo-randomized sequence. After each stimulus subjects/patients were asked to identify the nostril where the odorant had been presented. The sum of correct identifications was used for further statistical analyses (Berg et al., 1998). Testing of each odorant required approximately 30 min. To allow for recovery of the nasal sensitivity the two tests were separated by an additional interval of approximately 30 min.

2.3. Assessment of olfactory sensitivity

All of the participating subjects/patients underwent olfactory testing using the 'Sniffin' Sticks' test kit (Hummel et al., 1997a; Kobal et al., 2000). Odorants were presented in odor dispensers similar to felt-tip pens. The pens had a length of approximately 14 cm, and an inner diameter of 1.3 cm. Instead of liquid dye the tampon was filled with 4 ml of liquid odorants. For odor presentation the cap was removed by the experimenter for approximately 3 s and the pen's tip was placed approximately 2 cm in front of both nostrils. Testing involved assessment of *n*-butanol odor thresholds, odor discrimination, and odor identification. Odor thresholds were measured using a single-staircase, triple-forced choice procedure. Sixteen dilutions were prepared in a geometric series starting from a 4% *n*-butanol solution (dilution ratio 1:2 in aqua conservata as diluent). Three pens were presented in a randomized order, with two containing the solvent and the third the odorant at a certain dilution. The subject's task was to identify the odor-containing pen. Triplets were presented at intervals of 20 s. Reversal of the staircase was triggered when the odor was correctly identified in two successive trials. Threshold was defined as the mean of the last four out of seven staircase reversal points. In the odor discrimination task, 16 triplets of pens were presented. In each triplet two pens contained the same odorant, while the third pen had a different smell. Subjects had to determine which of three pens smelled different. The presentation of triplets was separated by 20–30 s. The interval between the presentations of individual pens of a triplet was approximately 3 s. Odor identification was assessed by means of 16 common odors. Using a multiple choice task identification of individual odorants was performed from a list of four descriptors. The interval between odor presentations was 20–30 s. In each of the three olfactory tests the subjects' scores ranged from 0 to 16. When measuring odor thresholds and odor discrimination, subjects were blindfolded to prevent visual identification of some of the odorant-containing pens. In order to categorize olfactory function in terms of functional anosmia, hyposmia, and normosmia, the sum of the three scores for odor thresholds, odor discrimination, and odor identification was used ('TDI score') (Kobal et al., 2000).

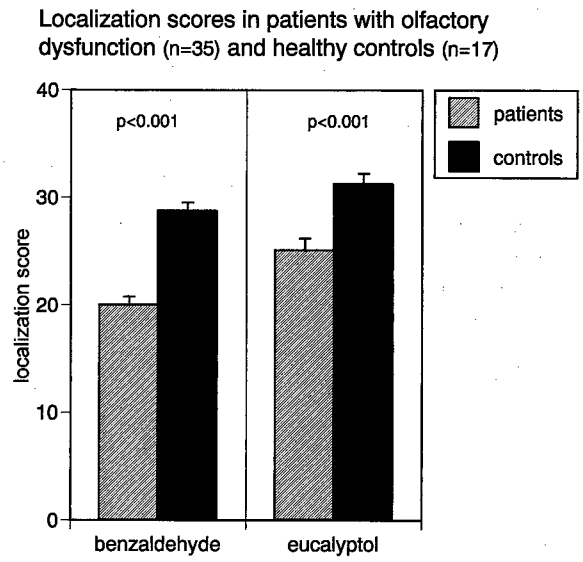

Fig. 1. Localization scores in patients with olfactory dysfunction ($n = 35$) and healthy controls ($n = 17$) (means, S.E.M.) shown separately for eucalyptol and benzaldehyde. When compared with normosmic controls patients had significantly smaller lateralization scores for both odorants ($P < 0.001$) indicating decreased trigeminal sensitivity.

2.4. Statistical analyses

Results were analyzed by means of SPSS 10.0 for WINDOWS™. To compare results from patients with different causes of olfactory dysfunction analyses of variance were employed ('diagnosis' as between-subject factor). For comparisons between patients and controls data were submitted to t-tests for independent samples. Spearman statistics were used for correlational analysis. The alpha level was set at 0.05.

3. Results

3.1. Effects of olfactory dysfunction of odor lateralization

When compared with healthy subjects patients with olfactory dysfunction had significantly lower scores in the odor lateralization task (eucalyptol: $t = 4.05$, $P < 0.001$, benzaldehyde: $t = 6.60$, $P < 0.001$) (Fig. 1). Scores in the odor lateralization task were not significantly different in relation to different causes of olfactory dysfunction (olfactory dysfunction following infections of the upper respiratory tract [$n = 18$], head trauma [$n = 8$], and idiopathic olfactory dysfunction [$n = 7$]; due to the small sample size results from the two patients with sino-nasal disease were not included in this analysis) ($F[2, 30] < 1.28$, $P > 0.29$) (Table 1). Interestingly, following removal of one outlier with a duration of disease of 61 years, in patients with olfactory dysfunction there was a weak, but significant, correlation between localization of eucalyptol and duration of olfactory dysfunction ($r_{34} = 0.41$, $P = 0.017$) (Fig. 2); however, this was not seen for benzaldehyde ($r_{34} = -0.18$, $P = 0.32$).

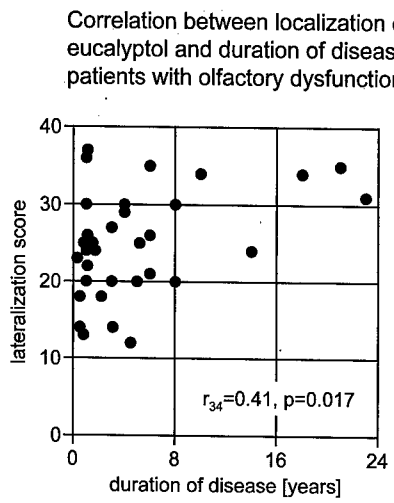

Fig. 2. Correlation between duration of disease and localization scores in patients with olfactory dysfunction ($n = 34$) for eucalyptol. After removal of one outlier with a duration of disease of 61 years, there was a weak, but significant, correlation between localization of eucalyptol and duration of olfactory dysfunction ($r_{34} = 0.41$, $P = 0.017$).

Table 1
Descriptive statistics (means, standards errors of means (S.E.M.)) of characteristics/results in patients with olfactory dysfunction due to the four different causes

Cause/context of olfactory dysfunction	Infection of upper respiratory tract ($n = 18$)		Trauma ($n = 8$)		Sino-nasal disease ($n = 2$)		Idiopathic ($n = 7$)	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Age (years)	58.4	2.0	53.8	5.7	49.0	–	52.9	5.5
Duration of olfactory dysfunction (years)	2.5	0.5	4.1	2.7	7.0	–	11.3	2.5
Odor threshold (dilution steps)	3.3	0.5	2.3	1.3	3.4	–	1.6	0.5
Odor discrimination (correct identifications)	8.4	0.8	6.0	0.9	8.5	–	8.9	1.5
Odor identification (correct identifications)	6.2	0.5	5.3	0.8	6.5	–	7.1	0.8
TDI score	17.8	1.5	13.0	2.6	18.4	–	17.4	1.9
Lateralization score: benzaldehyde	21.3	1.3	19.0	1.5	17.5	–	18.1	1.6
Lateralization score: eucalyptol	24.9	1.8	22.9	1.9	25.5	–	27.7	2.5

Due to the small sample size of patients with sino-nasal disease the S.E.M. is not presented.

3.2. Effects of gender and age in normosmic subjects

When investigating normosmic subjects only, there was no gender-related difference for lateralization thresholds (17 women, 24 men; $t < 0.39$, $P > 0.70$) (Table 2). However, age had an effect in that older subjects exhibited lower lateralization scores compared with younger subjects (subjects younger than 35 years of age: $n = 29$; subjects older than 35 years: $n = 12$; (Table 3) lateralization scores benzaldehyde: $t = 2.96$, $P = 0.008$; lateralization scores eucalyptol: $t = 4.11$, $P < 0.001$). The influence of age was further supported by the presence of negative correlations between lateralization scores and the subjects' age (benzaldehyde: $r_{41} = -0.39$, $P = 0.011$; eucalyptol: $r_{41} = -0.53$, $P < 0.001$) (Fig. 3).

3.3. Correlation between olfactory function and lateralization scores

Correlational analyses between olfactory function and lateralization scores were performed in all investigated subjects ($n = 73$). Using partial correlations age was controlled, because it significantly affects both olfactory and trigeminal sensitivity (Hummel et al., 2002). With the exception of eucalyptol lateralization and odor discrimination all correlations were significant ($0.59 > r_{73} > 0.31$, $P < 0.006$) indicating a close relation between the two chemosensory systems (Table 4).

Table 2
Descriptive statistics (means, S.E.M.) of characteristics/results in normosmic subjects, separately for men ($n = 24$) and women ($n = 17$)

	Gender	Mean	S.E.M.
Age (years)	Female	39.6	4.8
	Male	33.2	3.3
TDI score	Female	34.0	1.1
	Male	34.8	0.7
Lateralization score: benzaldehyde	Female	30.5	1.0
	Male	30.0	0.6
Lateralization score: eucalyptol	Female	33.2	0.9
	Male	32.8	0.8

Table 3
Descriptive statistics (means, S.E.M.) of characteristics/results in normosmic subjects, separately for subjects younger than 35 years of age ('young', $n = 29$) and subjects older than 35 years of age ('old', $n = 12$)

	Age group	Mean	S.E.M.
Age (years)	Young	25.4	0.5
	Old	61.0	3.6
TDI score	Young	35.1	0.7
	Old	32.8	1.0
Lateralization score: benzaldehyde	Young	31.2	0.6
	Old	27.8	0.0
Lateralization score: eucalyptol	Young	34.2	0.5
	Old	29.8	1.2

4. Discussion

Results of the present study indicate that patients with olfactory dysfunction have lower trigeminal sensitivity compared with normosmic controls. Importantly, this seems to be independent of the cause of the olfactory loss, because this deficit is found in patients with olfactory loss following trauma or infection of the upper respiratory tract, and in patients with idiopathic olfactory dysfunction.

The differences between normosmic and anosmic subjects are in line with previous reports. This has been observed clinically (Kobal and Hummel, 1988b; Van Toller, 1999), psychophysically (Gudziol et al., 2001; Walker and Jennings, 1991; Wysocki et al., 1997) behaviorally (Kendall-Reed and Walker, 1998), and with electrophysiological techniques (Hummel et al., 1996). In addition, comparable findings have been obtained in experimental animals (Henton et al., 1969; Silver et al., 1986; Walker et al., 1979).

The observed decrease in trigeminal sensitivity may be based on an interaction between the olfactory and the intranasal trigeminal system. It has been demonstrated in normosmic subjects that trigeminal stimuli are perceived as more intense when they were accompanied by olfactory stimulation (Kobal and Hummel, 1988a; Livermore et al., 1992). This assumption is also supported by the presently observed correlation between olfactory and trigeminal sensitivity. Interactions be-

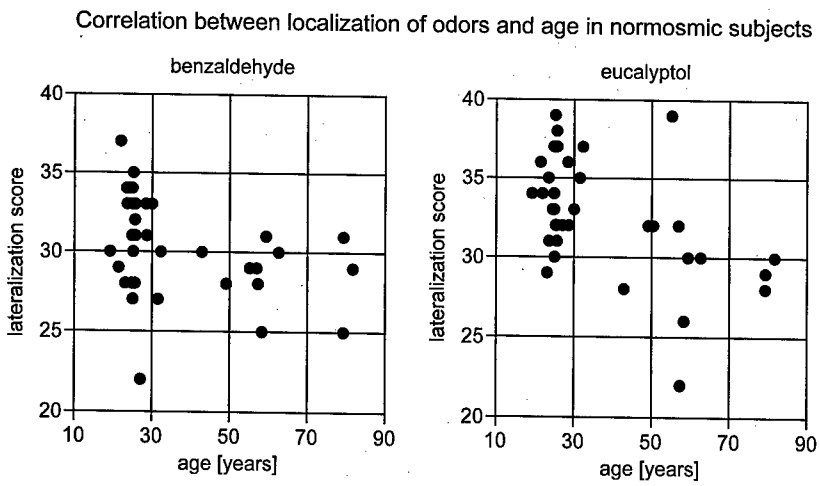

Fig. 3. Correlation between age of normosmic subjects and localization scores ($n = 41$) separately for eucalyptol and benzaldehyde. Benzaldehyde data of one subject are not shown for graphical reasons (age 50 years, lateralization score 19). There were significant correlations between the subjects' age and their ability to localize odors (benzaldehyde: $r_{41} = -0.39$, $P = 0.011$; eucalyptol: $r_{41} = -0.53$, $P < 0.001$).

Table 4
Correlation analyses between olfactory function and lateralization scores including all investigated subjects ($n = 73$)

	Benzaldehyde	Eucalyptol
Butanol odor threshold	$r_{73} = 0.57$	$r_{73} = 0.32$
	$P = 0.001$	$P = 0.005$
Odor discrimination	$r_{73} = 0.40$	$r_{73} = 0.22$
	$P = 0.001$	$P = 0.053$
Odor identification	$r_{73} = 0.53$	$r_{73} = 0.38$
	$P = 0.001$	$P = 0.001$
TDI score	$r_{73} = 0.58$	$r_{73} = 0.37$
	$P < 0.001$	$P = 0.001$

The table shows partial coefficients; age was controlled for as it significantly affects both olfactory and trigeminal sensitivity (Hummel et al., 2002). With the exception of eucalyptol lateralization and odor discrimination all correlations were significant.

tween the two intranasal chemosensory systems may take place in the thalamus (Inokuchi et al., 1993), in higher order of the central nervous system (Hummel et al., 1997b; Stone, 1969; Stone and Rebert, 1970), and at the mucosa where it might be considered that olfactory afferent activity could influence trigeminal input via neurosecretory changes (for review see Hummel and Livermore, 2002). Other reasons for the observed decrease in trigeminal sensitivity may relate to the cause of the olfactory loss. In posttraumatic anosmia one could easily imagine CNS lesions which may affect the processing of trigeminally mediated sensations (Lobbezoo et al., 2002; Sterner et al., 2001). However, because of the missing difference between results obtained in patients with postviral olfactory loss and those with posttraumatic olfactory dysfunction, the present data seem to argue against a specific influence of trauma on trigeminal sensitivity. Future studies in patients with olfactory dysfunction are needed to compare intranasal trigeminal sensitivity to different somatosensory stimuli and, e.g. cutaneous sensitivity in the trigeminal system.

In line with previous work (Wysocki et al., 1997) the present data indicate age-related loss of trigeminal function. Elevated thresholds to trigeminal stimuli have also been reported in elderly subjects (Minz, 1968; Murphy, 1983). At suprathreshold level an age-related decrease of intensity ratings of the (virtually odorless) trigeminal stimulant CO_2 has been reported (Stevens et al., 1982); this is supported by an age-related decrease of responsiveness to trigeminal stimulation as assessed through event-related potentials (Hummel et al., 1998). On behavioral level it has also been shown that the threshold for transitory apnea in response to CO_2 decreases in an age-related

manner (Stevens and Cain, 1986). In a more general context, however, the age-related decrease in trigeminal sensitivity may be related to a more general decrease of nociceptive function which seems to be more pronounced for the responsiveness of A_{delta}-fibers compared with C-fibers (Chakour et al., 1996; Harkins et al., 1996; Kenshalo, 1986; Ochoa and Mair, 1969).

Contrary to expectations (see Fillingim and Ness, 2000; McGrath, 1994), gender had no effect on trigeminal sensitivity. With regard to trigeminal chemoreceptive sensitivity, previous electrophysiological data indicate that healthy female subjects are more sensitive to trigeminal stimulants compared with males, i.e. women exhibit larger ERP amplitudes than men (Hummel et al., 1998; Opatz et al., 2000). Further, Dunn et al. (1982) reported that thresholds for trigeminally induced apnea is lower in females than in males. Thus, future studies are needed to investigate these potentially controversial observations.

It is interesting to note that the duration of olfactory loss exhibited a positive correlation with the trigeminal sensitivity for eucalyptol. This seems to confirm previous observations where amplitudes and latencies of trigeminally induced event-related potentials have been shown to correlate with the duration of olfactory dysfunction (Hummel, 1998). While this awaits further investigation, it may indicate adaptive changes of the intranasal trigeminal sensitivity.

In conclusion, results of the present study indicate that patients with olfactory dysfunction have lower trigeminal sensitivity compared with normosmic controls. This seems to be independent of the cause of the olfactory loss. Further, the present data suggest an age-related decrease of intranasal trigeminal sensitivity in normosmic subjects. Analyses controlling for the subjects' age additionally indicate a correlation between olfactory and trigeminal sensitivity.

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Review

Toxicity to nasal-associated lymphoid tissue

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Abstract

The mucosal membranes form a weak mechanical barrier, but they are provided with an extensive specific and non-specific defence system. Antigenic stimulation of the mucosal immune system of the oronasal passages induces specific, local immune responses, and activates immune components of mucosae elsewhere as well as the systemic immune system. Nasal lymphocytes are disseminated diffusely in the mucosa or are organised in structures at the entrance of the nasopharynx (nasal-associated lymphoid tissues, NALT). Nasal lymphatics, and possibly NALT, play an important role in drainage of brain fluid, especially in small animals. Little is known about toxicity to the NALT, despite its central role in mucosal immunity. Its strategic position in the nasal passages suggests that it comes easily into contact with inhaled nasal toxicants. Therefore, we recommend to include histopathological examination of NALT in standard guideline-driven inhalation toxicity studies.
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Keywords: NALT; Immunotoxicity; Mucosal immunity

1. Introduction

The mucosae and the skin are most often the first sites of contact with antigens and noxious substances. The mucosal membranes form a weak mechanical barrier when compared with skin, but they are provided with extensive non-specific and specific defence systems. Immune competent cells (lymphocytes, macrophages) are disseminated diffusely in the mucosa or are organised in mucosa-associated lymphoid structures or MALT.

Information is scarce about toxicity to the mucosal lymphoid tissues. The Organisation for Economic Cooperation and Development (OECD) is still developing its subacute toxicity testing guidelines to enhance the capability for detecting the immunotoxic potential of substances. The updated OECD guideline 407, under which rats or mice are exposed orally for 28 days to a test substance, includes the systemic lymphoid organs (thymus, bone marrow, spleen, blood, several lymph nodes) and only a few mucosal lymphoid organs (Peyer's patches and superior mesenteric lymph nodes). Indeed, most immunotoxic sub-

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stances appear to exert their effects predominantly on systemic lymphoid organs, in particular the thymus and bone marrow. These organs are the site of lymphopoiesis, a process which includes high cell proliferation and differentiation, gene amplification, transcription and translation, which makes these organs highly susceptible to toxic insults, especially till young adult age (Schuurman et al., 1992). It should be noted, however, that until recently only limited attention was paid to MALT and the mucosa-draining lymph nodes. Moreover, exposure-related effects such as granulomata formation, macrophage aggregates and lymphangiectasia, which have been observed in mucosa-draining lymph nodes and Peyer's patches, were not always discussed in terms of immunotoxicity.

Guidelines for toxicity studies do not address respiratory tract-associated lymphoid tissues and almost all immunotoxicity studies that include MALT only examine Peyer's patches. In a few inhalation studies bronchus-associated lymphoid tissues (BALT) but not nasal-associated lymphoid tissue (NALT) have been examined, probably because respiratory tract toxicity has long been focused on larynx and lungs. Larynx and lungs were the sites of concern in man, in carcinogenesis as well as in respiratory tract allergy (asthma and extrinsic allergic alveolitis or hypersensitivity pneumonitis). Finally, the lack of toxicity data concerning NALT may also be due to difficulty in sampling and examining the tissue: leaving NALT in situ allows detection of severe effects only, in the cross sections of the nose, and the diffuse nature of the single lymphocyte pool hampers sampling and examination altogether.

This paper outlines the nasal immune system including NALT and focuses on the question whether examination of NALT should be included in inhalation toxicity studies.

2. Nasal immune system

2.1. General

Antigenic stimulation of the mucosal immune system induces specific, local immune responses, notably involving secretory immunoglobulins (sIgA and sIgM) and the local production of IgG. Mucosal immunity is not a strictly local affair. The nasal immune system co-operates with immune components of mucosae elsewhere, namely those of the lungs, the eyes, the ears, the oral cavity, the gastrointestinal and urogenital tract (Brandtzaeg et al., 1999), as well as with the systemic immune system (Velin et al., 1999). The anatomy of the nasal passages demonstrates a close connection between the nasal tissues and ocular, oral and middle ear tissues via, respectively, the lachrymal and the nasopharyngeal duct and the Eustachian tube. These connections offer opportunities for pathogens and antigenic substances to reach ocular, oral and middle ear tissues via the nasal cavity. It is, therefore, understandable that nasal lymphoid tissues play a key role in the defence of its neighbour tissues (Filiaci et al., 1997; Kodama et al., 2000; Zierhut et al., 1997; Ridley Lathers et al., 1998; Saitoh-Inagawa et al., 2000). In addition, the nasal lymphatics, and possibly also NALT, have an important function in brain drainage, especially in small animals like the rat and the rabbit (Cserr and Knopf, 1992). Cerebrospinal fluid and interstitial brain fluid merge and a significant proportion of it drains along cranial and extracranial spaces of which the olfactory nerve is the most important one. Substances and cells draining via the olfactory pathway can be cleared from the nasal submucosa by passage into terminal lymphatics and then to the cervical lymph nodes or to blood capillaries. Proteins, lymphocytes and macrophages preferentially enter the lymphatics.

2.2. Nasal lymphoid tissues

In humans, the lymphoid structures associated with the oronasal mucosae are known as Waldeyer's ring and consist of a number of tonsils, lymphoid bands and the adenoid. In the rat nasal passages, tissues equivalent to the Waldeyer's ring have been found bilaterally at the entrance of the pharyngeal duct at the level of the ecto- and endoturbinates. NALT drains directly to the posterior cervical lymph nodes, whereas the nasal mucosa drains first to the superficial cervical

lymph nodes (Koornstra et al., 1991). The term NALT in rodents is often restricted to these nasopharyngeal structures; in larger species sometimes all organised lymphoid tissues associated with the nasal passages and accessory nasal structures like the lachrymal duct are denoted NALT (Mair et al., 1987). In addition, there is an abundance of intraepithelial and lamina propria lymphocytes (IEL and LPL, respectively). These single cells might also be placed under the term NALT, although we preferably describe these as nasal IEL and LPL.

2.3. Role of NALT

Although inhaled particulate impacting on the mucus layer of the nasal mucosa may be cleared rapidly by ciliary motion, it may also selectively be delivered to the organised NALT structures via the overlying specialised lymphoepithelium and induce an immune response (Kuper et al., 1992, 2001; Almeida and Alpar, 1996). Especially pathogens (e.g. intranasal applied mouse mammary retrovirus; Velin et al., 1997) or particles that are otherwise capable of affecting the ciliary clearance are taken up by the lymphoepithelium and induce a mucosal immune response. During a primary influenza virus infection, antibodies have been found in NALT (Tamura et al., 1998). Soluble antigen enters the nasal mucosa predominantly away from NALT to induce immunological non-responsiveness.

3. Toxicity to NALT

3.1. General

Despite the important role of nasal mucosal immunity and the key role of NALT therein, little is known about its vulnerability to toxicants. Yet there is reason for concern (Leopold, 1992). Firstly, epidemiology suggests that the incidence of allergic nasal disease is increasing, which has been related to an increase in certain air pollutants. Secondly, there is mounting evidence that airborne chemicals may cause a spectrum of immunologically mediated nasal disorders, which may involve NALT. There is also an increased use of intranasal applied drugs, which may not have been examined fully for a potential adverse effect on NALT. Interestingly, NALT is said to be strategically located for contact with aeroantigens, yet this apparently does not apply to inhaled toxicants. For instance, chronic inhalation of ozone induced lesions in the nasopharyngeal epithelium, i.e. epithelial hyperplasia and increased mucus production (Harkema et al., 1997). NALT was not examined, but it could have been affected considering the localisation of the effects, i.e. first part of the nasopharynx. On the other hand, the nature of the effect, i.e. increased mucus production, may have protected NALT. Indeed, substances can have an indirect effect on NALT, by impairing the mucociliary system and subsequently allowing toxicants to adhere to the lymphoepithelium and affect NALT. In contrast, increased mucociliary clearance may prevent substances to reach NALT.

Immunotoxicants may affect systemic as well as regional lymphoid organs in a similar way. The results found for Peyer's patches indicate that NALT is at risk as well. For example, cyclophosphamide given orally or parenterally has been demonstrated to suppress antibody production in both the spleen and GALT with similar dose response sensitivities (reviewed by Burchiel and Davila, 1997). Substances may affect also NALT indirectly via the systemic lymphoid organs by inducing prolonged lymphoid depletion in the bone marrow and/or thymus, or via influencing the endocrine organs, for example by increasing levels of corticosteroids, prolactin or sex hormones, as has been found for systemic lymphoid organs. The above-mentioned possibilities all involve the systemic lymphoid organs and this would imply that examination of systemic lymphoid organs would suffice. However, a few effects have been found in Peyer's patches as a result of direct, local action of orally administered toxicants, e.g. the formation of granulomata following ingestion of a quaternary ammonium compound (Kuper, unpublished observations) and it cannot be excluded that NALT can be affected in a similar way, i.e. directly by inhaled toxicants. Moreover, mucosal immunity differs distinctly from systemic

immunity: cellular trafficking is unique; numbers and types of cells in NALT and Peyer's patches differ from those found systemically; certain B-cell mediated responses in mucosal tissues do not undergo the same declines during ageing that have been measured in systemic lymphoid tissues (Burchiel and Davila, 1997). Therefore, immunotoxicity may be expressed differently in mucosal than in systemic lymphoid tissues.

3.2. Methodological aspects in examination of NALT

The evaluation of toxicant effects on NALT involves many of the same approaches and assays that are utilised to evaluate systemic and Peyer's patches immunotoxicity. In general, the use of standard histopathology, cell recovery and viability and certain immune function assays can be employed, albeit not without difficulty: the number of cells that can be recovered from the NALT, lamina propria and epithelium and the nasal-draining lymph nodes is limited or even very hard to collect. Analyses of nasal lavages may provide additional information. In infection models, it may be desirable to utilise relevant antigens, because one of the primary roles of NALT is to defend against aeroinfections and inhaled toxicants (Burleson, 2000).

Histopathology can be performed on NALT that is collected as part of or dissected from the nasal passages. The latter implies that nasal tissues are lost for common histopathological examination. Unfortunately, standard nasal cross sections transect NALT through its smallest diameter. Moreover, T- and B-cell areas in NALT are oriented more or less from apical to proximal. Thus, 1 or 2 cross-sections through NALT enable detection of severe effects only. Methods to isolate NALT have been described by Asanuma et al. (1997). The diffuse nature of the single lymphocyte pool (IEL and LPL) hampers sampling and examination altogether, although a method has been described for isolation of nasal IEL (Asanuma et al., 1997).

4. Discussion

The nasal immune system is extensive and extremely complex; first, due to its pivotal role in the defence of the middle ear, eye and oral cavity against antigenic substances, and, in obligate nose-breathers, in the defence of the lower respiratory tract; secondly, due to its impact on immune reactions in distant mucosae such as those of the urogenital tract and gut, its cooperation with systemic immunity, and in small animals like rodents its role in brain drainage. These aspects warrant a close examination of the nasal immune system in (immuno)toxicity studies. Nasal lymphocytes are roughly divided into two groups: the single lymphocyte pool or nasal IEL and LPL, and the organised lymphoid structures of which those at the entrance of the nasopharyngeal duct are most important in small laboratory animals. It has been demonstrated that mucosal lymphoid cells are sensitive to the effects of several toxicants (Burchiel and Davila, 1997; Bruder et al., 1999). However, there have been few immunotoxicology investigations that have actually focused on the effects of toxicants on mucosal immune responses. When one considers the many typical features of mucosal lymphoid tissues, it would not be surprising to find that certain toxicants will affect specifically these tissues and have unexpected consequences. For example, lymphoid depletion of NALT and submucosal nasal swelling due to inhaled toxicants might impair brain drainage.

With respect to the single lymphocyte pool, existing routine methods may not suffice. Up till now, we do not know what happens to the nasal IEL when their epithelial microenvironment is affected. Further studies will be necessary to determine whether there are unique mechanisms of toxicity to nasal mucosal single lymphoid cells and tissues. As to organised mucosal lymphoid tissues, existing methodologies are expected to be sufficiently sensitive to flag a substance as immunotoxic. In general Peyer's patches will be good representatives of MALT for detecting toxicity to mucosal lymphoid tissues. To detect local immunotoxic effects in inhalation studies, also NALT and nasal draining lymph nodes should be routinely investigated histopathologically.

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Short communication

Deoxynivalenol-induced IgA production and IgA nephropathy-aberrant mucosal immune response with systemic repercussions

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Abstract

Dietary exposure to the common foodborne mycotoxin deoxynivalenol (DON) selectively upregulates serum immunoglobulin A (IgA) in the mouse, most of which is polymeric, thus suggesting that the mucosal immune system is a primary target. When ingested, DON has no adjuvant or antigen properties but, rather, induces polyclonal IgA synthesis and serum elevation in an isotype-specific fashion. Resultant hyperelevated IgA is polyspecific, autoreactive and is likely to be involved in immune complex formation as well as kidney mesangial deposition. These latter effects mimic IgA nephropathy, the most common human glomerulonephritis. At the cellular level, DON upregulates production of T helper cytokines and enhances T cell help for IgA secretion. Analogous effects are observed in the macrophage with IL-6 being of particular importance based on ex vivo reconstitution and antibody ablation studies as well as experiments with IL-6 deficient mice. Upregulation of cytokines by DON involves both increased transcriptional activation and mRNA stability which are mediated by activation of mitogen-activated protein kinases. Interestingly, dietary omega-3 fatty acids can downregulate these processes and ameliorate DON-induced IgA nephropathy. From the perspective of gut mucosal immunotoxicology, these studies demonstrate that the capacity of a chemical to affect mucosal immune response can have systemic repercussions and, further, that these effects can be modulated by an appropriate nutritional intervention.
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Keywords: Mycotoxin; Trichothecene; IgA; Immunotoxicology; Peyer's patches; Cytokine; Transcription; mRNA stability; Mitogen-activated protein kinases; Omega-3 fatty acids

1. Introduction

Our laboratory has studied the effects of foodborne mycotoxins on immune function for the past 20 years. While conducting a Tier I immunotoxicology evaluation of the trichothecene deoxyni-

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valenol (DON), we observed that dietary exposure to this mycotoxin selectively upregulated serum immunoglobulin A (IgA) in the mouse suggesting that the mucosal immune system is a primary target of this mycotoxin. While primarily approached as a toxicological problem, this model provides mucosal immunologists with a unique chemical probe for studying in vivo regulation of IgA synthesis as well as putative pathogenic mechanisms in IgA nephropathy and attendant nutritional therapies. A key lesson derived from these studies is that because of the broad interface between components of the mucosal and systemic systems, immunotoxic chemicals that affect one of these arms will undoubtedly affect the other arm (Fig. 1).

2. Trichothecene mycotoxins

Trichothecene mycotoxins are a group of sesquiterpenoid metabolites produced by members of the genus *Fusarium* that include some of the most potent protein synthesis inhibitors known. Of over 180 trichothecenes that have been identified, several are frequently found in dietary staples such as wheat, corn, barley, rice and oats. Trichothecenes are recalcitrant to inactivation during milling and processing, and thus readily enter finished food products. DON, given the colloquial name 'vomitoxin' because of its emetic effects in swine, is encountered often in grain produced in the temperate areas in North America, Europe and

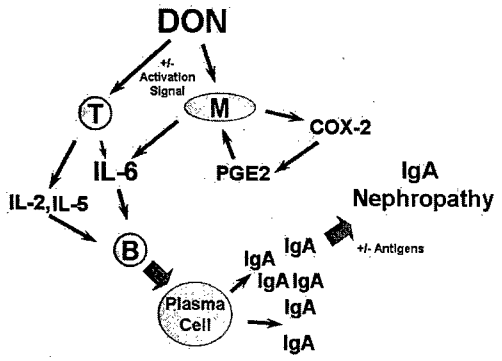

Fig. 1. Cellular mechanisms involved in DON-induced IgA production and IgA nephropathy.

Asia (Rotter et al., 1996). This is of toxicological significance because acute high-dose trichothecene poisoning has been observed in humans and animals. Acute toxicity is manifested as a multi-system shock-like syndrome, with symptoms that include dermal irritation, nausea, emesis, diarrhea, hemorrhage and hematological lesions such as leukopenia and anemia. While persons in developed countries are unlikely to encounter high levels of DON, they undoubtedly ingest low levels of this compound in chronic fashion. Key issues related to possible human health effects from chronic DON consumption are impaired growth and immunotoxicity (Canady et al., 2001).

3. DON-induced dysregulation of IgA production and IgA nephropathy in the mouse

In view of frequent occurrence in foods worldwide and anecdotal reports of DON-associated immunosuppression, we conducted a Tier I Immunotoxicology screen in the B6C3F1 mouse as developed by the National Toxicology Program at the US National Institute of Environmental Health Sciences (Luster et al., 1988). Surprisingly and seemingly inconsistent with its action as translational inhibitor, DON was found to cause a dramatic elevation in total serum IgA with concurrent decreases in total IgM and IgG (Forsell et al., 1986). In fact, after prolonged DON feeding, IgA became the major serum isotype and this effect was concurrent with marked elevation of serum IgA-immune complexes (IgA-IC) and polymeric IgA (Pestka et al., 1989). Notably, DON-exposed mice exhibit kidney mesangial IgA accumulation, electron dense mesangial deposits and hematuria (Dong et al., 1991) which are hallmarks of human IgA nephropathy, the most common type of glomerulonephritis worldwide (D'Amico, 1987). Elevated serum IgA, IgA-IC, mesangial IgA and hematuria persist for up to 3 months after removal of DON from the mouse diet (Dong and Pestka, 1993). Prolonged DON feeding for periods of 12 weeks or longer increases serum IgA levels by as much as 10- to 15-fold while fecal IgA levels only double (Greene et al., 1994a). This latter finding suggested that while the toxin targeted

regulation of an immunoglobulin primarily associated with the mucosal origin IgA was expressed in and impacted the systemic immune compartment. Intermittent DON exposure is less effective at inducing IgA nephropathy than continuous exposure (Banotai et al., 1999a). Dietary DON can induce IgA nephropathy in murine models of systemic lupus erythematosus but does not exacerbate the manifestations of lupus (Banotai et al., 1999b).

The Peyer's patches (PP) of mice are central to development and regulation of the mucosal IgA response (Beagley and Elson, 1992). PP from DON-fed mice exhibit elevated membrane IgA-bearing cells (Pestka et al., 1990a). PP lymphocytes and, to a lesser extent, splenic lymphocytes isolated from DON-fed mice produce significantly more IgA than control cultures prepared from mice fed clean diet ad lib or in a restricted fashion; this occurs with and without mitogen stimulation (Bondy and Pestka, 1991; Pestka et al., 1989, 1990b). IgA secretion in bone marrow cultures, generally considered to be a major source of serum IgA, is not affected by DON (unpublished findings). These data suggest that, in mice exposed to DON, there are rapid polyclonal activation of IgA-secreting cells in the gut at the PP level and that this is also reflected in the systemic compartment. It is interesting to note that in studies of DON disposition in the mouse, this compound diffuses freely throughout plasma and body tissue within 30 min after oral gavage (Azcona-Olivera et al., 1995). Thus, DON does not appear to be taken up by the PP in a selective fashion.

4. Specificity of DON-induced IgA

DON does not appear to exert adjuvant effects when orally administered with exogenous mucosal antigens (Pestka et al., 1990c). Rather, DON polyclonally induces production of IgAs that are reactive with a variety of intestinal and self antigens, while simultaneously down-regulating IgG and IgM reactive with the same antigens. A strong relationship exists between autoreactive IgA and DON-induced IgAN based on the specificity of serum IgA, IgA-secreting cells, and eluted kidney mesangial IgA for a diverse panel of antigens (Rasooly and Pestka, 1992, 1994). Furthermore, when 122 IgA-producing hybridoma clones were isolated from the PP of DON-fed mice and reactivities of the resultant antibodies were tested against a panel consisting of DNA, sphingomyelin, thyroglobulin, collagen, casein, cardiolipin and bovine serum albumin conjugates of phosphorylcholine, inulin and trinitrophenol, approximately 95% of the monoclonal IgAs bound to at least one of the panel antigens and 80% bound to more than one (Rasooly et al., 1994). Polyspecificity of some of the monoclonal IgAs was further suggested by demonstrating the capacity of one antigen to inhibit binding of monoclonal IgA to another antigen. Protein staining and Western blotting of gradient native polyacrylamide gels indicated that polymeric IgA predominated in the isolated monoclonal IgAs. Repeated injections of mice with representative monoclonal IgAs induced microhematuria (Yan et al., 1998a). Taken together, these data suggest that dietary DON promotes the polyclonal activation and expansion of IgA-secreting B cells at the PP level and that resultant polyspecific, autoreactive IgA may contribute to kidney pathogenesis via immune complex deposition or direct binding to the kidney mesangium.

Nivalenol (NIV), a trichothecene, structurally-related to DON occurs in some Asian countries including Japan, China and Korea. Hinoshita et al. (1997) reported that NIV ingestion reproducibly induces significant IgA deposits in the glomerular mesangium and elevates serum IgA levels in mice irrespective of the strain. The degree of immunopathological changes analogous to human IgAN was associated with the dose and duration of NIV treatment. Furthermore, a competitive enzyme-linked immunosorbent assay with an NIV analogue-protein conjugate suggested that the IgA antibodies in sera from NIV exposed mice had exhibited high affinity for this mycotoxin. We have also found that trichothecene-induced IgAN to be nonspecific relative to mouse strain (Greene et al., 1994b). Regarding specificity of the elevated IgA, however, our results argue against the suggestion that NIV or DON effects are 'antigen-specific'. Both DON and NIV have molecular

weights of approximately 300. There is no evidence to date that these haptens are metabolically altered to forms which react with proteins to render them immunogenic (Rotter et al., 1996). Using synthesized DON–bovine serum albumin conjugates linked at the 3′ or 15′ position of the toxin, we were unable to detect increases in DON-specific IgA from DON-exposed mice (unpublished data). Indeed, it is extremely difficult to obtain specific titers to DON following deliberate immunization with these conjugates (Casale et al., 1998).

5. Sexual dimorphism in DON-induced IgAN

Human IgAN is two to five times more common in men than women (D'Amico, 1987). Relatedly, male mice are more susceptible to DON-induced IgA hyperproduction and IgAN than their female counterparts when dose response, latency, and severity are evaluated (Greene et al., 1994b). Thus, a possible relationship exists between sex hormones and DON-induced IgAN. Greene et al. (1995) examined the effects of gonadectomy of male and female mice and supplementation with 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) on DON-induced IgAN. The results suggested that gonadectomy of males decreased the induction of IgAN by DON and that DHT supplementation of castrated male and female mice enhanced progression of the disease. Interestingly, although gonadectomy of females increased severity of DON-induced IgAN, supplementation of gonadectomized male or female mice with E_2 did not reverse this effect but rather increased disease severity. This model offers a novel strategy to study sexual dimorphism in IgAN.

6. Cellular mechanisms for DON-induced upregulation of IgA production

DON is unable to directly induce IgA secretion in primary B cells from PP or spleen or in cloned B cells (Warner et al., 1994). Rather, both T cells and macrophages have been implicated in the polyclonal expansion of IgA secreting cells. Evidence for T cell involvement is based on the findings in DON-fed mice that there is: (1) increased T cell numbers, $CD4^+$ cells and $CD4^+$:$CD8^+$ cell ratio in PP and spleen (Pestka et al., 1990a) and (2) increased help for terminal differentiation of naive PP B cells upon addition of T cells from PP of DON-fed mice (Bondy and Pestka, 1991), (3) increased help for IgA secretion by B cells when co-cultured with $CD4^+$ cells pulsed with DON (Warner et al., 1994), and (4) increased mRNAs and secreted proteins for IL-2, IL-4, IL-5 and/or IL-6 observed in activated $CD4^+$ cells exposed to DON (Dong et al., 1994; Ouyang et al., 1995).

To determine the potential role of macrophages in DON-induced IgA dysregulation, an ex vivo model was devised whereby PP and spleen cells were prepared from mice 2 h after acute oral exposure to DON, cultured, and then evaluated for IgA and cytokine IL-6 production (Yan et al., 1998b). Both PP and, to a lesser extent, spleen cells from treatment mice produced more IgA than did corresponding control cells when cultured without a co-stimulus or in the presence of either phorbol myristate acetate plus ionomycin (PMA+ION) or lipopolysaccharide (LPS). The DON effect was completely ablated in PP cultures that were depleted of macrophage cells. DON exposure similarly increased production of IL-6, an important helper factor for IgA secretion, in LPS-stimulated PP and spleen cell cultures. IL-6 production was also ablated by macrophage depletion. A potential costimulatory role for macrophage was further suggested because both IgA and IL-6 production increased when macrophage-depleted PP cells from DON-treated animals were co-cultured with peritoneal macrophages from DON-treated animals. Similar effects were observed when an analogous ex vivo approach was used with purified PP B cells and peritoneal macrophages. PP B cells from control animals also secreted elevated levels of IgA when co-cultured with splenic CD4(+) cells from DON-treated animals, thus confirming previous studies showing that T cell help also contributes to increased IgA production. Potential roles for soluble mediators and cell contact in this process were suggested when IgA production was measured in cultures of PP cells separated from DON-

treated macrophages by a semipermeable membrane. Thus, macrophages may play a key mechanistic role in elevating IgA production and IgA nephropathy in DON-exposed mice.

The aforementioned ex vivo approach was also employed to assess the roles of several cytokines in DON-induced IgA dysregulation (Yan et al., 1997). Specifically, the effects of a single oral exposure in mice to DON on production of IgA and cytokines in PP and spleen cell cultures were evaluated. IgA levels increased significantly in cultures prepared from mice at either 2 or 24 h after oral exposure to DON and subsequently stimulated with PMA+ION or with LPS. Supernatant IL-2 and IL-4 levels were unaffected by the prior treatment of animals with DON whereas IL-5 and IL-6 were elevated. Consistent with IL-5 and IL-6's previously documented roles in IgA production, antibodies to these cytokines decreased IgA to background levels in cultures of both control and DON-exposed PP cells. IgA levels were decreased to a lesser extent in PP cells cultured with LPS and in spleen cells cultured with PMA+ION from DON-exposed mice to which anti-IL-2 was added. Thus, the potential for enhanced IgA production exists in lymphoid tissue shortly after a single oral exposure to DON, which could be related to the increased capacity to secrete helper cytokines of T cell and macrophage origin, most notably IL-2, IL-5 and IL-6.

It is well established that IL-6 plays a critical role in driving differentiation of B cells to IgA production (Beagley and Elson, 1992). Given the finding that gene expression of this cytokine is increased in vivo and ex vivo upon DON exposure (Azcona-Olivera et al., 1995; Wong et al., 1998; Zhou et al., 1997, 1998), the effects of dietary DON on serum IgA levels and kidney mesangial IgA deposition in an IL-6-knockout (KO) mouse were compared with those in a corresponding wild-type strain that possesses the intact gene for this cytokine (Pestka and Zhou, 2000). DON-fed wild-type mice had significantly higher serum IgA, IgA immune complexes, kidney mesangial IgA and hematuria than did their corresponding controls which were fed clean diet, whereas significant differences were not observed between IL-6 KO mice, which were fed DON or control diets. In total, the results suggest that IL-6 is a requisite cytokine for DON-induced IgA production and resultant IgAN.

7. Molecular mechanisms for DON-induced cytokine gene expression

Based on the apparent relevance of cytokines to DON-induced IgA upregulation, the molecular basis by which DON induces cytokine gene expression has been studied. Transcriptional activation is one likely mechanism, based on the finding that DON activates binding of the transcription factors nuclear factor-kB (NF-kB) (Ouyang et al., 1996) and activator protein-1 (AP-1) (Li et al., 2000) in the EL-4 T cell model. Furthermore, DON upregulates NF-kB, AP-1 and CCAAT enhancer-binding protein (C/EBP, B) binding activity in nuclear extracts of the RAW 264.7 macrophage model (Wong et al., 2002) thus suggesting that this activity is not restricted to T cells and is consistent with the toxin's effects on cytokines in these two clonal models. Besides having transcriptional effects, DON may function post-transcriptionally. Using transcriptional inhibitors, Li et al. (1997) found that the superinduction of IL-2 mRNA expression by DON was due, in part, to markedly increased IL-2 mRNA stability. DON also stabilizes IL-6 and TNF-α mRNAs in RAW 264.7 cells (Wong et al., 2001). This is again consistent with the possibility that DON exerts its effects on multiple leukocyte targets.

A positive association exists between endogenous cyclooxygenase-2 (COX-2) metabolites and IL-6 synthesis based on in vitro and in vivo models of several inflammatory diseases (Dendorfer et al., 1994; Meyer et al., 1994; Anderson et al., 1996; Hinson et al., 1996; Williams et al., 2000). Prostaglandin E_2 (PGE_2) regulates production of macrophage and T cell cytokines which can, in turn, impact cell-mediated responses as well as immunoglobulin induction by B cells (Snijdewint et al., 1993; Fedyk et al., 1997). In some inflammatory response models, PGE_2 inhibits Th1 cytokine expression, whereas production of Th2 cytokines is unaffected or even enhanced by

PGE_2 (Betz and Fox, 1991; Kozawa et al., 1998; Kuroda et al., 2000). Macrophages are a major source of PGE_2 during inflammation and also have receptors for eicosanoids. Macrophage-generated PGE_2 is now recognized to modulate proinflammatory cytokines such as IL-6 in an autocrine or paracrine fashion (Arakawa et al., 1996; Williams et al., 2000). When the capacity of COX-2 to contribute to IL-6 induction by DON was evaluated, both the enzyme and its metabolites were found to mediate DON-induced upregulation of IL-6 expression in both macrophage cultures and in mice (Moon and Pestka, 2002; Moon and Pestka, in press). COX-2 might, therefore, play a critical role in IgA upregulation and other immunological effects associated with DON exposure. Interestingly, as with the proinflammatory cytokines, DON also appears to upregulate COX-2 gene expression by increasing transcription and mRNA stability.

What are the upstream signaling events that lead to induction of inflammatory genes by DON? The central mechanism of toxicity for trichothecenes has long been thought to relate to their ability to bind to ribosomes and inhibit protein synthesis (Carter and Cannon, 1977; Ueno, 1984). Consistent with this hypothesis, a 'ribotoxic stress response' has been described for related translation inhibitors such as anisomycin, ricin, α-sarcin and T-2 toxin (Iordanov et al., 1997). In this model, alteration of ribosome structure or function upon binding of these inhibitors has been postulated to be an initiation signal for activation of mitogen-activate protein kinases (MAPK). Trichothecenes induce JNK 1/2, ERK 1/2 and p38 (Shifrin and Anderson, 1999; Yang et al., 2000). Recently, we have observed that DON induces the rapid and transient phosphorylation of ERK, p38 and JNK in the mouse (Zhou et al., in press). These effects were concurrent with or were followed by time-dependent increases and decreases in transcription factor binding corresponding to nuclear translocation. Both timing and differential activation of MAPKs and transcription factors by DON were consistent with the profile, magnitude, and duration of expression of IL-6 and other cytokines. This study provides a novel model for studying the interrelationships among MAPKs, transcription factors, cytokines and IgA in intact animals exposed to DON.

8. Omega-3 polyunsaturated fatty acid consumption impairs DON-induced IgAN

Besides the obvious value in assessing hazards associated with ingestion of DON by humans, our studies of DON-induced upregulation of IgA production have potential biomedical importance for unraveling early upstream mechanisms of human IgAN as well as for evaluating potential ameliorative therapies for this important disease. Relative to possible nutritional therapies, fish oil contains a high level of omega-3 polyunsaturated fatty acids which inhibit expression of proinflammatory cytokines in both in vivo and in vitro models of inflammation and autoimmune disease (Calder, 1998; Sadeghi et al., 1999). We found that the replacement of corn oil with fish oil ameliorates DON-induced elevation of serum IgA and IgA-IC as well as kidney IgA deposition (Pestka et al., 2002). These findings are exciting because previous studies demonstrating that fish oil supplementation retards late-stage progression to renal failure in human IgAN patients (Donadio et al., 1994, 1999). Thus, with the DON model, it is now possible to evaluate the potential of omega-3 fatty acids as prophylactic agents for early stages of IgAN.

9. Summary

From the perspective of mucosal immunotoxicology, the studies on DON-induced dysregulation are informative because they demonstrate that an intimate connection exists between the mucosal and systemic immune compartments. DON-induced murine IgAN is a unique model to study the roles of early cellular/molecular events, aberrant IgA production, putative pathogenic IgA species and sexual dimorphism in this IgAN. When ingested, DON and other trichothecenes, induce polyclonal IgA synthesis. This, coupled with concurrent decreases in serum IgM and IgG, suggests that this event is isotype-specific

and mimics oral tolerance. Resultant hyperelevated IgA is polyspecific, autoreactive and is likely to be involved in immune complex formation as well as kidney mesangial deposition. At the cellular level, DON and other trichothecenes upregulate production of T helper cytokines and enhance T cell help for IgA secretion. Analogous effects are observed in the macrophage with IL-6 being of obvious importance. While similar effects can be observed in the mouse, further studies are necessary to merge in vitro and in vivo data into a cohesive mechanistic model. Since DON is frequently found in cereal-based foods, it raises the question whether it and other trichothecenes could be possible etiological factors in human IgAN. This question can be addressed only by determining critical mechanisms in the mouse, reconstituting these effects with human lymphocytes and conducting appropriate epidemiological studies in areas of the world where there are high concentrations of DON and other trichothecenes in the food supply.

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Review

Assessment of the allergenic potential of proteins

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Abstract

The development of novel foods, including foods derived from genetically modified plants, has generated considerable interest in the design and application of appropriate safety assurance measures. A specific focus of attention has been on allergenicity, and in particular the need to determine whether the products of novel genes introduced into food plants have the potential to cause allergic sensitisation. Among the approaches applied currently are considerations of whether a new protein has structural, sequence and/or antigenic similarities with known food allergens, and whether or not it displays resistance to digestion within a simulated gastric fluid, or by pepsin. Although such data are useful in an overall hazard assessment, they are neither individually, nor collectively, able to provide a direct evaluation of inherent sensitising potential. For this reason there is a need to develop and apply appropriate animal models that will offer a more holistic view of sensitising activity. Several methods have been suggested, but as yet none has been evaluated fully or validated. Nevertheless, significant progress has been made and in this article an experimental approach using BALB/c strain mice in which animals are exposed to the test protein via systemic (intraperitoneal, or in certain circumstances, intradermal) administration is described. Inherent sensitising potential is measured as a function of induced IgE antibody responses. Experience to date is encouraging and the data available reveal that this method is able to distinguish between proteins of different allergenic potential.
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Keywords: Allergic sensitisation; Animal models; Food allergy; IgE antibody

1. Introduction

There is considerable interest in the development of appropriate strategies for the safety assessment of novel foods, including foods and food products derived from genetically modified crops. Prominent among such considerations is the need to address concerns about allergenicity, and in particular whether the products of novel genes introduced into crop plants have the potential to induce allergic sensitisation (Kimber and Dearman, 2001a; Metcalfe et al., 1996; Taylor and Hefle, 2001).

The first detailed and systematic attempt to provide a structured approach to assessment of the allergenic potential of novel food proteins was developed jointly by the International Food Bio-

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technology Council (IFBC) and the International Life Sciences Institute (ILSI), Allergy and Immunology Institute (Metcalfe et al., 1996). The scheme proposed at that time was in the form of a decision tree in which the route taken was dependent upon whether or not the novel gene derived from a source was considered to be allergenic. The approaches identified included: (a) consideration of the serological identity of the novel protein with proteins known to be allergenic, (b) structural similarity to, or amino acid sequence homology with, allergenic proteins, and (c) resistance to proteolytic digestion in a simulated gastric fluid. This decision tree, and the investigations it comprises, provides useful information, particularly in those instances where proteins are found to share antigenic or structural properties with known human allergens. However, neither individually, nor collectively, do these parameters necessarily provide a definitive assessment of likely sensitising activity. As a consequence there has been a growing interest in the design, development and application of appropriate animal models (Kimber et al., 2000; Kimber and Dearman, 2001b).

At the time of the original IFBC/ILSI recommendations the view was that there were no suitable animal models available. However, it is now acknowledged that during the intervening period some important progress has been made. The Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) in 2001 convened a special consultation panel to consider food allergenicity testing and possible revisions to the original IFBC/ILSI decision tree (FAO/WHO, 2001). One conclusion reached was that, although animal models have not been evaluated extensively, there is sufficient evidence available to suggest that some such methods may contribute valuable information regarding the allergenic potential of foods derived from biotechnology.

This sentiment has provided further impetus to the development of appropriate animal models. Described briefly in this article is progress that has been made using mice, although other investigators have explored the utility of rats (Knippels et al., 1998, 1999), dogs (Ermel et al., 1997) and swine (Helm et al., 2002).

2. Assessment of allergenicity in mice: measurement of antibody responses

In the context of providing a robust approach to safety assessment the requirement is for a method that will allow the accurate identification of proteins that have the potential to induce allergic sensitisation. The assumption is, therefore, that proteins differ in their inherent allergenic activity, although the bases for such differences and the nature of the characteristics that confer on proteins the ability to cause sensitisation have yet to be defined (Aalberse, 2000; Bredehorst and David, 2001; Huby et al., 2000). Although mouse models of food allergy are available, including for instance those described recently by Li et al. (1999, 2000, 2001), there has not previously been a method suitable for hazard identification and characterisation. In developing such an approach in mice there are several important variables with respect to test design that must be addressed. These include the choice of end-point, strain selection, the route of exposure and the use of adjuvant.

The strategy we have adopted is to measure inherent allergenic potential as a function of the ability of proteins to induce the production of IgE; antibody of this isotype being the major effector of immediate-type allergic reactions. For this purpose we have chosen (initially at least) BALB/c mice; this being based on an understanding that mice of this strain have an 'atopic-like' phenotype characterised by a high IgE responder status. A contentious issue is the selection of route of exposure. Although in the context of food allergy it may appear appropriate to regard oral administration as the preferred route of exposure, there are compelling reasons to develop an alternative approach. Thus, exposure of rodents to novel proteins in the diet or in drinking water may result in tolerance or immunological hyporesponsiveness rather than sensitisation (Knippels et al., 1998). Even gavage exposure of rats or mice to proteins may be rather variable or insensitive with respect to the induction of IgE antibody responses (Dearman et al., 2001; Knippels et al., 1998). For these reasons we have chosen instead to expose mice to test proteins using systemic (intraperitoneal; i.p.) administration. Finally, some adjuvants have the

ability to augment IgE antibody responses and it is legitimate, therefore, to question whether their use would provide increased sensitivity. However, the danger is that such agents may have the potential to confer on otherwise non-allergenic proteins the ability to provoke IgE production, and in effect generate 'false positive' responses. Consequently we have chosen to measure antibody production induced by protein in the absence of adjuvant.

On the basis of these considerations the approach used currently can be summarised briefly as follows. Groups of young adult (8–12 weeks) female BALB/c strain mice are exposed by i.p. injection to selected concentrations of the test protein (two injections, 7 days apart). At various periods thereafter serum is collected and specific IgE antibody responses measured using homologous passive cutaneous anaphylaxis (PCA) assay, and IgG responses by enzyme-linked immunosorbent assays (ELISA). The strategy is to identify as potential allergens those proteins that have the ability to provoke IgE antibody responses, and to distinguish these from non-allergenic proteins that despite being antigenic (and which as a consequence are able to stimulate IgG antibody) either fail to elicit IgE responses or elicit only low grade and/or transient IgE production. Experience with this and similar approaches has been described previously (Dearman et al., 2000, 2001, 2002; Dearman and Kimber, 2001; Hilton et al., 1994, 1997).

Collectively, the results obtained to date have revealed that this method allows discrimination between proteins on the basis of their relative ability to induce IgE antibody responses. Under conditions of exposure where proteins were shown to elicit IgG antibody responses of comparable vigour, substantial differences were observed with respect to IgE antibody production.

Although these data are promising, it is important to draw attention to three important points. The first of these is that, in common with other proposed methods, experience has to date been limited to investigations conducted with a relatively small number of proteins (in this instance ca. 20). Clearly a more rigorous examination with additional allergens of confirmed sensitising potential, and with proteins known or suspected not to cause sensitisation, is now required. A second consideration is that the approach is designed to discriminate between proteins that have an inherent potential to provoke allergic responses from those that apparently do not. That is, the emphasis is on the identification of intrinsic hazard. In this context it must be acknowledged that an inherent potential to induce sensitisation as defined here will not necessarily translate into a risk of allergy and allergic disease under conditions of human exposure. The third related point is that assessment of sensitising activity deriving from measurement of antibody responses must be viewed in a broader context and as part of an holistic safety assessment which also incorporates information relating to the biochemical, structural and antigenic properties of the protein.

3. Assessment of allergenicity in mice: measurement of cytokine responses

As indicated above, the main focus of attention has been on characterisation of antibody production. More recently, however, parallel investigations have sought to examine the nature of cytokine responses induced by proteins. The rationale for this is that IgE antibody responses and the development of allergy are regulated by functional subpopulations of T lymphocytes and their cytokine products. It has been recognised for some time that there exists functional heterogeneity among $CD4^+$ T helper (Th) cells. The two most polar populations have been designated Th1 and Th2 and these differ with respect to their cytokine secretion patterns. The importance of this for the pathogenesis of allergic disease is that interleukin (IL) 4 (IL-4), a product of Th2 cells is required for the initiation and maintenance of IgE antibody production, while other cytokine products of Th2 cells (such as IL-5, IL-10 and IL-13) favour the elicitation of immediate-type allergic reactions. In contrast, Th1 cells antagonise allergic reactions and, importantly, the Th1 cell cytokine interferon γ (IFN-γ) inhibits IgE responses. A similar heterogeneity is also found among $CD8^+$ T cytotoxic (Tc) cells, with Tc1 and Tc2 cells

displaying cytokine expression phenotypes similar to those of the respective Th subsets (Corry and Kheradmand, 1999; Kimber and Dearman, 1997; Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Stevens et al., 1988). The prediction is, therefore, that proteins that are known to induce IgE antibody responses and to cause allergic sensitisation will induce in mice selective immune responses characterised by the preferential production of type 2 cytokines. Recent investigations have sought to test this prediction.

A representative experiment is illustrated in Fig. 1. Groups of mice were exposed by intradermal (i.d.) injection to peanut lectin, a minor, but nevertheless potent, allergen (Burks et al., 1994). Two weeks following the initiation of exposure, mice were sacrificed and the draining (auricular) lymph nodes isolated. Draining lymph node cells (LNC) were cultured in the presence of a general T lymphocyte mitogen, concanavalin A (con A), or purified peanut lectin, or were cultured in medium alone (Fig. 1). Culture of LNC in the presence of irrelevant protein (medium supplemented with 10% foetal calf serum (FCS)) resulted in only comparatively low levels of expression of most cytokines examined, with the exception of IL-12 that was secreted constitutively. Stimulation with the T lymphocyte mitogen con A induced vigorous up-regulation of the type 1 cytokines IFN-γ and IL-12, and some enhancement of type 2 cytokine production. In contrast, culture of LNC from peanut-primed mice with the relevant allergen (peanut lectin) caused a very marked elevation in production of the Th2 cytokine products IL-4, IL-5, IL-10 and IL-13, with a concomitant decrease in Th1-type cytokines.

Although as yet unconfirmed with other antigens, these preliminary data suggest that it may

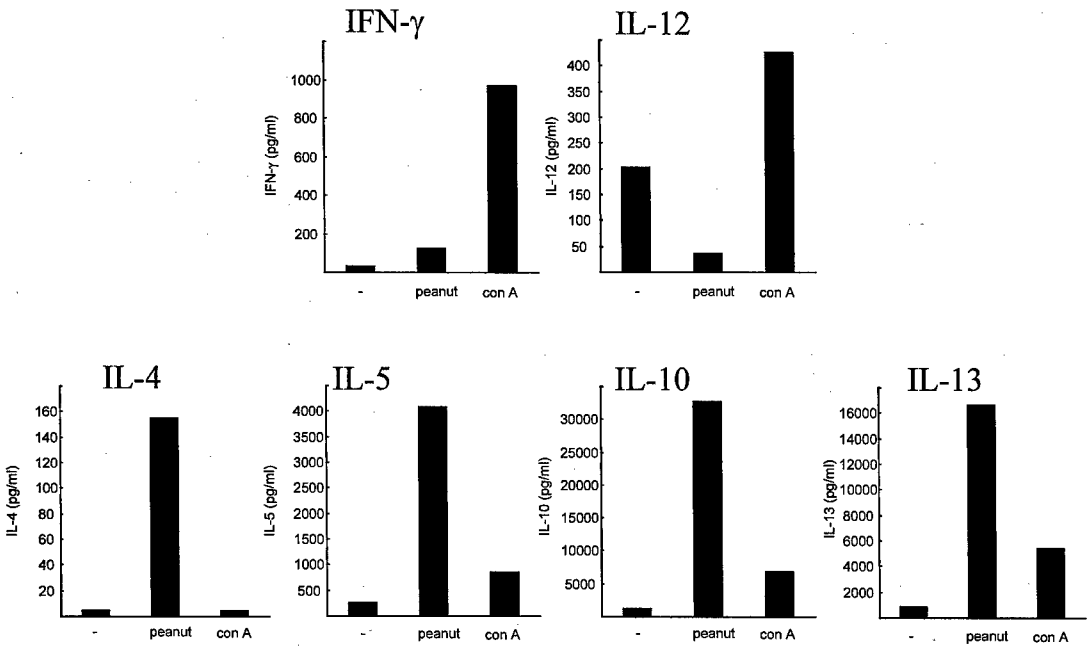

Fig. 1. Cytokine secretion profile induced by exposure to peanut allergen. BALB/c strain mice ($n = 10$) received 30 μl of 0.2% peanut (*Arachis hypogea*) lectin in phosphate buffered saline by i.d. injection in the dorsum of both ears on days 0 and 7. Fourteen days after the initiation of exposure, draining auricular lymph nodes were excised and a single cell suspension of LNC was prepared by mechanical disaggregation. LNC were cultured at 10^7 cells per ml in the presence of the T cell mitogen con A (2 μg/ml), purified peanut lectin (200 μg/ml) or in the presence of medium alone (RPMI 1640 medium supplemented with 10% FCS) for 120 h. Supernatants were harvested and the concentrations of interferon γ (IFN-γ), IL-12, IL-4, IL-5, IL-10 and IL-13 were measured by cytokine-specific ELISA. Results of a single representative experiment are displayed.

prove possible in the future to use cytokine expression profiling as an adjunct or an alternative to serological analyses.

4. Concluding comments

We have provided here one example of progress that has been made in developing approaches to the identification of potential protein allergens based on responses induced in animals. Although such methods require more searching evaluation with respect to sensitivity, selectivity and overall accuracy, there is reason to believe that some approaches at least will in the future form an important element of the safety assessment process. It is important to re-emphasise, however, that effective assessment of potential allergenicity will require an integrated approach where the inherent sensitising activity as judged from appropriate animal models is considered together with the antigenic, structural and biochemical properties of the protein and an understanding of expression patterns and likely levels of exposure.

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Short communication

Animal models in food allergy: assessment of allergenicity and preventive activity of infant formulas

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Abstract

Food allergies occur in about 5–10% of the overall infant and small-child population. Cow's milk protein allergy (CMPA) is the most common in young infants, with a 2–4% incidence. When breastfeeding is not possible, hypoallergenic (HA) cow's milk based formulas are usually given during the first months of life for prevention of CMPA. Depending on primary (sensitization) or secondary (triggering) prevention, the requested quality of HA formulas may be different. Besides in vitro methods, in vivo and ex vivo animal models are helpful in assessing residual allergenicity and the preventive effect of HA formulas. The sensitizing capacity of a formula can be examined by either the parenteral rat (IgE), the guinea pig (IgG1a mediated) or the oral mouse (IgE) models. The triggering IgE mediated allergenicity is tested by a parenteral rat model with oral gavage for intestinal mast cell protease (RMCPII) release. These animal models are also used for testing the oral tolerance inducing capacities of formulas. Together with cellular in vitro assays, animal models are very helpful in predicting allergenicity and the tolerogenic potential of HA infant formulas.
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Keywords: Food allergy; Oral tolerance; Animal models; Infant formulas

1. Background

Atopic diseases in infants have a prevalence of about 30% and are among the most important morbidity factors in industrialized countries (Tariq et al., 1998) where their incidence has roughly doubled in recent decades. Food allergies occur in about 5–10% of the overall infant and small-child population (Bock, 1987). Most common food allergens are cow's milk, eggs, peanuts, nuts, soy, wheat and fish.

In a number of epidemiological studies, cow's milk protein allergy (CMPA) is reported with an incidence of 2–4% and is, therefore, the most common allergy in early infancy. This incidence raises further sharply in infants with a genetically elevated risk of allergy: about 20% of them will develop CMPA during their first year of life if fed on cow's milk proteins.

Many infants present with skin and gastro-intestinal symptoms of CMPA. In the majority

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of infants with CMPA, more than two organ systems are involved. The severity of symptoms varies from mild to life threatening (anaphylactic shock).

To diagnose CMPA, in vitro tests (RAST, CAP) and in vivo tests (Skin-Prick, patch) are frequently used but the gold standard of CMPA diagnosis is still the double-blind placebo-controlled food challenge (DBPCFC).

CMPA is mainly an IgE mediated hypersensitivity reaction where multiple sensitization to different cow's milk proteins is observed in about 75% of milk allergic patients (Wal, 1998). Cow's milk contains at least 20 proteins that may be allergens. Although caseins and β-lactoglobulin are the most frequent allergens of cow's milk, other proteins like α-lactalbumin, serum albumin and even lactoferrin (present in trace amounts) are potent allergens. Three dimensional structure is important in cow's milk protein allergenicity, but besides the conformational epitopes, IgE binding studies also showed the presence of sequential epitopes. IgE epitope analysis of cow's milk proteins is usually made by direct or competitive in vitro ELISA inhibition tests with sera from allergic patients but also with the help of animal sera. For example, IgE epitopes of bovine β-lactoglobulin have been studied by several groups mainly by using tryptic or synthetic peptides. Major IgE binding epitopes of this protein have now been identified to be within amino acid sequences 41–60, 102–124 and 149–162 (Tokita, 1985; Takahashi et al., 1990; Selo et al., 1998). Similar analyses on α-lactalbumin showed sequences 17–58 and 59–94 to be mostly recognized by patient sera (Maynard et al., 1997). In β-casein, the IgE epitopes are found in sequences 1–60, 110–144, 157–185 and 186–209. Less information is available on T cell epitopes of milk proteins. For β-lactoglobulin, the region 119–133 was identified whereas for αs1-casein several sequences (61–80, 91–110, 136–155, 151–170) were described.

Breastfeeding is certainly the best way for preventing sensitization of babies to cow's milk proteins. When breastfeeding is not possible, hypoallergenic (HA) cow's milk based formulas are usually given during the first 6 months of life for prevention of CMPA, mainly to infants from atopic families (at risk infants). Depending on primary (sensitization) or secondary (triggering) prevention of CMPA, the approach with HA formulas is different. When infants are already sensitized to CMP, minute amounts of cow's milk protein allergenicity may trigger an allergic reaction and it is generally agreed that only so-called extensively hydrolyzed hypoallergenic formulas (eHF) or amino acid formulas can be given to infants with established CMPA. On the other hand, for primary prevention in non-sensitized at risk infants and for a long-term protection to CMPA, partially hydrolyzed hypoallergenic formulas (pHF) should be recommended. Such formulas are sufficiently reduced in allergenicity for not sensitizing at risk infants but contain still enough immunogenicity for deviating the immune system to a hyporesponsiveness against cow's milk proteins, called 'oral tolerance'.

2. Allergenicity determination

For reducing the allergenicity of cow's milk proteins, IgE-binding and T cell epitopes have to be destroyed or inactivated. Different processes are used currently to achieve this. For example heating cow's milk proteins above 80 °C will destroy the globular structure of proteins, and consequently conformational epitopes but not sequential ones. Enzymatic hydrolysis is by far the most efficient process for allergenicity reduction. Depending on the type of enzymes used and the conditions of hydrolysis, peptides of different length may be obtained carrying more or less allergenicity. Porcine trypsin/chymotrypsin are frequently used for producing hypoallergenic formulas but proteases extracted from bacteria or of fungal origin are increasingly also used.

Physico-chemical methods are often used for examining the amount of proteins/peptides obtained after heat and enzymatic hydrolysis processing. SDS-PAGE and peptide profiles give usually a good picture on allergenicity reduction. This correlation is, however, not always true and trying to define a molecular weight below which a peptide is not allergenic is very difficult if not impossible. Immunological methods have, there-

fore, to be used for reliable allergenicity determinations.

2.1. ELISA tests

ELISA inhibition or uptake methods are frequently used for the determination of IgG and IgE *binding* epitopes, using respectively allergen specific polyclonal animal sera or human patient sera. These methods are very sensitive, allowing the detection of minute amounts of antigen (nano/picogram levels). ELISA inhibition tests measure monovalent and polyvalent IgG and IgE epitopes that may be potentially allergenic. ELISA uptake tests determine usually bivalent and polyvalent IgG epitopes. None of these methods determines exclusively IgE epitopes leading to allergic triggering.

2.2. Cellular assays

The IgE mediated mast cell *triggering* capacity of allergenic epitopes can be measured with the help of a functional in vitro assay. We have set up such an assay in our laboratory, based on peritoneal rat mast cells passively sensitized with specific rat IgE and labeled with 3H-serotonin (Fig. 1). Cells are triggered for mediator release with standard dilutions of the allergen or test formula dilutions (Fritsché and Bonzon, 1990). Results show that very different amounts of residual allergenicity can be determined in hypoallergenic infant formulas and that residual traces of allergenicity are found even in extensively hydrolyzed formulas to be used by allergic infants (Table 1).

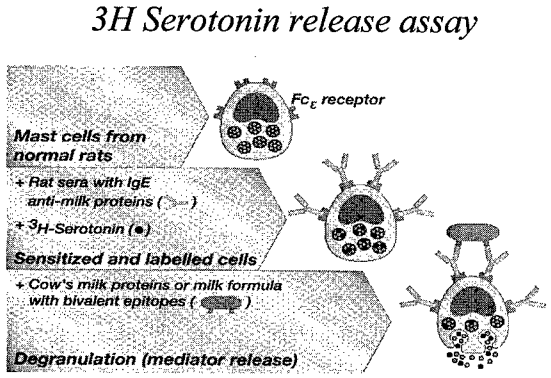

Fig. 1. Determination of allergenicity by in vitro triggering of IgE sensitized and 3H-serotonin labeled mast cells.

2.3. In vivo animal models

The IgE dependent allergic reaction is composed of two phases: an inducing step, where the immune system of the host is sensitized by the allergen, ending up in specific IgE anti-allergen antibodies production which are then fixed by mast cells in target organs; the second step is a triggering phase mediated by the allergen binding to these IgE and stimulating mediator (histamine) release from mast cells. For evaluating allergenicity of food antigens, both phases should be examined by appropriate tests. In vivo animal models help in achieving this goal.

Historically, guinea pigs (Devey et al., 1976) and rats (Fritsché and Bonzon, 1990) have been used to investigate allergenicity of food proteins. Guinea pigs can be sensitized by the oral route without adjuvants making it a good model, close to the human situation. Difficulties associated with passive cutaneous anaphylaxis (PCA) testing and the fact that the reaginic antibody response is of the IgG1a subtype limit the use of guinea pigs as a suitable model with which to study CMPA (Poulsen and Hau, 1987). The Brown Norway rat has been reported to be a useful model for the investigation of food allergy because intraperitoneal sensitization generates IgG and IgE antibodies to a range of milk proteins that are of similar specificity to those produced by humans (Atkinson and Miller, 1994).

There is a significant obstacle to the development of oral murine models of food allergy, namely the strong innate tendency to develop oral tolerance to ingested antigens.

2.3.1. Rat parenteral model

The standard protocol we are using is the following. Brown Norway high IgE responder rats are injected subcutaneously with the allergen in presence of $Al(OH)_3$. Fourteen days later the primary IgE response is determined by ELISA in animal sera. For the evaluation of the in vivo triggering activity of a product containing the allergen, above sensitized rats are gavaged with

Table 1
Allergenicity of HA formulas determined by mast cell triggering

Product	MW med. (Da)	Amino-N (%N tot)	Residual BLG (mcg/g prot)
HUMANA-HA	500	36.89	3100
ENFASTART	850	27.45	550
FRISOPEP 1	1090	31.68	150
NAN HA	1820	21.63	300
E-AKACHAN	850	35.37	550
ADAPTA HA	NT	NT	150
APTAMIL HYP	500	39.85	15
DAMIRA	NT	NT	10
FRISOPEP 2	850	23.92	10
PROFYLAC	850	28.79	5.5
PEPDIETT	390	59	2
PEPTI JUNI	660	42.56	1.2
NUTRILON PE	500	46.15	0.8
ALFARE	500	33.35	0.5
EPITOLESS	300	59.47	0.3
HIPP HA	660	36.95	0.2
MA-1	390	64.79	0.15
NUTRAMIGEN	390	61.21	0.15
HA THERA	425	44.7	<0.1
ELEMENT F.	140	70.79	<0.1

the test product, bled after 2 h and the level of rat mast cell protease II (RMCPII) determined in the serum. After a booster injection of the allergen, the secondary IgE response as well as spleen/lymph node lymphocyte proliferation and culture supernatant cytokine determinations are done.

This parenteral rat model provides a good indication on the IgE inducing capacity of standard and hypopallergenic infant formulas: moderately and extensively hydrolyzed cow's milk formulas induced, respectively, 100 to 10 000 times less IgE antibodies than a standard milk formula (Fritsché and Bonzon, 1990). Further, intestinal mast cells are primary targets of food allergens in IgE dependent hypersensitivity. In the rat model, the specific protease (RMCPII) is released into blood after intestinal mast cell triggering. This protease, determined by ELISA in serum, is a good indicator of the IgE mediated allergic triggering capacity of infant formulas at the intestinal level (Fig. 2). Standard formulas stimulated the highest specific release in our model, followed by moderately and extensively hydrolyzed formulas (Fig. 3).

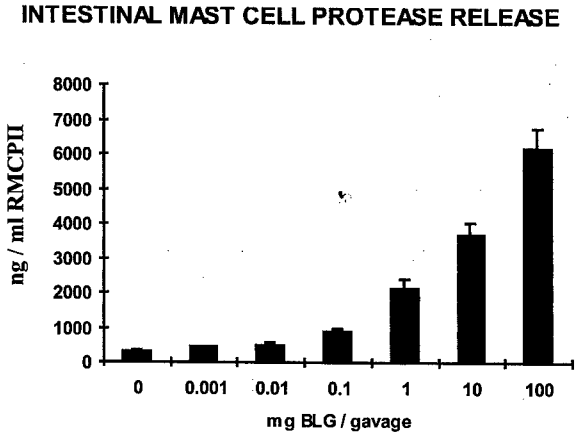

Fig. 2. βLG mediated rat mast cell protease II (RMCPII) release from βLG sensitized rats.

2.3.2. Oral mouse model

Several tests in mice, using adjuvants like cholera toxin, have been published which succeeded in inducing oral IgE mediated sensitization to cow's milk proteins (Li et al., 1999; Von-derweid et al., 2001).

In our experiments, for oral sensitization, 6-week-old Balb/c mice were given weekly gavages

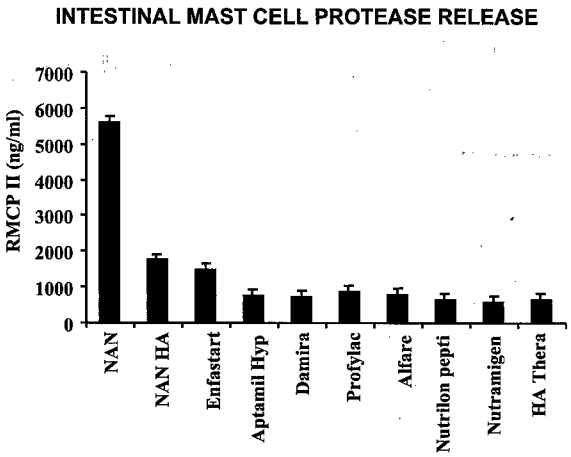

Fig. 3. Oral stimulation by infant formulas of rat mast cell protease II (RMCPII) release from cow's milk sensitized rats.

during 4 weeks of cow's milk proteins (e.g. 2 mg β-lactoglobulin) mixed with cholera toxin (10 μg). Specific IgE, IgA, IgG1 and IgG2 anti-βLG antibodies were raised in this model. Although this model requests also an adjuvant, its main advantages are to be oral and IgE-mediated. It certainly deserves to be fully developed in the future.

3. Evaluation of tolerizing activity

While it is mandatory for safety reasons to know the residual allergenicity of an infant formula, it is also important to determine its positive immune modulatory capacities. One of these is its ability to induce oral tolerance to food antigens (e.g. cow's milk proteins). Oral tolerance is a basic mechanism commonly accepted to be the normal immune reactivity of the host to foods. Its breakdown leads to food allergy. Oral administration of protein antigens induces specific immunologic hyporesponsiveness to these antigens. Induction of oral tolerance with intact proteins has been well documented with a number of antigens in several animal models (Wells and Osborne, 1911; Thomas and Parrott, 1974; Hanson et al., 1977) and in humans (Husby et al., 1994). Various studies have shown that oral tolerance is influenced by strain (Ito et al., 1997; Kiyono et al., 1982), age of first feeding (Hanson, 1981; Strobel and Ferguson, 1984; Strobel, 1996) and the dose as well as the nature of antigen (Mowat, 1987; Lamont et al., 1989).

The following models are helpful for evaluating the tolerizing capacity of a product.

3.1. Rat parenteral ad lib feeding

For induction of oral tolerance to cow's milk proteins, Sprague–Dawley rats are given different experimental liquid milk formulas ad libitum in their drinking bottles and a solid 'milk-free' pellet diet from days 1 to 9 of the experiment. For challenge, rats are immunized on day 11 by subcutaneous injection of a selected cow's milk protein (e.g. βLG) in the presence of aluminum hydroxide. Animals are sacrificed on day 24 and sera analyzed for specific IgE antibodies.

We have shown with the help of this model that partially hydrolyzed whey proteins (pHF) are able to induce oral tolerance to intact whey proteins whereas extensively hydrolyzed whey proteins (eHF) are unable to achieve this (Fritsché et al., 1997). This was demonstrated at the levels of the IgE response, lymphocyte stimulation and intestinal mast cell secretion (Fig. 4).

3.2. Mouse oral gavage model

Orally sensitized mice mounted a βLG specific IgE response when gavaged with βLG in the presence of cholera toxin. A single gavage of whey proteins given prior to the onset of oral sensitization resulted in the suppression of both specific and bystander IgE (Von-der-weid et al., 2001).

3.3. Guinea pig oral ad lib model

Residual antigenicity, age of onset and length of feeding are important parameters for either sensitizing or inducing oral tolerance in guinea pigs. We have for example shown that oral ad libitum administration of moderately hydrolyzed whey proteins to Dunkin–Hartley guinea pigs during 5 weeks before an oral challenge during 2 weeks with intact whey proteins prevents the induction of

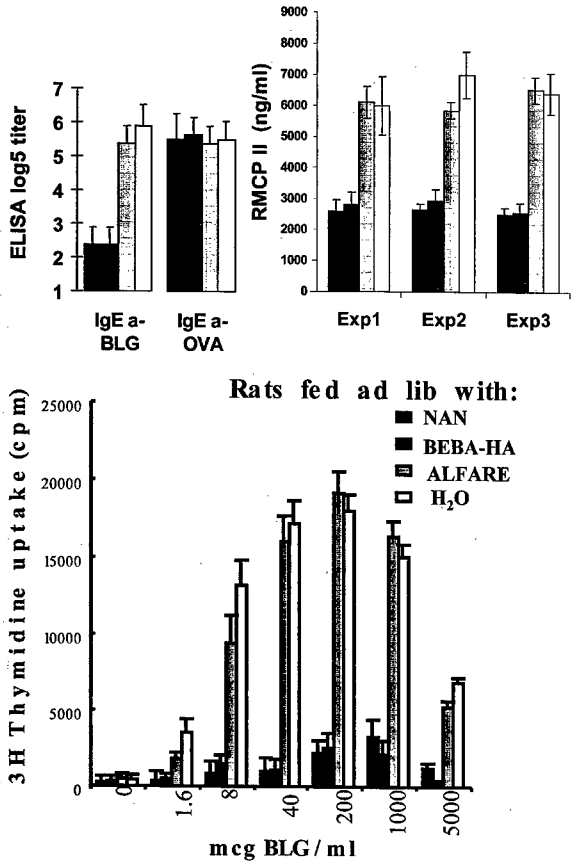

Fig. 4. IgE, rat mast cell protease II (RMCPII) and lymphocyte suppression following oral tolerance induction to βLG with intact or moderately hydrolyzed whey based infant formulas (βLG, β-lactoglobulin; OVA, ovalbumin).

specific anti-cow milk protein reaginic IgG1a antibodies. This demonstrated that oral tolerance could be induced by prefeeding with a tolerogenic non-sensitizing formula, in a similar approach as the one occurring in infant feeding.

4. Discussion

In vitro and cellular assays are very helpful tools for evaluating residual allergenicity in infant formulas, mainly for the determination of IgG and IgE binding and triggering epitopes. The sensitizing capacity as well as the residual tolerogenicity of a formula can, however, be evaluated only with in vivo animal models.

The rat parenteral model appears to be adequate for measuring both the IgE specific sensitizing (production of IgE antibodies) and triggering (IgE-mediated RMCPII release) capacities of food allergens. It further allows the determination of the tolerizing capacity of an infant formula. One major disadvantage of this model is that sensitization is done by the parenteral route. In this respect, the oral mouse and guinea pig models are closer to the physiologic sensitization to food proteins. The mouse model, however, still requires an adjuvant and in guinea pigs, oral sensitization produces IgG1a reaginic antibodies, not IgE.

As no ideal animal model exists for food allergy, it is recommended to use several selected ones simultaneously for preclinical testing of infant formulas.

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Review

Principles in toxicological risk analysis

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Abstract

Toxicological risk analysis comprises a process of hazard identification, dose–response assessment and exposure assessment, providing as an output the estimation of the incidence and severity of adverse effects likely to occur in a human population in relation to actual or eventual exposure to hazardous compounds. Within this respect, toxicological risk analysis represents a scientific activity, which uses data from toxicological research, and serves as a basis for risk management which is the decision-making process which is performed by regulators and politicians, considering also non-scientific aspects such as socioeconomic and political factors. The predominant step in hazard identification is the answer to the question whether a compound must be classified as a genotoxic carcinogen, epigenetic carcinogen or non-carcinogen. Additional important aspects concern the questions how to extrapolate from high to low dose, as well as how to deal with exposures to complex mixtures of carcinogens. Also, the question is addressed whether it is required in toxicological risk analysis to consider susceptible subgroups in the population, for instance on the basis of specific genetic predispositions.
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Keywords: Toxicological risk assessment; Carcinogenic compounds

1. Introduction

Toxicological risk can be defined as the toxic consequence of a particular activity in relation to the likelihood that it may occur (Van Leeuwen and Hermens, 1995).

Toxicological risks should be characterized in terms of:

- Type of the hazard.
- Magnitude of the hazard.
- Probability of the hazard.

Fig. 1 shows the paradigm of toxicological risk assessment (adapted from Committee on the Institutional Means for Assessment of Risks to Public Health. Commission on Life Sciences. National Research Council, 1983). With respect to hazard identification, the question has to be answered whether the agent under study induces an adverse health effect. In this paper, the difference in hazard identification between geno-

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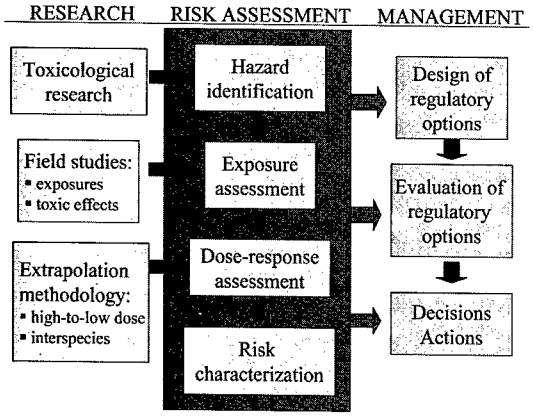

Fig. 1. Research risk assessment management.

toxic carcinogens and other compounds will be extensively addressed. Subsequently, the magnitude of the hazard has to be estimated on the basis of dose–response relationships as available from the toxicological data base. Most importantly, it has to be assessed whether established or calculated exposures are above critical doses at which adverse health effects are induced. Risk assessors in general rely on so-called safety limits which have been designed in such a way that if not exceeded, no intolerable health risks are likely to occur among the exposed population. This paper will specifically pay attention to certain aspects of setting safety limits to exposures to (mixtures of) genotoxic carcinogens. Human exposure can follow simple routes, for instance via inhalation of toxic compounds containing air, of rather complex schemes, for instance via food (Fig. 2) and can be

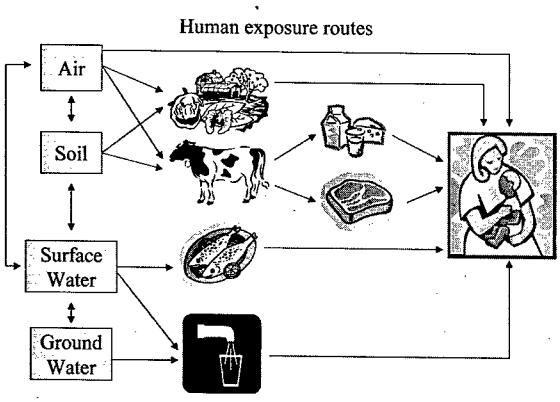

Fig. 2. Human exposure routes.

estimated through measuring contamination levels of relevant environmental compartments, and multiply these with the calculated intake of the involved environmental media. To discuss available models for exposure assessment, is however, considered beyond the scope of this paper.

Risk managers use the outcome of the risk assessment process in order to design and to imply policy measures, but also implicate social/economic/political factors in their decision making process (Fig. 1). As a consequence, risk managers have the final word on for instance, environmental health standard setting. Therefore, it can be argued that risk assessment is a scientific activity while risk management is not: alternatively, scientists should refrain from risk management, and decision makers should leave the risk assessment to scientists/scientific advisory boards.

2. Toxic effect assessment

2.1. Linear non-threshold extrapolation for genotoxic carcinogens

For most compounds, it is assumed that their toxic capacity is only expressed beyond a certain threshold and that this toxic effect increases with increasing dose till a certain maximum level, e.g. dose–response relationships in general show a non-linear sigmoid curve. Below this observed threshold dose, no chronic toxic effect is induced, implying that this dose can be considered safe in case of lifetime exposure. For assessing a safety limit with regard to human health for such a compound, this threshold dose is divided by a factor of 10 in order to account for interspecies differences in susceptibility, assuming humans to be more susceptible than laboratory animals, and another factor of 10 to account for interindividual differences, e.g. related to differences in genetic background between humans which are assumed to be much larger than in any inbred strain of laboratory rodents. This yields for instance the acceptable daily intake (ADI), representing that particular dose of the compound under study to which humans can be exposed daily during their life time without experiencing any adverse health

effect: the so-called zero-tolerance risk model derived for non-genotoxic chemicals. If in a certain case of environmental exposure to such a compound the outcome of the exposure assessment shows that the ADI is not likely to be exceeded, the risk of toxic effects is considered absent. Exposure to higher doses also does not necessarily imply that persons will suffer from adverse health effects, in view of the safety factors applied in deriving the ADI from apparent threshold doses: however, caution is warranted and some policy measure, in general aiming at reducing exposure, is indicated.

By contrast, the notion that one single hit by one molecule of a DNA-damaging agent may, under the worst conditions, induce a point mutation in a crucial gene and lead to tumorigenesis on longer term—however, unlikely this may be-underlies the effect assessment for genotoxic agents. Consequently, the concept of a safe threshold dose is no longer applicable since cancer risk in case of exposure is unavoidable; furthermore, dose–response relationships for genotoxic compounds, are therefore, considered linear. Compounds are defined as genotoxic carcinogens if they demonstrate their inherent capacity of inducing genetic damage as apparent from positive results obtained in dedicated in vitro tests (mutation assays, chromosomal damage tests) as well as in cancer bioassays, in general involving two different biological species (Van Leeuwen and Hermens, 1995). Fig. 3 demonstrates the calculation of tolerable doses for given genotoxic carcinogens, applying linear non-threshold extrapolation. This extrapolation uses the lowest dose of the genotoxic carcinogen under study which appears to be capable of inducing tumorigenesis significantly beyond the 'spontaneous' background of cancer incidence down to a level of cancer risk which is considered to be tolerable, e.g. one additional case of cancer due to the exposure to this particular carcinogen, among 1 million individuals exposed during their life time. From Fig. 3, it becomes obvious that because of the fact that the tumor incidence in the bioassay has to be significantly increased above background, this has to be in the order of several per cent, implying that this extrapolation towards a tolerable risk spans four to five orders of magnitude on which no factual observations are available (Lutz, 1990). The real shape of the dose–response curve at these low doses, is therefore, unknown.

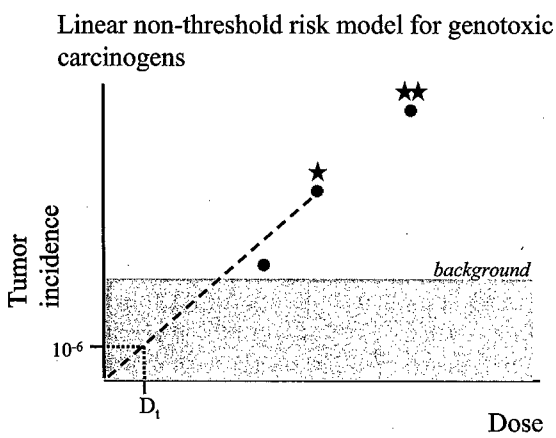

Fig. 3. Linear non-threshold risk model for genotoxic carcinogens.

2.2. Thresholded mechanisms for the action of genotoxic carcinogens

Although the so-called one hit model for chemical mutation induction as described in the previous paragraph, has a sound theoretical basis, it is also obvious from our understanding of the biological fate of chemical compounds that the default assumption of dose–response linearity, specifically at low dose of genotoxins, is questionable. During a recent ECETOC symposium devoted to this discussion, several mechanisms involved in threshold effects of genotoxins, have been suggested (Speit et al., 2000):

- Protection of DNA by membranes, proteins, etc. against attack.
- Metabolic inactivation of reactive intermediates.
- Repair of induced DNA lesions.
- Apoptosis of too heavily damaged cells.

Although there are data from animal carcinogenesis studies which challenge the linearity of the dose–response curve, e.g. for liver carcinogens (Williams et al., 2000), in general, whether or not

such thresholds at low dose really exist is difficult to demonstrate experimentally beyond any doubt. An alternative approach has been suggested (Speit et al., 2000): In view of the fact that genes encoding for these thresholded pathways, happen to be polymorphic in the human population (Brennan, 2002) it can be hypothesized that in humans with defective alleles, no threshold mechanisms are in operation, and consequently, a linearity of the dose–response curve becomes apparent. However, although both in vitro as in vivo studies have shown ample evidence for increased susceptibility among these subjects for genotoxic effects in relation to carcinogen exposure (Xiong et al., 2001; Hou et al., 2002; Thier et al., 2002), till now, no data have been reported on the shape of the dose–response curve at low dose in (cells derived from) polymorphic humans.

The method of linear non-threshold extrapolation to assess chemical cancer risk is considered to be very conservative probably leading to some over-estimation of the risk, and therefore, no additional safety factors to account for interspecies and interindividual differences in susceptibility as is done for non-genotoxic compounds, are implied. It may, however, be questioned whether genetically predisposed differences in genotoxic susceptibility between humans is that large that the model has to be consequently adjusted. However, up till now, results within this respect are conflicting: for instance, a recently published meta-analysis summarizing 43 case-control studies on the impact of the *GSTM1* polymorphism on lung cancer risk involving more than 18.000 individuals showed a slightly higher risk in relation to *GSTM1* deletion, but when in a smaller set of 21 studies on pooled original data of about 9.500 subjects the level of smoking was taken into account, no interaction with the *GSTM1* genotype became apparent (Benhamon et al., 2002).

2.3. Risk assessment of mixtures of genotoxic agents

In general, people are exposed to mixtures of genotoxic agents, for instance through food, through air pollution, through passive smoking. However, cancer risk assessment as described above, usually refers to exposure to single compounds. How to deal with mixtures, still is matter of debate. In case of polycyclic aromatic hydrocarbons, it has been proposed (Nisbet and LaGoy, 1992) to assign toxic equivalency factors (TEFs) to individual PAHs as has also been done for polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and certain polychlorinated biphenyls. Benzo(a)pyrene was used as reference compound and the following end points were considered for assessing the relative potency of other PAHs and for assigning TEFs:

- Carcinomas in lungs of rats exposed via intrapulmonary administration.
- Complete carcinogenesis in mouse skin.
- Papillomas and/or carcinomas in mouse skin in initiation-promotion studies.
- PAH–DNA adducts in in vitro studies.

We applied this approach for instance, to assess cancer risks of PAHs present in whiskies (Kleinjans et al., 1996).

This TEF approach uses additive interaction between individual genotoxic compounds as a default assumption. However, also non-additive interactions in a mixture of genotoxic carcinogens have been reported, for instance between cigarette smoke and radon exposure, between asbestos and cadmium, and in a mixture of thyroid carcinogens (Hasegawa et al., 1994).

It is obvious that we need further information on the pathways of multiple carcinogen interactions in order to achieve a reliable cancer risk assessment in case of exposure to mixtures of DNA-damaging agents. This may come from toxicogenomics. It is proposed to apply microarray technology in various ways in order to try and understand carcinogenic effects of mixtures:

- In defined animal models, interactions between carcinogens can be quantitatively evaluated by comparing changes in gene expression of the mixtures with the sum of changes for each individual carcinogen.
- Gene expression profiles of mixtures of carcinogens can be qualitatively compared with expression patterns of individual carcinogens.

This new molecular tools also including molecular markers for individual susceptibility, may improve the basis for cancer risk assessment in the near future.

3. Summarizing

Rather than with a set of clear-cut conclusions, this paper ends with a series of statements with regard to the state-of-the-art of toxicological risk assessment:

- Many things are still unknown or uncertain in cancer risk assessment.
- Cancer risk assessment is not a routine job yet.
- Risk assessors may not need to be at the front of toxicological research, but certainly need to be in close contact.

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Review

The role of mode of action studies in extrapolating to human risks in toxicology

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Abstract

The US Environmental Protection Agency (EPA) in 1999 issued draft guidelines on carcinogen risk assessment, which included the use mode of action information in the risk assessment process. We have used the five stages of induction of toxicity as described by Aldridge to illustrate in the case of two drugs, tamoxifen and NTBC, how mode of action information played a key role in assessing the risk of cancer and target organ toxicity, respectively.
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Keywords: Risk assessment; Mode of action; Tamoxifen; NTBC; Liver cancer; Ocular toxicity

1. Introduction

In 1999 the United States Environmental Protection Agency (EPA) issued draft Guidelines for Carcinogen Risk Assessment, these were very detailed and in particular discussed a framework for evaluating a postulated mode of action. A mode of carcinogenic action is composed of the key events and processes starting with the interaction of the agent with a cell, leading to functional and anatomical changes, and finally resulting in cancer formation. 'Mode' of action was contrasted with 'mechanism' of action, which implies a more detailed description of molecular events than is meant by mode of action. There are many examples of modes of carcinogenic action, such as mutagenicity, mitogenesis, inhibition of cell death, immune suppression and reparative cell proliferation. When analysing mode of action all pertinent information is reviewed, an overall weighing of evidence is then performed, laying out the strengths, weaknesses and uncertainties of the case. This process may then help identify data gaps and research needs. This approach which, was developed for cancer risk assessment, can also be applied to risk assessment for target organ toxicity, where key steps in the mode of action can be identified to help determine whether there is a casual relationship between these events and organ damage.

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2. Framework for the use of mode of action data in risk assessment

Mode of action information forms part of hazard identification—can the agent present a carcinogenic (toxicity) hazard to humans, and if so under what circumstances? Analysis of mode of action information should include the following key pieces of information (USEPA, 1999).

1) The sequence of events and processes that are considered to lead to target organ toxicity or cancer formation.
2) The strength, consistency and specificity of the association.
3) Dose response relationship.
4) Temporal relationship.
5) Biological plausibility and coherence.
6) Other modes of action.
7) Conclusion.
8) Human relevance, including subpopulations, i.e. is the mode of action operative in animals also operative in humans, and are there any human subpopulations likely to respond qualitatively differently than the general population?

In this brief paper we have used the five stages of induction of toxicity (Aldridge, 1981, 1986) to illustrate, with two examples, how the USEPA guidelines on mode of action can be used for a carcinogen and for target organ toxicity. The five stages of induction of toxicity are:

1) Entry of the drug into the organism, whether by ingestion, inhalation, through the skin or more directly by injection into the blood stream.
2) Once the drug has gained access to the body many factors can influence whether it reaches a potential target and the concentration at which it does so. Some such processes include the physical and chemical properties of the drug and its routes and rates of metabolism. Metabolism may lead to detoxification or in some cases to bioactivation.
3) The third stage involves the identification of the primary target, which the drug or its metabolite interacts with, thus triggering the cascade of events culminating in signs or symptoms of toxicity. This may be a reversible or a covalent interaction. The primary reaction needs to be separated from those reactions, which cause non-hazardous changes in biochemistry, physiology or morphology.
4) The next stage involves the secondary changes that follow as a consequence of the primary reaction.
5) The fifth stage is the consequence to the organ or tissue, such as a pathological change, e.g. a pre-neoplastic lesion or morphologic changes in the target organ.

The International Life Sciences Institute/Health and Environmental Sciences Institute, Washington, US first used this approach to explore the application of the proposed EPA guidelines to toxicity testing of chemicals. In this article we outline how mode of action studies can assist in the risk assessment process, using the drugs tamoxifen and NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione] as examples.

3. Case study with tamoxifen

The anti-oestrogen tamoxifen is currently the most clinically tested drug used in the adjuvant therapy of breast cancer treatment. In the US tamoxifen has also been used as a prophylactic treatment of breast cancer in women at high risk of the disease (Smith et al., 2000). Rats given daily doses of tamoxifen (5–35 mg/kg per day) for 2 years developed a dose related increase in the incidence of liver tumours, which was similar in males and females (Greaves et al., 1993). Chronic exposure of mice to tamoxifen has been negative with regard to liver tumours (Tucker et al., 1984).

The issue: are humans at risk from liver cancer (or other cancers) when taking tamoxifen and is it the mouse or rat response that is more relevant to humans?

The available information on tamoxifen will now be discussed in terms of the five stages of toxicity outlined by Aldridge (1981, 1986).

3.1. Route of exposure

In rats, the exposure route is usually dietary or by gavage, the dose levels used vary quite considerably from study to study but in the bioassay the top dose was ~40 mg/kg per day. In women, exposure is via a tablet usually once or twice a day, the vast majority of women receiving only 20–40 mg/day.

3.2. Distribution and metabolism

In both rats and humans tamoxifen is rapidly absorbed, however, elimination in the rat is relatively fast compared with humans. The elimination in humans is biphasic with initial and terminal half-lives of 7–14 h and over 7 days, respectively (Adam et al., 1980). In contrast, the terminal plasma half-life in rats is about 14 h (Fromson et al., 1973). Following daily doses of 20 mg tamoxifen per person, mean serum levels of about 300 ng/ml were found. Due to the faster elimination of tamoxifen in rats, similar doses (0.4–1 mg/kg tamoxifen) resulted in plasma concentrations of only 1–20 ng/ml. At least a 20-fold higher dose is required in rats (20 mg/kg) to obtain steady-state plasma concentrations approaching those of women.

Tamoxifen is metabolised by phase I enzymes more rapidly in rats and mice compared with humans. The major phase I routes of metabolism are N-demethylation, N-oxidation and alkyl and ring hydroxylation. (Smith et al., 2000 and references therein). Phase II metabolism also differs significantly between species. Mice and humans generate less α-OH tamoxifen than the rat (rat > human, 3 ×) and the former species both glucuronidate tamoxifen to a greater extent than the rat (human ≫ rat, 100 ×). The rat sulphates α-OH tamoxifen (rat > human 5 ×), and this is the key species difference as this pathway leads to the generation of reactive metabolites (Smith et al., 2000 and references therein).

3.3. Primary biochemical response

Metabolism in the rat to α-OH tamoxifen followed by sulphation generates a reactive electrophile that binds to protein and DNA (see Smith et al., 2000). Consequently tamoxifen can produce large quantities of DNA adducts in the liver of rats (up to 3000 adducts per 10^8 nucleotides), and their formation is both time and dose dependant. In contrast, few DNA adducts are formed in mouse liver following tamoxifen exposure and there is no evidence for the formation of adducts in hepatic DNA from women taking tamoxifen (Martin et al., 1995).

Thus there are major differences between rats, mice and humans both in the metabolism of tamoxifen and in the formation of adducts. Human subpopulations with putative polymorphisms in the glucuronidation of α-OH tamoxifen, which is the detoxification route, need to be considered in the risk assessment process.

3.4. Secondary biochemical response

Treatment of rats with tamoxifen also leads to an increase in cell proliferation in the liver, but it is not clear if this is a primary or secondary response. Mice show no increase in hepatic cell proliferation after exposure to tamoxifen, suggesting that the response in the rat may be a mitogenic effect provoked by the partial estrogenic properties of tamoxifen, a consequence of liver damage or a combination of both. Both DNA adduct formation and cell proliferation appear to be required for rat liver tumour formation (Fig. 1).

3.5. Pathology or consequence to organ or tissue

Tamoxifen produces hepatocellular carcinomas in rats, with some metastasis. At high doses (40 mg/kg per day) rats can develop tumours within 6 months and few will survive to 15 months. There is no evidence of liver cancer in mice, while extensive epidemiology studies in women failed to demonstrate evidence of liver cancer associated with tamoxifen (IARC, 1996). Epidemiology studies have, however, shown an increased incidence of endometrial cancer in women on tamoxifen, which is small but real. There is some evidence for a low incidence of ^{14}C-tamoxifen adducts in human uterus DNA (< 1 adduct per 10^8 nucleotides). A plausible explanation is that tamoxifen acts as an

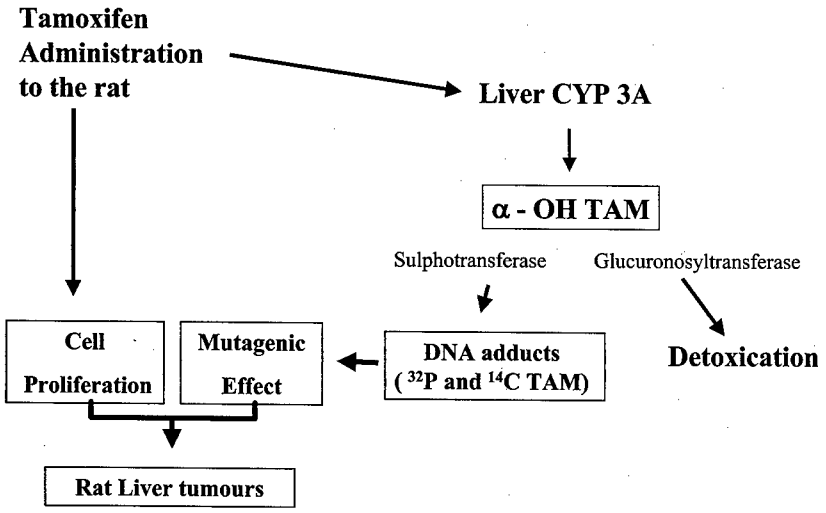

Fig. 1. Proposed mode of action for the production of liver tumours in rats administered tamoxifen.

oestrogen in the uterus, rather than an anti-oestrogen, inducing cell proliferation in endometrial tissue and promoting tumours already initiated.

Thus, based on routes and rates of metabolism of tamoxifen and the lack of DNA adduct formation in human liver, women are at very low risk of developing liver cancer.

4. Case study with NTBC

NTBC (Nitisinone) is currently registered as an orphan drug for the treatment of tyrosinaemia type 1 (OMIM 276700), a recessive inherited metabolic liver and kidney disease caused by deficiency of fumarylacetoacetate hydrolase (Lindstedt et al., 1992; Holme and Lindstedt, 2000). Rats given daily doses of NTBC developed lesions on the surface of the cornea of the eye, which are similar in males and females (Lock et al., 1996). Chronic exposure of mice to NTBC has been negative with regard to corneal lesions (Lock et al., 2000).

The issue: are children at risk from corneal lesions (or other toxicity) when taking NTBC and is it the mouse or rat response that is more relevant to humans?

The available information on NTBC will now be discussed in terms of the five stages of toxicity outlined by Aldridge (1981, 1986).

4.1. Route of exposure

In rats, the exposure route was by gavage or in the diet; the dose levels used covered a wide range but an incidence of ocular lesions, of about 80%, was seen at daily doses of 0.5 mg/kg per day for 6 weeks. In children, exposure is via a tablet usually twice a day, the vast majority of children receiving 1 mg/kg per day, which in some cases may be increased to 2 mg/kg per day.

4.2. Distribution and metabolism

In both rats and humans NTBC is rapidly absorbed, with plasma elimination being relatively slow. The plasma half-life in humans is 54 h and in rats it is biphasic with initial and terminal half-lives of about 6 and 74 h, respectively (Hall et al., 2001; Lock et al., 1996). Following a single oral dose of 1 mg/kg peak plasma levels of about 10 μg/ml were attained in humans, while in rats peak plasma values were about 33 μg/ml following a single oral dose of 10 mg/kg. Thus the plasma values in the rat at 3–4 mg/kg will be similar to those attained in children.

NTBC undergoes metabolism by phases I and II, primarily ring hydroxylation and conjugation with glucuronic acid. The pharmacological activity resides in the parent compound and, based on the blood levels, no major metabolic differences are expected between rats and humans. The compound is distributed throughout the body with no evidence for selective retention in the rat eye, although some retention is seen in the liver and kidneys. A similar distribution profile is seen in both rats and mice (Lock et al., 1996, 2000).

4.3. Primary biochemical response

NTBC is a potent, tight-binding reversible inhibitor of hepatic 4-hydroxyphenyl pyruvate dioxygenase (HPPD), an enzyme involved in the catabolism of the amino acid tyrosine (Ellis et al., 1995). No major species difference in enzyme sensitivity is seen; the rat, mouse and human liver enzymes all being inhibited in vitro by nanomolar concentrations of NTBC.

4.4. Secondary biochemical response

As a consequence of inhibition of HPPD, NTBC produces a time and dose dependent increase in the concentration of tyrosine in both the plasma and ocular fluid in rats and mice (Table 2a and b). Dietary supplementation of tyrosine also causes corneal lesions in rats (Rich et al., 1973), which are very similar to those seen with NTBC. The extent of the tyrosinaemia induced by NTBC is more marked in the rat than mouse (Table 2a). These findings show that there is a threshold level for tyrosinaemia in the eye (approximately 3000 nmol tyrosine/ml ocular fluid) above which corneal lesions are seen; in mice this threshold is never breeched and no corneal lesions have been observed. (Fig. 2b). Thus the corneal lesions are secondary to the tyrosinaemia produced by NTBC.

4.5. Pathology or consequence to organ or tissue

NTBC produces corneal erosion and opacity in rats with some vascularisation, which is reversible upon cessation of NTBC administration. No such

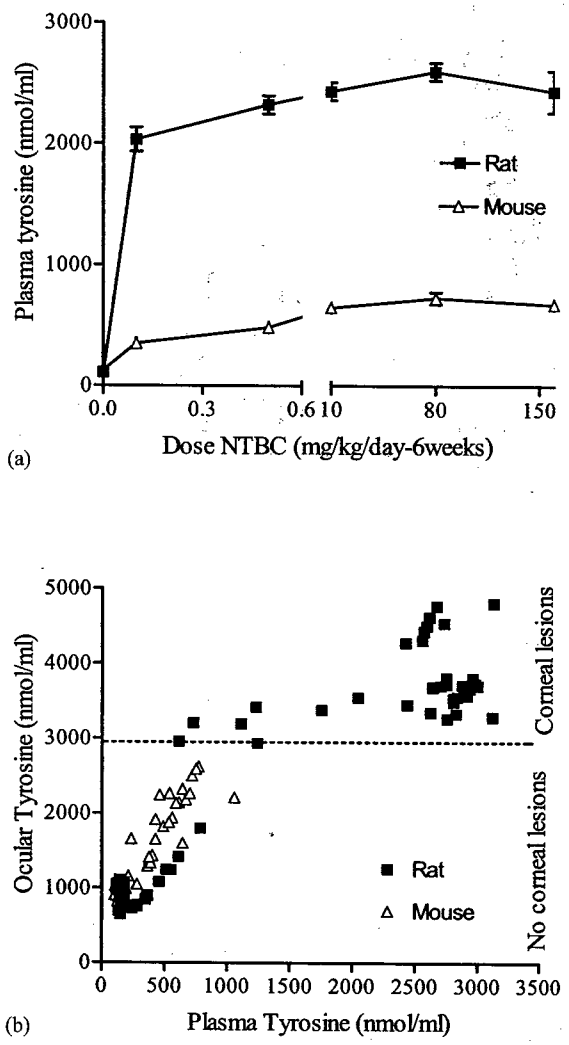

Fig. 2. The extent of tyrosinaemia produced in the rat and mouse following NTBC administration and its relationship to corneal lesions.

lesions were seen in mice given doses at least two orders of magnitude above those at which lesions are seen in rats. Experience with over 300 patients typically receiving 1 mg/kg per day, with over 100 children receiving treatment for 7–11 years, have reported no major adverse side effects. The metabolic block produced by NTBC leads to an increase in plasma tyrosine, usually of about 500–700 nmol/ml, although in some patients with poor dietary control values as high as 1000–1300 nmol/ml has been reported. The most

common adverse findings reported (17 occasions) are for the eye, namely itching, burning, photophobia and corneal clouding (Holme and Lindstedt, 2000). It is known that subpopulations with the metabolic disorder tyrosinaemia type II, a deficiency of tyrosine aminotransferase, can develop corneal lesions analogous to those seen in rats. These children have a marked tyrosinaemia, resulting in plasma tyrosine values of around 2400 nmol/ml. Placing these children on a low tyrosine/phenylalanine diet lowers plasma tyrosine and the eye lesions resolve (Goldsmith, 1983). The aim with NTBC treatment is to keep plasma tyrosine < 500 nmol/ml to avoid adverse effects on the eye.

Thus, based on the extent of tyrosinaemia produced by NTBC it is clear that children and mice can remove excess tyrosine from the body more effectively than rats, thereby reducing the risk of adverse ocular effects.

In summary, both these cases illustrate how important mode of action information is for the risk assessment process and how it can be used in the context of the five stages of induction of toxicity. In the future, greater use of human tissue in vitro, or human enzymes/proteins expressed in cellular systems, is likely to help with the extrapolation of animal findings to humans. We can also anticipate an increasing use of genomic and proteomic approaches to impact on the identification of primary modes of actions. In both the cases we have discussed it is also clear that the benefit to the patients clearly out way the risks.

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Short communication

Accumulative heavy metal patterns in the sediment and biotic compartments of the Tisza watershed

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Abstract

The paper presents data on toxic heavy metals in sediments measured after two large chemical spills on the Tisza watershed area. On the basis of the results, it is concluded that significant volume, high concentration heavy metals reached the Upper Tisza and Szamos River sections. Based on the longitudinal distribution of the heavy metals in sediments, the primary sedimentation zones and concentration increase compared with reference site was determined. Results verified arrival of fresh spills of mining origin and superimposed pollution. Regarding to chronic ecotoxicological effects, degradation and bioaccumulation rates of heavy metals priority problems are associated with arsenic, lead, mercury and cadmium in the sediments of the Tisza and Szamos Rivers after the spills of Romanian origin in 2000. Results indicate that the biological availability of the various heavy metals significantly differ along the river, particularly upstream and downstream of the Tisza Lake. The recent investigation did not identify one single sample in which muscular metal concentration of pike (*Esox lucius* L.) exceeded the present Hungarian consumer guidelines. The investigated pike population on the Tisza River could be divided into characteristic subgroups based on muscular tissue metal concentrations (Cd, Hg, Pb, Cu and As), depending on the bioavailability of the metalloids at the different river sections. On the basis of the data evaluation, it is concluded that the present state of pollution on Tisza River indicates the potential for deterioration and need for further biomonitoring.
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Keywords: Heavy metals; Bioaccumulation; Biomonitoring; Ecotoxicology

1. Introduction

Dam failure of the mine slurry of Baie Mare (Romania) caused major environmental disaster in Hungary and other downstream countries at the end of January 2000. According to official estimates, the spill carried 100 tons of cyanide and equal amounts of heavy metals (silver, copper, zinc, cadmium, etc). The toxic plume reached the Szamos River, then via the Tisza River, it entered to the Danube on Yugoslavian territory. Measured cyanide concentrations were as high as 20–

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30 mg/l on the Szamos River and 5–10 mg/l on the Hungarian section of the Tisza River, respectively. Due to the prevailing hydrometeorological conditions and poor management, yet another dam (Baia Borşa, Romania) was broken in only 2 weeks to the first environmental disaster. The latter spill carried about 20 000 tons of heavy metal rich mine slurry that eventually reached Hungarian territory via another tributary of the Tisza River. Acute toxic consequences of the first spill (cyanide) were regional-scale damages in the aquatic ecosystem of the Tisza River, mass kills of several Vertebrata and Invertebrata taxa. The second spill received less attention particularly in terms of potentially occurring long-term, chronic ecotoxicological effects.

The paper aims to provide preliminary assessment on the state of the sediment following the two large spills and to initiate to build a comparative baseline data on the heavy metal contents of some biotic compartments (pike muscular tissue) on the exposed river sections. Preliminary data of the environmental authorities and involved international agencies indicated that significant volume of potentially hazardous heavy metal has been accumulated in the Upper Tisza and Szamos sediment. Consequently, future tasks are to be focused on monitoring the chronic effects of the sediment accumulated heavy metals for setting priorities of rehabilitation actions.

The European Environmental Agency set 12 environmental priorities in 1995, as it was issued in the Dobris Report. One priority related to the large-scale, regional industrial spills. Regional-scale spills of transboundary character, having long-term ecotoxicological consequences occurred on the Tisza River limiting vital water uses (i.e. drinking water supply) in 2000. The high volume of the transported heavy metals in the mine slurry underlines the need to focus on the non-aqueous phases (i.e. sediment and suspended solids) when assessing and predicting the future state of the exposed aquatic ecosystems.

The Water Framework Directive of the European Union (Water Framework Directive, 2000) sets priorities in maintaining and preserving the natural conditions of freshwater ecosystems and when it is necessary, the rehabilitation. Rehabilitation actions have to be based on the evaluation of the state of the various environmental compartments (aqueous phase, sediment and biotic elements). Assessment of the bioavailability of the sediment accumulated heavy metals is a major element in setting rehabilitation priorities.

The paper discusses two data groups:

- Distribution of heavy metals in the sediment of Szamos and Tisza Rivers in the follow-up period of the two large spills.
- Heavy metal contents in pike tissues (*Esox lucius* L.) on the Tisza River after about 8 months exposure time.

Complex physical, biochemical and microbiological processes occurring in and between the solid phase, interstitial water and the water body regulate distribution dynamics of the metals and metalloids (Hansen et al., 1996). Ultimately, these mechanisms determine the environmental fate and hence the biological availability of the heavy metals accumulated in sediment.

Envisaged scenarios on the Tisza watershed implicate multiple and dynamically changing discharges of various chemicals, fractionated sedimentation patterns, varying adsorption–desorption mechanisms depending on the transportation processes of suspended solids. These processes have not yet described. Consequently the results of the sediment survey presented here are considered as a momentary picture on the primary sedimentation zones (hot-spots), a changing scene with largely unknown regulatory mechanisms that will determine future water quality and long-term state of the aquatic ecosystem.

2. Materials and methods

2.1. Sediment survey

Two sampling campaigns were launched, one is after the cyanide spill and yet another after the heavy metal discharge. Fig. 1 shows the Tisza watershed and the watercourses carrying the spills.

Sediment samples were collected at 11 sites following the cyanide spill from which three were

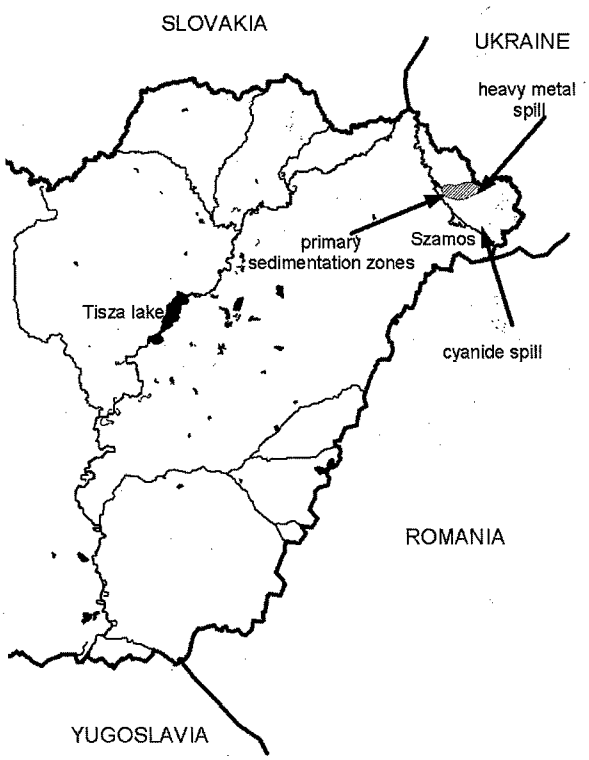

Fig. 1. The watershed of the Tisza and the watercourses carrying the spills.

on the Szamos and eight on the Tisza River. One sampling site on the Tisza River served as reference site, located upstream of the confluence point of the Szamos River carrying the cyanide spill.

Following the second spill (heavy metal loaded mine-tailing slurry) eight sampling sites were selected. At three locations, stratified sediment sampling was conducted to reveal differences between recent and historical heavy metal concentrations. One particular location (near to Tivadar, at 705 river km (rkm), that served as reference site in case of the cyanide pollution) was used as comparative background to detect differences for it was sampled before and after the heavy metal spill as well. Another reference point was also added from the vicinity of this location that has been isolated from the river on the flood-protected area to measure natural background levels of geochemical origin.

2.2. Fish tissue sampling

Three fish populations were investigated. Two control groups were selected for comparison, one is within and one is outside the Tisza watershed. On the Tisza River, the 'exposed population' was sampled in the period of October–November 2000. This allowed us to investigate heavy metals patterns in fish tissues following 8 months exposure time.

Captured fish were deep-frozen and dissected to various tissues (muscle, gonads, scales, etc.). Altogether, 117 fish sample were collected on the Tisza from which the results of the 25 pike specimen are discussed herein. Presented results are referring to fish muscle samples on the Tisza section between Tiszaadony (668 rkm) and Szeged-Tápé (180 rkm). Average body mass was 239.2 g with the average (standard) body length of 327.5 mm. Standard deviation of body mass was 135.9 and 58.3 regarding to standard body length.

2.3. Analytical methods

Quantitative and qualitative chemical analyses were conducted at the internationally accredited laboratory of the Bálint Analitika Ltd., Budapest. Each measurement was made in two parallels. Data presented are based on the arithmetic average of two measurements. Metal determination was made according to Hungarian Standard 21470-50:1998 (corresponding to US EPA 6020 method) using ICP-MS instrumentation (MSZ, 1998; US EPA, 1990).

Metal analysis from fish used 2.50 g tissue sample measured to a Teflon bomb with 0.1 mg accuracy. Two cm^3 65% nitric acid (Merck, Suprapur) and 6 cm^3 30% hydrochloric acid was added to the sample followed by destruction in microwave oven at low power. Following full evaporation the same volume of acid and 25 μl 50 mg/l Sc, Rh, Lu inner standard-solution was added to the bomb. Upon full destruction, the sample was washed to 25 ml flasks. Analysis of the metals was made by inductively coupled plasma mass spectrometer (ICP-MS method). Tissue results are expressed in wet weight units. Sediment results are referring to dry material contents.

Assessment of data was based on applicable Hungarian environmental standards in force. For sediments, the authority limits for polluted soils were used as given in Table 1 (Hungarian Ministry for Environment, 2000). Intervention values are indicating concentration levels when clean up is obligatorily ordered by the environmental authorities. For fish allowed concentrations are set (Hungarian Ministry of Health, 1999) by a ministerial order (As 1.0, Hg 0.3, Pb 0.5 and Cd 0.1 mg/kg). It is noted that neither ecological sediment quality criteria nor fish consumption guidelines for sport fish are in force in Hungary at present.

3. Results

3.1. Longitudinal sediment characteristic

Chronic ecological effects of the pollutants reaching surface water bodies are profoundly affected by sediment-water body interactions often showing site-specific character.

Based on the results, the following observations were made on the heavy metal contents of the river sediments after the first (cyanide) spill in 2000:

- The Hungarian intervention limit value for soils was reached in the sediment of the Szamos at the national border by the following elements: arsenic (71.4 mg/kg), zinc (660 mg/kg), cadmium (4.8 mg/kg) and copper (345 mg/kg). Values in bracket are the means of two parallel measurements.
- Concentration of four toxic heavy metals (As, Zn, Cd and Cu) was above the polluted level on the Szamos River sediment at all three sampling sites.
- In the sediment of Tisza River, the cadmium reached the Hungarian intervention limit value at three sampling locations, the arsenic at two locations and the zinc at one sampling site.

Results of the sediment survey after the second (heavy metal) spill originated indicated the following:

- The primary sedimentation zones on the Upper Tisza section were at the Milota and Tivadar regions on the basis of measured concentration ranges (zinc, cadmium, lead and copper). Extremely high values were measured on the river section upstream of Vásárosnamény (between 733–705 rkms) indicating the primary sedimentation zones of heavy metals.
- Concentrations of cadmium, copper, lead and mercury were elevated significantly at the reference site (Tivadar, 705 rkm) indicating superimposed sediment pollution: cadmium $0.33 \rightarrow 1.89$, copper $23 \rightarrow 147$, lead $14.7 \rightarrow 212$, mercury $0.05 \rightarrow 0.16$ mg/kg.

Fig. 2 shows the longitudinal profile of lead concentrations on the Upper Tisza section, indicating the locations of metal accumulation in surface sediment layer.

3.2. Vertical sediment profiles

Elevated heavy metal concentrations were measured in surface sediment samples at the national border (Tiszabecs, 742 rkm). Concentration values

Table 1
Hungarian authority standards for soil (values in mg/kg unit, expressed in dry weight)

Element	*A* value (natural background)	*B* value (polluted)	*C* value (intervention)
Cd	0.5	1.0	2.0
Pb	25	100	150
Hg	0.15	0.5	1.0
As	10	15	20
Ni	25	40	150
Zn	100	200	500

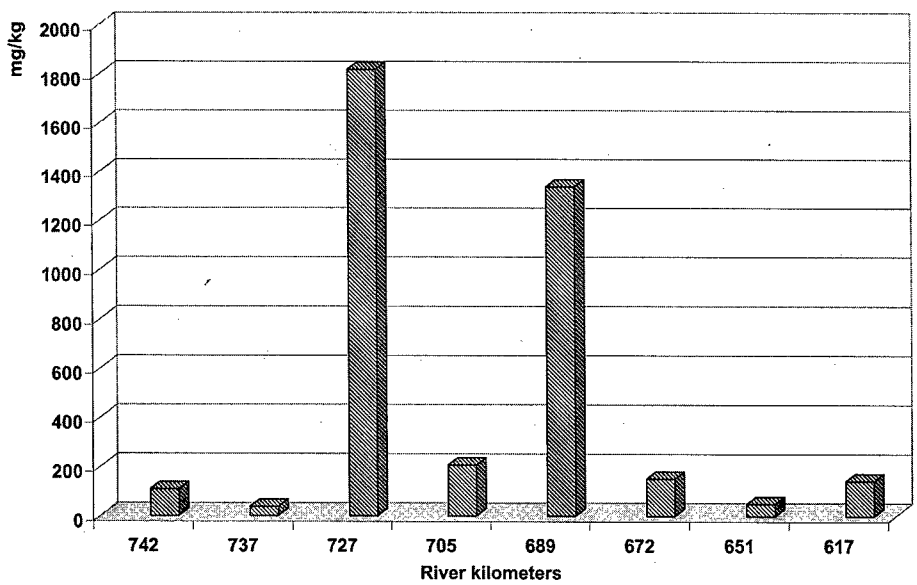

Fig. 2. Longitudinal distribution of lead concentrations on the Upper Tisza sediment (from Tiszabecs 742 rkm to Tuzsér 617 rkm).

in the surface sediment layer compared with the lower laying sediment layer (in depth of 10 cm) revealed an increase of about a magnitude of order in many heavy metal (lead, silver, bismuth, cadmium, copper and zinc) concentrations indicating newly arrived pollution of mining origin. Sampling sites near to national border (Milota, 733 rkm and Tivadar, 705 rkm) revealed the presence of hot spots (heavy metal accumulation sites) due to the hydromorphology of the river. Copper, cadmium, lead and zinc concentrations were close to or above the intervention limit (See Table 1).

At one sampling point at Tiszakórod (728 rkm), the lead concentration was so high that we repeated the measurements (see also Fig. 2). The results indicated lead concentrations of 1810 mg/kg dry material sediment. This sample originated from an inundated riverbank that became dry during the cessation of the flood that carried the spill. Stratified samples taken at Jánd (688 rkm) proved that in spite of the high metal concentrations measured at the surface, no significant heavy metal accumulation occurred in deeper laying sediment layers of the downstream situated river section at the period of sampling (March 2000).

Further downstream at Aranyosapáti (670 rkm) concentrations were still high, around the Hungarian intervention value (zinc, arsenic, cadmium, etc.). Downstream of the confluence point of the Szamos River that carried the cyanide plume no particular stratification could have been observed in sediment values. Sediment of the Tisza River downstream to the confluence point of the Szamos River showed significant pollution in the investigated vertical sediment strata (0–10 cm) due to mixing, bioturbation during the past years.

Fig. 3 shows the vertical distribution of heavy metals in sediments as measured at the national border (Tiszabecs, 742 rkm).

3.3. Heavy metals in muscular tissue of pikes of the Tisza River

3.3.1. Arsenic

Measured arsenic concentrations in pikes are depicted on Fig. 4. Results indicate that the pike population of the Tisza River can be divided into two separate groups. Pikes downstream of the Tisza Lake (Kisköre Reservoir) contain elevated arsenic levels in their tissues, compared with those that were captured upstream of the reservoir.

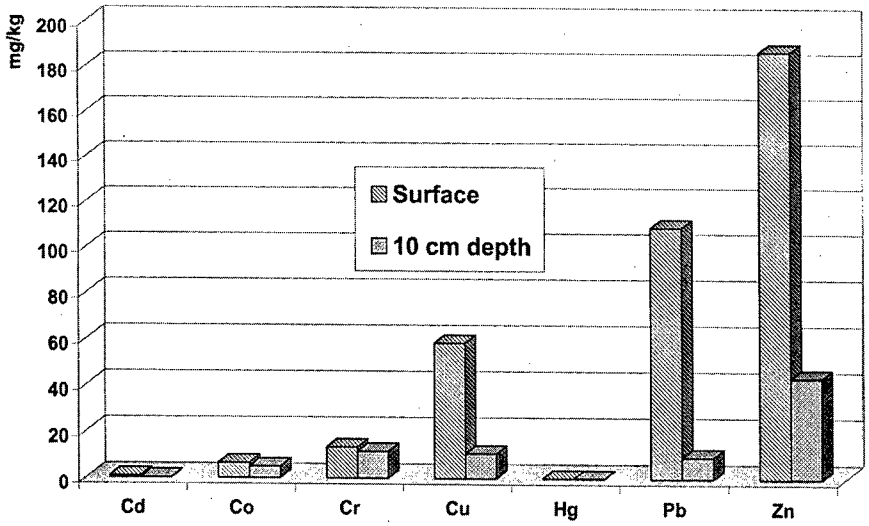

Fig. 3. Vertical distribution of heavy metals in the sediment at the national border (Tiszabecs).

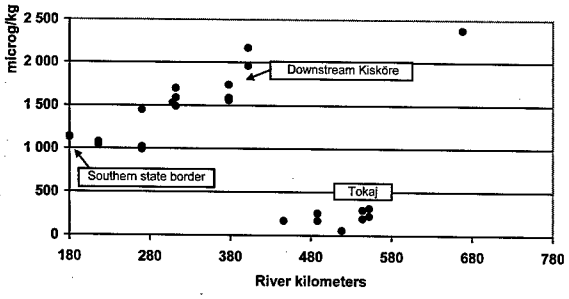

Fig. 4. Changes of arsenic concentration in pike muscle on the Tisza River.

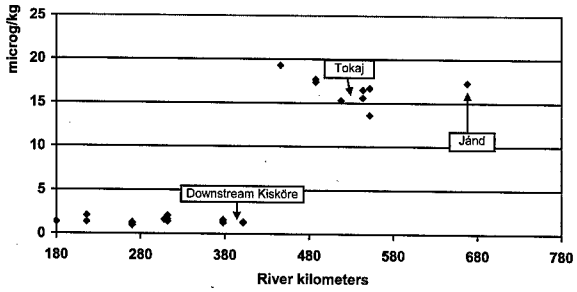

Fig. 5. Changes of cadmium concentration in pike muscle on the Tisza River.

Higher arsenic concentrations in muscle might be the results of higher geochemical background and/or anthropogenic loads. Several arsenic compounds are accumulating in fish tissues but arsenic is not toxic to fish (ATSDR, 1999).

3.3.2. Cadmium

Fig. 5 shows the measured cadmium concentrations in pike muscle. As opposed to the concentration pattern revealed in arsenic distribution, the cadmium contents of pike muscle shows different layout. Higher cadmium exposure level was observed in middle and upper river sections. It is also noted that the measured (ppb) of cadmium levels are in the low to moderate concentration range.

The range of the measured cadmium concentrations is far below the human consumption guideline values (Hungarian Ministry of Health, 1999).

3.3.3. Copper

Fig. 6 shows copper concentrations in pike muscle on the Tisza River. Copper contents in pike revealed linearly decreasing tendency towards to downstream sections. Value of the linear regression coefficient is $r = 0.79$. The only elements that showed linearly decreasing tendency in pike tissues along the longitude of the river was the copper. This would make us to hypothesize that the copper was mainly present in soluble and complexated form and the discharge was literally washed out to the downstream river sections.

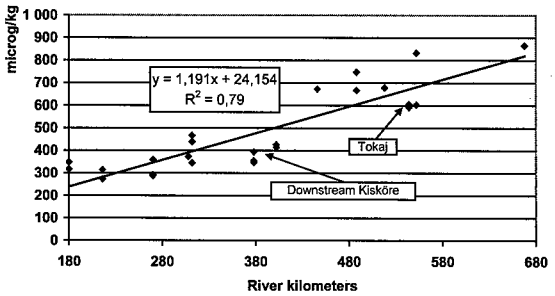

Fig. 6. Changes of copper concentration in pike muscle on the Tisza River.

Table 3
Heavy metal concentrations in pike muscle downstream of the Tisza Lake

Parameter	Arithmetic mean	Median	S.D.
As (μg/kg)	1448.75	1510.00	354.44
Cd (μg/kg)	1.45	1.36	0.30
Cu (μg/kg)	359.50	351.50	56.67
Hg (μg/kg)	303.19	277.00	93.22
Pb (μg/kg)	18.02	17.30	3.49
Body mass (g)	258.25	224.00	146.57
Body length (mm)	336.38	334.00	60.53

Relatively high concentrations in pike muscle were measured in case of four other components but only on the upstream river section: *palladium, tellurium, thulium and thorium* (Pd, Te, Tm, Th). The concentrations of these elements were elevated by factors of 10–80 regarding to the control populations within and outside of the watershed. No physiological function is described so far on these elements except the tellurium that is toxic. Elevated concentrations of these rare earth elements are attributed to the hydrogeological background as well as past discharges of mining and industrial origin.

Data of primary statistical analysis on measurement results are given in Tables 2 and 3.

Level of priority pollutants (As, Cd, Cu, Hg, Pb) in muscular tissues were analyzed by using non-parametric statistical methods. Results achieved by the Wilcoxon-test and the two-sided Kolmogorov–Smirnov test (Table 4) lead us to the following conclusions.

Table 2
Heavy metal concentrations in pike muscle upstream of the Tisza Lake

Parameter	Arithmetic mean	Median	S.D.
As (μg/kg)	417.55	210.00	694.00
Cd (μg/kg)	16.97	17.00	1.93
Cu (μg/kg)	697.70	676.00	93.90
Hg (μg/kg)	149.94	146.50	49.40
Pb (μg/kg)	31.28	30.50	4.06
Body mass (g)	205.22	179.00	114.44
Body length (mm)	311.78	295.00	53.72

Both statistical tests verified that for the five elements (As, Cd, Cu, Hg and Pb), the null-hypothesis of homogeneity has to be rejected. Measured heavy metal concentrations in the muscular tissues of the Tisza River pikes upstream and downstream of the Tisza Lake were originated from different distributions.

Opposed to the above conclusion, it was justified that the body length and mass values of the collected pikes were originated from a homogenous statistical population, and the difference observed was no significant with respect to these morphometric parameters.

Connecting these two conclusions, it is envisaged that the length and mass of the sampled pike population (as related to age and predatory strategy) played little or no role in establishing characteristically different heavy metal patterns in muscular tissues. Consequently, experienced differences are due to external factors, as results of different exposure conditions indicating highly different bioavailability of the metals along the longitude of river. Regarding to the muscular concentrations of As, Cd, Cu, Hg and Pb, the region of the Tisza Lake divides the pike population into two, significantly different sub-populations, a division that cannot be explained by differences of the body weight and length.

This result concludes us that there are significant differences in exposure conditions upstream and downstream of the Tisza Lake. The biological

Table 4
Results of the Wilcoxon and Kolmogorov–Smirnov non-parametric statistical analyses

Parameter	Wilcoxon-test (risk of rejecting the null-hypothesis in [0...1] interval)	Kolmogorov–Smirnov test (risk of rejecting the null-hypothesis in [0...1] interval)
As	0.20	1.20×10^{-4}
Cd	0.00	1.23×10^{-5}
Cu	0.00	1.23×10^{-5}
Hg	8.0×10^{-2}	4.81×10^{-4}
Pb	0.00	1.23×10^{-5}
Body length	0.69	0.29
Body mass	0.57	0.15

availability of the sediment accumulated heavy metals is different on the particular river sections and/or the absolute load is also differing.

4. Discussion

Environmental effects of mine tailings on the aquatic ecosystems are discussed in details in United States Geological Survey (USGS) reviews. Data indicate (Fey, 1999) that metal contents of the slurry of secondary utilization mines can be as high as 1–8 % having relatively high bioavailability due to the particle size which is typically in the colloid range (100–500 μm). Applying the lower estimate (1%), it is presumed that the released 20 000 tons of slurry might contribute to around 2×10^{11} mg additional lead load (200 tons of lead) on the watershed. Theoretically, this discharge could pollute 2×10^{9} dm^3 sediment volume regarding to the Hungarian intervention value for lead in soils (100 mg/kg). This assumption is based on zero lead contents prior to the mine accidents. Realistically, the exposed floodplain and riverbed area might stretch to several hundred square kilometers as internationally applied sediment quality criteria are lower than intervention standards for soil, and the potential site of exposure (grazing and feeding on the uppermost sediment layer, bioturbation, etc.) is only a few centimeters deep in the vertical profile of the sediment. This indicates that expected long-term ecological effects are on the regional scale. A particularly important factor in relation to suspended solid bound metal transport processes is the existence of two man-made reservoirs on the exposed river section (Tiszalök and Kisköre, alias Tisza Lake). On these reservoirs, the fractionated sedimentation of suspended solids of various particle sizes occurs and the surface adsorbed metals are deposited in these locations. Different exposure conditions are evidenced in the muscular tissues of predatory fish population as it was described in this paper.

Comparison of sediment data from other Hungarian rivers showed that apart from nickel, chromium and zinc, the concentration of other toxic heavy metal concentrations in the Tisza sediment were above of the Danube, Sajó and the Soroksári–Danube branch values. Average values of cadmium, lead and copper were about twice (Cd), or 4-fold (Pb, Cu) compared with other Hungarian reference sediment values. Considering long-term ecological effects, the most hazardous two elements (Cd and Pb) represent a long-term, special problem on Tisza River.

It is of interest to compare our results with historical data of other European rivers. In earlier studies (Beurkens et al., 1994), measured time trends of heavy metals in the sedimentation zone of the Rhine. Historical data of the heavy metal contents of Rhine sediment and the recent findings on the Tisza River are compared in Table 5.

Comparison of the two data sets shows that on average the present concentrations of two metals (copper and lead) in the Tisza exceeded historical levels measured in Rhine sediment in the middle eighties. All the other measured metals were below the Rhine pollution level. It is also noted that the

Table 5
Historical average concentrations of River Rhine and of the Tisza River sediments in 2000

Heavy metals (mg/kg)	Rhine (1945)[a]	Rhine (1965)[a]	Rhine (1985)[a]	Tisza (2000)
Cd	4	20	11	2.5
Pb	170	400	170	261
Cu	80	300	100	161
Ni	35	60	40	36
Cr	110	500	200	26
Zn	1100	2500	1000	476
As	50	140	25	29
Hg	2	11	2	0.1

[a] Beurkens et al. (1994).

results of the international water quality management program of the Rhine started to show up in sediment quality during the middle eighties.

Fish survey revealed no heavy metal concentration in muscular tissues that was above the applicable Hungarian fish consumption guidelines. Hungarian regulation, however, is concerned on commercial fish and not on fish from natural water. Considering the US guideline values for mercury (US EPA, 1997) for example, the measured average mercury concentrations in pike of Tisza River (247 μg/kg mercury, wet weight) would refer only to three pike meals per month (3 × 227 g) not to exceed recommended mercury intake. It is, therefore, strongly suggested to establish Hungarian consumption guidelines for fish considering non-commercial fishing in natural aquatic environment. Based on the heavy metal concentrations in fish tissues, the environmental authorities could map and survey regularly the problems of 'small concentrations-chronic exposure' water quality problems that are still going unnoticed due to the applied monitoring system (focused largely on aqueous phase, with relatively low sampling frequency).

Regarding the Tisza and particularly the Szamos River, it cannot be concluded that the ecological state and exposure levels of the two rivers are solely attributable to the large-scale Romanian mine accidents in 2000. One of the main conclusion of the survey was that sediment as well as fish in their tissues reflects the past industrial and urban effects of national and international origin. Measured data, therefore, necessarily reflects to the integrated results of the past and present pollutant loads of conservative pollutants. In case of some heavy metals, the effects of the Romanian discharges are already detectable in the elevated heavy metal levels of predatory fish population of the Upper Tisza section and it is anticipated that the expression of the ecotoxicological effects require decades to develop. The future fate of the already accumulated metals in sediment is largely dependent on the initiation of rehabilitation projects and introduction of environmentally sound management practices on the entire watershed.

Acknowledgements

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Ecotoxicological studies and risk assessment on the cyanide contamination in Tisza river

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Abstract

As a result of the dam failure of January 30, 2000 in Rumania, water and a huge amount of sediment contaminated with cyanide and later heavy metals entered the Tisza river system. In order to determine the chronic consequences of the contamination, periphyton and sediment samples were collected from River Tisza and her tributaries over the next 2 years. After flooding periods, the sediment deposited in the foreshore was also sampled. Applying the ICP-AES analysis method, the amounts of major heavy metals in the periphyton and sediment samples were measured, and the related concentration factors were calculated. Attempts were also made to find a correlation with the toxicity data. Ecotoxicological analyses were performed on the sediment using the following test techniques: Daphnia test, static fish test, alga test (chlorophyll content measurements), seedling test (*Sinapis*) and *Lemna* test (increase in mass and measurements on the chlorophyll concentration). Examinations on the chronic effects of heavy metals deposited in the periphyton and sediment can be regarded as an important factor in assessing ecological–conservational disasters and in carrying out biomonitoring activities in the future.

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Keywords: Biomonitoring; Periphyton; Sediment; Lemna test; Cyanide; Heavy metals

1. Introduction

In Hungary and particularly in her Great Plain, the year of 2000 can certainly be designated as the year of rivers. Fear and anxiety over the ecological disaster hitting River Szamos and Tisza from the end of January had hardly faded when in March a new wave of heavy-metal contamination entered River Tisza. Impacting the two rivers, the ecological catastrophe unequivocally revealed that local damages to the environment, such as clear-cutting the forests, can easily expand to a regional scale, consequently the treatment, and principally the

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prevention of environmental problems call for national and international cooperation.

When regarding the experience of the previous 2 years, it is the monitoring of contamination effects with its sphere of operation embracing all the elements of the aquatic ecosystem that is to be considered as of general validity and top priority. To work out and accomplish the proposed rehabilitation programme, River Tisza and her catchment are to be subjected to drawing faunistic and floristic inventories. Used as background factors, hydrochemical, hydrobiological and ecotoxicological research information should be compiled into a risk assessment system that offers a realistic view on the pre-contamination ecological conditions and the occurring changes.

The littoral zone of rivers, as a transitional region between watercourses and lands, has remarkable conservational and environmental significance. Periphyton is the complex of organisms found on submerged substrates that are of materials different from those of the water bottom and clearly distinguishable from it (Behning, 1924; Dussart, 1966; Lakatos, 1976). The autotrophic constituents of the periphyton produce organic material and oxygen by binding light energy and taking up inorganic plant nutrients. The organic material yielded by them constitutes an essential nutrient source for the zoo-organisms of the periphyton, as well as for the animals of other heterotrophic communities, such as some fish.

Periphyton has been used in the monitoring of water quality for decades. Initial attempts were based on the conception of indicator organisms, and species of known tolerance were involved in the process of determining water quality. One of the earliest efforts is represented by Kolkwitz and Marsson's (1908) saprobic system. Periphyton can be regarded as an ideal monitoring agent in watercourses, perfectly suitable for the continuous investigation of environmental and water-quality conditions, because sampling is relatively simple and the method reliably indicates the occurring changes having a rapid process in water flows. Periphyton has a taxonomically diverse composition, short regeneration period, and owing to its common occurrence it offers an outstanding opportunity to compare different areas (Patrick, 1973; Round, 1991; Friedrich et al., 1992).

Periphyton can be analyzed by taxonomic composition and characteristics, as well as by non-taxonomic traits. Taxonomic parameters, such as the diversity index, species diversity, indicator organisms have frequently been used by a number of experts (Patrick, 1973; Palmer, 1977; Weitzel, 1979). On the other hand, non-taxonomic factors, like ash-free dry weight (AFDW) or chlorophyll-*a* content (chl-*a*) are applied to expose effects that have not been revealed by taxonomical analyses, e.g., sub-lethal or physiological consequences of toxic substances that cannot be shed light on immediately or directly by taxonomic parameters, but a smaller biomass or chlorophyll concentration can show altered conditions.

Hill et al.'s (2000) work overviews the use of periphyton for test purposes in watercourses; examining control and contaminated water flows, the study compares the taxonomic indexes of the periphyton to non-taxonomic data with respect to the response given to metal contamination. As a conclusion, indicator organisms, such as *Achnanthes* and *Fragilaria* diatoms occurring in large masses are suitable for establishing the degree of heavy-metal contamination and loading in the investigated streams.

Unfortunately, the cyanide and heavy-metal contamination hitting the aquatic living world of River Szamos and Tisza had spectacular consequences, such as large-scale fish mortality, that were presented in the media in quite a sensationalistic fashion, yet much less attention was paid to the other, microscopic and invertebrate organisms of the rivers; thus, investigating the periphyton and benthic organisms of the offshore sediment is to be attributed high priority.

The present study intends to report on the non-taxonomic research results obtained in the course of investigating the periphyton collected after the cyanide and heavy-metal contamination from the submerged substrates of River Tisza and her tributaries, as well as on the taxonomic analysis data as focusing primarily on hazard and risk communication issues. After flooding periods, the sediment newly deposited in the foreshore was also sampled and studied. Further insight is to be given

on the results of ecotoxicological tests on sediment samples taken from River Tisza, in the framework of "Joint Danube Survey—Investigation of the Tisza River" (ITR samples), as well as on their classification as concerning toxicity.

2. Materials and methods

Sampling was performed on a seasonal basis, in summer and autumn, at 20 sampling sites from the Tiszabecs border reach to Tiszasziget in the south, and at 10 sampling sites on the Tisza tributaries. Periphyton samples were collected from the off-shore aquatic plants (epiphyton), living and dead trees (epixylon), stone surfaces (epilithon) and metal structures (epimetallon). In the case of River Tisza and her major tributaries, it was the submerged green branches of willow trees that constituted the substrates, while in the still tracts of smaller water flows epiphyton samples could be taken from the submerged parts of plants living near the shore. Where it was possible, epilithon samples were also collected from stone surfaces.

The collected periphyton samples were subjected to investigations and measurements on their fresh and dry weight, inorganic and organic chemical constituents, chlorophyll-*a* concentration, phytotecton and zootecton composition, as well as on the community structure, biodiversity and similarity of this latter element.

Dates for periphyton sampling were the following: May 31, June 21, July 4, July 18, July 24, September 21 in 2000, and July 30, July 31, August 8, October 18, October 19, October 29 in 2001. In laboratory, the suspended material content of the water, as well as its concentration of inorganic orthophosphate and nitrogen forms were established. The permanganate oxygen consumption and planktonic chlorophyll-*a* concentration of the water samples were also detected. Element analyses from the water, periphyton, plants serving as substrates and sediment were carried out as applying the ICP-AES method. Sampling sites on River Tisza and her tributaries are shown in Table 1.

Table 1
Sampling sites on River Tisza and her tributaries

Periphyton and sediment newly deposited sampling sites	
C1	Szamos, Csenger
C2	Szamos, Tunyogmatolcs
C3	Tisza, Tiszabecs
C4	Túr, Sonkád
C5	Kraszna, Kocsord
C6	Tisza, Kisar
C7	Tisza, Aranyosapáti
C8	Tisza, Lónya
C9	Tisza, Tuzsér
C10	Tisza Dombrád
C11	Tisza, Tiszabercel
C12	Tisza, Balsa
C13	Tisza, Tokaj
C14	Bodrog, Tokaj
C15	Tisza, Tiszalök
C16	Tisza, Polgár
C17	Tisza, Tiszacsege
C18	Tisza, Tiszafüred
C19	Tisza, Kisköre
C20	Tisza, Tiszaroff
C21	Tisza Tiszapüspöki
C22	Tisza, Tiszaug
C23	Berettyó, Szeghalom
C24	Maros, Makó
C25	Tisza, Tiszasziget
C26	Tisza, Tápé
C27	Tisza, Mindszent
C28	Hármas-Körös, Magyartés
C29	Lónyai Canal, Nyírbogdány
C30	Zagyva, Újszáz
Sediment sampling sites	
ITR1	Titel
ITR2	Bega Canal
ITR3	Novi Bejec
ITR4	Novi Nezevac
ITR9	Tiszaug
ITR10	Szolnok
ITR11	Zagyva, Újszász
ITR12	Kisköre
ITR13	Aranyos-sziget
ITR14	Tiszafüred
ITR15	Tiszacsege
ITR16	Polgár
ITR17	Sajó, Kesznyéten
ITR18	Rakamaz, Tokaj
ITR17	Bodrogkeresztúr
ITR21	Tiszabercel
ITR22	Dombrád
ITR23	Tuzsér

Examining the chronic effects of heavy metals deposited in the sediment is also an essential part in the complete assessment of the ecological disaster and the possible elimination of its after-

maths. In 2001, in the framework of "Joint Danube Survey—Investigation of the Tisza River" (ITR samples), the ecotoxicological examination of 18 sediment samples collected in the Yugoslavian and Hungarian reaches of River Tisza and her tributaries was carried out. From the samples, the chemical analysis of major heavy metals was completed as applying the ICP-AES analysis method, the related concentration factors were calculated and any correspondence to the ecotoxicological toxicity data was inquired for.

Sediment originating from River Tisza and her tributaries were air-dried, powdered and homogenized. For toxicological investigations, sediment samples were subjected to extraction with distilled water as conforming to Standard MSZ 21978/10–85, the element content of the extract was measured. The distilled-water decant is first strained through a Filtrak 289 filter paper, then a Whatman cellulose acetate membrane filter of 0.45 μm pore diameter, and the distilled-water solutions is used for *Daphnia* tests in accordance with Standard MSZ 21978–13, fish tests in accordance with Standard MSZ 21978/3–86, as well as seedling tests in accordance with Standard MSZ 21978–8. Alga tests and *Lemna* tests, a method regarded as a novel technique in Hungary, were also performed (Lakatos et al., 1993).

3. Results and discussion

3.1. Results and discussion of background investigations

On the basis of chlorophyll-*a* concentration values, it can be stated that the planktonic chlorophyll-*a* concentration could always be measured and flooding had a significant reducing effect, since the high water level in July showed a concentration of 0.2 mg m^{-3} (Tiszabercel), while the most outstanding, 9.4 mg m^{-3} value for River Tisza was measured in the sample collected at Lónya. The flooding water of River Szamos was detected to have a suspended material content of 700 mg l^{-1}, which observation was also reinforced by the thick layer of fresh sediment deposited on the river bank. The considerable suspended material content obtained for the July water samples—and that is also responsible for the special colouration of the water, the so-called phenomenon of "blonde Tisza"—also largely decreased by the arrival of the low water level, and the related value was measured to be around 10 mg l^{-1}. Due to the process of deposition and the simultaneously occurring filtering effect, the suspended material content basically influences the chemical composition of the periphyton; flooding increases the dominance of the inorganic fraction as it is also supported by the present analysis results.

The dissolved orthophosphate concentration was large, especially in the water samples from River Kraszna, Szamos and Hármas-Körös, and these watercourses also showed high inorganic nitrogen concentrations. COD values extensively ranged among the sampling sites, and properly reflected the fluctuations in the water level, as well as the effects of anthropogenic organic pollution. The examination results also suggest that the measured concentrations of plant nutrients could potentially sustain the reproduction of planktonic and periphytic algae in the river.

The dominant ion of cations in River Tisza and her tributaries was invariably calcium. There was a significant magnesium content in the water samples, while the connected observations found iron ion only on few occasions and just traces of manganese. It is worth to note the lead concentration data obtained for the autumn with the ICP-AES method. Copper ion could sporadically be measured in trace concentrations, but was practically detectable just in autumn. In the water of the rivers, dissolved heavy metals could be observed at the detection threshold, but the presence of planktonic algae, as well as of periphytic organisms made incidental phytotoxical effects unjustifiable.

3.2. Results and discussion of periphyton investigations

Periphyton samples were taken from natural (plants, branches, etc.) and artificial substrates (various engineering structures), too. In 2001, after the prolonged period of spring floods, the summer periphyton samples collected from the willow trees

(*Salix* ssp.) of River Tisza could be characterized by a small- (occasionally medium-) massed, heterotrophic periphyton type of dominantly inorganic constituents, with these basic features lasting even to the autumn sampling period, though there was an increase observed in the dry mass. In the case of epilithon having a 10 times larger mass, owing to the scale of measurement being different from that of the epiphyton (Lakatos et al., 1998), also a small- and medium-massed periphyton type could be established; at the same time, high ash content indicated inorganic composition and heterotrophic type. As compared with samples collected from willows, stones and iron engineering structures yielded larger periphyton masses (452 and 182 dw g m^{-2}, respectively).

Both the 600 μg AFDW cm^{-2} mass of the willow periphyton taken from River Szamos and the average value determined for River Tisza (620 μg AFDW cm^{-2}) notably exceeded the values between 2 and 39 μg AFDW cm^{-2} that was obtained for the periphyton biomass (Hill et al., 2000). The authors referred to above pointed out that in the second year biomass showed a significantly negative correlation to the dissolved Cd concentration. Weber (1973) and Crossey and LaPointe (1988) reported on the increase in the periphyton biomass alongside with the rise of metal concentration, though their studies did not explain this phenomenon. Other experts stated that as a consequence of a larger heavy-metal concentration in aquatic microcosm the periphyton biomass decreased (Hedtke, 1984; Dean-Ross, 1991).

Weber (1973) suggested that it was the autotrophy index (AI, biomass/chl-*a*) that should be used to examine water quality, and pointed out that the rise in the organic material content and copper contamination had a rising effect on AI. At the same time, periphyton investigations revealed that chlorophyll-*a* and AI had a strong correlation to the concentrations of dissolved metals, which clearly proved that non-taxonomic parameters could be effectively used in monitoring the metal loading of water flows. AI value (9500) calculated for the periphyton sample taken from a willow at Csenger, in River Szamos considerably exceeded AI values for periphyton samples collected from the same substrate in River Tisza, at Tiszabecs (1700), Tokaj (2800), Tiszazug (6077) and Tiszasziget (3190), yet a tendency, either strictly increasing, or strictly decreasing could not be established for the river.

In summer, the chlorophyll-*a* concentration of the periphyton collected from willows in River Szamos was 0.60 μg cm^{-2}, while in River Tisza the two extreme value for the samples from the same substrate was detected to be 0.03 and 0.46 μg cm^{-2}, which properly agrees with the data of the related literature. According to Hill et al.'s (2000) publication, in the first investigation year the chlorophyll-*a* concentration of the periphyton ranged between 0.02 and 1.21 μg cm^{-2}, while the following year, in 1992 quantitative chlorophyll data was 0.00–4.37 μg cm^{-2}. Similarly to the results described by other authors, the chlorophyll content of the periphyton reflected a negative correlation to the heavy-metal concentration (Hill et al., 2000), which the present studies could just partly support, because it was the periphyton collected from willows in River Szamos that yielded the smallest chlorophyll-*a* contents both in summer and autumn.

Corresponding to their large ash contents, periphyton samples had significant element contents, a fact confirmed by concentration factors calculated for some of the heavy metals from the periphyton samples taken from willows in the upper reaches of River Szamos and Tisza, in both years (Table 2).

The copper and manganese content notably fluctuate with no clear tendencies detected except for the significant copper content in the periphyton of River Szamos and the considerable manganese contents established for the periphyton of River Túr and Kraszna. As opposed to copper, chromium did not show similar results, as the periphyton could be characterized by high-chromium concentrations neither in River Szamos, nor in the upper reach of River Tisza. Both the calculated concentration factor values and heavy-metal concentration data of the periphyton support the assumption on the periphyton playing a bioaccumulation role, as well as on its suitability for bioindication, to be applied in biological monitoring.

Table 2
Concentration factors calculated for some heavy metals of the epixylon samples taken in the upper reaches of River Szamos and Tisza

River	Concentration factor in year					
	Fe		Cu		Cr	
	2000	2001	2000	2001	2000	2001
Szamos	4.1×10^2	8.9×10^2	1.1×10	–	2.1	–
Tisza	2.1×10^2	15.3×10^2	1.1×10	1.2×10	1.9	10.7

The dry mass and remarkable ash content of the samples justify that periphyton has a filtering, depositing function. Apart from the physico-chemical processes taking place in the periphyton, living organisms also play a major role that can be traced, e.g., in the feeding of caseless caddis larvae or *Chironomidae* etc. Metabolic processes in the periphyton are described by autotrophic and heterotrophic characteristics, as well as by the autotrophy index.

3.3. Results and discussion of the newly deposited sediment

The calcium and aluminium contents of the newly deposited sediment fluctuate, the obtained results are quite heterogeneous. The iron content of this sediment is larger than that of the plant and periphyton samples, and a similar outcome was observed for manganese. The Tiszabecs and Lónya samples produced larger quantities of copper and chromium in the newly deposited sediment than the other sampling sites, yet the copper content in the sediment of River Szamos at Csenger was also significant. The smallest copper, chromium and lead contents were detected in the sediment of River Túr at Sonkád. In the sediment samples from River Szamos and the Upper Tisza region, heavy-metal concentrations were measured to be higher than in the lower sections of the rivers, but it can be concluded that local influences constituted an important factor in the final outcome.

3.4. Results and discussion of phytotecton and zootecton analyses

From the samples, alga (phytotecton) species lists were recorded, since they were regarded to provide appropriate ground information to examine and reveal further changes. Qualitative processing was always completed with quantitative procedures, counting and calculations on the related living organisms. The largest individual number in unit area was established for the samples collected from willows in River Túr (1 083 466 ind cm^{-2}). The smallest individual number belonged to the periphyton taken from willows in the flooding River Kraszna, in summer (45 ind cm^{-2}).

In 2000, it was the May sample from the stone surfaces of the Tiszabecs sampling site in River Tisza that yielded the smallest individual number (77 ind cm^{-2}), while in the autumn of 2001 hundreds of thousand algae were found on the surfaces of the collected stones. In the above cases, the registered individual numbers belonged to 9 and 19 taxa, respectively, with the dominance of *Achnanthes*; this result indicates that stone surfaces have started to be colonized by algae after the heavy-metal contamination, consequently there is no phytotoxic effect observed. In summer, the value of the total individual number remained under 500 ind cm^{-2} even in the periphyton sample from willows in River Szamos.

Periphyton communities have been used to monitor metal contamination for a while, and the connected investigations have demonstrated

that the proportion of deformed cells rises as corresponding to the increase in the metal loading. On the basis of analyses on periphyton communities, the authors involved (Clements, 1994; Clements and Kiffney, 1994; Kiffney and Clements, 1994) obtained results that are properly agreeable with the data provided by experts who used aquatic invertebrate organisms (macro-invertebrates) to indicate the effects of heavy-metal loading. The former works report on the decrease in the relative frequency of *Ephemeropterae* with a parallel growth in the individual number of *Chironomidae*, when zinc was added in an increasing concentration.

Hill et al. (2000) pointed out that all the sampling sites were dominated by the diatom genera of *Fragilaria* and *Achnanthes*; nevertheless, *Fragilaria* was found to be predominant only in areas not contaminated with heavy metals, while the genus of *Achnanthes* existed at every site. As concerning alga dominance, Besch et al. (1972) and Medly and Clements (1998) arrived at similar observations when investigating Canadian rivers contaminated with copper and zinc, respectively. Besch et al. (1972) and Hill et al. (1997) described a number of *Achnanthes* species as outstandingly or moderately resistant, succeeded in distinguishing various taxa with discrimination analysis, and confirmed that the dominance of *Achnanthes* was detectable at sites contaminated with zinc, while areas of lower zinc concentration was primarily inhabited by *Fragilaria* and *Nitzschia*.

Following the conception of the above experts, a comparison of genera *Achnanthes* and *Fragilaria* was performed for the periphyton of the willow, and it was concluded that the summer samples from River Szamos did not hold *Fragilaria*. In the Tisza samples, the proportion of this latter genus reflected a drop towards the lower sections of the river, thus could be characterized by an increasing individual number. As for the 2000 samples, *Achnanthes lanceolata* was frequently present in large masses in the periphyton, consequently regarded as the dominant species, while in 2001 *Synedra ulna* was found only at one sampling site. Considering diatoms, *Melosira varians*, *Navicula capitatoradiata*, *Cocconeis placentula*, *Nitzschia palea* and *Gyrosigma scalproides* could be detected on just few occasions.

In the case of samples collected from willows, it was the diatoms that dominated the 2000 samples with a 75% proportion, while the following year this figure was 73% as concerning all the samples (Fig. 1). During the study, 134 algal taxa were identified with 52 of them being common for both sampling procedures; in summer 111 taxa were found, and in autumn 75 taxa were classified.

From the zootecton and sediment samples, 71 taxa were identified. The species number for 2000 is identical to that of 2001, yet favourable differences include the detection of 17 *Mollusca* and five *Trichoptera* species, as well as the occurrence of *Dreissena polymorpha* and *Corophium curvispinum*.

All the above outcomes of the investigations call for the continuation of the efforts to monitor the conditions in the rivers having been impacted by the cyanide contamination, as studying the periphyton and sediment, in order to gain a realistic notion on the populations of various species and the composition of the local communities, as well as to meet the Hungarian and EU expectations that have been drawn upon Hungarian experts of hydrobiology to steadily perform up to a standard being internationally acknowledged. We are to learn from the mistakes and defects of the past, thus acquire proper research results, facts of professional approach and relevant arguments.

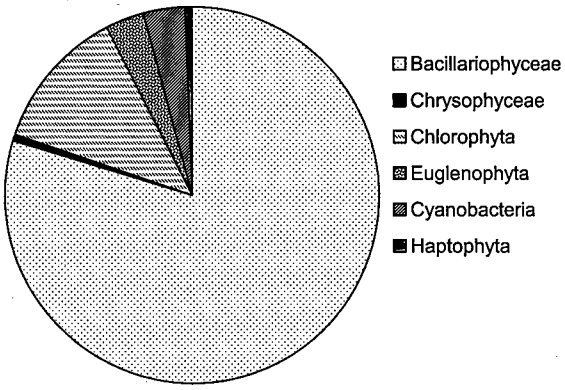

Fig. 1. Composition of phytotecton, 2001.

3.5. Results and discussion of ecotoxicological testing on the sediment

In 2001, in the framework of "Joint Danube Survey—Investigation of the Tisza River" (ITR), the ecotoxicological examination of 18 sediment samples collected in the Yugoslavian and Hungarian reaches of River Tisza and her tributaries was carried out. On the sediment samples, dry material and ash content investigations were made, then major heavy metals were subjected to chemical analyses with the use of the ICP-AES method. Higher chromium and lead concentrations were found for ITR samples 4, 11 and 12 with the first one collected beyond the southern border of Hungary, while the other two originating from the middle sections of River Tisza. A little amount of heavy metals, chiefly zinc could be detected in samples 1 and 20, of which latter one was taken from River Bodrog.

Throughout the performed Daphnia tests, immotile individuals were not observed, no effect indicating toxicity in the samples was experienced. During the fish tests, no individual died; consequently toxic and lethal outcomes could not be registered.

Average values for the length of seedlings are expressed in the percentage of the control organisms. One test brought about better results than the control values, which implied that the solution had stimulating effects, while for the other two sediment samples the calculated result was around 95%, therefore they could not be regarded as toxic. As concerning the other cases, a slight phytotoxic effect could be established, and on three occasions the length of the treated seedlings was measured to be less than 80% of the control ones (ITR samples 10, 12 and 16). Generally, it is indicatory that one-sixth of the sediment samples had to be identified as slightly toxic (Fig. 2).

The 24 and 48 h data of the alga test, as well as the calculated percentage values were considered as favourable, since slightly inhibiting effects could only be registered for ITR sediment samples 2 and 3, and these samples originated from outside the boundaries of Hungary. On the other hand, several samples showed pronouncedly stimulating effects.

The mass growth of *Lemna* plants was typically stimulated by the majority of sediment samples; ITR samples 4, 17 and 18, however, could be described by slight toxic effects, while ITR sediment sample 22 reflected strong toxicity. This latter sample yielded smaller individual and leaf numbers, as well as a reduced amount of organic material. The most adverse conditions were revealed when analyzing the content and amount of chlorophyll-*a*, because in this respect ITR samples 1, 2, 10, 13, 14 and 17 also belonged to the sediment groups of slight and strong toxicity that were established on the basis of mass conditions (Fig. 3). As relying on the *Lemna* tests, more than half of the examined sediment samples could be characterized by slight or strong toxicity. Accordingly, the results obtained from the seedling, alga and *Lemna* tests are rather exhortative, and pose the need, raise the demand for further ecotoxicological investigations.

4. Conclusions

A realistic notion on the actual ecological conditions prevailing before the contamination, as well as on the occurring changes can be analyzed in the framework of a risk assessment system that is to be based on hydrochemical, ecotoxicological, hydrobiological, algological and hydrofaunistical research information.

It can be stated that the measured concentrations of plant nutrients are capable of potentially sustaining the reproduction of planktonic and periphytic algae in the rivers. In the water of the rivers, dissolved heavy metals can be measured at the detection threshold, but the occurrence of planktonic algae makes incidental phytotoxical effects unjustifiable, and the presence of algal organisms in the periphyton also supports this view.

The dry mass and substantial ash content of the periphyton samples indicate important filtering and depositing functions, and apart from the physico-chemical processes taking place in the periphyton the contribution of living organisms to the favourable processes is essential. Both the calculated concentration factor values and heavy-

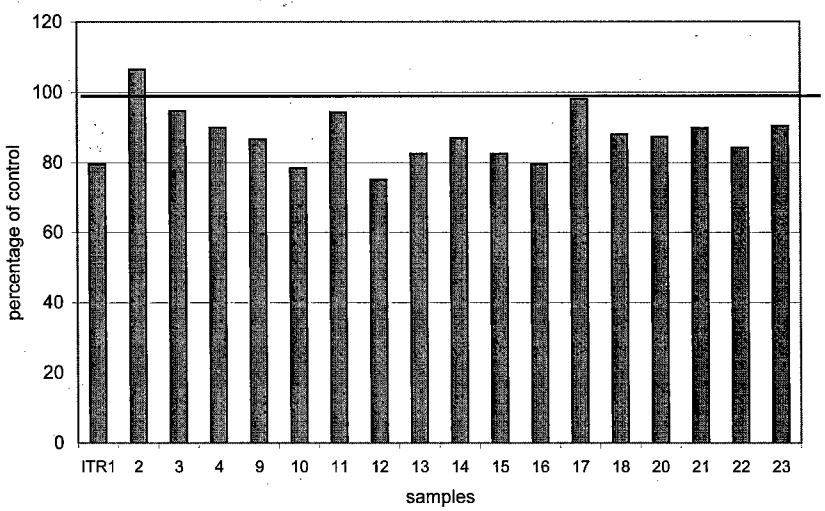

Fig. 2. Results of Sinapis test (length of seed bud in percentage of control).

metal concentration data of the periphyton support the assumption on the periphyton playing a bioaccumulation role, as well as its suitability for bioindication, to be applied in biological monitoring.

In revealing the effects of heavy-metal loading, several non-taxonomic and taxonomic parameters of the structure of the periphyton community can be used as excellent indicators for the conditions in the examined aquatic ecosystem. Consequently, periphyton as an outstanding source of information on water quality has a vital part in the examination and monitoring of water flows.

Periphyton is an important bioindicator of the occurring processes, because its organisms fixed to the substrates are not able to escape from any impact. At the same time, they are basic constituents of the aquatic food network, and to be attributed a principal role in the biological monitoring of water quality and risk assessment.

All the above results of the investigations call for the continuation of the efforts to monitor the

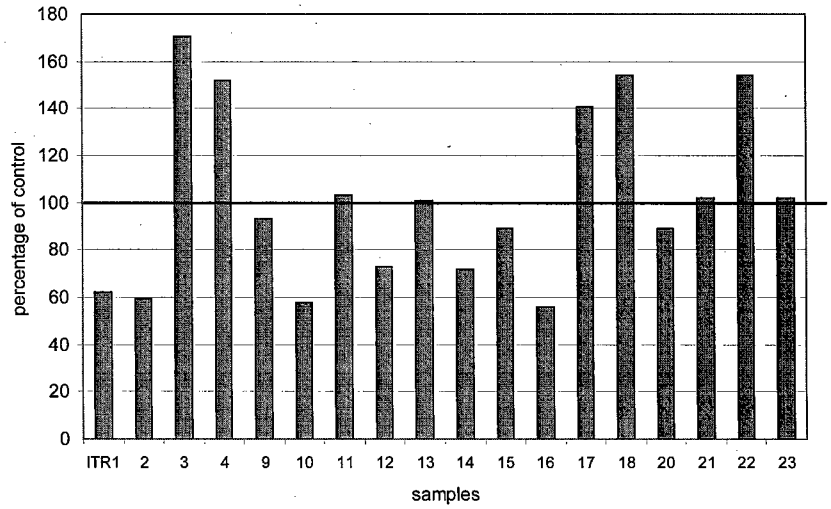

Fig. 3. Results of Lemna test (chl-*a* % in percentage of control).

conditions in the rivers having been impacted by the cyanide contamination, as studying the periphyton and sediment, in order to gain a realistic notion on the population of various species and the composition of the local communities, as well as to meet the Hungarian and EU expectations that have been drawn upon Hungarian experts of hydrobiology to steadily perform up to a standard being internationally acknowledged.

The outcomes of the seedling, alga and *Lemna* tests performed as parts of the sediment investigations, are exhortative, and pose the need to raise the demand for further ecotoxicological investigations on River Tisza and her tributaries.

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Review

Groundwater pollution and remediation options for multi-source contaminated aquifers (Bitterfeld/Wolfen, Germany)

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Abstract

Large-scale contaminated megasites like Bitterfeld/Wolfen in the eastern part of Germany are characterized by a regional pollution of soil, surface water and groundwater due to the long and varied history of the chemical industry on location. The pollutants in groundwater may spread to uncontaminated areas and endanger receptors like surface water and drinking water wells according to the site-specific hydrologic regime. In addition, the sheer extension of the contamination at megasites as well as the existence of large densely populated areas and land of high-reuse value prevent a simple risk management strategy of use restriction for the whole area. Since a complete clean-up of the groundwater on a megasite is neither economically feasible nor technically possible within a reasonable time-frame, a multi-approach remediation strategy is needed, taking into account the immediate risks for human health, ecosystem and so-called "protectable goods". Moreover, the contaminants at megasites typically represent a dangerous cocktail of multiple harmful substances stemming from a variety of sources, which may interact with each other and complicate the search for an appropriate remediation strategy. At the SAFIRA-project site in Bitterfeld approaches for in situ remediation of multiple contaminants in groundwater are being tested. Alternatives in local implementation strategies as well as consequences of long-term restrictions for megasites like Bitterfeld need an independent evaluation of the situation using a risk-based approach. For this reason, a GIS-based 3D model of the area including geology, contaminants, hydrogeology, land-use and protected areas has been built. The regional groundwater pollution is characterized by contamination profiles of all monitored substances. In the area of investigation, e.g. threefold and fourfold threshold levels of chlorinated methane, ethane and ethene as well as HCH-isomers, mono-, di- and tetrachlorobenzene, DDT-isomers and benzene are frequently detected in groundwater, that means in at least 60% of the wells that were sampled. High median values of more than 10 μg/l were calculated for *cis/trans*-1,2-dichloroethene, 1,2-dichloroethane, chloroethene and monochlorobenzene. In general, the regional distribution of contaminants reflect the different sources and pathways, and give first results from a regional point of view, depending on a land-use classification of specific areas.
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1. Introduction

So-called megasites of a regional scale represent a complex problem in the field of integrated contamination site management and risk assessment for the site owners and stakeholders involved. Megasites can result from former industrial and mining areas, harbors and military areas and are commonly found inside and outside Europe, as we know today. Due to the large amount of contaminants that have entered the soil and groundwater on a regional scale, it must be assumed that these sites will emit pollutants for a long time to come, if preventive measures are not undertaken. Megasites like Bitterfeld/Wolfen with a long industrial history have a multi-source origin. These, in general, intractable site history of contaminant inputs in addition to a large variety of contaminants of different chemical properties complicate the assessment of potential health effects and represent a challenging situation for the remediator (Krapp and Ruske, 1992; Walkow et al., 2000).

Post-research in the field of risk assessment and groundwater remediation techniques has been mainly restricted to single-source contamination sites with individual plumes of contaminated groundwater. Multi-source megasites are characterized, in the case study of Bitterfeld/Wolfen, by an extended mixture of organic pollutants, which is typical for the situation of the industrial site, and possible synergistic and antagonistic effects, which have not yet been examined to the necessary details. Since a complete clean-up of groundwater of the megasite including the removal of the contaminated source areas >10 km^2 is neither technically nor economically feasible within an intermediate time-frame, an effective remediation strategy must focus on risk minimization (Wycisk et al., 1997; Wycisk, 1998). Land-use restriction measures as well as simple relocation are in many cases not a desired option for a regional scale contamination, due to the given high land-reuse value within the region. Human and ecosystem risk management must be compliant to the national and governmental (state) regulations and will present an ongoing challenge within the European Water Framework in the years to come.

2. Bitterfeld/Wolfen megasite

The industrial region of Leipzig–Halle–Bitterfeld in eastern Germany is characterized by overlapping environmental impacts of the chemical industry, an extensive devastation of the landscape, and a lowering of the groundwater table caused by lignite mining. Among the different tasks of land reclamation, the management of hazardous-waste deposits and groundwater remediation are the most urgent. Underground and open-cast mining activities since 1830 and the concentration of chemical industry sites led to an extensive lowering of the groundwater table and a change of the groundwater dynamics. The major part of industrial-waste deposits is in contact with the groundwater. As a consequence of this hydrogeological situation and the low-flow velocities, the landfills currently show a stagnating emission pattern.

An area of about 235 km^2 is affected in the Bitterfeld region, of which an area of 25 km^2 shows a significant groundwater contamination, containing a volume of about 200 million m^3. The restoration of pre-mining groundwater conditions is the major task in re-development of post-mining landscapes. This will have considerable consequences for the assessment of the polluted areas and the landfills.

The increase of the groundwater level will also cause considerable problems for the basements of the buildings in the region. During the last hundred years, the mean groundwater level was kept noticeably below the surface. The consequences of the currently rising groundwater level are not only damages to buildings, but also the

mobilizing effect on contaminants and their transport into basements of inhabited structures (buildings, houses).

In northwest of Bitterfeld, several former open-cast lignite mines have been used as landfills for chemical wastes. Hydrogeclogically, the waste sites are situated in a region with severely disturbed groundwater conditions due to the mining activities. Since the industrial dumps were incompletely sealed, the contaminates affected the groundwater directly. Fig. 1 gives an overview of the groundwater-flow direction and the abundant distribution of large contaminated sites.

The "Antonie" landfill, for example, contains about 6 million tons of various industrial residues, including waste material from pesticide production. The groundwater below the site is extremely polluted by HCH-isomers (Walkow et al., 2000). The groundwater pollution downstream, in contrast, is characterized by the more mobile contaminants like chlorinated aliphatic and aromatic hydrocarbons. The concentrations reach several hundreds of milligrams per liter, exceeding effluent standards by several orders of magnitude. Conventional "pump and treat" technologies are economically unfeasible in the long term, due to the fact of the continuous output from the landfills and the necessary volumes of about 4 million m^3 per year. Presently, the groundwater is discharging into the "Goitzsche" open-cast, but after complete flooding, the whole floodplain area of the Mulde River will be increasingly affected in the future.

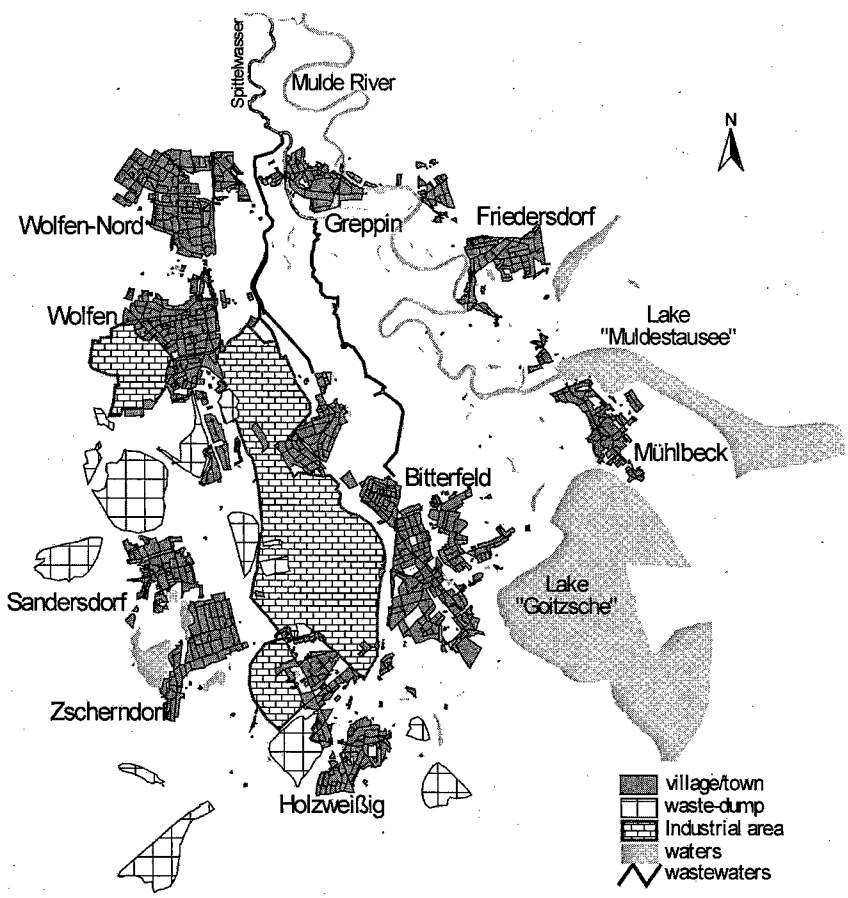

Fig. 1. Overview of the Bitterfeld area with the distribution of contamination sites and the major groundwater-flow direction, 1995.

2.1. Geological and hydrogeological background

The contaminated area of Bitterfeld is located in the floodplain of the Mulde River and can be described by the following generalized hydrogeological situation. Fig. 2 shows a schematic geological/hydrogeological overview of the area.

The upper aquifer consists of Quaternary sands and gravels. The Quaternary unit can be divided into a lower part, represented by lower terrace sediments of the Weichselian Mulde and overlying sediments, composed of braided river deposits of a smaller tributary stream. Both are separated by a hydraulically effective clay layer (Krapp and Ruske, 1992; Ruske et al., 1997).

This aquifer is in parts underlain by the upper oligocene lignite seam acting as a local aquitard. The lignite seam has been intensively mined in the southern part of Bitterfeld. The lower aquifer consists of the upper oligocene micaceous sands of different hydraulic conductivity in its upper and lower part. The base of this hydrogeological section is represented by middle Oligocene clays (Rupelian clay). The latter unit is considered to be the regional scale aquitard, hence corresponding to the base of the groundwater pollution.

Identification, assessment and remediation of a large-scale groundwater contamination require a profound knowledge of the geological structure to predict the fate of contaminants in the subsurface. Due to the distinct heterogeneity of the upper aquifer (Quaternary) and the large artificial mining dumps, a detailed 3D digital geological model of the subsurface geology was built (Wycisk et al., 2002). Using 125 selected drill holes as a base of 28 networked cross-sections, small-scale lithological and structural heterogeneities, in particular of the Quaternary layers, could be assigned to 31 litho-stratigraphic sedimentation units and depicted using a $10 \times 10\ m^2$ GIS grid. An assignment of hydraulic parameters to individual sedimentary bodies allows a combination with flow and transport models. The structural model was generated by combining gridding sections and additional point and lateral information of sediment distribution and allows—beyond visualization purposes—volumetric calculations of distinct sedimentary units, which are relevant for an assessment of retardation processes in the remaining lignite seam. The present structural model is the base for a "Spatial Model Bitterfeld" (Wycisk et al., 2002) and enables further investigations on the

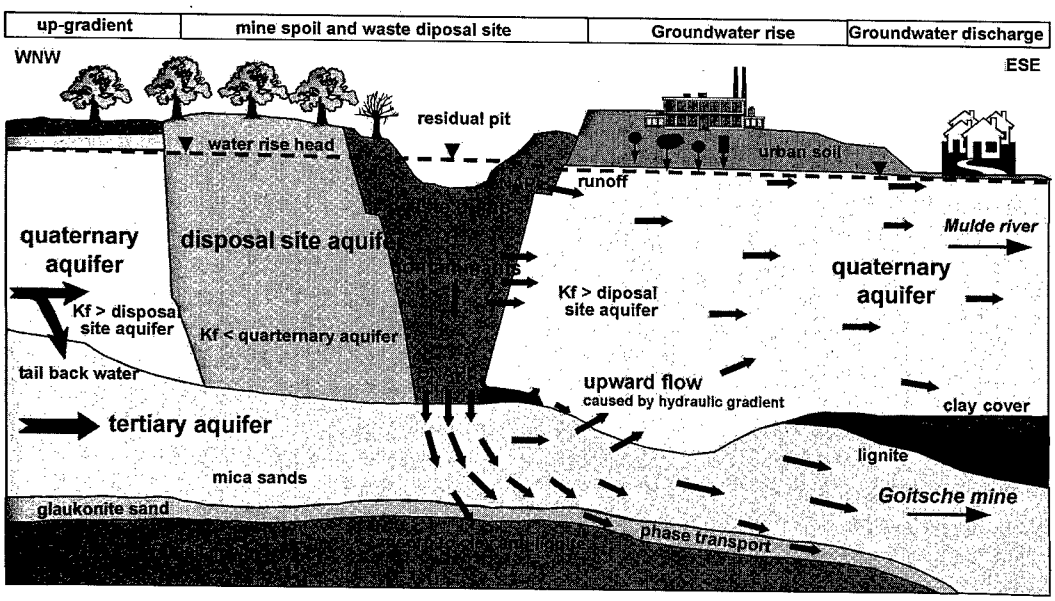

Fig. 2. Schematic geological and hydrogeological setting of the pathway of contaminants at the Bitterfeld area, which has been dominated by industrial and mining activities for more than 100 years.

environmental impact of distinct scenarios of toxicological exposure assessment.

2.2. Groundwater pollution at the local scale

In order to characterize the organic pollution of the groundwater at various water levels, several samples were examined at a local scale from different depths at the remediation SAFIRA pilot plant. The 'main components' of organic contamination were found to be chlorobenzene, 1,2-dichlorobenzene, 1,4-dichlorobenzene, benzene, trichloroethene, *cis*-1,2-dichloroethene and *trans*-1,2-dichloroethene. Despite the fact that high organic pollution with halogenated hydrocarbons was confirmed in both aquifers, contamination varied considerably in terms of quality and quantity. The lower aquifer is dominated by aliphatic chlorinated hydrocarbons (*trans*-1,2-DCE, *cis*-1,2-DCE and TCE), whereas the main component of the upper aquifer is chlorobenzene. During depth-oriented sampling, the contamination was found to display a significant stratification, whereas the sample from a depth of 7–8 m only contains very low amounts of pollutants; samples from depths between 16 and 20 m were found to contain high levels of chlorobenzene (8–51 mg/l), as well as substantial concentrations of dichlorobenzenes (up to 1 mg/l).

One striking aspect is that the aliphatic halogenated hydrocarbons and benzene chiefly occur in the strata between 19.5 and 24.5 m, whereas chlorobenzene and dichlorobenzene are found in higher concentrations in the strata between 12 and 22.65 m. The main components are *trans*-1,2-dichloroethene, *cis*-1,2-dichloroethene, chlorobenzene, trichloroethene and benzene. Bromobenzenes and perchloroethene were also identified by mass spectrometry. Contamination of the groundwater with inorganic pollutants (e.g. heavy metals, arsenic, etc.) has proven to be of minor importance. The only noteworthy feature is the high levels of sulfate (up to 1000 mg/l) and chloride (about 1300 mg/l).

To sum up the local situation of the Bitterfeld site, the contaminants show a distinct vertical stratification and high concentration levels alternate with low or zero levels. This shows very clearly that such a differentiated situation can only be described in detail by multi-level wells, which are usually not state of the art. Secondly, due to the hydrogeological heterogeneity in the subsurface, the experience during the last years of groundwater sampling gives a clear evidence of a strong local variability of the detected contaminants. These two facts make it very difficult to state distinct concentration levels as well as certain organic substances or compounds.

2.3. Groundwater pollution at regional scale

Due to the environmental importance of risk assessment in the region of Bitterfeld, a groundwater-monitoring program has been implemented by the site owners and carried out continuously since 1990. Based on parts of this data set (290 wells, 1200 samples, each up to 180 contamination parameters), a specific contamination profile for this region was derived on the base of available monitoring data within the SAFIRA project (Thieken, 2001). Before doing that the influence of important parameters like detection limits and the statistical measures to estimate the average detected concentration on the results of the ranking procedure were investigated and led to a modification of the method. Furthermore, a cluster analysis with the two criteria, detection frequency and average-detected concentration, reveals substances with similar behavior in the environment in terms of persistence and mobility, and improves the contamination profile at the regional level. In the area of investigation, e.g. threefold and fourfold detection frequency of chlorinated methane, ethane and ethene as well as HCH-isomers, mono-, di- and tetrachlorobenzene, DDT-isomers and benzene are detected frequently in groundwater, that means in at least 60% of the wells that were sampled. Various statistical measures were used to estimate the average-detected concentration for the monitored contamination data. Fig. 3 shows the regional distribution and total frequency of the organic compounds like BTEX, volatile chlorinated organic compounds and chlorobenzene from the upper aquifer. It was concluded from the monitoring data that the median is the most suitable

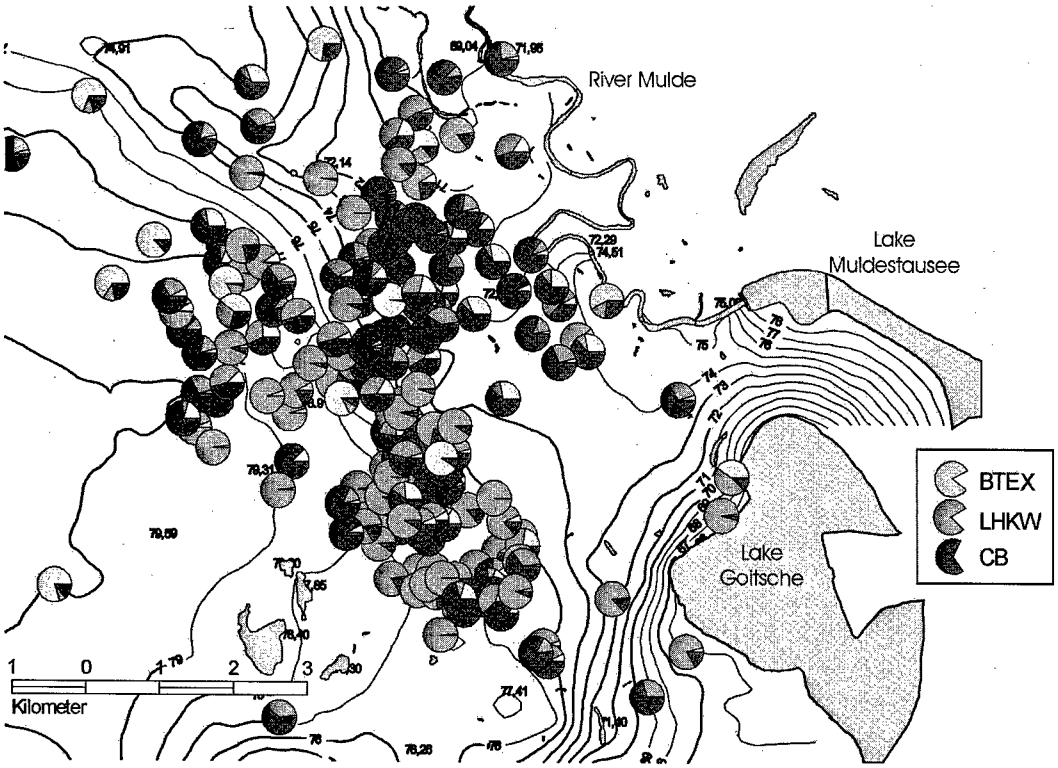

Fig. 3. Regional distribution and total frequency of selected organic compounds and substances from the upper aquifer, like BTEX, volatile chlorinated organic compounds (LHKW), and chlorobenzene (CB). Groundwater contours from the year 2000.

measure for this purpose. High median values of more than 10 μg/l were calculated for *cis/trans*-1,2-dichloroethene, 1,2-dichloroethane, chloroethene and monochlorobenzene. The regional distribution of contaminants reflects in general their different sources and pathways and gives first results from a regional point of view, depending on the land-use classification of specific areas, e.g. industry, mining, settlements, agricultural and alluvial plains/meadows (see Table 1).

2.4. Remediation options for megasites

The large-scale contaminated megasite of Bitterfeld/Wolfen is characterized by a regional pollution of soil, surface water and groundwater, due to the long and varied history of the chemical industry. The pollutants in groundwater may spread to uncontaminated areas and endanger receptors like the surface water of the Mulde River and to some extent drinking water wells according to the site-specific hydrologic regime. The extension of the contamination at the multi-source megasite as well as the existence of large densely populated areas and land of high-reuse value prevent a risk management strategy of use restriction for the whole area. Since a complete clean-up of the groundwater on a megasite is neither economically feasible nor technically possible within a reasonable time-frame, a multi-approach remediation strategy is needed taking into account the immediate risks for human health, ecosystem and so-called "protectable goods". Moreover, the contaminants at the megasite typically represent a cocktail of multiple harmful substances stemming from a variety of sources, which may interact with each other and complicate the search for an appropriate remediation strategy. Fig. 4 gives a schematic overview of possible remediation options at the megasite.

To stop the uncontrolled spreading and extension of the contaminants in the groundwater

Table 1
Average detected concentration in μg/l of indicated organic compounds per classified land-use unit

	Alluvial plain		Mining areas		Urban areas		Industrial areas		Agricultural		Total area	
	$n > RL$	MEC	$n > RL$	MEC	$n > RL$	MEC	$n > RL$	MEC	$n > RL$	MEC	$n > RL$	MEC
LHKW	51	16.7	107	22.1	36	66.0	155	89.4	16	5.8	365	42.7
BTEX	40	4.8	62	2.7	24	11.2	130	15.7	13	2.5	269	8.2
CB	46	17.0	84	15.8	34	13.2	149	89.2	16	10.2	329	26.5
HCH	28	1.8	58	2.6	11	2.0	124	2.6	1	–	222	2.5
DDT/D/E	27	0.2	32	0.1	10	0.2	97	0.3	0	–	166	0.2

The measured concentrations (MEC) reflect the source–receptor (Mulde River) relation of the pathway of contaminants. Selective data set 9198M. MEC, mean emission concentration; RL, relevance level; LHKW, volatile chlorinated organic compounds; CB, chlorobenzene (modified after Thieken, 2001).

towards uncontaminated areas and receptors, including the surface waters, a common approach consists of "pump-and-treat" measures. The sufficiently treated water can be channeled into the surface water (channels, rivers, creeks). If the quantity of water pumped is large enough, the groundwater flow regime may be altered and a further expansion of the contaminated zone is avoided. However, this approach requires that relevant technologies exist for treating the particular contamination encountered at the site. Pump-and-treat measures are being currently employed and their expansion considered at the Bitterfeld site within the framework of the Ökologisches Großprojekt (Ecological Mega Project) Bitterfeld–Wolfen. Barriers consisting of pumping wells are used to stop the groundwater flow in northern, northeastern and eastern direction. The NE barrier, for example, has been in operation since 1994, using 20 pumping wells to extract an annual amount of 1.3×10^6 m^3 contaminated water from the Quaternary aquifer (Lücke and Großmann, 2002).

In order to provide the cost-effective remediation schemes, efficient monitoring strategies need to be developed. These strategies include integral sampling methods such as time integrating sampling devices for the assessment of contaminant fluxes over longer periods of time. In addition, depth-specific information on contaminant distribution is required to efficiently place active remediation measures or permeable reactive barriers (Schirmer et al., 1995; Merkel et al., 2001; Weiß et al., 2001).

At the SAFIRA-project site in Bitterfeld, different approaches for in situ remediation for multiple

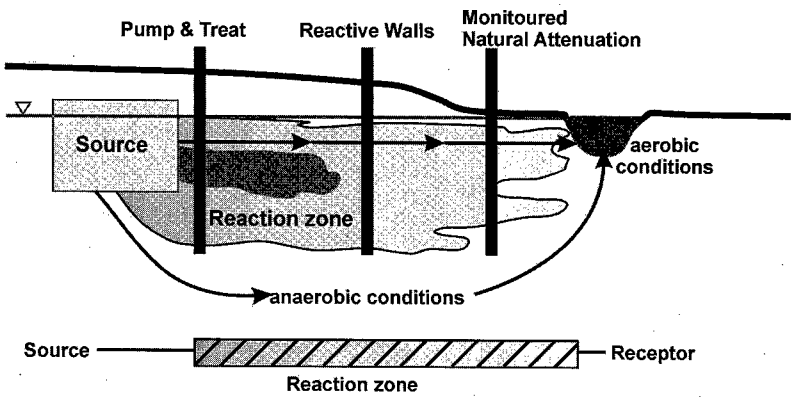

Fig. 4. Sketch of the long-distance related fate processes of organic contaminants at megasites and alternative remediation options for groundwater.

contaminants in groundwater are being tested. SAFIRA is the German acronym for "Remediation Research in Regionally Contaminated Aquifers" (Merkel et al., 2001; Weiß et al., 2001, 2002).

The following systems are being tested as part of in situ technologies:

- biodegradation of chlorinated contaminants in an anaerobic/microaerobic system,
- adsorption and simultaneous microbial degradation on activated carbon,
- zeolite-supported palladium catalysts,
- membrane-supported palladium catalysts,
- oxidative solid metal catalysts,
- activated carbon filtration,
- anaerobic microbial degradation of pollutants,
- combination of redox reactors.

These technologies which have been used in the pilot plant reactors are directly fed by groundwater from the Quaternary aquifer. The technologies may be upscaled and used subsequently in the so-called "sorptive barriers" or "permeable reactive barriers", which are installed directly in the aquifer (Fig. 4). Reactive barriers are located in the main direction of contaminant flow and decrease the contaminant loading by, e.g. adsorbing reactions (e.g. activated carbon) or through redox reactions (Fe°, ORC) (Teutsch et al., 1996; Weiß et al., 2002). A variation of reactive barriers is the "funnel-and-gate" approach, where the contaminant plume is funneled by impermeable funnel walls towards the reactive gate inside the aquifer. The gate reacts as a zone, where the contaminants are broken down or adsorbed.

For the area of the Bitterfeld megasite lying downstream of the main contaminant zone, monitored natural attenuation (MNA) may prove to be a valuable and additional remediation option and should be included in the overall strategy. MNA is based upon a variety of physical, chemical or biological processes that, under favorable conditions reduce mass, toxicity, mobility, volume or concentration of contaminants in soil or groundwater. After sufficient NA rates have been confirmed on-site, long-term monitoring must ensure the persistence and relevance of these rates to minimize all relevant risks, in order to guarantee a permanent protection of man and the environment.

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Review

Ecotoxicological problems associated with contaminated sites

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Abstract

Contamination sites pose significant environmental hazards for terrestrial and aquatic ecosystems. They are important sources of pollution and may result in ecotoxicological effects. At severely contaminated sites acute effects occur, but the core problem lies in possible long-term chronic effects. Ecotoxicological effects occur at all levels of the biological organization, from the molecular to the ecosystem level. Not only certain organisms may be affected, but the ecosystems as a whole in its function and structure. Contaminants at large contaminated sites often share critical properties such as toxicity, high environmental persistence, often high mobility prone to contamination of groundwater, and high lipophilicity resulting in bioaccumulation in food webs. Contaminants present at polluted sites occur as mixtures; therefore, interactions between individual compounds may be of importance. The bioavailability is a key factor responsible for ecotoxicological effects of contaminants: only the bioavailable fraction induces ecotoxicological effects, as shown for organotin compounds. Organotins belong to the most toxic pollutants known so far for aquatic life. Widespread contamination of harbor sediments occurs globally due to the ongoing use of organotins in antifouling paints on large ships. In lake sediments, tributyl- and triphenyltin are very persistent and bioavailable to biota even after a long time. The bioavailability of organotins is dependent on the pH and the content of organic matter. Organotins accumulate in sediments, but remobilization occurs during disturbance and dredging. A key question in dealing with contaminated sites is whether, and to what extent ecotoxicity occurs. Usually, established OECD tests and whole effluent toxicity tests are performed for an ecotoxicological evaluation and for risk assessment. However, these assays are often expensive, laborious and sometimes not sensitive enough. As a consequence, we have used rapid and inexpensive in vitro systems such as fish cell lines for the evaluation of sediments and landfill leachates, which were contaminated by polycyclic aromatic hydrocarbons (PAHs). The determination of cytotoxicity as a measure for acute toxicity, and the induction of cytochrome P4501A (CYP1A) as a biomarker of exposure and effects were found to be important measures, which can be used for hazard and risk assessment. We have developed a concept for the ecotoxicological evaluation of PAH contamination based on induction equivalents, which can be applied for aquatic and terrestrial ecosystems. One of the key question and present gaps, however, includes the long-term chronic ecotoxicological effects of single compounds and mixtures on soil and aquatic biota at contaminated sites. This should be addressed in the future.
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Keywords: Ecotoxicology; Bioavailability; Organotins; Fish cell lines; Cytochrome P450

1. Introduction

Ecotoxicology as an interdisciplinary environmental science has evolved mainly from toxicology, applied ecology and environmental chemistry. It is focused on interactions between environmental chemicals and biota, thereby addressing adverse effects at different levels of the biological organization. Toxic effects of anthropogenic compounds in biota and ecosystems are regarded in relation to their chemistry and fate in the environment. The bioavailability of chemicals, which is dependent on biogeochemical and physiological processes, is an important factor, often neglected in ecotoxicological evaluation and hazard assessment. The bioavailable fraction is critical for uptake, and ultimately, for the concentration at the target site in organisms (Fig. 1). Ecotoxicological research requires an interdisciplinary approach considering physicochemical, molecular, toxicological, physiological and ecological processes. Whereas ecotoxicology in practice deals with regulatory issues (registration of chemicals), and thus to testing of chemicals in standardized tests; the focus of ecotoxicological research is aimed at an understanding of toxicological phenomena in a variety of biota of different complexity, populations and the ecosystem as a whole. Thereby, diverse aspects such as mechanisms of toxic action and ecological processes in contaminated systems are regarded (Fent, 1998).

Ecotoxicological studies also focus on ecological and toxicological effects observed in the field in retrospective studies, whereby a causative correlation between effects and chemical residue analysis is, however, often difficult to establish. Ecological investigations, such as biomonitoring studies, alone do not have sufficient resolving power to identify causative agents. Likewise, chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicological consequences in biota. An integrated approach considering environmental, chemical, toxicological and ecological concepts is needed for the understanding of ecotoxicological effects in contaminated ecosystems. One strategy to at least assess the contamination and its potential effects is the use of biomarkers in ecological surveys to verify the bioavailability and presence of relevant concentrations in biota (Bucheli and Fent, 1996). A selection

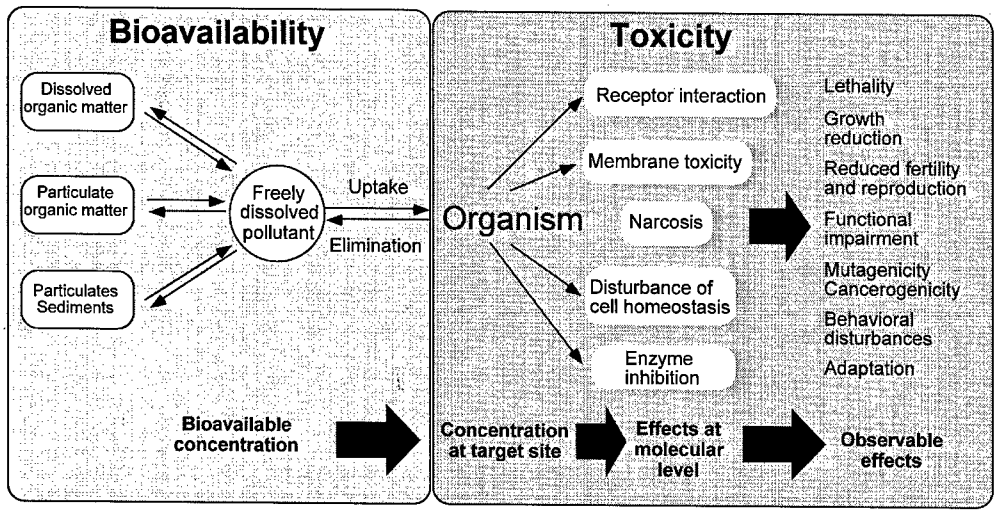

Fig. 1. Ecotoxicological effects are dependent on the bioavailable fraction of pollutants, and concentrations at the target sites induce molecular effects that propagate to a variety of toxic manifestations in organisms. Adapted from Escher et al., 1997.

of the type of biomarker allows a gross discrimination between certain groups of contaminants (e.g. polyaromatic and dioxin-like pollutants versus heavy metals), and toxicological mechanisms or biological functions affected.

A more prospective approach is based on investigations of potential toxicological effects in laboratory assays that may be used for extrapolation to the field. Bioassays play an important role in this process; however, more comprehensive studies on contaminated systems and ecotoxicological effects are needed in addition. Often, bioassays do not consider the processes in ecosystems and neglect environmental factors that influence toxicity. However, they are valuable tools in the characterization of the toxic action of chemicals, and in the understanding of associated toxicity. Despite the usefulness of these tools, the multitude of chemicals in ecosystems, species diversity, biological and ecological functions and structures makes extrapolations necessary for estimating possible effects of contaminants in ecosystems. An important task, therefore, is the improvement of the predictive power and quality of experimental systems for use in risk assessment.

In ecotoxicological research, cellular and biochemical effect studies including mechanisms of toxic action are equally important as studies in laboratory organisms, because the primary interaction between chemicals and biota occurs at the surface of, or in cells (Fig. 1). Whether chemical-induced alterations in cell structure and physiology will develop into an adverse toxic effect depends on many parameters, including adaptive responses. However, the hypothesis that cellular changes may ultimately influence biological parameters important for populations such as growth, development, health and reproduction is obvious. Cellular toxicology provides an essential concept in the understanding of ecotoxicological processes by elucidation of the toxic modes of action and toxicological effects. Its value will be strongly increased, when it can be integrated more closely with ecological effects. Here, the applicability of in vitro cell systems for the assessment of sites contaminated with chemicals having different toxicological modes of action is presented.

1.1. Ecotoxicological evaluation of contaminated sites and risk assessment

A frequently used approach to evaluate ecological impacts of contaminated sites is field monitoring. This includes structural and functional measurements of resident biota, and sometimes in situ (caged) studies. In contaminated aquatic systems, structural measures include algal and aquatic plant density, biomass, benthic macroinvertebrate and fish surveys. In the terrestrial part, structural parameters such as plant density, soil invertebrate and biomass are determined. Structural indices include total abundance, taxa richness, diversity indices and various ratios of different taxonomic groupings. Functional measurements monitor rate processes over time such as algal carbon uptake, photosynthesis and rates of reproduction or growth. Monitoring should be complemented with in situ or experimental whole effluent toxicity assessments aimed at the estimation of the ecotoxicological potential of pollutants and about cause relationships. In situ toxicity testing, as an experimental approach, can be used to measure variation in exposure under actual receiving system conditions. In addition, whole effluent tests assessment of water samples or their extracts.

Evaluation of the ecotoxicological potential of contaminated sites includes several steps. In a first phase of an ecological risk assessment, in which sources and contaminants of potential concern are identified, toxicity is assessed using relatively simple ecotoxicity tests. The aim is to search for potential causal relationships among contaminants, receptors and ecotoxicological endpoints. Further stages of risk assessment—exposure and effects assessment, then risk characterization—require additional information and can include other tools such as measures of exposed populations of animals and plants, long-term laboratory or field bioassays, toxicity identification evaluations, etc. Finally, risk characterization builds upon the results of the analysis phase to develop an estimate of risk. Fig. 2 illustrates the commonly used risk assessment concept.

Leachates from sediments, soil and groundwater at contaminated sites are being tested in whole

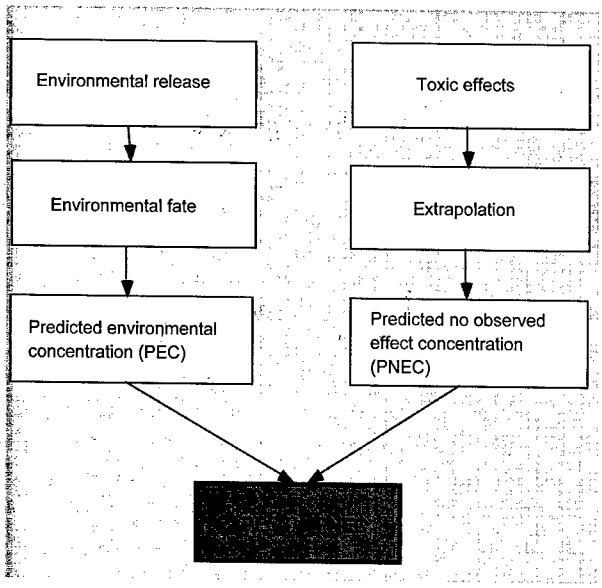

Fig. 2. Environmental risk assessment. PEC, predicted environmental concentration; PNEC, predicted no-observed-effect concentration. Risk analysis is necessary when PEC/PNEC > 1. Adapted from Ecetoc, 1993.

effluent toxicity tests, which integrate interactions among complex mixtures of contaminants. Thereby, the total toxic effect, regardless of physical and chemical composition, is assessed. This is a powerful integrative measure of the toxicity of chemicals not being achievable by analytical chemical measurements. Usually, direct toxic effects on survival, growth or reproduction are determined using bacteria, algae or periphyton, water flea and fish. Although these tests have limitations, they give important hints to the ecotoxicological potential of contaminated sites. Often, however, there is a discrepancy between results derived from both standardized laboratory tests and whole effluent tests compared with known biological impacts. For example, a set of 250 discharges from contaminated river systems across the United States were tested in standardized *Daphnia* and fathead minnows tests, whole effluent toxicity tests and in-stream biological condition as measured by benthic macroinvertebrate species composition and abundance. The results indicated that whole effluent toxicity testing was more predictive of biological effects in the rivers, if several tests addressing different types of endpoints were used (Diamond and Daley, 2000). Fish acute and chronic endpoints were mostly related to in-stream condition, but no one endpoint was capable of accurately reflecting conditions of all discharges. This indicates that whole effluent toxicity testing is a tool which is useful for ecotoxicological evaluation of contaminated sites, but as all tools, has its limitations such as test variability, species differences and extrapolation from laboratory to the field. Whole effluent tests underestimate field effects in some cases (Clements and Kiffney, 1994); in other cases they serve as good predictors of fish response, but poor predictors of invertebrate response (Birge et al., 1989). With regard to reliability in predicting biological community effects, laboratory single-species toxicity tests are suggested, in a majority of cases, reliable qualitative predictors.

Environmental risk assessment is based on laboratory tests for mainly practical reasons. Extrapolating effects of toxicants from a limited number of test species to ecosystems as a whole is a difficult, but essential part of environmental risk assessment (Koller et al., 2000). If toxicity test results are available for very few species, the lowest toxicity value is divided by an application factor or safety factor, which varies from 10 to 1000, depending on the number of species tested, and whether the endpoint is based on acute mortality or effects (LC_{50} or EC_{50}), or chronic no-observed-effect concentrations (NOEC). A comparison of 248 studies on 34 substances, involving both model ecosystems studies and chronic single-species studies, indicate that an assessment factor of about 10 would be appropriate in the extrapolation from the lowest chronic single-species NOEC-value in a model ecosystem (Chemicals, 1997). This risk assessment concept (Fig. 2) using the ratio between predicted exposure concentration (PEC) and predicted no-observed-effect concentration (PNEC) is widely used (Ecetoc, 1993). However, hazard and risk assessment using ecotoxicological tests with laboratory species may provide an uncertain level of protection due to the variation in bioavailability of toxicants, contaminant concentrations and interactions in compound mixtures. In addition, bioaccumulation in food webs is not regarded and

effects on populations or community responses may differ from the situation in the field. Moreover, adaptation to contaminants can occur at contaminated sites after long-term exposure. Adaptive reactions include actively pumping out incoming toxicants, detoxification mechanisms, damage repair and avoidance of contamination (Hansen et al., 1999), and they may have ecological costs such as energy consumption that reduces the organism's fitness. In this paper, two ecotoxicological case studies are presented, in which the applicability of rapid and rather inexpensive in vitro cell culture systems for the assessment of ecotoxicity is demonstrated, and in which the importance of the bioavailability of contaminants is shown.

2. Organotins

Organotin compounds are among the most hazardous pollutants known so far in aquatic ecosystems (Fent, 1996). Tributyltin (TBT) is of particular importance because of its widespread use as a biocide in antifouling paints on ships and in wood protection. Organotin pollution of aquatic environments is of global concern. Restrictions on its use have been implemented in many countries (Fent and Hunn, 1995), but contamination of harbor sediments persists due to the low degradation in anoxic sediments (Biselli et al., 2000; Fent and Hunn, 1991) (Fig. 3). Organotin speciation shows a strong pH dependence. TBT and triphenyltin (TPT) are present as cations at low pH and as hydroxides at higher pH. Hydroxides and cations, however, exhibit very different partitioning and sorption behavior. The octanol–water partition coefficients of TBT and TPT are more than an order of magnitude higher at pH 8 (log K_{ow} of TBTOH and TPTOH is 4.10 and 3.53, respectively) than at pH 3 (Arnold, 1998). This is related to the fact that TBTOH and TPTOH, but not TBT^+ and TPT^+, readily partition into the octanol phase.

What is the influence of pH and humic acids on the bioconcentration of TBT and TPT in different aquatic biota and at different conditions? In *Daphnia magna*, *Chironomus riparius* larvae and fish TBT and TPT bioconcentration was dependent on the speciation (Fent and Looser, 1995; Looser et al., 1998, 2000). Significantly higher bioconcentration occurred at pH 8 than at 6 or 5, which is related to the fact that these compounds mainly, but not exclusively, accumulate as hydroxides which predominate at pH 8. At pH 8, TBT occurs predominantly as TBTOH (95% of total TBT), whereas the fraction of TPTOH is more than 99%. At pH 5, TBT is present primarily as positively charged TBT^+ (95%), whereas in the case of TPT, both species are present in about similar fractions (61 and 39%, respectively; Arnold, 1998). Dissolved organic matter such as humic substances result in a significant reduction in the bioavailability due to hydrophobic sorption of organotins (Fent and Looser, 1995; Looser et al., 1998, 2000). The effect of Aldrich humic acids (AHA) on the bioconcentration of TBT and TPT is shown in Fig. 4. AHA reduce the bioconcentration of TBT and TPT. These results clearly demonstrate that the bioavailability of organotins is a key parameter for bioconcentration, and also for toxicity. It is a function of pH and concentration of dissolved organic matter.

Many studies on the ecotoxicity of organotins have been reported (Alzieu and Heral, 1984; Bryan et al., 1986; Fent, 1996; Fent and Meier, 1992; Fioramonti et al., 1997; Hamasaki et al., 1993; Horiguchi et al., 1997). However, the long-term ecotoxicological effects on the structure and function of aquatic ecosystems are still not well understood, particularly with respect to biomagnification in food webs (Guruge et al., 1996; Kannan et al., 1997; Kim et al., 1996; Stäb et al., 1996). Organotins are extremely toxic to aquatic biota as demonstrated for a variety of different organisms in vivo and in vitro (Fent, 1996). TBT and TPT act via different modes of action. Perturbation of calcium homeostasis, inhibition of mitochondrial oxidative phosphorylation and ATP synthesis, inhibition of photophosphorylation in chloroplasts, and inhibition of enzymes, such as ATPases and cytochrome P450 (CYP) monooxygenases, are among the key processes (Fent, 1996). Inhibition of CYP enzymes, in particular the aromatase, responsible for the conversion of testosterone to estradiol, has ecological

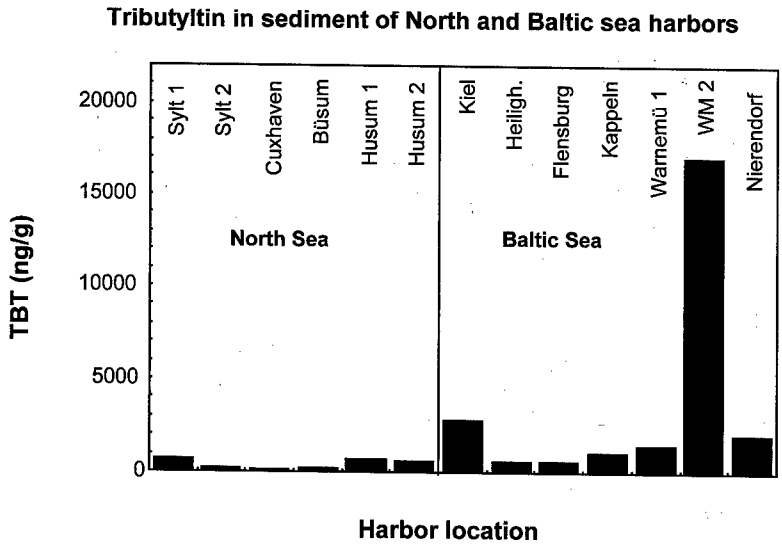

Fig. 3. Contamination of pleasure boat harbors in Germany in the North Sea and in the Baltic Sea. Harbor locations: Heiligh., Heilighafen; Warnemü, Warnemünde; WM2, Warnemünde. Data after Biselli et al., 2000.

significance. Among the most susceptible organisms affected by organotins are gastropods, of which over 100 species are known to be affected by TBT. The females develop male reproductive organs at trace concentrations of a few ng/L TBT, due to inhibition of aromatase and associated disturbance of steroid metabolism (Bettin et al., 1996). Inhibition of CYP by TBT and TPT has also been demonstrated in fish in vivo (Fent and Stegeman, 1993) and in vitro (Fent and Bucheli,

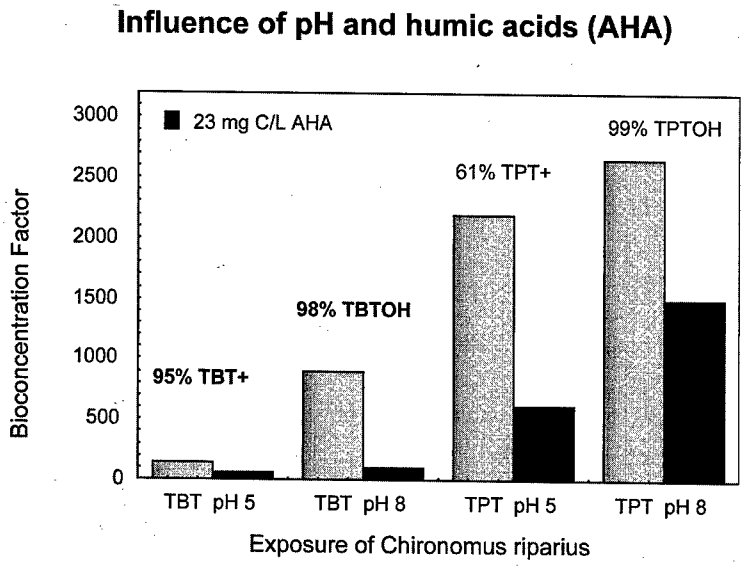

Fig. 4. Bioaccumulation of TBT and TPT in *Chironomus riparius* larvae at pH 8 and 5 in the absence or presence of 23 mg C/L AHA. Exposure concentrations 2.4 μg/L TBT and 3.0 μg/L TPT. Percentage of organotin species at respective pH is also given. Data after Looser et al., 2000.

1994; Fent et al., 1998). Recently, inhibition of aromatase has also been demonstrated in rats, where testis weights, spermatid and sperm count were also reduced at 125 mg TBT/kg (Omura et al., 2001).

Interaction of anthropogenic chemicals with biota takes place first at the cellular level making cellular responses not only the first manifestation of toxicity, but also suitable tools for the early and sensitive detection of chemical exposures. As an alternative for acute fish toxicity testing, or as a supplementary bioassay for toxicity ranking of chemicals, in vitro cytotoxicity assays have been applied (Babich and Borenfreund, 1987, 1991; Brüschweiler et al., 1995; Fent and Hunn, 1996; Ryan and Hightower, 1994). Cytotoxicity in different cell lines was found to be correlated with in vivo acute fish toxicity (Babich and Borenfreund, 1991; Brüschweiler et al., 1995; Fent and Hunn, 1996), and correlated to the octanol–water partition coefficient (Brüschweiler et al., 1995). We have also monitored aquatic environments employing in vitro systems with fish cell lines. In particular, estrogenic activity could be assessed in treated sewage using a new fish cell line reporter gene system (Ackermann et al., 2002; Fent, 2001). These studies lead to the conclusion that fish cell line-based assays are important in contributing to the evaluation of the environmental hazard of chemicals and environmental samples. Ideally, they should be used in combination with other ecotoxicological test systems representing organisms of different ecology.

3. Case study: polycyclic aromatic hydrocarbons and related CYP inducers

Environmental compounds that elicit toxic effects similar to that of polychlorinated dioxins (dioxin-like chemicals) are of concern due to their high chronic toxicity to organisms of different evolutionary levels. Several toxic and biochemical effects of such chemicals are mediated through the aryl hydrocarbon receptor (AHR), which is highly conserved in evolution and found in numerous taxa (Hahn, 1998). AHR is a ligand-dependent transcription factor located in the cytosol of the cell. The ligands for AHR are hydrophobic aromatic compounds with planar structure of particular size, which fit the binding site. Upon binding of an agonist, AHR is translocated to the nucleus, where it forms a heterodimer with the AHR nuclear translocator protein and binds to dioxin-responsive elements in the promoter region of certain genes, which are then upregulated. One of the expressed genes is CYP1A, a key enzyme in the metabolism of xenobiotics. It has been shown that the binding strength of a ligand to the AHR is roughly directly proportional to the enhanced gene transcription and associated toxicity.

3.1. An induction equivalency concept for the toxicity assessment of polycyclic aromatic hydrocarbons

CYP1A induction is a sensitive and specific adaptive response of organisms exposed to an important series of environmental pollutants such as planar congeners of polychlorinated dibenzodioxins and dibenzofurans (PCDD and PCDF), polychlorinated biphenyls (PCBs), several polycyclic aromatic hydrocarbons (PAHs), polychlorinated naphthalenes and related compounds. CYP1A induction is used as a biomarker indicative for exposure to such pollutants in organisms such as fish and fish cell systems (Bucheli and Fent, 1995). Fish liver cells contain an AHR, which is very similar to mammalian AHR, but less expressed. We studied the induction potential of a diverse category of environmental chemicals and environmental samples in the fish liver cell line PLHC-1 to determine their induction potential and evaluate its toxicity (Fent, 2001). These cells, derived from topminnows *Poeciliopsis lucida* (Hightower and Renfro, 1988), bear metabolic activity (Babich et al., 1991) and contain an AHR (Hahn, 1998). Recently, we demonstrated that they also express very low levels of estrogen receptor mRNA (Fent, 2001; Haugg et al., 2000). This cell line has the capacity to induce the expression of CYP1A after exposure to environmental pollutants such as PCBs (Brüschweiler et al., 1996; Hahn et al., 1993, 1996). As an indicator of toxicant-induced changes in biological systems,

CYP1A induction is regarded as one of the most sensitive and specific biomarkers for such pollutants (Bucheli and Fent, 1995; Stegeman and Hahn, 1994).

Cost-effective bioassays that integrate biological effects of complex mixtures of PAH and other pollutants are needed. The toxic potential of halogenated aromatic hydrocarbons (HAHs) can be estimated by applying a toxic equivalency (TEQ) concept (Safe, 1994). Thereby, the toxic potency of a compound or mixture of these pollutants in biological systems is compared with the potency of the most toxic compound, e.g. 2,3,7,8-TCDD. TEQs are the sum of the products of the concentrations of the pollutants and their toxic equivalency factors (TEF), which are estimated relative to TCDD. This concept is based on in vivo and in vitro studies with cultured cells that indicate that these compounds cause similar effects, but differ in potency. The TEF concept has also been developed for other important AHR-mediated endpoints such as receptor binding, and fish early life stage mortality. By using this concept, environmental concentrations can be expressed in terms of biological responses that are bioassay derived, and are therefore more meaningful than concentration figures alone.

PAHs have only rarely been analyzed for CYP1A induction in fish in vitro systems. By employing PLHC-1 cells, we have derived fish-specific TEFs, or as better called, induction equivalency factors (IEFs), for 19 PAHs (Fent and Bätscher, 2000), 12 nitrated PAHs (NPAHs) and 12 azaarenes (Jung et al., 2001). The relative CYP1A induction potencies, determined as ethoxyresorufin *O*-deethylase (EROD) activity, and the cytotoxicities of 19 compounds with 1–6 benzene rings, mixtures of PAHs, and contaminated landfill leachates have been determined in PLHC-1 cells (Fent and Bätscher, 2000). No CYP1A induction was observed with benzene, naphthalene, anthracene, acenaphthene, benzo[*g*,*h*,*i*]perylene and fluorene, but low induction was found with fluoranthene and phenanthrene. All other PAHs with three and more benzene rings led to a concentration-related induction of CYP1A, with rebound decreases at high concentrations resulting in bell-shaped concentration-activity curves (Fig. 5). Fish-related IEFs were estimated for all PAHs on the basis of EC_{50} values of their EROD activities, thereby taking the most active compound, dibenz[*a*,*h*]anthracene as the reference compound (Table 1). In contrast to the EROD activity showing a bell-shaped concentration–activity curve due to competitive substrate inhibition (Hestermann et al., 2000; Jung et al., 2001), the immunodetectable protein content determined by ELISA showed a concentration-dependent increase. Moreover, we have determined the cytotoxicity, and the CYP1A-induction potential of NPAHs and azaarenes (Jung et al., 2001). The induction potency was highly dependent on the compound's structural properties, reflected by significant correlation between the half-maximal EROD induction ($-\log EC_{50}$) and the molecular descriptors lipophilicity ($\log K_{ow}$) and maximal molecular length (L_{MAX}).

3.2. *In vitro assessment of the ecotoxicological potential of contaminated sites*

At contaminated sites, generally compound mixtures occur, but little is known about their activity. We found that the interaction of PAHs in mixtures of up to eight individual compounds is additive based on their EROD activities (Fent and Bätscher, 2000). Therefore, an evaluation of the CYP1A induction potential of environmental samples can be performed taking into account an additive behavior of individual compounds. The advantage of such in vitro systems is that the total mixture of all contaminants is evaluated, which allows a more appropriate ecological risk assessment. This is shown here for a severely mineral oil-contaminated river system in Estonia. Among watersheds at the oil shale area of Estonia, River Purtse with its tributaries is heavily polluted with wastewater from coke-ash dumps of oil shale processing plants and with oil shale drainage water from underground mines and open-cast pits. Impacts have been recorded not only in this river and its tributaries, but also the Gulf of Finland (Huuskonen et al., 2000). One of the benefits of using fish cells for assessment of mineral oil pollution is rapidity, which is necessary in making

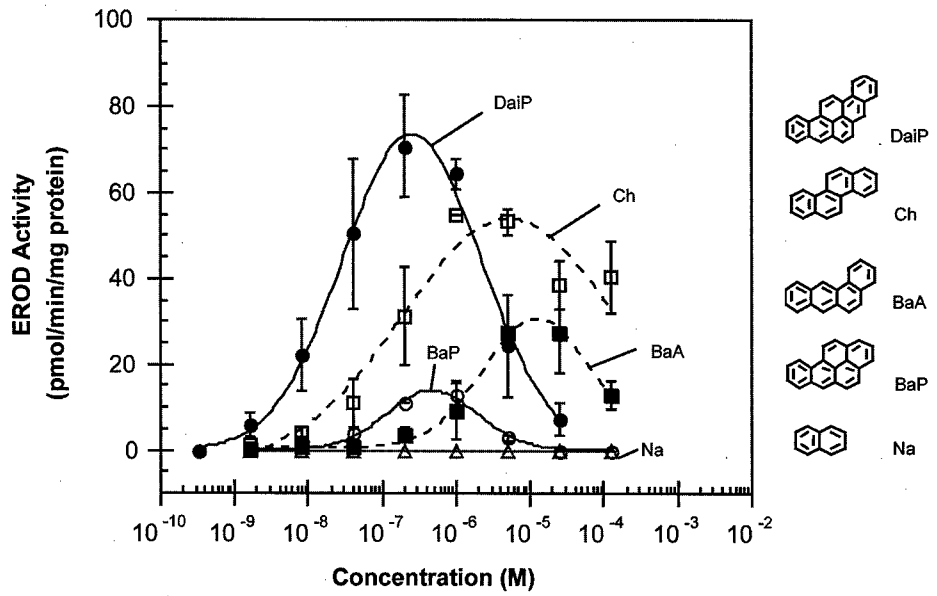

Fig. 5. Induction of CYP1A in PLHC-1 cells determined by catalytic activity. Concentration–EROD activity curves of representative polycyclic aromatic hydrocarbons in PLHC-1 cells. DaiP, dibenz[*a*,*i*]pyrene; Ch, chrysene; BaP, benzo[*a*]pyrene; BaA benz[*a*]anthracene; Na, naphthalene. Adapted from Fent and Bätscher, 2000.

Table 1
IEF of PAH in PLHC-1 cells[a]

Compound	IEF
Dibenz[*a*,*h*]anthracene	1.0[b]
Dibenzo[*a*,*i*]pyrene	0.42
Benzo[*k*]fluoranthene	0.30
3-Methylcholanthrene	0.13
Benzo[*a*]pyrene	0.050
Benzo[*e*]pyrene[c]	0.040
Chrysene	0.029
7,12-Dimethylbenz[*a*]anthracene	0.011
Perylene[c]	0.0091
Benz[a]anthracene	0.0053
Pyrene	0.0022
Benzo[*g*,*h*,*i*]perylene	–
Fluoranthene	–
Phenanthrene	–
Anthracene	–
Acenaphthene	–
Fluorene	–
Naphthalene	–
Benzene	–

–, no significant EROD induction.
[a] Data after Fent and Bätscher, 2000.
[b] Arithmetic mean of at least three experiments relative to dibenz[*a*,*h*]anthracene.
[c] Substances with low maximal EROD activities.

predictions based on a larger data set in risk assessment. The evaluation of the CYP1A induction potential of sediments from River Purtse and River Kohtla, Estonia, was performed in determining CYP1A induction and porphyrin accumulation of hexane extracts (Huuskonen et al., 2000). In contrast to aqueous extracts, with which the bioavailable fraction of pollutants is assessed, organic solvent extracts give a worst-case estimate as all lipophilic residues are dissolved and evaluated. All sediment extracts led to CYP1A induction and porphyrin accumulation in cells, the most active sediments originated near the oil shale processing plants, which contained very high concentrations of PAH. The biological potency in cells and PAH contamination of the samples showed the same rank order, with some exceptions. These and other studies indicate that in vitro cell systems provide a sensitive bioanalytical tool for sediment analysis contaminated with PAH-type pollutants (Villeneuve et al., 1997; Vondracek et al., 2001).

We have also been using PLHC-1 cells for the assessment of cytotoxicity and CYP1A induction

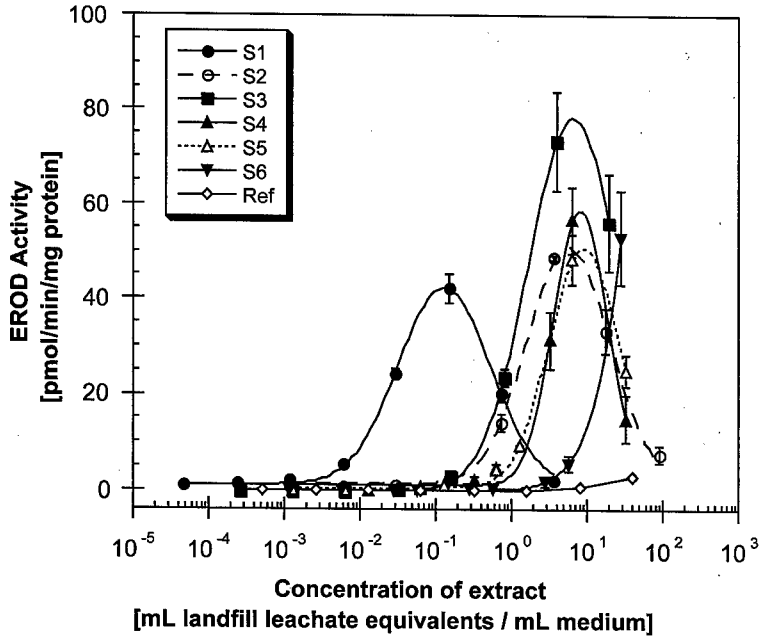

Fig. 6. CYP1A induction potency of organic landfill leachate extracts (S1–S6), contaminated with a variety of PAH. Leachates, but not uncontaminated reference extracts (Ref), lead to a concentration-related induction of CYP1A activity in PLHC-1 cells. As with pure compounds, inhibition of EROD activity occurred at high concentrations. After Fent and Bätscher, 2000.

potential of PAH-contaminated landfill leachates (Fig. 6). By applying the induction equivalent (IEQ) concept, the ecotoxicological potential of several organic extracts of landfill leachates, contaminated sediments and motorway-runoffs were assessed. In landfill leachates, contaminated with a variety of PAHs, dose-dependent EROD induction curves were determined, EC_{50} values derived and IEQs calculated on the basis of both the analytically measured PAHs and cell-derived IEFs. There was a discrepancy between the determined IEQs in PLHC-1 cells and the calculated IEQs: bioassay derived IEQs were between >4 to 112 times higher than the calculated IEQs based on chemical analysis of PAH (Fent and Bätscher, 2000). In order to test whether additional inducing compounds are responsible for this discrepancy, or whether PAHs in mixtures interact synergistically, reconstituted mixtures were analyzed consisting of eight different PAHs in concentrations found in landfill leachates. The good correlation between the bioassay-derived and calculated IEQs indicates that PAH mixtures show an additive behavior. The demonstration of additive interactions is important in hazard and risk assessment of PAH-contaminated environmental samples and supports the additive model for the prediction of TEQs. It is impossible to identify all the compounds in environmental samples such as landfill leachates by chemical analysis. Therefore, unidentified compounds will increase the bioassay-derived IEQs compared to calculated IEQs. Taken together, the increased IEQs are likely caused by additional unknown inducing compounds including unidentified PAHs, substituted PAHs, PCBs, PCDDs, PCDFs or dibenzothiophenes that may be present in the landfill leachates. Similar observations have been made in other studies using mammalian cell lines (Willet et al., 1997). This shows the advantage of bioanalytical tools such as the PLHC-1 cell system over chemical determinations alone for the ecotoxicological hazard and

risk assessment of environmental samples, as the sum of contaminants are regarded and their interactions assessed.

4. Conclusion

There are many important classes of contaminants at large contamination sites that are not studied in detail, partly due to the lack of suitable instrumental analytical techniques. As chemical analysis is aimed at identifying and quantifying specific environmental chemicals, others not covered by the analytical technique are neglected. Another problem is the interaction of chemicals in complex mixtures, which is not yet fully understood. Currently, environmental contamination is assessed mainly by chemical analysis of environmental samples. However, concentrations analyzed provide only part of the knowledge necessary to evaluate and assess the toxic potential of pollutants at contaminated sites. This is partly because the bioavailability of the compounds is not considered and because each of the compounds has different biological activities. Moreover, the complex interactions between different environmental chemicals are not considered when hazard assessments and predictions of possible ecotoxicological effects are made based on concentrations alone.

The use of selective bioanalytical tools, particularly in connection with chemical analysis, can circumvent these limitations. In vitro cell systems, used as bioassays, offer a rapid and sensitive solution to some of the limitations of chemical analysis. They enable an estimate of the total biological activity of chemicals in environmental matrices or extracts thereof that act through the same mode of action. They also integrate the interaction among the different chemicals present in the tested sample from contaminated sites. We have derived an induction equivalency concept for PAHs normalized to the most active compound, dibenz[*a*,*h*]anthracene, for assessing contaminated sites. We demonstrate that fish cell in vitro systems may serve as an integrative bioanalytical tool in the ecotoxicological evaluation. However, as in vitro systems focus only on certain toxicological aspects, an ecotoxicological hazard and risk assessment of contaminated sites should be based on a set of different in vitro and relatively simple in vivo bioassays that can be used for extrapolation to populations and the ecosystem. In the future, ecotoxicological risk assessment and evaluation of contaminated sites should rely on ecotoxicological meaningful bioassays in addition to the current analytical chemical measurements alone.

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Review

Exposure assessment of environmental organic chemicals at contaminated sites: a multicompartment modelling approach

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Abstract

For the prevention of future damages from chemicals at large contaminated sites, all transfer pathways leading to the exposure of man and vulnerable ecosystems have to be taken into account. For organic contaminants, the uptake into vegetation is the major entry route for the transfer into the food chains. Lipophilic substances are taken up by roots but are not translocated with the transpiration stream. Atmospheric background concentrations have a significant impact on foliage contamination due to the effective gaseous and particle deposition. Vegetables can also be contaminated after irrigation with contaminated water supplied by groundwater wells. By means of a multicompartment model, the various uptake processes into roots and foliage as well as the transformation and translocation processes are described and the concentration pattern resulting from daily irrigation with methyl-*t*-butyl ether in the edible parts is simulated. The results demonstrate the advantage of a dynamic multicompartment model over the static environmental quality standard approach in terms of derivation of possible exposure reduction measures for organic chemicals.
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Keywords: Contaminated soil and groundwater; Vegetables; Ingestion; Irrigation; MTBE

1. Introduction

Soil and groundwater contamination with organic chemicals can pose risks to soil organisms as well as to other ecosystems and man by transferring substances out of the contaminated soil. Regulation standards for soil contaminants have only been issued recently in European countries. The German federal soil protection act was passed in 1998 and regulates both soil contamination and toxic waste from the past (BBodSchG, 2001). When we refer to toxic waste from the past, we mean 'toxic waste depositions', i.e. abandoned and disused disposal sites, rubbish dumps, etc. as well as 'toxic waste locations', i.e. the sites of disused plants. In Germany, about 200 000 sites of disused plants were known in 1998. In addition, it can be assumed that there are approximately 100 000 sites which are suspected locations of toxic waste from the past (UBA, 2001). If polluted groundwater is used for irrigation or if natural or artificial flooding occurs, chemical pollutants can enter the soil. Another important pathway which has led to

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considerable amounts of heavy metals and persistent xenobiotics in soil is the application of sewage sludge to soil as a fertiliser (Drescher-Kaden et al., 1992).

Soil pollution is evaluated by taking into consideration the various exposure and utilisation pathways. A distinction is made between precautionary, test and intervention values. If a test value is exceeded, a site investigation has to be carried out to ascertain whether a harmful change to the soil has occurred. Intervention values involve measures which need to be taken to dispose of, secure or cleanup the contamination in question. The list of precautionary, test and intervention values contains mostly metals, several inorganic compounds and only a few organic compounds that are graded as being dangerous or potentially dangerous to soil, or those which have been proven to be important persistent harmful substances in the soil/plant/animal/man system. Groundwater should be protected against any anthropogenic impact. Drinking water quality standards were issued by European and national laws for pesticides and many industrial chemicals such as chlorinated hydrocarbons (e.g. TrinkwV, 1990).

A risk assessment approach is an alternative methodology to the static regulation standards which can, in principle, be applied to all kinds of organic substances in soil and groundwater (and any other environmental compartment). Such a risk-based methodology is already used in EU for the regulation of new and existing chemicals including pesticides and biocides (EU, 1996a). Risk is being characterised by the risk characterisation ratio (RCR) which is the quotient of the predicted environmental exposure (PEC) and the predicted no-effect concentration (PNEC). Exposure is either determined by monitoring data from environmental compartments or by exposure models (EU, 1996b). Measured levels in soil and groundwater are good indicators of the potential harm to man and ecosystems but do not say anything about the movement, transfer, uptake and the resulting dose. Mechanistic process-based models offer a powerful tool to predict the potential dose of dangerous substances and to protect vulnerable parts of ecosystems as well as food chains leading to man, including drinking water (van Leeuwen and Hermens, 1995). They provide insight into the relative role of various processes to derive possible exposure reduction measures for organic chemicals.

The paper deals with the transfer of organic compounds from soil and groundwater into food chains. Uptake into plants such as vegetables or pasture is the entry into the ingestion pathway. For many toxic substances, such as polychlorinated dioxins and -furans (PCDD/F), PCBs and pesticides, the ingestion of contaminated food is the dominant exposure route.

2. Transfer and exposure pathways

The various transfer and exposure pathways of chemicals leading to man are shown in Fig. 1. Soil is connected by the dynamic exchange processes to the air and water compartments. The transport and fate of organic toxicants in contaminated soil are governed by various environmental processes such as

- partitioning between air, soil and water,
- sorption to the soil matrix,
- abiotic and biotic degradation/transformation,
- volatilisation into the atmosphere from soil and foliage,
- wet and dry deposition to soil and plant foliage, and
- uptake into plants via roots and foliage including metabolism in plants.

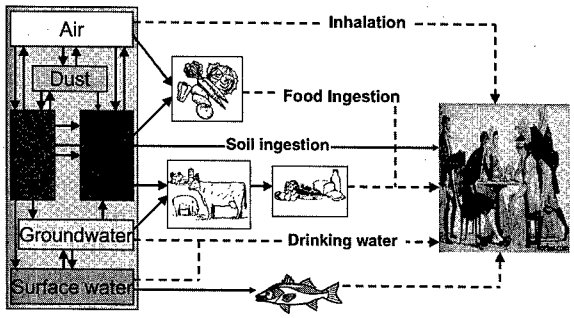

Fig. 1. Exposure pathways for health and environmental risk assessment.

Liquids such as oil may form their own soil phase which is not considered here. Sorption to the soil matrix plays a major role for the mobility. Soil residence times of sorbing substances can be in the order of years. Abiotic and biotic degradation processes remove substances from soil but can produce reaction products with unknown properties and risks. Substances can be volatilised into air or resuspended by soil erosion. Inhalation of gases and particles has mainly to be considered in the vicinity of contaminated sites. Airborne substances can be transported over long distances and deposited in remote areas such as the arctic. Such substances called persistent organic pollutants (POPs) are the subject of international protocols to reduce their global emissions. The environmental long-range transport potential and the overall persistence of organic substances can be determined with the multimedia model ELPOS which was developed to support the selection of substances with POP-like properties and global behaviour (Beyer and Matthies, 2002).

Soils are nearly always covered by vegetation, and roots take up water and chemicals. For organic contaminants, the uptake into vegetation is the major entry route for the transfer into the food chains. Crop plants are directly ingested or used to feed cattle for meat and milk production. Transfer factors are usually applied to calculate the uptake from soil into plants. They are experimentally determined by dividing the measured concentration in the edible plant parts (leaves or fruits) at the time of harvest by the soil concentration (Matthies and Trapp, 1994). Another method is the linear regression between soil and plant concentrations, which can be derived from a concentration series. Transfer factors have been successfully applied to inorganic substances including heavy metals and radionuclides. However, for organic chemicals this experimental approach can lead to erroneous results if the uptake from air is not explicitly taken into consideration. Several studies show that persistent chemicals such as PCDD/F, PCBs or PAHs can effectively partition between air and foliage (Riederer, 1995; McLachlan, 1999). To avoid any misinterpretation of measured transfer factors, all contamination sources and pathways have to be taken into account. Models can help to distinct between the various transfer routes and to quantify their contribution to the resulting dose.

Samsoe-Petersen et al. (2002) studied the uptake of trace elements and PAHs by fruit and vegetables from contaminated soils. The results of this field experiment showed elevated levels of several elements and PAHs in the vegetables grown in contaminated soil. The linear regression between concentration of most metals in soil and vegetables showed a good correlation, which was generally not found for PAHs. Regressions were not considered to be an appropriate tool for predicting concentrations of PAHs in vegetables at advisory interval soil concentrations. Ambient air concentrations or deposition were not measured. Delschen et al. (1999) investigated the importance of different pollution sources for the PAH contamination of cultivated plants in a long-term field lysimeter experiment. Their results demonstrate that the PAH pollution may be caused by both the atmospheric deposition and the direct contamination of plant leaves with resuspended soil particles and subsequent PAH turnover by ad/desorption processes. Systemic PAH transfer via root uptake could generally not be observed. They concluded that the soil as well as the deposition pathway must be integrated into a complete risk assessment of locations with food plant production, particularly in urban areas. Trapp et al. (1997) used the same experiments to investigate the various transfer pathways of PCBs and PAHs by means of a multicompartment model (Delschen et al., 1996). They found that volatilisation from soil and subsequent uptake into leaves could also contribute to the contamination of plants, in particular to the outer leaves. Only the low-chlorinated PCBs and the 3- and 4-ring PAHs such as phenanthren and fluoranthen have a small potential to be taken up via roots. Maddalena et al. (2002) investigated the mass transfer and partitioning of four PAHs at the plant/air interface by exposure chamber measurements. The four PAHs were taken up within a few days. With a probabilistic two-compartment model they could calculate the plant/air partition coefficients, the overall mass transfer rates and the reactive loss rates. A generic one-compartment model was developed by Trapp and Matthies

(1995) for the uptake of organic chemicals into aerial plant compartment from soil and air and applied to the herbicide bromacil and the Seveso-dioxin 2,3,7,8-TCDD. Bromacil has a log K_{OW} of 2.02 and is transported with the transpiration stream into leaves. The major loss process is metabolisation. For the lipophilic 2,4,7,8-TCDD with a log K_{OW} of 6.76 the mass transfer from air dominates and the systemic uptake can be neglected.

3. Irrigation of groundwater contaminated with MTBE

Groundwater is contaminated with various organic compounds in many European urban-industrialised regions. Often chlorinated hydrocarbons, BTXE (benzene, toluene, xylene, ethylbenzene) were found. Recently methyl-*t*-butyl ether (MTBE) has become an issue of surface and groundwater contamination (Achten et al., 2002). MTBE is produced in large amounts in EU and mainly used as a fuel oxygenate. It is frequently found in groundwater in USA (Pankow et al., 1997; Squillace et al., 1999), Germany (Brauch et al., 2000; Klinger et al., 2002) and many other European countries (EU, 2002) up to concentrations of a few hundred mg/l. Groundwater is contaminated by leaking underground petrol storage tanks and spillage from overfilling the tanks. MTBE is water-soluble and persistent in soil and groundwater since it is almost not biodegradable. Only in air, it is photochemically degraded with a half-life of 3–6 days. Shallow groundwater is still taken as drinking water without purification or for irrigation of vegetables in house gardens (Berlekamp et al., 2000).

3.1. Multicompartment model of uptake

A dynamic model was developed to calculate the simultaneous uptake of organic chemical from air and soil. It is a modification of the PLANTX model (Trapp et al., 1994; Stephan, 1994; Trapp, 1995). It consists of soil, roots, leaves and air (Fig. 2). Soil has one layer with of a fixed depth. It is assumed that the chemicals partition instantaneously between soil matrix, soil solution and soil air. Only leafy vegetables such as lettuce and spinach and root vegetables such as radish are regarded making the model structure rather simple. Similar model versions were applied to barley, wheat or various vegetables (Matthies and Behrendt, 1995; Trapp et al., 1997). The roots are in equilibrium with the soil solution. The organic substance is taken up with the transpiration stream. Substances can also be transported to the fruits and roots by the phloem stream. The exchange of soil with air by volatilisation from and deposition to the soil surface is supplemented. Also the partitioning of substances between the gaseous and the particle-bound states are explicitly considered. Foliage is exposed by dry and wet deposition processes. Metabolism in plant is modelled by first-order degradation constant. However, individual metabolites are not explicitly simulated because physical–chemical and degradation data are rarely available. Ambient air fluxes are taken into account. The model allows any kind of substance input and initial values in soil, plant and air. Irrigation is usually expressed as a pulse function. The ordinary differential equation system is numerically solved by a Runge–Kutta integration method of fourth-order with variable time step (Powersim, 2000). It calculates the chemical mass and concentration in all compartments and the fluxes between them within each time step.

3.2. Substance, plant, soil and environmental data

The substance data of MTBE were taken from published literature (Table 1). The metabolic half-life was estimated from the laboratory experiments of Dörfler and Scheunert (1993) who investigated the uptake and transformation in lettuce and radish with ^{14}C-labeled MTBE. The experimental and analytical design is described in Schroll and Scheunert (1992). They planted almost fully grown seedlings in homogeneously contaminated soil, put them into a closed system of 0.2 m^3 and harvested leaves and radish after 7 days. Air rapidly equilibrated with soil and plant. They found only polar and non-polar ^{14}C residues but did not characterise their chemical structure. Assuming that all ^{14}C

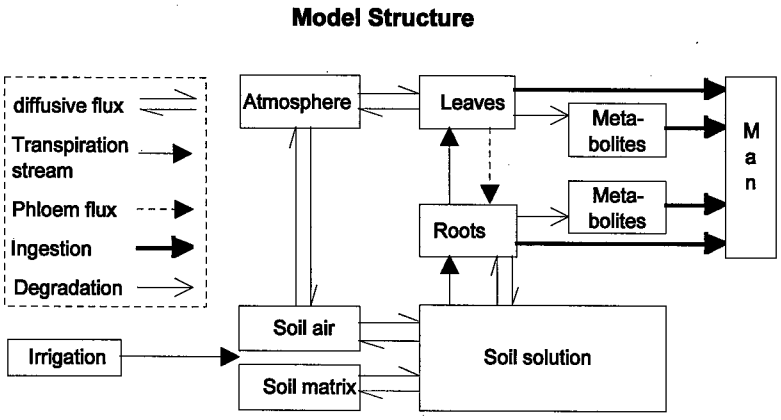

Fig. 2. Model structure.

Table 1
Substance data of MTBE

Substance data	Value	Reference
Molecular mass (g/mol)	88.15	Rippen (2002)
Vapor pressure at 25 °C (Pa)	32 700	Rippen (2002)
log K_{OW}	0.94–1.2	Rippen (2002)
K_{AW} (at 25 °C)	0.024	Rippen (2002)
TSCF	0.68	Calculated from Briggs et al. (1982)
Photodegradation half-life (days)	3–5	Rippen (2002)
Degradation half-life in soil	No degradation	–
Metabolic half-life in plant	3.2 days lettuce	Derived from Dörfler and Scheunert (1993)
Ambient air concentration (μg/m^3)	0.25	Estimated from Achten et al. (2001)
Concentration in irrigation water (mg/l)	10	Default

below the detection limit of 20 μg/g fresh mass is MTBE, half-lives of 3.2 days could be derived for a first-order metabolic reaction for lettuce and 3.7 days for radish (Table 1). Substance data were taken without temperature corrections although model calculations demonstrate the temperature dependence of the chemical's environmental distribution and fate (Beyer et al., in press).

Only the uptake into lettuce as a surrogate for other leafy vegetables was regarded here. Root vegetables were not considered. Dörfler and Scheunert (1993) found similar mass distribution of MTBE in lettuce and radish. The measurement time of 7 days was too short to investigate the uptake in root tissue of radish. Trapp et al. (1997) observed higher PCB and PAH concentrations in carrots which they assigned to enhanced oil content of carrot roots. Delschen et al. (1996) showed that PAHs are transported from carrot leaves to the roots. Schroll and Scheunert (1992) distinguished between the outer and inner parts of carrot roots. The properties of lettuce were taken from various references or measurements (Table 2; Stephan, 1994). There are typical values for home garden fresh vegetables. A loamy/sandy soil was assumed (Table 3). Climate data are average long-term values of German locations. The soil water content is kept constant, i.e. irrigation, precipitation, evaporation and transpiration are in a steady

Table 2
Plant properties

	Lettuce (leaves)	Reference
Dry mass percentage (%)	5.4	Dörfler and Scheunert (1993)
Yield (kg/m^2)	2.0	Stephan (1994)
Water content (%)	94.6	Dörfler and Scheunert (1993)
Vegetation period (days)	ca. 50	Default
Total irrigation water (l/m^2)	150	Stephan (1994)

Table 3
Soil properties

Soil organic content (%)	1	Default
Dry soil density (kg/l)	1.3	Default
Soil water content (%)	15	Default
Soil air content (%)	35	Default
Soil depth (m)	0.2	Default

state. Percolation of water and hence substance leaching is neglected. Biomass is growing linearly. Transpiration stream, leaf area index and soil cover are correlated to growth. A concentration of 10 mg/l in the irrigation water with a daily application over 50 days is assumed. The daily amount of irrigation water is related to the growth of the vegetables with a total amount of 150 l. After 50 days, the irrigation is stopped and further 5 days are simulated to study the MTBE decline in the harvested vegetable. All other environmental data were taken as realistic as possible. Achten et al. (2001) measured MTBE in rural and urban precipitation in Germany. They found great differences between summer and winter time as well as rural and urban areas. An initial ambient air concentration of 0.25 μg/m^3 was assumed which is equal to the equilibrium concentration of 10 μg/l in precipitation. The initial concentration in soil and plant was set to the equilibrium concentration with the ambient air level which is 6.1 μg/kg dry soil and 0.014 μg/kg fresh plants. Two scenarios were simulated: one scenario assuming no metabolism in plant and the other with the formation of metabolites. Since the properties of the metabolites are not known, no mass transfer and degradation of the metabolites were assumed.

4. Results and discussion

4.1. MTBE in soil

The simulated concentration pattern of MTBE in soil after the irrigation with MTBE is shown in Fig. 3a. After each daily irrigation pulse, MTBE concentration in soil increases immediately but decreases due to the losses by volatilisation and root uptake. Volatilisation from soil is very rapid. Dörfler and Scheunert (1993) found volatilisation half-lives between 18 and 24 h in all their test series. Can volatilised MTBE enhance the air concentration and thus the uptake into foliage from air? The answer is no, since the additional amount of MTBE to the ambient air concentration is negligible. Ambient air concentration and thus deposition is not enhanced by the additional mass from volatilisation due to the rapid mixing in the air boundary layer. Soil concentration oscillates between the two extremes with increasing amplitude. Since the substance input into soil by irrigation is greater than the losses, the soil concentration increases up to approximately 750 μg/kg DM. When irrigation is stopped, soil concentration decreases rapidly to its background level with a half-life of less than 3 days due to volatilisation.

4.2. MTBE in plant (no metabolites)

Fig. 3b shows the temporal concentration pattern of MTBE in plant leaves assuming no metabolism in plant. The pattern follows the oscillations of concentrations in soil but reaches asymptotically a maximum value of approximately 6.5 μg/kg fresh mass. The amplitude is about 1.5 μg/kg fresh mass. Systemic uptake from soil via roots, deposition from and volatilisation into air determine the up and down of the MTBE level in lettuce leaves. After irrigation is stopped, the concentration decreases with a half-life of approximately 3 days which is due to volatilisation from the leaves and similar to the elimination half-life in soil (see above). It is interesting to compare the uptake process via roots with the deposition pathway from air. Fig. 4c shows that the uptake of MTBE with the transpiration stream contaminates the leaves. The transpiration stream concentration factor (TSCF) is approximately 0.689 according to the regression equation of Briggs et al. (1982). Both together, the rather large fraction of MTBE in soil solution and the TSCF, reveals a considerable uptake from soil. The uptaken MTBE is then transported to the leaves and volatilises into air due to its low log K_{OA} of 2.56 which is rather low compared with those of PCBs

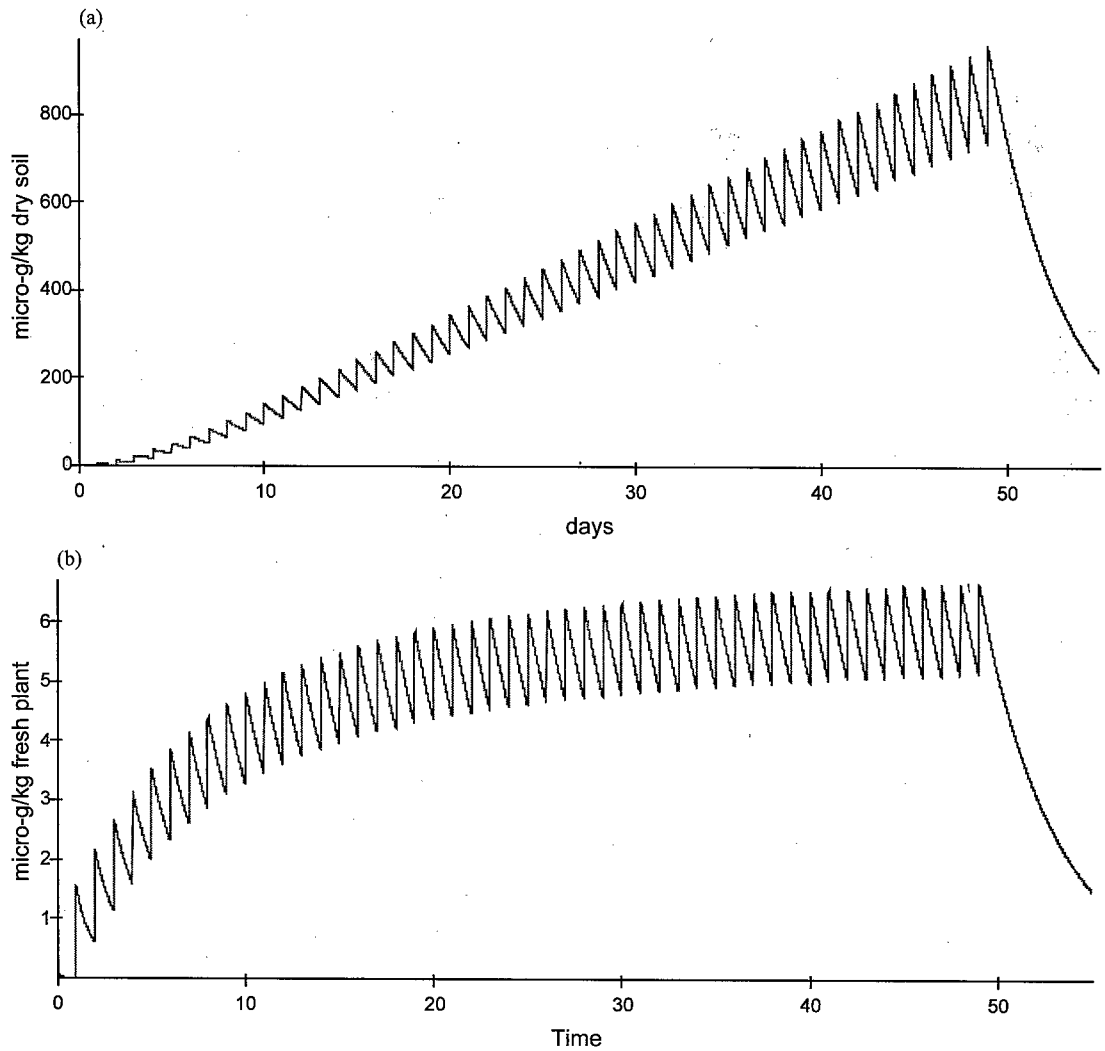

Fig. 3. Concentration of MTBE (no metabolism in plant) in (a) soil and (b) plant after irrigation with groundwater contaminated with MTBE.

or PAHs. (PCBs have log K_{OA} from 7.6 to 10.3; PAHs those from 6.1 to 10.2; Beyer et al., 2002.) This is the reason that MTBE does not accumulate in lettuce leaves. After harvesting, there is no further uptake from soil and MTBE is lost to the ambient air. In steady state, the systemic uptake and the deposition flux equal the volatilisation loss (no reactive losses in plant). Deposition is approximately 65 μg per day and constant over time because the ambient air concentration is constant (Fig. 4a). (Similar reasoning as with soil due to the rapid mixing in air.) The systemic uptake reaches a maximum of 31 000 μg per day which is much higher than the deposition flux. This finding is in contrast to the given examples of the uptake of lipophilic substances such as PCBs, PAHs and PCDD/Fs, which are almost solely determined by the uptake from air. However, this value holds only for the defined initial concentrations and the assumed irrigation input flux.

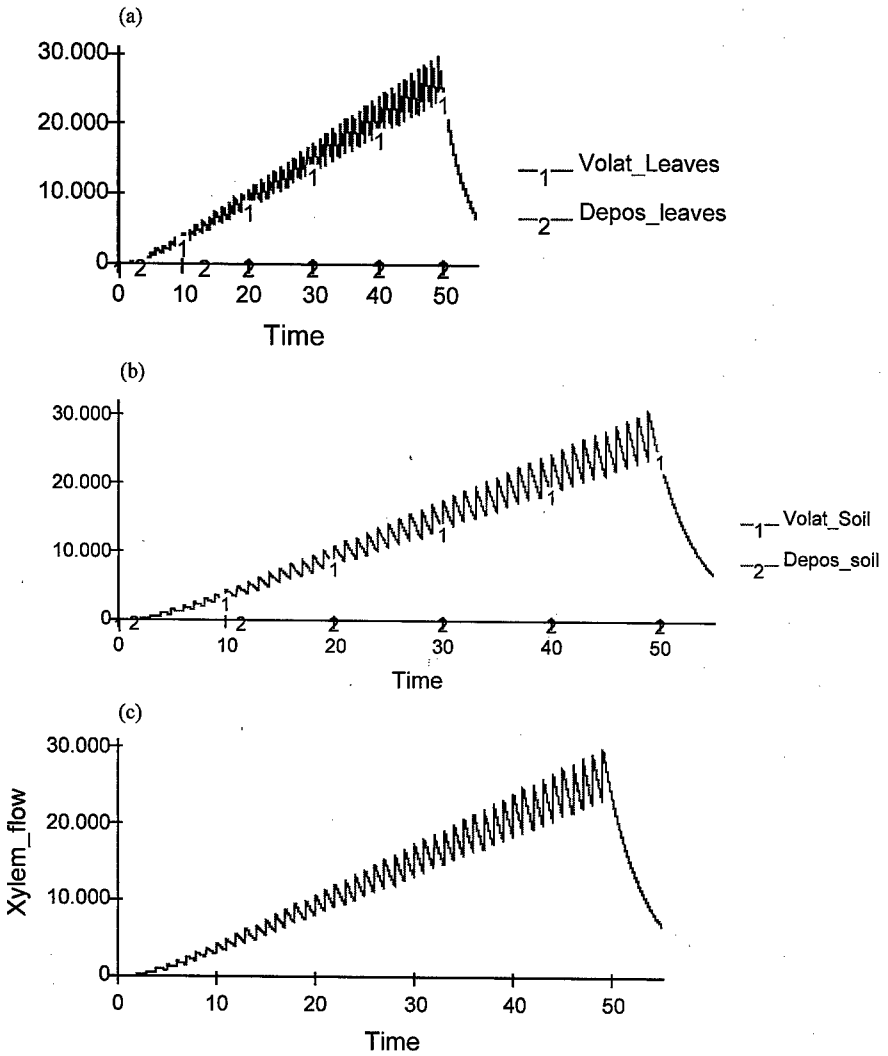

Fig. 4. Fluxes of MTBE: (a) volatilisation from and deposition to plant leaves, (b) volatilisation from and deposition to soil, and (c) transpiration stream.

4.3. MTBE in plant (with metabolites)

What is about the metabolism of MTBE in the plant? The concentration pattern with the metabolism in plant is shown in Fig. 5. Now a considerable part of MTBE is transformed into metabolites, which accumulate in the plant up to more than 30 μg/kg. No loss process such as volatilisation or further metabolisation is considered due to the unknown chemical identity of the metabolites. However, various metabolites are known to be toxic, e.g. *t*-butyl formiate (TBA), *t*-butanol or formaldehyde. Although the toxic *t*-butanol could not be detected by Dörfler and Scheunert (1993), the question of the health risk of MTBE in plants consumed by man remains including its metabolites.

4.4. Ingestion dose

What is the daily dose resulting from the ingestion of vegetables contaminated with

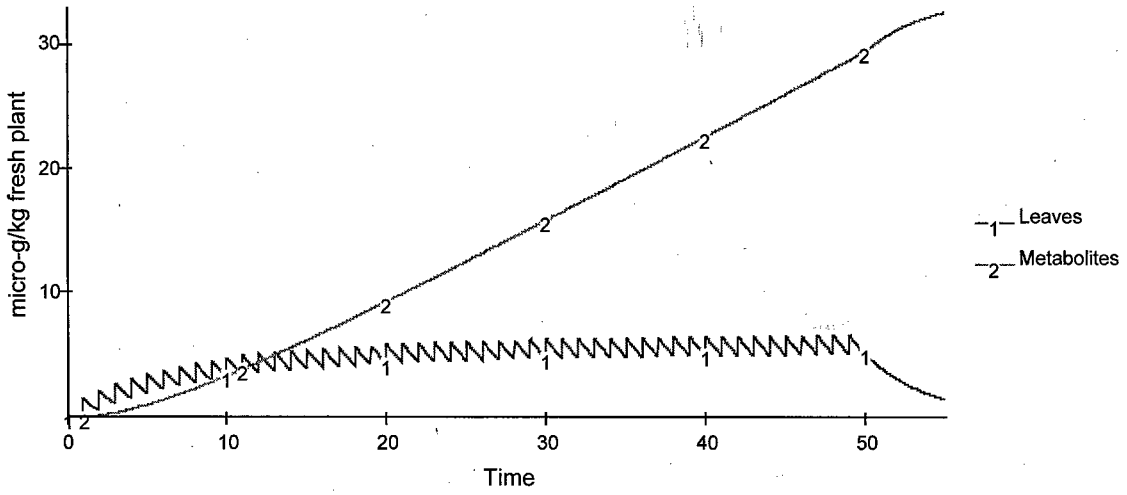

Fig. 5. Concentration of MTBE and metabolites in plant after irrigation with contaminated groundwater.

MTBE? Delschen et al. (1999) assume an average daily consumption of 300 g total all vegetables (fresh matter) in Germany which is approximately 34 g DM per day. Considerably lower values are reported for leaf vegetables. The compilation by ECETOC (2001) gives values for the consumption of fresh leaf vegetables between 26 and 36 g per day. The immediate consumption of the harvested vegetables assuming no metabolites causes a daily MTBE dose of 169 μg per day (only fresh leaf vegetables) and 1950 μg per day (total all vegetables). Three days delay before consumption brings the dose down to 85 μg per day (only fresh leaf vegetables) and 975 μg per day (total all vegetables). With the consideration of metabolites, the total dose from MTBE and its metabolites increases to approximately 980 μg per day (only fresh leaf vegetables) and 11 400 μg per day (total all vegetables) which is the maximum ingestion dose (Table 4). Waiting before consumption decreases the dose to half the values after 3 days. A delay between harvesting and consumption is thus recommended. It is interesting to compare the doses from the ingestion of contaminated vegetables with those from drinking the contaminated water from local water well and inhalation of ambient air. A daily mean dose of 10 mg per day (= 10 000 μg per day) is caused assuming a total tapwater intake of approximately 1.0 l per day (ECETOC, 2001) which is higher than almost any of the ingestion doses calculated for the consumption of vegetables contaminated with MTBE (Table 4). Only the 'worst-case scenario' with the intake of MTBE and metabolites by the consumption of total all vegetables is higher (11.4 mg per day). No volatilisation from tapwater is considered leading to an upper dose estimate. Usually tapwater is boiled or otherwise treated before it is drunken or used for food preparation. Table 4 also shows the mean inhalation dose of 2.8 μg per day for female adults and 3.8 μg per day for male

Table 4
Mean daily exposure from various pathways

Daily dose (μg per day)	MTBE	MTBE including metabolites
Ingestion of fresh vegetables		
Home garden (26 g per day)	169	980
Total (36 g per day)	234	1357
Ingestion of total all vegetables		
300 g per day	1950	11400
Inhalation of ambient air		
Inhalation rate (11.3 m^3 per day (female))	2.8	2.8
Inhalation rate (15.2 m^3 per day (male))	3.8	31.8
Tapwater		
Mean daily consumption (1 l per day)	10000	10000

adults assuming 11.3 and 15.2 m^3 per day long-term inhalation rates (ECETOC, 2001). For infants and children, the inhalation rates are lower thus leading to a lower inhalation dose. If we compare the exposure from various pathways, the dominating mean daily dose comes from the drinking of contaminated water. Next is the consumption of contaminated vegetables and then inhalation of ambient air. This finding only holds for the presumed initial concentrations and additional input flux with irrigation water. Moreover, the estimated doses are conservative in that sense that no delay between harvesting and consumption as well as for tapwater use was taken into account which would reveal a remarkable exposure reduction.

5. Conclusions

Soil and groundwater contamination is a serious problem in most industrialised countries. The assessment of existing pollution and the reduction of continued input require the knowledge about the governing processes in soil, groundwater and plant which influence concentration and transport. Mechanistic models offer a powerful tool to predict the potential exposure of dangerous substances and to protect vulnerable parts of ecosystems as well as food chains leading to man including drinking water.

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Review

Long-term ecological assessment of West African rivers treated with insecticides: methodological considerations on quantitative analyses

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Abstract

For the control of the aquatic stage of the blackfly vector of the onchocerciasis in West Africa, rivers had been partially sprayed weekly with insecticides from 1974 to 2002 on an area over 1 million km^2, as part of the onchocerciasis control programme (OCP). To evaluate the possible short- and long-term effects of insecticides on non-target fauna, an aquatic monitoring programme was set-up and carried on for over 20 years. A number of papers have been published concluding that biological variations found are ecologically acceptable. In this paper, by means of a new elaboration of a subset of data, the authors discuss value and limitations of the data analysis methodologies utilised in the ecological assessment. Multivariate analysis, rank-abundance curves and diversity indices are applied to evaluate the modification occurred in invertebrate community taxa richness and evenness. These methodologies allow to decompose the variance between insecticides effects and seasonal biological variations, to evidence the relative toxicological potency and to show the fingerprint of action among various insecticides.
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Keywords: River quality; Biological monitoring; Insecticides ecological assessment; Onchocerciasis; Vector control

1. Introduction

Human onchocerciasis (river blindness) is caused by a filarial worm *Onchocerca volvulus*; the microfilariae live in the skin and cause debilitating skin lesions and ultimately blindness. West Africa used to be the most affected area in terms of distribution and severity of clinical manifestations of the disease. Since 1974, control has been undertaken through the onchocerciasis control programme (OCP) that successfully ended in 2002.

The strategy of larviciding was designed to interrupt the transmission of the parasite for longer than the longevity of the adult worms in a human host (estimated to be about 14 years) by destroying larval stages of the vector, the blackfly *Simulium damnosum*, through aerial application of insecticides at river breeding sites. The develop-

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ment of aquatic stage from egg to pupae is around 1 week, hence insecticide application is undertaken weekly. At the peak of larviciding activities, during the years 1986–1991, about 50 000 km of rivers were treated in an area of over 1 million km^2.

Since prolonged and regular use of insecticides presents a potential risk to the aquatic environment, an ecological group was set-up, prior to launching the OCP, with the objectives to organise a long-term monitoring programme of the aquatic fauna, to identify criteria for the selection of insecticides for operational use and to determine the conditions by which insecticides use can be optimised in relation to seasonal and environmental factors in different areas of the programme.

The criteria for the evaluation of insecticide impact on aquatic environment include: (1) the vector control activities should not reduce the number of invertebrate species or cause a marked shift in the relative abundance of species; (2) the insecticides applied should have neither a direct impact on fish nor effect on the life cycle of fish species; (3) bioaccumulation and biomagnification through food webs should be avoided; (4) human activities in the control area should not be impaired; (5) temporary and seasonal variations in non-target invertebrate populations were acceptable.

Seven insecticides were used in rotation, six chemicals (temephos, phoxim, pyraclofos, permethrin, etofenprox and carbosulfan), while the seventh was a biological insecticide, *Bacillus thuringiensis* H-14. The rotational use of insecticides has been particularly effective, with only limited resistance to the organophosphates, while the susceptibility of the *Simulium* populations to other classes of compounds remains unchanged (Calamari et al., 1998).

A recent evaluation of 20 years of OCP monitoring data showed some effects of the insecticides applied on the invertebrate fauna, particularly the rarefaction of a few taxa and, for example, in the case of permethrin, a biomass reduction. The ability of the aquatic biota to recover was, however, demonstrated. The taxonomic and functional variability in non-target invertebrate fauna would therefore seem to be compatible with the range of biological variation that would normally occur in these river systems (Crosa et al., 2001a,b; Yaméogo et al., 2001). Similar biological monitoring activities were conducted on fish population and show no evidence of effects (Paugy et al., 1999).

Using a subset of invertebrate data acquired during the extensive programme of insecticide application in West African rivers and the published results of the measured biological side effects (as quoted above), we comment, in this paper, by means of an example, the appropriateness, the limits and the descriptive power of the invertebrates data analysis methodologies utilised in the programme.

The importance of evaluating the effects of chemicals by in situ monitoring aquatic invertebrate community is justified by considering that, also if all the information about a chemical and the interacting ecosystem are known, the prediction of the effects of the chemical on the ecosystem could be biased because of the vast amount of data required and the ecological uncertainty principle (Giesy and Graney, 1989).

The aquatic macroinvertebrates received a general agreement as appropriate biological indicators because their metabolic and ecological properties allow to control pollutants side effects on both biological structures (reduction of the taxonomical biodiversity) and ecological processes (energy transfer within the food web, post-disturbance recovery).

The advantages of macroinvertebrates as quality indicators are well reviewed (Rosemberg and Resh, 1993; Chessman, 1995; Karr, 1999) and these organisms are now incorporated in standard monitoring protocols developed by numerous national environmental agencies for local use such as biotic index (e.g. Ghetti and Bonazzi, 1980) or predictive invertebrate models such as, e.g. RIVPACS (Wright, 1995).

In spite of their demonstrated usefulness, the applicability of the biotic index or predictive invertebrate models is limited by their high biogeography specificity and high demanding efforts required for their development on regional scale.

As an alternative to the use of biotic index or predictive invertebrate models, a number of data analyses methodologies have been developed and applied to facilitate the measure of the changes of

the invertebrate community structures due to stressing factors in rivers for which specific ecological knowledge is lacking. These methodologies, most of which were developed in the field of community structures studies, are based on the assumption that any modification of the natural environmental factors, acting selectively on the aquatic organisms, modify the relative abundance of the taxonomic community composition.

The data analysis methodologies considered here have been selected to be the representative of three strategies of analyses: multivariate analysis (principal component analysis), graphical analysis (rank-abundance curves) and numerical synthetic descriptors of the community taxa richness and evenness (diversity indices).

Objectives of the paper are to report our experience and to discuss value and limitations of methodologies applicable in ecosystems, where the biological and ecological information are poor or unavailable and therefore do not consent the use of predictive biological models or biotic indices.

2. Material and methods

2.1. Biological data

The biological data used, for example, consist of a subset of the benthic invertebrate data collected during extensive monitoring programme organised within the OCP from October 1984 to January 1998 in the Niandan river at Sasambaya (Guinea).

Since during the rainy season, the treatments were suspended being not useful for vector control, to better evidence the biological effects of the applied insecticides only the samples collected after the insecticides applications were taken into account for the analyses.

Invertebrates were quantitatively collected over rock substrate using the classical Surber technique. The systematic units used for the numerical analyses are the following: Beatidae, Caenidae, Leptophlebiidae, Heptageniidae, Tricorythidae, Oligoneuriidae, Hydropsychidae, Leptoceridae, Philopotamidae, Chironomini, Orthocladiinae and Tanypodiinae. For sampling details, see Dejoux et al. (1979) and the OCP-related papers (Crosa et al., 2001a,b; Yaméogo et al., 2001).

2.2. Data analyses

The numerical analyses methodologies were selected to be the representative of the most common techniques applied in the studies of the biological communities and are all oriented in measuring the variation of two structural attributes of the communities diversity: taxa richness and evenness.

Principal component analysis was applied to the log-transformed abundances of the collected invertebrates using the CANOCO programme for Windows (Ter Braak and Smilauer, 1998).

Rank-abundance curves (sensu Marsh-Mathews and Mathews, 2000) were generated using the average densities of the invertebrate assemblages sampled after each insecticide application. Curves consisted of proportional abundance for each taxon plotted against the corresponding taxonomic abundance ranking.

Indices used to assess the communities diversity included the Margalef and Shannon-Weiner diversity indices.

3. Results

The community structural variation extracted by the first two components of the PCA accounting for 42% of the total variance (27% first axis and 14% second axis) is illustrated in Fig. 1. In the traditional biplot ordination plot (Fig. 1(a)), the samples classified according to the treatment conditions result arranged along two directions represented in the figure by two bold lines named directions 1 and 2 (the same result could be attained by using the original PCA axes after a 45° rotation).

Direction 1 is showing that most of the pretreatment data result separated by the treated samples. These last, in turn, are ordered along direction 2 mainly according to the potency of the applied insecticides (i.e. permethrin, phoxim and B.t.). Examining the correlation between the taxonomic units and the ordination axes (Fig.

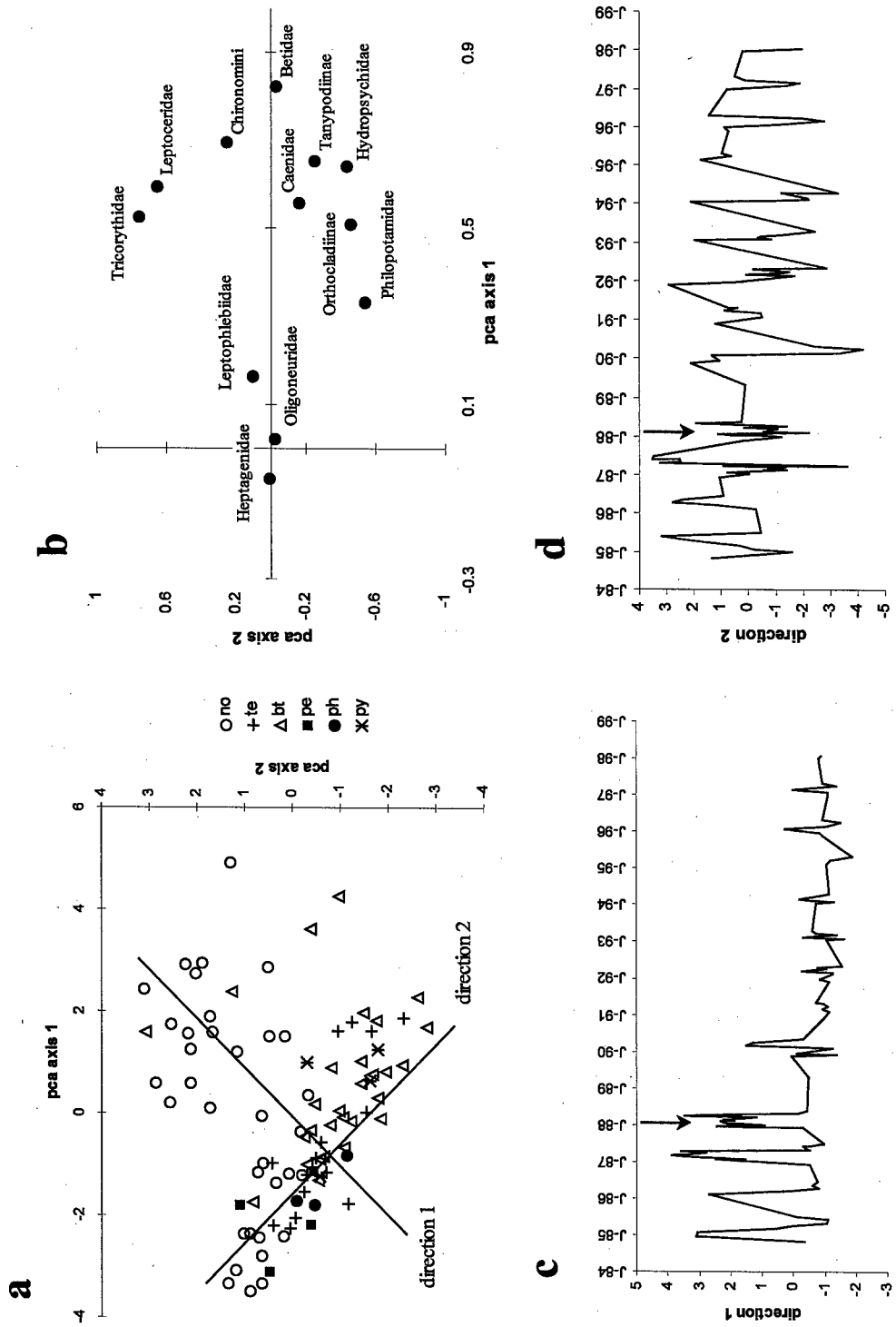

Fig. 1. Ordination analysis of invertebrate communities sampled in Niandan river; treatments code: no = pre-treatments, te = temephos, bt = B.t., pe = permethrin, ph = phoxim, py = pyraclofos. (a) Ordination plot of the invertebrate communities sampled during different treatments as resulting from the first two axes of the PCA; two variance patterns are identified by directions 1 and 2 lines. (b) Correlation between the taxonomic units and the first two axes of the PCA. (c) Samples projection scores on direction 1 arranged according to the sampling time, the arrow marks the beginning of the insecticides application. (d) Samples projection scores on direction 2 arranged according to the sampling time, the arrow marks the beginning of the insecticides application.

1(b)), it can be evidenced that direction 1 accounts for the variation of Tricorythidae and Leptoceridae and direction 2 for Philopotamidae, Orthocladiinae and Hydropsychidae. The other taxonomic units do not contribute at the biological variance described by direction 1 and 2 axes due to their low correlation with both of these axes.

Representing the sample projections on these two directions along the time gradient (Fig. 1(c) and (d)) two patterns can be recognised. Direction 1 evidences a long-term change of the invertebrate communities beginning after the insecticide treatments (insecticides chronic effects). Direction 2 shows a cyclic pattern along all the years examined (seasonal biological variations).

Considering the low contribute of Heptageniidae, Leptophlebiidae and Oligoneuriidae to the overall variance explained by the first two principal components (these families are located near the axes origin in Fig. 1(b)) and considering the lack of correlation between Caenide, Chironomini, Betidae and Tanypodiinae and directions 1 and 2 axes, PCA has been repeated on the same data set without considering these taxonomic units (Fig. 2).

In the new ordination plot accounting for 70% of the total variance (Fig. 2(a)), the samples present the same patterns previously commented with the advantage to be arranged along the first two PCA ordination axes (no rotation is required). Similar to the first PCA, the taxonomic units considered result strongly correlated to the ordination axes: Tricorythidae and Leptoceridae ordination axis 1 (previously direction 1); Hydropsychidae, Orthocladiinae and Philapotamidae ordination axis 2 (previously direction 2).

Examining the ordination plot shown in Fig. 2(a), a different location of the samples can be observed in relation to the insecticide applied before the invertebrate sampling. For example, the communities sampled after permethrin and phoxim application result separated from those sampled after B.t. and temephos application. This different location of the samples in the ordination plot can be interpreted as a "fingerprint" showing the selective toxicity of the chemicals on the taxonomic units analysed. A "fingerprint" is observed because of the selective action on particular groups of insects, according to the mode of action of different types of larvicides. In the presented case, permethrin and phoxim determine a sensible mortality on taxonomic units not greatly affected by B.t. or temephos application (Hydropsychidae, Orthocladiinae and Philapotamidae) as results considering the correlation of these taxonomic units with the second ordination axis (Fig. 2(b)).

Moreover, since the non-structured biological variation has been removed by analysing only selected taxonomic units, the samples ordination scores arranged with respect to the sampling time (Fig. 2(c) and (d)) show a reduction of the background noise and facilitate the following considerations:

the community structure presents strong seasonal variation justified by the relevant seasonal pattern of the river discharges (as results from the cyclic variation of the ordination score before the treatments);
a long-term change of the invertebrate structure can be detected soon after the beginning of the treatments. This change represents the abundance reduction of Leptoceridae and Tricorythidae (as results from the positive correlations between these taxonomic units and the first ordination axis);
the seasonal variation of the examined taxonomic units remains evident during all the examined years;
the slight increase of the second ordination axis scores suggest that the abundance of insecticide-resistant taxa (Hydropsychidae, Orthocladiinae and Philopotamidae) increases in relation of the rarefaction of less tolerant taxa (Leptoceridae and Tricorythidae).

To examine the biological effects emerging at medium-term (less than 1 year), rank-abundance curves were draft by using invertebrates assemblages data sampled after different insecticides application (Fig. 3). The slopes of the curves provide information on the invertebrate assemblages heterogeneity: slopes become increasingly negative when the proportional abundance of the more dominant taxonomic units increase, or when taxon richness decrease.

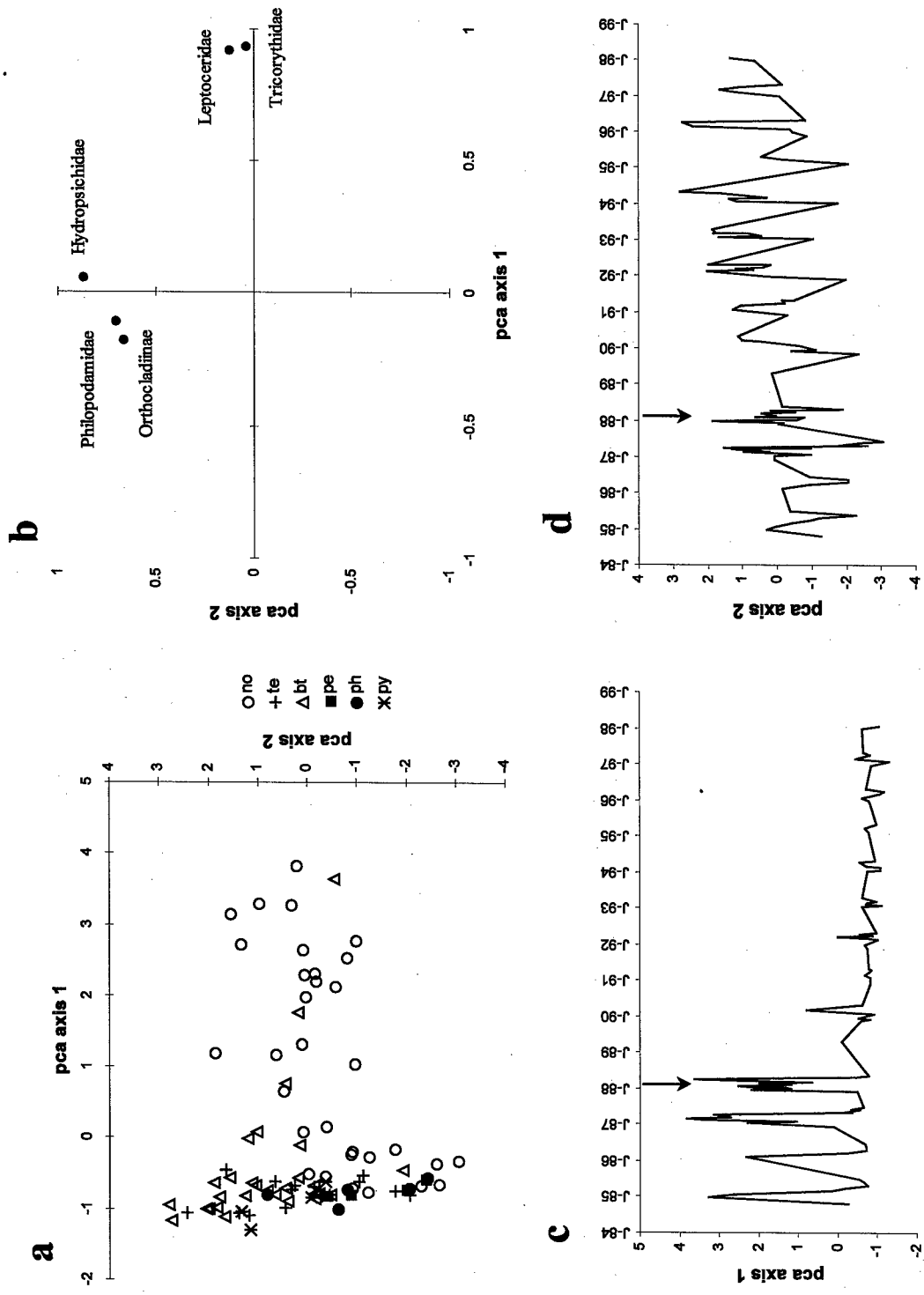

Fig. 2. Ordination analysis of selected invertebrate taxonomic units sampled in Niandan river; treatments code: no = pre-treatments, te = temephos, bt = B.t., pe = permethrin, ph = phoxim, py = pyraclofos. (a) Ordination plot of the selected invertebrate taxonomic units sampled during different treatments as resulting from the first two axes of the PCA. (b) Correlation between the taxonomic units and the first two axes of the PCA. (c) Projection scores of the first axis of the PCA arranged according to the sampling time, the arrow marks the beginning of the insecticides application. (d) Samples projection scores on direction 2 arranged according to the sampling time, the arrow marks the beginning of the insecticides application.

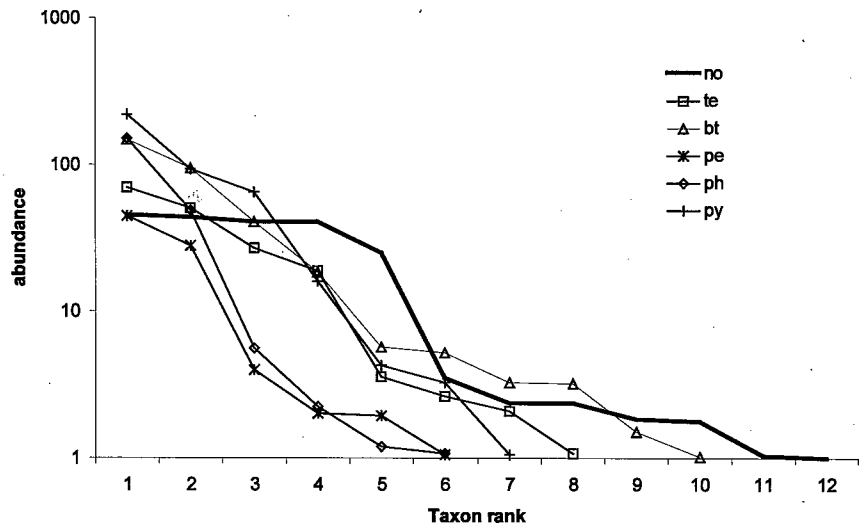

Fig. 3. Rank-abundance plot of invertebrate assemblages sampled in Niandan river during pre-treatments and different insecticides application. Code treatments: no = pre-treatments, te = temephos, bt = B.t., pe = permethrin, ph = phoxim, py = pyraclofos.

Having, as a reference condition, the taxonomic structure of the invertebrates sampled before the insecticides applications (bold line), two types of biological responses can be observed. The taxonomic structures of the invertebrates sampled after phoxim and permethrin applications show the highest alterations both in terms of taxa richness (x-axis) and evenness (y-axis), while the invertebrates sampled after temephos, B.t. and pyraclofos application present the lowest structural alterations. These different and chemical-specific biological effects shown by the curves represent the "relative toxicological potency" of the applied insecticides.

The numerical expression of the invertebrates diversity calculated by applying the Shannon and Margalef's diversity indices to each invertebrate sample are time ordered in Fig. 4. No long-term trends are evident from their graphical representation and no differences can be detected between pre-treatments and during-treatments samples.

Similarly to the PCA results (Figs. 1(d) and 2(d)), the Shannon diversity indices show a cyclic pattern defined by high seasonal differences which can be explained considering the discharge pattern.

The lack of time-related trends in the calculated diversity indices do not necessarily attest the inadequateness to detect structural changes of the sampled communities. In fact when the Shannon and Margalef's diversity indices are applied to the communities sampled soon after the treatments (<8 weeks) irrespectively of the sampling date (i.e. all the samples are classified according to the insecticide application and the average values of the diversity indices are calculated for each insecticide classes), a sensible decrease of the community diversity can be evidenced (Table 1). From Table 1, it can also be noted that the two indices provide a similar rank of the effects on the community structures of the applied insecticides.

Two factors could possibly explain this apparently contrasting result: the time-related recovery of the structural changes and the appearance and increase of insecticide-resistant taxa in relation of the rarefaction of less tolerant taxa (as suggested by PCA).

4. Discussion

The side effects induced by insecticides on river biota can be monitored by examining the variation of the taxonomic structure of the invertebrate communities defined by two attributes: taxa richness and evenness.

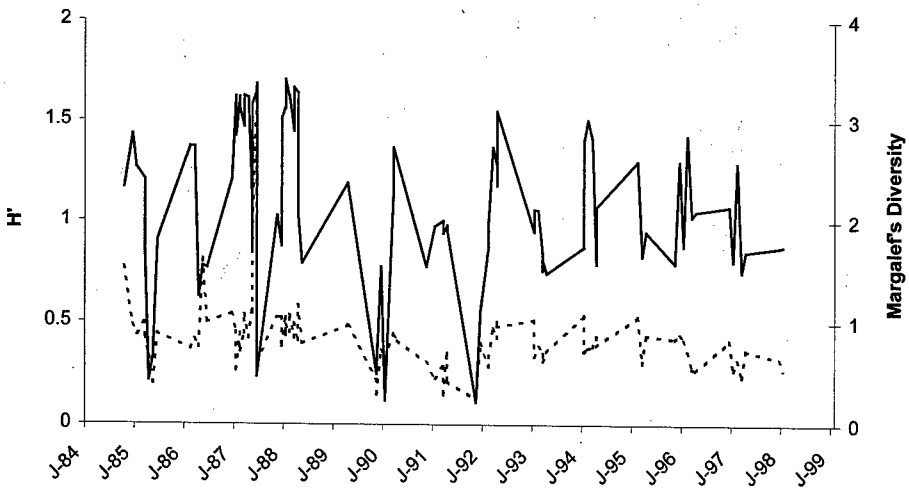

Fig. 4. Shannon (H') and Margalef (dotted line) diversity indices calculated for invertebrate communities sampled in Niandan river. The arrow marks the beginning of the insecticides application.

Table 1
Average values of the Shannon (H') and Margalef diversity indices calculated for invertebrate communities sampled in Niandan river during different insecticide application

	H'	Margalef
Pre-treatments	1.22	1.02
Temephos	1.08	0.79
B.t.	1.07	0.75
Pyraclofos	0.90	0.69
Phoxim	0.63	0.55
Permethrin	0.35	0.45

Since the relative abundance of the invertebrate species, other than by insecticides, is strongly regulated by natural driving forces (river discharge in the present case) and presents a short-term random component (i.e. variance not structured along any environmental or stressing factor gradient), an appropriate numerical analyses techniques should allow to decompose the total data variance in subset of not interrelated components. With this procedure, the biological variation attributed at each component can be subsequently outlined with respect to the sampling time in order to describe different biological patterns.

In general, as for the study-case we considered, two time-related biological patterns result from the analysis: long- and medium-term. The long-term pattern can be considered representing the chronic effect of the insecticides on the biota structures and the continuing load pressure on time scale, while the medium-term pattern can be regarded as the seasonal biological variation mainly due to the river flow dynamic.

For this task, PCA can be considered a pertinent data analysis tool, highly informative since, by decomposing the total variance, it allows to identify the "fingerprint" of the insecticide effects on aquatic communities and provides a detailed quantitative measure of the biological changes. It is easily applicable considering its widespread use and does not require sophisticated data transformation and manipulation procedures (variance normality can be attained by logarithmic transformation of data). Moreover, these techniques do not assume specific data properties unrealistic for most of the environmental monitoring activities such as, e.g. regular time-spaced samples. To facilitate the examination of the biological patterns, a rotation of the PCA axes or a selection of a subset of taxonomic unit could be required.

The rank-abundance models result appropriate tools for describing the short-term variation of the biological communities by providing a graphic representation of the "relative toxicological potency" of chemicals on a high level of organisms organisation.

The rank-abundance models present the advantage of producing a description of the total community structural changes without any loss of biological variance or any numerical manipulation. The limit of these techniques is related to the number of taxa considered since having a low taxonomic classification (indicatively less than 10 systematic units) it can result difficult to interpret the pattern of the generated curves.

The Shannon and Margalef's diversity indices are two of the most used tools among the traditional ecological diversity indices and allow to summarise the information about the community structures without regard to taxonomic classification or difference between species (they are low cost monitoring tools). In spite of such advantages, their application to aquatic communities for measuring river quality leads to contrasting results and the debate on their usefulness in monitoring programme is still open. In some studies, they result relatively weak in diagnosing water pollution (Katoh, 1991, 1992); in other situations, their application provides suitable information on water quality changes (Garcia-Criado et al., 1999; Sponseller et al., 2001).

Our results indicate that their descriptive power can significantly decrease when they are applied to communities sampled during a long-time scale. This finding can be explained considering that the long-term effects of chemicals on aquatic macroinvertebrates can be expressed by contrasting changes in the abundance of aquatic species that do not sensibly modify the numerical structure of the communities. For example, monitoring the long-term effects of permethrin on the aquatic macroinvertebrates of Guinean rivers it results that the abundance reduction of sensible taxa can be "shadowed" by a corresponding density increase of taxa less sensible to the applied chemical (Crosa et al., 2001b). In this context, it has to be considered that the measure of the communities structure by means of diversity indices assumes that the species are all equally different. This rationale strongly contrasts the species selective response to the chemical exposure (see the concept of chemical "fingerprint" previously discussed). Not considering the qualitative information of the biological component (taxonomic classification) can thus be a factor leading to misinterpretations of long-term monitoring data.

A more general constrain limiting the applicability of the diversity indices can also be the low number of the taxonomic unit that is usually classified in extensive and large-scale monitoring programmes.

5. Conclusions

Long-term monitoring data usually are not suited for most of the parametric tests used in ecotoxicological studies (i.e. ANOVA, MANOVA and t-test) because of the lack of normal distribution, homogeneous variance and independence. Non-parametric tests, such as the Mann–Whitney U-test as an alternative to the t-test, are sometimes criticised because they do not utilise all the information potentially provided by the sample. As an alternative, the descriptive techniques, as those presented, result as appropriate tools for a detailed analysis of the biological variation.

As demonstrated in the illustrated example, where only 12 taxonomic units classified above the family level were used, for monitoring insecticides effects on invertebrate assemblage a relatively small taxonomic efforts could be adequate. Reduction of the activities addressed to the biological classification of the samples can be an invaluable option in situations for which basic ecological informations are poorly available or the monitoring project encompasses broad time or spatial scales.

Rank-abundance models and ordination techniques (i.e. PCA) are two easily applicable data analyses tools that, although do not allow the statistical test of any hypothesis (i.e. deviation from a non-impacted situation), provide a valuable and synthetic description of the two community structural attributes potentially affected by the toxicants: taxa richness and evenness.

The rank-abundance models can be used to graphically represent the "relative toxicological potency" of the chemical on aquatic communities provided the samples have been collected within the time range of community recovery that can be short especially at tropical latitudes where many

invertebrates show several generations per year. In this graphical representation, the slope of the curves describes the potency of the compound biological impact (i.e. increasing slopes correspond to low evenness and taxa richness).

Long-term changes in the macroinvertebrates community structures can be better measured by examining the patterns of the ordination samples scores obtained by PCA along the sampling time gradient instead to use biplot ordination graphs. To optimise the time biological changes, a preliminary rotation of the ordination axes can be required. Usually two ordination axes allow to detect structured invertebrate changes. A long-term structured variation can be related to the chronic effects of toxicants while a medium-term (cyclic) biological variation reveals the effect of the natural diving force acting on aquatic communities at seasonal time scale.

Shannon-Wiener and Margalef diversity indices result poorly informative numerical descriptors of the long-term community structures changes because of the low number of taxonomic units that are usually classified in extensive biomonitoring designs and because these indices do not take into account taxonomic replacements within the community (shadow effect) as a consequence of "fingerprint" type of action of the chemical substances.

Finally, it has to be considered that the commented analyses techniques are intended to provide synthetic descriptors (graphical or numerical) of the biological changes occurring in insecticide-treated rivers. The role of these descriptors is to facilitate the formulation of hypotheses on the chemical effects at ecosystem level provided the biological variation due to natural driving factors have been accounted for. Results can therefore be used to improve treatment strategies for vector control or river management actions in relation to pollutant loads.

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Long-term effects of heavy metals and microelements on nematode assemblage

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Abstract

Effects of Cd, Cr, Se and Zn at a maximum rate of 270 mg kg^{-1} were studied on a nematode assemblage after 6–10 years of application. Winter wheat, sunflower, sorrel, barley and rape were grown on the experimental field. Cd had a moderate effect on nematodes in spite of the fact that this element significantly decreased plant biomass. Cr was harmful to plants only in the first year of the study. However, Cr decreased Aporcelaimellus density and maturity index, increased Pratylenchus density and bacterial–fungal ratio, changed the c-p structure and feeding type composition. Se proved to be very toxic at a concentration of 11 mg kg^{-1} (NH_4-acetate+EDTA soluble form). Some advantageous effects of Zn were found in the first year. These disappeared later on. Remarkable between-year fluctuations of the nematode assemblage composition were observed.
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Keywords: Soil pollution; Heavy metals; Microelements; Nematode assemblage

1. Introduction

Heavy metals and microelements from different sources, mainly from industry, traffic, wet or dry deposition, sewage sludge, fertilisation, and pesticide application pollute agroecosystems. Besides the crop plants other components of the system, as nematode assemblage are also affected (Bongers and Bongers, 1998; Bongers and Ferris, 1999; Yeates and Bongers, 1999; Ferris et al., 2001).

There is relatively little information on the long-term effects of heavy metals and microelements on the nematode assemblage of the agroecosystems. Weiss and Larink (1991) examined the effect of heavy metal contaminated sewage sludge on agricultural field. They found that a mixture of heavy metals in sewage sludge increased nematode density. This was a consequence of growth of bacterial (especially Rhabditid) and fungal feeder nematode populations. On the other side, density

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of the omnivores decreased owing to the presence of heavy metals.

Korthals et al. (1996a) examined Cu effects at different pH levels after 10 years of exposure in an arable field which was used by normal agricultural practice in the meantime. They found that nematode assemblage indicates sensitively Cu pollution. The increasing Cu concentration significantly decreased the number of Acrobeloides, Acrobeles, Basiria, Cervidellus, Diphterophora, Merlinius and Trichodorus, but increased the number of Chiloplacus. The abundance of feeding groups was also altered. The density of bacterial feeders and omnivores decreased and that of fungal feeders increased owing to Cu application. No such effect was found in respect to plant feeder and carnivore nematodes. The effect of Cu was influenced indirectly by pH.

The effects of the Cu, Ni, Cu+Zn, Ni+Zn and Zn-enriched sewage sludge on nematodes were studied by Georgieva et al. (2002). Sludge was applied 12 and 8 years before the nematode sampling. A positive correlation was found between the density of plant feeder Criconemoides and Paratylenchus. Bacterial feeder Alaimus, Acrobeles and Bastiania negatively correlated with Cu and Zn. Omnivores and predatory nematodes proved to be sensitive to heavy metal contamination. Indirect effects explained the higher density of plant feeder nematodes in Zn treatment. Cu and Zn effect was discovered at a relatively low contamination rate with the help of the life history analyses of nematode taxa developed by Bongers (1990, 1999).

In a previous analysis of nematode assemblage on the Nagyhörcsök site Nagy (1999) detected negative effects of Cr and Se on nematode density, taxon richness and maturity index (MI) 6 years after the contamination. Zn treatment had an increasing effect on these parameters. This finding was explained by the fact that Zn application acted as microelement fertilisation on this Zn depleted soil. Cu increased the density of the nematodes.

The objective of this study was: (1) to analyse Cd, Cr, Se and Zn effects on nematode assemblage composition after 6–10 years of the application, and (2) to examine whether any heavy metal effect is to be found on nematode assemblage in treatments of no observed phytotoxic effects.

2. Materials and methods

The sampling location was the experimental field of the Research Institute for Soil Science and Agricultural Chemistry (RISSAC) of the Hungarian Academy of Sciences at Nagyhörcsök, Hungary (UTM-code: CT 00). The soil of the experimental site is a calcareous loamy chernozem with medium to deep humus layer formed on loess. A long-term field experiment was commenced in 1991 to study the effects of heavy metals and microelements on crop plants. The experiment was arranged in a split-plot design, with two replicates. The size of each plot was 21 m^2. The samples for the present study were taken from plots at maximum contaminant level. In these plots Cd, Cr, Se or Zn was applied at a rate of 810 kg ha^{-1} (approximately 270 mg kg^{-1}) as $CdSO_4 \cdot 3H_2O$, K_2CrO_4, Na_2SeO_3 and $ZnSO_4 \cdot 7H_2O$ salt, respectively. For further description of the site and experiment, see Kádár (1995) and Nagy (1999). The NH_4-acetate+EDTA soluble (mobile) fraction of elements was determined according to Lakanen and Erviö (1971). These analyses were performed in 1991, 1992, 1994, 1997, and 2000.

Subsequent plant species grown according to regular agricultural practice every year were as follows: winter wheat, sunflower, sorrel, winter barley and winter oil rape from 1997 to 2001, respectively. Agrotechnical intervention was kept to the minimum every year.

Nematodes were sampled between 1997 and 2001 once a year during the period 1–15 June. Composite nematode samples (20 cores per plot of approximately 300–400 g) were taken using a soil corer of 2 cm in diameter. The top 10 cm of the soil were sampled. Nematodes were extracted from 100 g soil per plot using Cobb's decanting and sieving method (modified according to s'Jacob and van Bezooijen, 1984), counted (typically approx. 20% of the obtained suspension), fixed in 80–90 °C hot formalin (cc. 8%) and processed by identifying at least 150 specimens per sample to genus level in mass slides. In certain Se treatments where this

amount of nematodes was not available, samples were processed totally.

Two levels of the responses of nematode communities were analysed by different methods. Assemblage level structure was investigated by MI calculation (Bongers, 1990; Korthals et al., 1996b). Abundance, genus number and species turnover were measures to analyse the changes of population level structure. Besides the structural responses of the assemblage, the composition of the feeding groups (Yeates et al., 1993) and c-p groups (Bongers, 1990, 1999), as functional variables, were investigated.

Since nematode samples were taken from the same plots each year from 1997 to 2001, repeated measures ANOVA followed by Fisher's LSD comparison of means were performed to examine the effects of Cd, Cr, Se and Zn treatments on the nematode density and generic richness values. Effects of time, treatments and the interaction of these factors were examined. Nematode density data were ln transformed before the analysis in order to achieve normality of the data. Significant difference of means was tested at LSD. Taxon similarity (S) of the consecutive years was calculated by the Sorensen formula. Taxon turnover is expressed as $(1-S) \times 100$ (%). Relative data as c-p groups and feeding groups were compared by χ^2 analysis. All these analyses were carried out by the STATISTICA for Windows 5.0 software package.

3. Results

The total applied element concentration in the soil of the 0–20 cm plow layer was theoretically 270 mg kg^{-1} which load was about 100 times over the allowable threshold total limit values in the case of Cd, Cr(VI) and Se. This concentration did not change significantly during the course of the study in the case of Cd and Zn. Only the deeper plowing diluted the plow layer and contaminated the 20–30 cm soil. However, Cr and Se given in anion form were partly leached down to 3–4 m depth. Plant uptake was negligible, but fixation and leaching resulted in dropping of the NH_4-acetate+EDTA soluble element fractions are remarkable. This decrease of mobile fractions in plow layer was dramatic in the case of the leached Cr and Se elements (Table 1).

Phytotoxic effects were observed on the Cd, Cr and Se-treated plots. No such effect was found in the case of the Zn. Se was highly toxic in the given concentration every year irrespective of the cultivated crop species. There was practically no plant growth on these plots throughout the studied period. Cd decreased the crop yield significantly every year during the study period. Cr was phytotoxic in the first year (1997) of this study and in the previous years as well. From 1998 onwards, no phytotoxic effects were observed in Cr-treated plots. Cr(VI) partly leached in deeper layers and partly turned into non-toxic Cr(III) form.

Se was very toxic for nematodes. This microelement decreased the average nematode density and genus number (Table 2). No other element used in this experiment has a similar effect. Analysing the data for each year it was found that Se significantly decreased nematode density in the years 1997, 2000 and 2001. The other elements did not influence nematode density in any year.

Only a few significant differences were found concerning the density of different taxa owing to element application (Table 3). Eucephalobus density was higher in Cd treatment as opposed to the control. Cr decreased the density of Aporcelaimellus and Pratylenchus. Aphelencoides, Aporcelaimellus, Ditylenchus, Filenchus and Tylencholaimus decreased in numbers in the case of Se treatment. The density of Pratylenchus was higher on the Zn-treated than on the control plot.

There were six taxa which dominated all the experimental plots. Acrobeloides, Aphelenchus, Chiloplacus, Pratylenchus, Rhabditida (including dauer larve), and Tylenchorhynchus occurred in each treatment every year. Acrobeloides formed the most abundant genus in all treatments. Acrobeloides, Tylenchorhynchus and Pratylenchus dominated the control and Zn treatment. Chiloplacus dominated the Se treatment. In the Cr and Cd treatments, it was the second and third most abundant nematode genus, respectively. It has to be pointed out that considerable inter-year fluctuations of nematode density values were detected. The lowest coefficient of variation (CV%) was

Table 1
NH_4-acetate+EDTA soluble (available) fraction (as mg kg^{-1}) of the studied elements in soil as a function of the year of application

Years after application	Loading in spring 1991, 810 kg ha^{-1} (270 mg kg^{-1})			
	Cd	Cr	Se	Zn
0 (1991)	249	19.5	103.5	136.5
1 (1992)	228	10.1	81	213
3 (1994)	164	4.0	89	147
6 (1997)	190	1.4	36	143
9 (2000)	124	1.6	11	85
Background level	0.1–0.2	0.1	0.1–0.2	1–2
Threshold level in Hungary	1	75	1	200

Allowable threshold levels in Hungary for total amount in soil are as follows: Cd, Cr(VI) and Se: 1 mg kg^{-1}, Cr(III): 75 mg kg^{-1}, Zn: 200 mg kg^{-1}.

34.1% for Ditylenchus on control plot. The highest fluctuation was measured for Rhabditida (144.1) in Cd treatment. CV was more than 65% on average for all treatments.

The turnover of the taxa between years was considerable (Table 4). About 40% of the taxa changed yearly in the control. To some extent, less taxa changed on average in Cd (32.1%), Cr (31.0%) and Zn (33.9%) treatments. Average turnover values of the Cd, Cr and Zn treatments are very similar to each other. The highest taxon turnover was found in Se treatment (62.7%). It was a general trend to decrease turnover in all treatments from 1997 to 2000. Severe transformation in composition of the taxa was observed in the year 2000. This is shown by the high turnover values between 2000 and 2001 (Table 4).

Analysing c-p group composition on a yearly basis it may be found that Cd treatment differed from the control in the year 2000 and Zn treatment in the years 1997, 1999, 2001 (Table 5). The c-p group composition of the Cr and Se treatments was different from the control every year. The dynamics of the c-p group pattern is shown very clearly in Table 5. The relative importance of the c-p3–5 group increased from 1997 to 1999 in each treatment. In line with this, the proportion of the c-p2 group decreased except the Se treatment where the c-pl group decreased and the c-p2 group increased. This general trend stopped in the year 2000. An important rearrangement of c-p groups occurred this year. The relative importance of the c-p2 group becomes very high in each treatment. Next year the c-p structure of the Co, Cd and Cr treatments becomes quite similar to that of the year 1999. In the case of Se and Zn treatments a recovery to the c-p structure in 1997 is to be observed.

Comparing the c-p composition of the treatments in the consecutive years 1999, 2000 and 2001 highly significant differences (at least $P < 0.01$) were found in each case. The c-p3–5 group

Table 2
Total number (100 g soil per plot) and genus number of nematodes in the different treatments

	Control	Cd	Cr	Se	Zn
Total density	1085.4 (±390.0)	929.4 (±401.7)	720.6 (±177.2)	90.5 (±59.0)[a]	1391.3 (±476.4)
Genus number	20.8 (±2.5)	22.0 (±3.9)	18.0 (±2.0)	10.8 (±5.4)[a]	23.0 (±2.0)

Average number (±S.D.) of 5 years (1997–2001).
[a] Significant difference to the control at $P < 0.001$.

Table 3
Five years average density data (±S.D.) of the different taxa (100 g soil)

	Control	Cd	Cr	Se	Zn
Acrobeles	3.4 (±3.4)	4.7 (±5.0)	6.5 (±5.3)	0.0 (±0.0)	14.1 (±20.1)
Acrobeloides	156.7 (±154.7)	209.6 (±224.0)	134.4 (±111.9)	26.6 (±23.9)	230.1 (±288.8)
Alaimidae	3.5 (±6.0)	0.8 (±1.8)	3.4 (±6.1)	0.8 (±1.8)	13.7 (±15.4)
Aphelenchoides	51.3 (±67.3)	43.4 (±41.7)	32.5 (±37.0)	3.4 (±5.0)[a]	25.7 (±25.5)
Aphelenchus	112.9 (±121.0)	129.2 (±108.8)	66.7 (±62.1)	2.2 (±2.4)	119.5 (±95.4)
Aporcelaimellus	29.5 (±27.1)	31.9 (±28.9)	8.7 (±11.0)[a]	2.6 (±5.1)[b]	34.4 (±27.2)
Aporcelaimus	1.8 (±3.9)	3.7 (±10.8)	0.0 (±0.0)	0.0 (±0.0)	2.8 (±8.8)
Aulolaimus	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.6 (±1.8)
Cephalobus	5.3 (±9.4)	5.6 (±6.4)	3.7 (±9.5)	1.5 (±2.9)	6.6 (±9.7)
Cervidellus	10.7 (±16.9)	8.3 (±9.8)	8.2 (±10.4)	0.2 (±0.4)	10.4 (±9.9)
Chiloplacus	86.6 (±53.5)	93.5 (±76.6)	95.9 (±71.6)	37.8 (±30.7)	122.5 (±53.5)
Diploscapter	0.0 (±0.0)	4.7 (±14.8)	20.2 (±46.3)	0.2 (±0.6)	0.0 (±0.0)
Diplogasteridae	0.0 (±0.0)	0.4 (±1.4)	0.0 (±0.0)	0.0 (±0.0)	0.3 (±1.0)
Discolaimium	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	2.3 (±5.5)
Discolaimus	1.9 (±4.3)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	1.0 (±3.3)
Ditylenchus	68.2 (±39.7)	87.3 (±75.7)	40.4 (±41.4)	3.4 (±6.7)[c]	76.8 (±38.2)
Ecumenicus	17.2 (±15.2)	19.4 (±22.5)	13.5 (±20.7)	0.5 (±1.5)	36.4 (±34.3)
Eucephalobus	1.1 (±2.4)	6.8 (±9.3)[a]	2.0 (±4.6)	0.1 (±0.3)	4.7 (±5.5)
Eudorylaimus	5.4 (±11.9)	5.9 (±9.0)	0.7 (±2.2)	0.0 (±0.0)	5.2 (±6.4)
Filenchus	28.5 (±16.1)	17.8 (±17.0)	18.7 (±19.6)	0.5 (±1.0)[c]	49.6 (±36.2)
Helicotylenchus	9.4 (±23.7)	1.7 (±3.1)	18.8 (±28.0)	0.7 (±1.3)	8.1 (±11.1)
Heterocephalobus	56.6 (±60.2)	30.8 (±30.4)	15.7 (±13.2)	1.5 (±3.5)	20.9 (±22.4)
Malenchus	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	2.0 (±6.4)
Monhysteridae	0.0 (±0.0)	0.0 (±0.0)	0.6 (±2.0)	0.0 (±0.0)	0.0 (±0.0)
Meloidogyne J2	0.3 (±0.9)	0.6 (±2.0)	0.5 (±1.6)	0.0 (±0.0)	0.0 (±0.0)
Paraphelenchus	3.2 (±7.5)	2.7 (±4.3)	5.9 (±16.4)	0.0 (±0.0)	1.9 (±4.2)
Paratylenchus	7.8 (±15.4)	8.2 (±15.4)	43.8 (±96.3)	0.6 (±1.3)	29.2 (±35.7)
Pratylenchus	180.3 (±91.6)	62.3 (±50.8)	39.4 (±48.2)[c]	3.2 (±5.7)	299.0 (±193.9)[a]
Prismatolaimus	24.1 (±30.1)	18.2 (±37.2)	51.2 (±76.3)	0.3 (±0.7)	29.9 (±25.0)
Pungentus	1.1 (±3.6)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	1.1 (±3.4)
Rhabditida (including dauer larve)	24.7 (±23.3)	23.5 (±33.1)	14.8 (±19.3)	3.8 (±4.7)	13.7 (±13.9)
Tylencholaimus	9.1 (±9.2)	13.2 (±25.0)	2.4 (±2.9)	0.4 (±1.3)[b]	5.9 (±10.3)
Tylenchorhynchus	183.9 (±197.5)	37.2 (±66.0)	24.0 (±39.7)	0.8 (±1.7)	204.4 (±266.2)
Tylenchus	3.1 (±5.8)	25.9 (±48.1)	48.2 (±105.6)	0.2 (±0.4)	0.8 (±1.6)
Diplogastridae	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.3 (±1.0)
Monhysteridae	0.0 (±0.0)	10.8 (±21.0)	0.0 (±0.0)	0.0 (±0.0)	9.4 (±14.8)
Wilsonema	1.7 (±4.1)	1.4 (±2.3)	0.0 (±0.0)	0.2 (±0.5)	3.1 (±5.9)
Xiphinema	0.3 (±1.1)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)

[a] Significant differences are indicated as $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

decreased to the largest extent in Cr and Zn treatments in 2000. Recovery of this group was most expressed in the Cr treatment.

Change of MI showed a clear trend in all treatments (Table 6). This trend was similar in all cases except the Zn treatment, where MI was higher in 1997 than in 1998. MI increased in the first 3 years. Thereafter it markedly decreased in 2000 and started to increase again in 2001. It was not possible to calculate MI for the Se treatment because of the low abundance of nematodes in the given samples.

The dominance of the plant feeder nematodes increased in all but the Se treatment during the study period (Table 7). Bacterial feeder dominance decreased more or less in all treatments in the first

Table 4
Taxon turnover (%) between consecutive years

Between years	Control	Cd	Cr	Se	Zn
1997 and 1998	48.2	41.7	28.6	72.7	41.4
1998 and 1999	50	32.1	26.1	75	38.7
1999 and 2000	25	25.9	19.1	42.9	20.7
2000 and 2001	32	28.7	50	60	34.6
Average (±S.D.)	38.8 (±12.3)	32.1 (±6.9)	31.0 (±13.3)	62.7 (±14.7)	33.9 (±9.2)

3 years. This trend was especially obvious in the Se treatment. The dominance of this group became greater in 2000 and decreased thereafter except the Cr treatment. As opposed to the plant feeders, fungal feeder nematode dominance decreased continuously, except the Se treatment. The proportion of the omnivore and carnivore nematodes increased in the first 3 years, decreased in 2000 and started to increase in 2001. This trend was similar in all treatments. The bacterial feeder to fungal feeder ratio was significantly higher in Cr and Se treatments than in the control (Table 8).

Table 5
Nematode c-p group composition of the different treatments (%) in the years from 1997 to 2001

	Control	Cd	Cr	Se	Zn
1997					
c-p1	5.5	0.5	1.1	47.5	5.1
c-p2	86.9	92.0	97.4	52.5	76.8
c-p3-5	7.6	7.5	1.4	0.0	18.2
		n.s.	$P<0.01$	$P<0.001$	$P<0.001$
1998					
c-p1	3.3	0.0	1.9	30.9	0.5
c-p2	81.0	80.0	97.5	60.5	87.5
c-p3-5	15.7	20.0	0.6	8.6	12.0
		n.s.	$P<0.001$	$P<0.001$	n.s.
1999					
c-p1	1.0	0.7	7.2	21.4	0.3
c-p2	70.8	75.6	56.7	67.5	58.5
c-p3-5	28.2	23.7	36.1	11.1	41.2
		n.s.	$P<0.001$	$P<0.001$	$P<0.05$
2000					
c-p1	3.8	0.7	12.5	25.1	1.3
c-p2	86.9	95.6	86.4	74.1	90.7
c-p3-5	9.3	3.8	1.1	0.8	8.0
		$P<0.05$	$P<0.001$	$P<0.001$	n.s.
2001					
c-p1	0.9	0.6	2.6	39.9	13.8
c-p2	79.5	85.9	43.9	59.4	73.3
c-p3-5	19.6	13.5	53.5	0.7	12.9
		n.s.	$P<0.001$	$P<0.001$	$P<0.001$

Nematode c-p group composition of each treatment was compared to the control of the same year. Results of the χ^2 analysis are given in last rows of each year data. n.s.: no significant difference.

Table 6
Average MI values of the five sampling years from 1997 to 2001

	Control	Cd	Cr	Zn
1997	2.08	2.10	1.98	2.44
1998	2.40	2.55	2.02	2.30
1999	2.88	2.48	2.85	2.96
2000	2.14	2.07	1.99	2.11
2001	2.50	2.24	2.16	2.38
Average	2.40	2.29	2.20	2.43

4. Discussion

Contamination of a field with plant microelements (including heavy metals) decreased nematode abundance and generic richness in several studies (Korthals et al., 1996a; Yeates et al., 1993; Georgieva et al., 2002). Similar results were found in this experiment. Se proved especially toxic for nematode in the applied concentration. The increase in nematode density in the Zn treatment is owing to the higher density of plant feeder and bacterial feeder nematodes in this treatment. This may be a consequence of the Zn-depleted character of the soil on the Nagyhörcsök site. Zn application probably stimulated plant growth and microbial biomass thus indirectly favoured plant feeding and bacterial feeding nematodes. Similar effect was observed if Zn-enriched sewage sludge was applied (Weiss and Larink, 1991; Georgieva et al., 2002). The same conclusion may be drawn for genus richness as well.

The increase of the Eucephalobus density in the Cd treatment is in agreement with the data of Popovici (1994) and Popovici and Korthals (1995), who found that the density of r-strategist bacterivore nematodes increased on heavy metal polluted sites. Eucephalobus species are short-lived, small, bacterial feeder nematodes. The lower Pratylenchus density in Cr treatment may be a direct effect of the heavy metal. The higher density of the same genus in the Zn treatment may be a consequence of indirect effects as discussed above. It is known that omnivore and carnivore nematodes are the most sensitive groups to heavy metal pollution (Bongers and Bongers, 1998). In this experiment Cr and Se decreased Aporcelaimellus density even after 10 years of application. The results of Georgieva et al. (2002) support our finding. Although Weiss and Larink (1991) found increased abundance or proportion of predatory nematodes on heavy metal polluted sites it is not comparable with our results because a mix of heavy metals were used in their experiments.

It has to be pointed out that the yearly fluctuation of the density and genus composition in an arable field is relatively great compared with other ecosystems. The taxon turnover in this study was comparable to that in an agriculturally cultivated meadow at Kjettslinge in Sweden (Boström and Sohlenius, 1998). The only exception lies in the Se treatment. The extremely high turnover found in this treatment may be explained by the highly toxic effect of Se. Therefore, the situation on this plot is similar to the early phase of a succession, which is characterised by high turnover of the species.

The relatively lower proportion of plant feeder nematodes in Cd and Se treatments may be in connection with the significantly reduced plant biomass on these plots. It is known that the density of the plant feeder nematodes can be related to biomass and vigour of plants (Yeates, 1987; Bongers and Ferris, 1999; Yeates and Bongers, 1999).

Analysis of the c-p group composition of the nematode assemblage is a valuable tool to detect heavy metal pollution (de Goede et al., 1993; Bongers and Ferris, 1999). A related method is the MI analysis (Bongers, 1990; Korthals et al., 1996b). Results gained with both methods showed that Cd had a moderate, while Cr had a more pronounced effect on nematode assemblage in this experiment. The modest effect of Cd on nematodes is in accordance with the findings of Korthals et al. (1996c) who did not find acute effects of Cd up to 160 mg kg^{-1} (highest rate in their experiment) after 1 week of the contamination. Haight et al. (1982) and Kammenga et al. (1994) proved low susceptibility of nematodes to Cd as well. However, Bengtsson and Tranvik (1989) found decreased density and lower diversity of nematodes at a rate of 26 mg kg^{-1} Cd level in a forest soil.

The effects of the Cr on the nematode assemblage are not well known. In this experiment, Cr

Table 7
Nematode feeding group composition of the different treatments (%)

	Control	Cd	Cr	Se	Zn
1997					
PF	15.9	4.2	9.1	2.9	29.2
BF	42.9	54.5	55.2	95.4	34.3
FF	37.8	35.9	33.8	2.1	27.4
O	1.8	3.4	0.9	0.0	6.6
C	1.5	2.0	0.9	0.0	2.4
		$P<0.01$	n.s.	$P<0.001$	$P<0.001$
1998					
PF	32.9	21.5	13.1	20.0	41.5
BF	30.8	25.6	43.2	70.0	19.3
FF	23.0	31.2	42.9	10.0	30.0
O	8.4	12.1	0.5	0.0	5.7
C	4.9	9.7	0.3	0.0	3.5
		$P<0.01$	$P<0.001$	$P<0.001$	$P<0.05$
1999					
PF	44.4	19.8	31.1	12.3	38.7
BF	22.6	36.4	39.7	54.9	28.0
FF	22.5	29.4	13.8	17.7	11.2
O	7.0	10.4	11.3	8.3	14.7
C	3.6	3.9	4.0	6.9	7.4
		$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$
2000					
PF	45.1	17.1	45.4	1.0	35.9
BF	39.7	60.1	46.2	92.7	52.3
FF	11.9	20.0	8.4	4.8	9.2
O	1.9	1.5	0.0	0.7	1.4
C	1.3	1.2	0.0	0.7	1.2
		$P<0.001$	n.s.	$P<0.001$	n.s.
2001					
PF	61.5	29.8	34.8	0.0	59.2
BF	20.6	42.9	55.0	88.4	22.8
FF	11.5	19.7	5.8	11.6	11.4
O	5.6	5.4	3.5	0.0	4.8
C	1.0	2.3	0.9	0.0	1.8
		$P<0.001$	$P<0.001$	$P<0.001$	n.s.

Nematode feeding group composition of each treatment was compared to the control of the same year. Results of the χ^2 analysis are given in last rows of each year data. n.s.: no significant difference. PP: plant feeder, BF: bacterial feeder, FF: fungal feeder, O: omnivore, C: carnivore.

effect on nematodes was detectable during the whole experimental period. In spite of the fact that Cr was not toxic for plants after 1997, the c-p structure of this treatment differed significantly from the control every year. Besides, c-p composition of the Cr treatment reacted more intensely to the disturbance in 2000 and the recovery to the status in 1999 was most expressed in this treatment compared with others. All these facts show that the long-term effect of Cr is more pronounced on nematode assemblage than on plants. Therefore, nematodes are more sensitive indicators of Cr contamination than several species of agricultural crops. Bardgett et al. (1994) and Yeates et al. (1995) studied the Cr effect on nematode assemblage on the field. However, their data are not comparable with ours because Cr was mixed with other heavy metals in those experiments.

Table 8
Dominance of the bacterial feeder and fungal feeder nematode (%) and the bacterial feeder-fungal feeder ratio in the different treatments

	Control	Cd	Cr	Se	Zn
BF	58.8	59.2	68.7	88.3	66.7
FF	41.2	40.8	31.3	11.7	33.3
BF:FF	1.43	1.45	2.18	7.3	2.0
		n.s.	$P < 0.05$	$P < 0.01$	n.s.

BF: bacterial feeder, FF: fungal feeder.

Se is extremely toxic for plants and nematodes if NH_4-acetate + EDTA-exchangable concentration is at least around and above 10 mg kg^{-1}. Acrobeloides and Chiloplacus seem to be resistant to Se pollution, because these two genera occurred every year in relatively high density. This is in agreement with the results of Nagy (1999). Mobile Se concentration decreased rapidly during the study period. Three taxa, Alaimidae, Aphelenchus and Ditylenchus appeared in 1999 and made stable populations in the consecutive years. That is, why it is supposed that these taxa are more Se-tolerant than the others are. Concerning the lacking taxa it is not possible to determine whether Se pollution or absent plant biomass is responsible for this circumstance. Virtually no other data are present about Se effect on nematodes.

Zn had a positive effect on nematode assemblage in terms of nematode density, richness and MI in the years 1996 and 1997 (Nagy, 1999). In the subsequent years (except 1999) this effect disappeared. However, deleterious effect of Zn was not detected in our experiment. Korthals et al. (1996c) found Zn toxic on nematodes at a rate of 100 mg kg^{-1} 1 week after the contamination but their results are not comparable with ours because of the short duration of the experiment. Zn had adverse effect at a rate of about 150 mg kg^{-1} on nematode assemblage (Georgieva et al., 2002) in a long-term experiment. This contradicts our results. This inconsistency draws attention to the importance of soil type because Georgieva et al. (2002) worked on sandy loam and our experiment was carried out on chernozem soil of higher buffering capacity. The presence of plants on Zn treated plots also contribute to the absence of toxicity in the case of this heavy metal (Korthals et al., 1998). The presence of plants may especially be important because Smit et al. (2002) found NOECs based on $CaCl_2$-exchangable Zn as low as 9.4 for nematode richness, 9.9 for Shannon–Weaver diversity index and 0.67 for principal response curve on bare sandy soil. Nematode richness was not decreased in Zn treatment in our experiment in spite of the fact that NH_4-acetate + EDTA-exchangable concentration of Zn was 85 mg kg^{-1}. Aphelenchus sp. was found in the experiment of Smit et al. (2002) as a zinc-sensitive species. Aphelenchus occurred in high density in our experiment but no Zn effect was found. This may be because either another species was found on our site or the bioavailability of Zn was lower. Paratylenchus density was higher in Zn treatment as it was found by Georgieva et al. (2002).

High ratio of fungal feeder compared with bacterial feeder nematodes may be a sign of heavy metal contamination (Sturhan, 1986; Bongers and Bongers, 1998; Korthals et al., 1996a). However, the opposite situation was found in this experiment. Cd, Cr and Se treatments were characterised by the dominance of bacterial feeding nematodes. This may be because heavy metal contamination was in connection with decreasing pH in the above-cited experiments. Such environment is favourable for fungi over bacteria (de Goede et al., 1993). Soil on our site is a calcareous loamy chernozem with neutral pH, which is advantageous for the growth of bacterial populations. Besides, regular agricultural activity promotes the increase of bacterial feeder nematode (Sohlenius and Boström, 1984; Ettema and Bongers, 1993). Popovici (1994) and Popovici and Korthals (1995) found also higher density of bacterivore nematodes on heavy metal polluted sites. Therefore, change of the fungal:bacterial ratio owing to heavy metal pollution seems to have a site-specific character.

5. Conclusions

Cd, Cr and Se application in a rate of 270 mg kg^{-1} had a deleterious effect on soil nematode assemblage 6–10 years later. Zn treatment caused

no such effect. Heavy metal and microelement effects were the most pronounced on c-p3–5 group and on carnivore nematodes. In the first 3 years of the study a succession of nematode assemblages were observed in all treatments. Due to a detectable disturbance for which we have no explanation however, this process regressed in 2000. The succession seemed to continue again in 2001. A considerable effect of the Cr treatment on nematode assemblage was found between 1998 and 2001 in spite of the fact that Cr has not decreased plant biomass at that time. Zn was not harmful to nematodes in this period.

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Molluscs in biological monitoring of water quality

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Abstract

Molluscs living in Lake Balaton accumulate persistent toxic substances, namely heavy metals, to a greater extent, than other organisms, and can serve as excellent passive biomonitors. Especially gills are good accumulators. Regular sampling showed that the level of Cd and Hg concentrations increased, while Pb contamination decreased during the past 20 years in mussels, corresponding probably to changes in pollution of the Lake. In functional, active monitoring various behavioral patterns of molluscs were employed. In mussels the periodicity of activity and rest (pumping activity vs. valve closure) is a sensitive indicator of unfavorable conditions and so of toxic substances. Low concentrations of inorganic and organic toxicants (heavy metals, PCBs, PAH compounds) cause reduction of the active and increase of the rest periods in a concentration dependent manner in a few hours. A second, suitable test for evaluating toxicity of chemicals is the measurement of the water flow through the exhalant siphon. Under the effect of toxicants the siphon activity, both the strength and duration of water flow changes characteristically within a few minutes. For both behavioral tests special techniques have been developed suitable for long duration recording, supported by mechano-electrical transduction and computerized data evaluation. In case of the pond snail (*Lymnaea stagnalis* L.) the behavior (positive/negative geotaxis and orientation) is disturbed in the presence of the above mentioned toxic chemicals. The execution and evaluation of the changes in the movement of the snail is based on video-recording and measurement of the direction and distance the animal performs in uncontaminated water and in the presence of the pollutants during the same period of time (0.5–4 h).
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Keywords: Mussel; Pond snail; Heavy metals; Organic pollutants; Behavior; Accumulation

1. Introduction

Aquatic ecosystems are under permanent pressure of anthropogenic pollutants originating from various sources located at the catchment area, or at distant places, polluting the environment through the air. Many of the pollutants are toxic to aquatic organisms causing their lethal or sub-lethal deterioration. The toxic effect depends mainly on the type of the pollutant and on its concentration. In most cases the concentrations are low, causing only sub-lethal or chronic disease,

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nevertheless, acute massive pollution resulting fish-kill or death of various organisms, may also occur in rivers or lakes. This happened 2 years ago 2000 on River Tisza (Hungary), due to a high cyanide and heavy metal pollution originating from an accidental release of waste water produced during gold mining and processing and stored in reservoirs at a nearby area, in Romania (Csépes et al., 2001). The level of contamination in surface waters is mostly low, like in Lake Balaton, which is a recreational area at Hungary (Farkas et al., 2001). Nevertheless, a permanent control of water quality is indispensable. To reveal the presence of pollutants and to measure their toxic effect biological indicators can be used, which are also suitable for prediction of the expectable toxic influence of known or unknown substances.

In biological indication passive and active monitoring are accepted as a general approach, at different levels of organization. In passive monitoring degradation of the ecosystem, elimination of sensitive species and reduction of biodiversity can be revealed as adverse consequences of pollution at the level of populations, while at the level of individuals accumulation of toxic substances in specimen, in organs and tissues indicative of pollution in the environment can be traced. In active monitoring the response of artificial or modified populations, behavioral patterns of specimen, specific function of organs like movement, feeding, respiration, reproduction and the neural regulation, as well as cellular and subcellular events are studied under the effect of toxic substances.

Among aquatic organisms suitable for biological monitoring molluscs occupy a prominent place (Goldberg, 1986; Salánki, 1989), and they are often used both for passive and active bio-monitoring and in hazard and risk assessment (Borcherding and Volpers, 1994). In our studies bivalves and snails are under study for evaluating the level of pollution in Lake Balaton and for assessing the sub-lethal effects of existing and potential toxic substances on some physiological functions. In the present paper selected results obtained with mussels and three molluscan behavioral models are presented.

2. Materials and methods

2.1. Determination of the concentration of toxic heavy metals

The concentration of cadmium, copper, mercury and lead were measured in organs of mussels and fish collected in lake Balaton. For the determination of non-volatile elements tissues were digested according to the technique applied by Krishnamurthy et al. (1976) and Farkas (1993), while for mercury according to Paus (1972). The concentration of metals was measured with a Perkin–Elmer 5100 atomic absorption spectrophotometer equipped with a HGA 60 graphite furnace and deuterium background corrector. Mercury was determined by the cold-vapor technique, according to Hatch and Ott (1968).

2.2. Recording of the rhythmic and periodic activity of the valves in the mussel

The general feature of mussel behavior is the rhythmic and periodic character of the shell movements, which is accomplished by the active force of the adductor muscles and the oppositely directed passive tension of the ligament connecting the two shells. Recording the shell movements of fixed or free moving animals is performed by electromechanical transmission, based on the inductive attenuator principle (Véró and Salánki, 1969). A small source and sensor are mounted on the two shells having two parallel tuned circuits on the same frequency. The inductive coupling of the primary and secondary circuits is dependent on the distance between the two shells. Driving the primary circuit with a generator tuned to the resonant frequency, the secondary voltage will be proportional to the coupling distance between the two circuits and thus to the shell movement of the animal. The changes of the secondary voltage are recorded by using appropriate software and computer (Kontreczky et al., 1997). Collecting and evaluation of data is performed by using an appropriate program.

The activity of the valves is characterized by the periodicity of long lasting, 10–20 h openings with shorter, 4–6 h closure. During open position due

to fast adductor contractions and relaxation water pumping takes place, therefore this period is called as active period, in contrast, the closed time called rest period (Salánki, 1992).

In monitoring the effect of pollutants self-control is applied. The activity of mussels is recorded for several days (for 1 week) in clean water and the average duration of active and rest periods is calculated. Following the control period the activity is recorded in polluted water for several days, and the mean duration of active and rest periods is compared to the control values. The changes are expressed in per cent of the control.

2.3. Recording of the water flow through the exhalant siphon in the mussel

At the posterior (anal) part of the mussels two apertures are located, the inhalant and the exhalant siphon, through which water passes into and out of the mantle cavity. The activity of the exhalant siphon provides mechanisms for the control of filtration (Foster-Smith, 1976). The outflow of the water is a strong stream, characterized by a detectable mechanical force. Since both the inflow and exhalant siphon are sensitive to chemicals, the system provides a reliable measure for screening toxic substances.

The mussel applied for testing is placed in a 2-l vessel, fixed to a stage at the bottom. The setup used for recording the water flow from the exhalant siphon (Kontreczky et al., 1997) consists of a flow-sensor in the form of a small plastic umbrella, located against the exhalant siphon. The water flow causes dislocation of the sensor, which can be monitored by using a mechano-electric transducer, amplifier, and recording system (Salánki et al., 1991). The water emission through the exhalant siphon is characterized by three basic parameters: (a) the duration of the active period, when the water is pushed out; (b) the intensity of the water pumped which can be evaluated in terms of the amplitude of the dislocation of the umbrella; and (c) the rest period, when no water flow occurs.

Testing of the pollutants takes place in self-control experiments. After recording the water outflow in clean lake water for 30–60 min the toxic chemical is added and consecutive recording takes place for similar time duration. The mean values of the parameters are calculated both for the control and the testing period, and changes are expressed in per cent of the control.

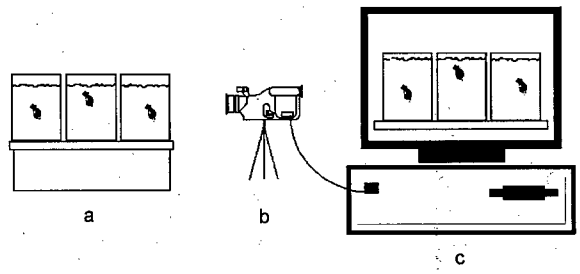

Fig. 1. Method of recording the locomotory activity of the snail (*L. stagnalis* L.): (a) three aquarium with animals; (b) video recorder; (c) monitor.

2.4. Recording of the locomotion in the pond snail (*Lymnaea stagnalis L.*)

Snails are placed individually in a narrow (20 × 10 × 3 cm) aquarium allowing upward, downward and two-directional horizontal movements. The locomotion is recorded on a video tape for 30 or 60 min, and evaluated according to the direction, distance and duration of the movements of the animal (Fig. 1). In testing the effect of substances either self control can be applied, when the same animal is treated with clean water and with waters of different quality (dissolved chemicals), or parallel animals are used for control and for checking the effect. Using a perfusion system the water with tested chemicals can be changed automatically according to a programmed protocol.

For evaluation of the results the movements of the animal can be copied to a transparent paper at a fast forward speed of the tape, and the upward, downward and horizontal path performed by the animal can be measured separately, as a distance (mm). Using time measurements the mean speed can be calculated. Data of control and test measurements can be compared and analyzed.

In the course of studies the effect of both inorganic and organic pollutants were tested, namely heavy metals (Cd, Cu, Hg, Pb, Sn), pesticides, PAHs, PCBs. In control measurements

Table 1
Heavy metals in aquatic organisms inhabiting Lake Balaton (1996–2001) and in the fresh water mussel (1978–80)

Species	Organs	Cadmium	Copper	Mercury	Lead
Eel	Muscle	0.55–1.25[a]	2.18–2.43	0.08–0.10	0.34–0.40
	Gill	1.31–3.96	4.08–7.06	0.02–0.06	0.69–1.48
	Liver	3.06–4.09	51.9–88.0	0.12–0.05	1.31–2.04
Bream	Muscle	0.34–1.46	1.88–2.61	0.13–0.16	0.83–0.89
	Gill	0.95–8.26	3.59–3.73	0.03–0.11	1.62–1.92
	Liver	3.13–4.85	30.2–50.9	0.08–0.16	0.98–0.99
Pike perch	Muscle	0.36–0.61	1.12–2.05	0.25–0.28	0.43–0.53
	Gill	0.48–4.09	2.42–3.03	0.06–0.09	0.70–0.79
	Liver	1.47–2.34	5.29–7.97	0.12–0.17	0.54–0.71
Crustaceans	Biomass	0.10–0.50	4.44–40.3	n.d.	2.61–23.04
A. cygnea	Mantle	14.7±3.2[b]	10.7±3.3	1.12±0.04	4.2±1.1
	Outer gill	13.1±2.1	14.9±3.6	1.37±0.07	8.9±2.3
	Inner gill	13.4±2.1	28.3±6.0	2.04±0.03	12.4±3.1
	Foot tissue	9.0±1.6	4.2±0.9	0.71±0.09	3.2±1.4
A. cygnea[c]	Mantle	3.7±0.38	11.9±2.24	0.62±0.33	21.7±6.42
	Gill	3.7±0.26	12.3±1.37	1.22±0.54	48.5±10.3
	Foot tissue	1.7±0.25	7.0±0.80	0.39±0.15	11.4±2.91

n.d., not determined.
[a] Average heavy metal concentration intervals (min–max) mg kg^{-1} dry weight.
[b] Average heavy metal concentrations (mean±S.D.) mg kg^{-1} dry weight.
[c] Results obtained during 1978–80.

natural Balaton water was used, and the tested chemicals were dissolved also in Balaton water.

3. Results and discussion

3.1. Concentration of toxic metals in mussel and fish of Lake Balaton

To reveal the contamination of the animals living in Lake Balaton with toxic metals regular sampling and measurements were carried out since 1978 on various species. Based on these measurements static bioindicators, characterized by high accumulator capacity were selected, and used for monitoring the pollution of the Lake.

Supporting earlier suggestions (Goldberg, 1986), mussels proved to be best monitors for heavy metal contamination of the area. Data of Table 1 show, that in comparison with fish tissues and the crustaceans organs of the mussel contain in most cases one order higher metal concentrations. Exception is the fish liver, which contains highest copper concentration among all studied organs. Nevertheless, using mussels for regular studies in consecutive years one can obtain good information on the level and possible changes in the contamination of the environment.

Heavy metal concentrations measured in mussels in the last 5 years (Table 1) were compared with values obtained between 1978 and 80. It became obvious, that during the past 20 years the pollution of the mussels has changed, reflecting probably that for Lake Balaton. Cd and Hg contamination increased by five and two times, respectively, while concentrations of Pb reduced by about five times. Cu concentration did not change.

3.2. Periodic activity of mussels in monitoring toxic substances in the water

The periodic activity, namely the duration of active and rest periods proved to be a simple and reliable behavioral phenomenon in testing the harmful, toxic influence of a number of environ-

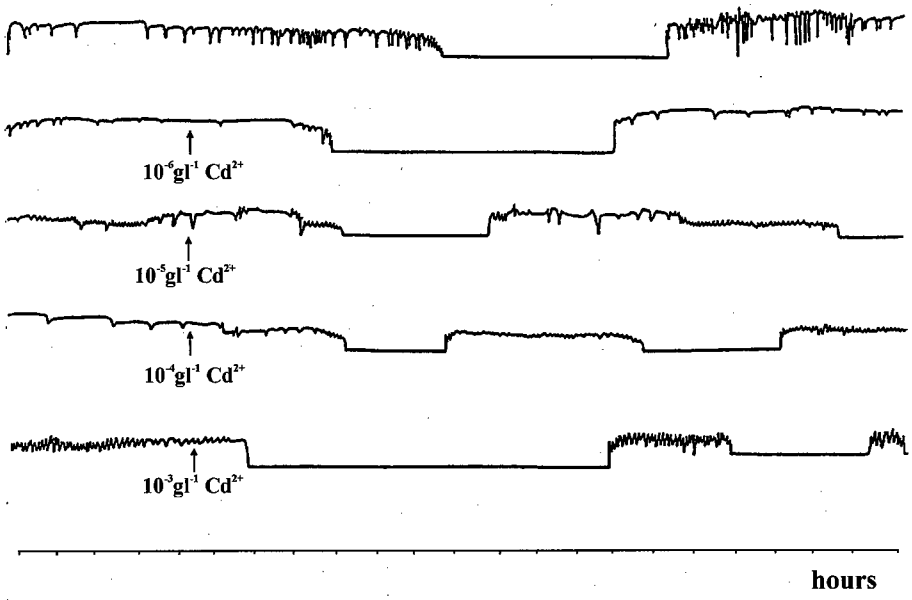

Fig. 2. Effect of cadmium on the periodic activity of the mussel (*Anodonta cygnea* L). Upper—control activity; lover records—the effect of increasing concentrations.

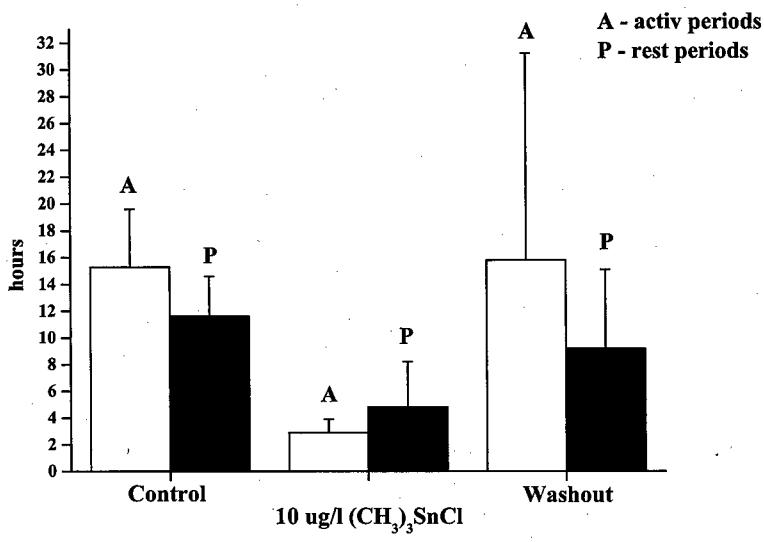

Fig. 3. Effect of organic Sn on the duration of the active and rest periods in the mussel.

mental pollutants. In our studies the effect of heavy metals, namely Cd, Cu, Hg, Pb, Sn, as well as organic pollutants, like pesticides, herbicides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls were investigated. In general, toxic substances caused shortening of active, and lengthening of rest periods. The effect was concentration dependent. Mercury and cadmium (Fig. 2) proved to be most effective pollutants. Higher concentrations or other pollutants may result in shortening

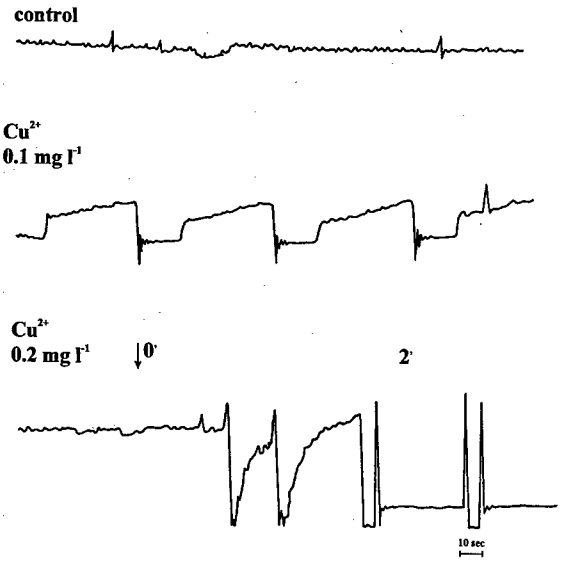

Fig. 4. Effect of copper on the activity of the outflow siphon in the mussel. Upper—control; middle and lower—effect of two concentrations of Cu.

of both active and rest periods, but the effect is more pronounced on activity (organic tin; Fig. 3).

3.3. Use of the water outflow through the exhalant siphon in monitoring toxicity

To the presence of pollutants in the water the siphon may respond with a depression of water emission or by total closure, and as a result the water flow becomes weaker or stops, which is reflected in the position and movement of the umbrella. In uncontaminated water, under open position of the valves, the water flow from the outflow siphon is continuous or performs several minutes long activity, interrupted with flow-free periods. In contrast to the periodic activity of the valves, this case time relations can be measured in minutes, instead of hours. Under the effect of toxic substances the long duration siphon activity is modified, short filtering and rest periods alternate (Fig. 4). Often not only the duration of the water flow, but also the strength is reduced, which can be calculated from the dislocation of the umbrella (Kontreczky et al., 1997). Most heavy metals and organic pollutants influence the water outflow in a concentration dependent manner, as seen in case of the mosquito killer pyretroid deltamethrin

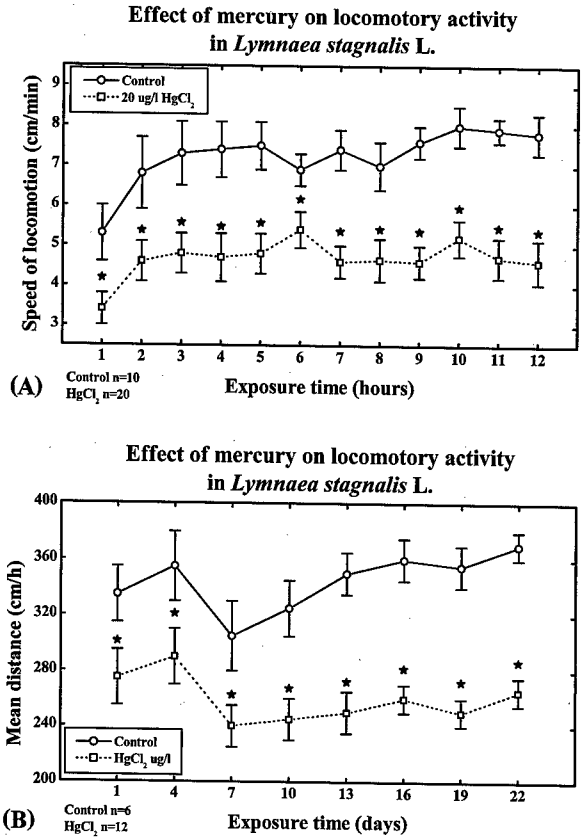

Fig. 5. Effect of mercury on the locomotory activity of the snail, *L. stagnalis* L. (A) acute, 12 h treatment; (B) chronic, 22 days long treatment.

(Kontreczky et al., 1997). With the method the toxicity of unknown or newly produces substances can also be studied.

3.4. Use of snail locomotion in monitoring water pollution

The effects of heavy metals (Hg, Cu, Pb and Sn) on the locomotory activity of *Lymnaea* were studied in acute and chronic experiments. In acute experiments lasting for 12 h the movement of the animals was recorded for 30 min in each hour, started after adding heavy metals. In chronic experiments the movement of heavy metal treated animals was recorded also for 30 min in the same period of every consecutive 3rd day.

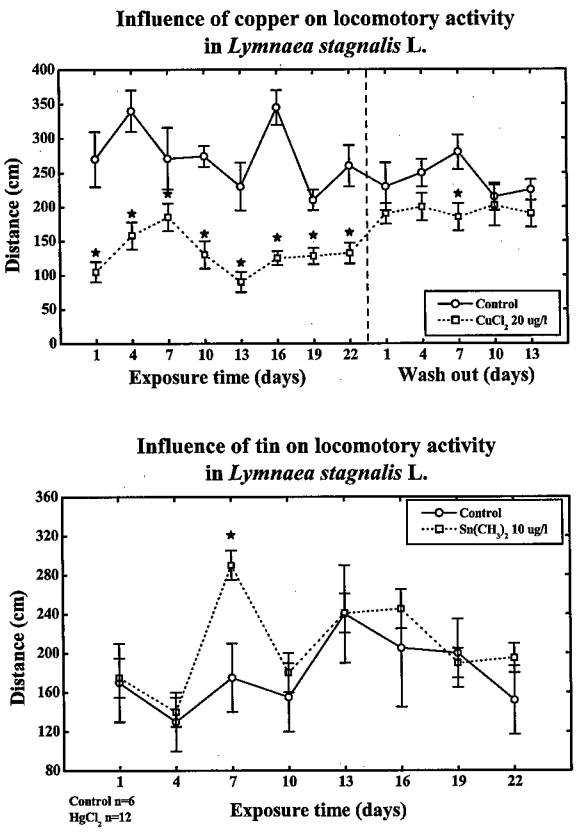

Fig. 6. Effects of copper (upper) and tin (lower) on the locomotory activity of *Lymnaea*.

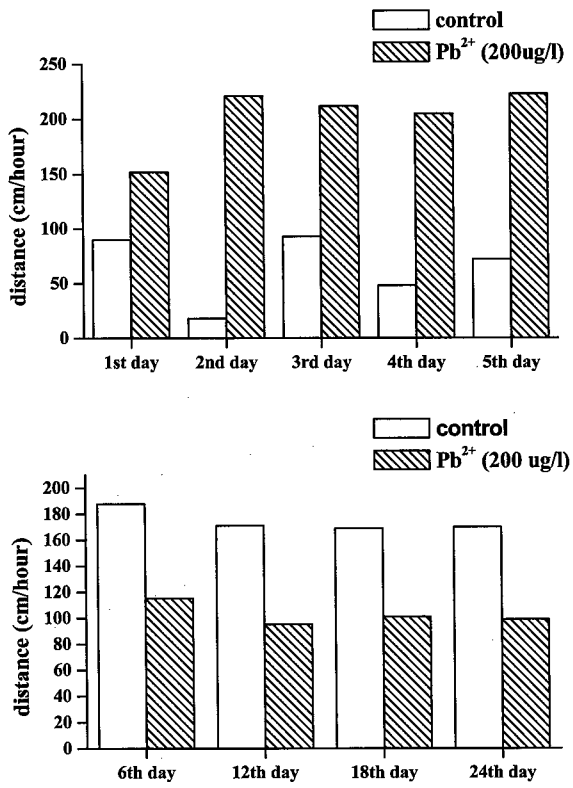

Fig. 7. Biphasic effect of lead on the locomotion of the snail during chronic treatment.

The locomotory activity of *Lymnaea* was disturbed both by acute and chronic treatments. In most cases a strong depression was observed (Hg and Cu treatment; Figs. 5 and 6), which could be eliminated by wash out (Fig. 6, upper). Some metals did not cause reduction in the locomotion, in contrary, stimulated the movements (Sn; Fig. 6, lower). In chronic exposure Pb exerted a biphasic effect: during the first 5 days it stimulated the locomotion, but depressed afterwards (Fig. 7). The disfunction in animal locomotion can be connected to the functioning of the chemosensory organ, the osphradium, or directly to the effect of the metals on the central regulation (Kamardin et al., 1999).

The results proved that *L. stagnalis* is a relevant indicator species for studying the effect of heavy metals and possibly of other pollutants. This model can contribute to monitor sublethal effects of chemicals in the aquatic environment.

4. Conclusions

Among aquatic animals molluscs, both bivalves and snails are excellent objects in monitoring the presence and effects of toxic substances representing danger for livings in aquatic ecosystems. Molluscs can be applied in passive and active monitoring alike. The methods presented in this paper are easy to install and introduce in any biological or toxicological laboratory, where biological monitoring seems to be necessary in testing the level of pollution of natural waters or the toxicity of substances polluting surface waters.

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Evaluation of some alternative guidelines for risk assessment of various habitats

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Abstract

Recently, some relatively inexpensive and simple methods suitable for classification of dangerous wastes and pollution of surface water have been developed. In order to compare the performance of the various alternative bioassays a series of examinations have been carried out with *Thamnotox* kits and *Daphnia* heart test. In addition, duckweed, as a relatively new bioindicator has been used. The alternative methods have been used for the monitoring of river Tisza, and for detection of the pollution of some watersheds. The results revealed that the alternative tests are sensitive to indicate the combined effect of various pollutants in aquatic ecosystems. It seems to be that the tests using crustacean species would give realistic results in well-defined point sources, but they can be accepted somewhat more sceptic way when comparing samples taken from different habitats. *Daphnia* heart rate were specific only to a limited number of compounds like pyrethroid type pesticides. For some mixtures of pollutants there was a correlation between *Thamnotox* and *Daphnia* heart data. In the case of *Lemna minor* at some sites instead of growth inhibition a stimulation can be detected. The toxicity estimation with *Lemna* strains will give us a habitat-specific indication of the pollution. An important conclusion of the results is that there is no universal bioassay available for detection of all types of pollution. The best results can be obtained with employment of a battery of bioassays.
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Keywords: Risk assessment; Bioindicators; Pollution; Surface water; *Lemna* test; *Daphnia* heart test

1. Introduction

Recently the use of bioindicators has been more and more employed in the ecotoxicology (Moriarty, 1990; Lopez-Barera, 1995). They give us possibility of an easy estimation of the environmental toxicity at low (sublethal) doses. In a natural ecosystem, all the interacting biological components of the system at risk are exposed either simultaneously or successively to chemicals under variable, site-specific environmental conditions, and bioindication seems to be suitable for testing of these summarized effects.

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Some relatively inexpensive and simple methods suitable for classification of the pollution of surface water have been developed (Persoon and Jansen, 1998; Persoon, 2000). These biological assays, especially *Microbio-test kits* required a validation procedure before any acceptance for environmental risk assessment. To compare the performance of the various bioassays and that of the conventional toxicity tests a series of examinations have been carried out with Thamnotox kits being presently prepared for standardization in Hungary (Törökné and Takács, 2000; Kiss et al., 2002).

Duckweed (*Lemna minor*) is a relatively new bioindicator suggested by United States Environmental Protection Agency (1996) and OECD experts (Jenner and Jansen-Momnmen, 1989; Huebert and Shay, 1993). The objective of this toxicity test is to quantify substance-related effects on vegetative growth. The main advantage is *L. minor* is a species easy to collect and cultivate in laboratory, and can give rapid test results. (Wang, 1990). However, a validation procedure is required before the final standardization. For this reason international ring tests were established (Fairchild et al., 1997).

Daphnia magna is a standard organism to use in bioassays because it is sensitive to changes in water chemistry and is simple and inexpensive assay to establish in laboratory (Kuehn et al., 1989). It is common to conduct bioassays using endpoints other than death, e.g., the heart rate of *Daphnia* is measured under microscope (Kiss, 2001).

For all of the above test systems a certain degree of habitat-specificity has been demonstrated (Moriarty, 1990; Kiss et al., 2001). In addition to collecting data for the standardization, our aim was to clear up the extent of this habitat-specific indication of the pollution.

2. Materials and methods

2.1. Thamnotox kit F

Acute (24 h) bioassay is performed in a multiwell test plate using instar II–III larvae, which are hatched from dormant eggs. Hatching of *Thamnocephalus* eggs was initiated 24 h before the start of the toxicity test under standard conditions (25 °C under continuous illumination). The hatching medium was prepared by transferring standard freshwater into a vial and adding an appropriate amount of distilled water. Dilution series of the test compound was prepared in the case of surface water. When acute toxicity of a known chemical compound was measured, the percentage mortality for 24 h LC_{50} value was defined. In this case the test design was based on one control and five toxicant concentrations, each with three replicates of 10 animals. The results are presented as percentage mortality.

After 24 h incubation of the test plate at 25 °C in darkness the dead animals in each test well were counted, then LC_{50} values were calculated. Potassium dichromate ($K_2Cr_2O_7$) was used as reference chemical.

2.2. Lemna test

For cultivation standard SIS nutrient (Swedish Standard Institute, 1995) was used. The colonies consisted of 10–10 fronds each were exposed in 150 ml glass vessels for 7 days, in 8000 lx continuous illumination and at a temperature 23±2 °C. From the natural logarithm of fronds—ln(F)—specific growth rate (μ) were calculated. From the same curve we can derive the doubling time (T_D) = ln 2/μ. For the *Lemna* strain used for water quality assessment the value of T_D was 4.2.

2.3. Daphnia heart rate

One *Daphnia* was placed in a drop of standard solution to a microscope slide. The control heart rate was recorded, then the measurement was repeated at 30, 60 and 90 min. The same observation was made at each concentration of the test material. 5–5 replicates were used at each concentration level. The observed heart rates were plotted into a graph against time and a regression line was drawn. The slope of the control line was calculated, as well as that obtained at the various treatments. If there is a significant change in the slope, it indicates a toxic effect.

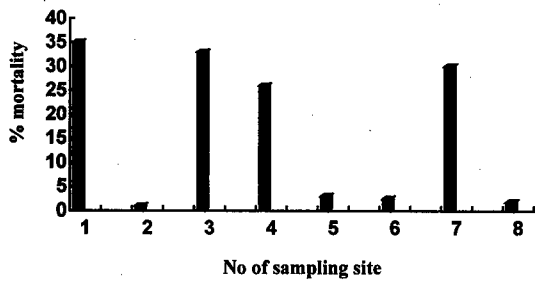

Fig. 1. Pollution of some inflows into Lake Balaton detected by Thamnotox kit (summer of the year 2000).

2.4. Statistical analysis of the data

When applicable, multiple comparison of dose groups and evaluating the significance level of the effects was made by ANOVA, then Duncan test.

3. Results

The alternative methods have been used for the monitoring of river Tisza, one of the main rivers of Hungary, as well as for detection of the pollution of some watersheds and inflows into Lake Balaton. The inflows were selected in the catchment area between Balatonfûzfô and Aszófô. The tests were performed in the period 2000–2001.

3.1. Thamnotox F

3.1.1. Lake Balaton

Results are presented in Fig. 1 as percentage mortality. The inflows are numbered from 1 to 8. Relatively high percentages of mortalities have been found at four sites indicating toxic level of pollution.

3.1.2. River Tisza

The well known cyanide and metal pollutions were in January 30, 2000 and March 10, 2000, respectively. The first water samples from the river Tisza were taken in the year of 2000 and 2001. Accordingly, the sampling dates were as follows: 12 October 2000 (1st sampling period), 24 April 2001 (2nd sampling period) and 29 October 2001 (3rd sampling period).

Table 1
Summary result of the Thamnotox test with water samples from the river Tisza

Samples	Average of living larvae	SD	% inhibition
Control	10.00	0.00	0.00
1st sampling period			
1. cc.	8.67	0.58	13.33
2. cc.	8.67	1.15	13.33
3. cc.	9.00	0.00	10.00
4. cc.	8.00	2.00	20.00
5. cc.	8.67	0.58	13.33
1. 10 × dilution	9.33	0.58	6.67
2. 10 × dilution	9.00	1.00	10.00
3. 10 × dilution	10.00	0.00	0.00
4. 10 × dilution	9.67	0.58	3.33
5. 10 × dilution	8.67	0.58	13.33
Control	8.67	2.08	13.33
2nd sampling period			
1. cc.	7.67	0.58	23.30
2. cc.	8.00	1.00	20.00
3. cc.	8.67	1.53	13.33
4. cc.	8.33	0.58	16.67
5. cc.	8.33	1.53	16.67
1. 10 × dilution	8.67	0.58	13.33
2. 10 × dilution	8.33	0.58	16.67
3. 10 × dilution	9.00	1.00	10.00
4. 10 × dilution	8.33	1.53	16.67
5. 10 × dilution	9.67	0.58	3.33
Control	9.67	0.58	3.33
3rd sampling period			
1. cc.	9.33	0.58	6.67
2. cc.	10.00	0.00	0.00
3. cc.	10.00	0.00	0.00
4. cc.	10.00	0.00	0.00
5. cc.	8.67	1.53	13.33
1. 10 × dilution	9.00	0.00	10.00
2. 10 × dilution	9.67	0.58	3.33
3. 10 × dilution	9.33	1.15	6.67
4. 10 × dilution	10.00	0.00	0.00
5. 10 × dilution	9.33	0.58	6.67

The initial number of the treated larvae was 10 in each case.

The sampling points were located from upwards the extended reservoir Tisza-tó (indicated as Nos. 1 and 2), at Tisza-tó (indicated as Nos. 3 and 4) and down the river, at Kisköre (indicated as No. 5).

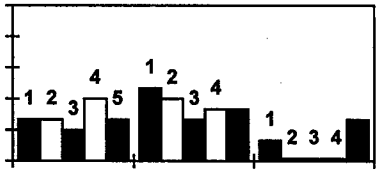

Fig. 2. Testing of water samples (concentrated) from the river Tisza with Thamnotox F. Arabic numbers: sampling points; Roman numbers: sampling periods.

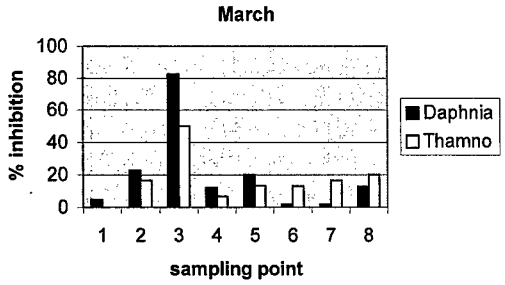

Fig. 3. Data obtained in *Daphnia* and Thamnotox test in different sampling points of Veszprém Séd (March 2001).

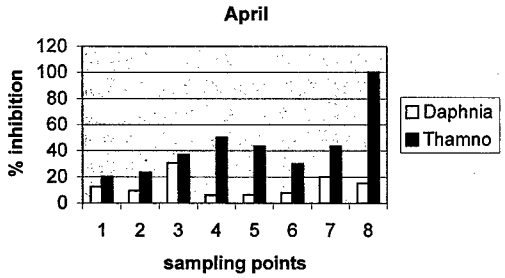

Fig. 4. Data obtained in *Daphnia* and Thamnotox test in different sampling points of Veszprém Séd (April 2001).

Samples taken from the points listed above were tested in triplicate. Both the original concentrations of the water samples without dilution and their 10 × dilutions (10% Tisza water and 90% standard freshwater) were used.

As shown in the Table 1 and Fig. 2, only one sample taken at the first sampling and two sample taken at the second sampling period (Nos. 1 and 2) caused inhibition slightly exceeding the critical 20% level of mortality, which indicated a toxic effect based on the earlier experiences.

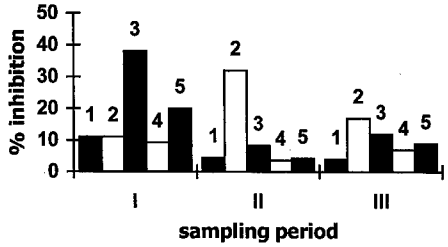

Fig. 5. Inhibitory effects of the samples from the river Tisza on *Daphnia* heart rate. Arabic numbers: sampling points; Roman numbers: sampling periods.

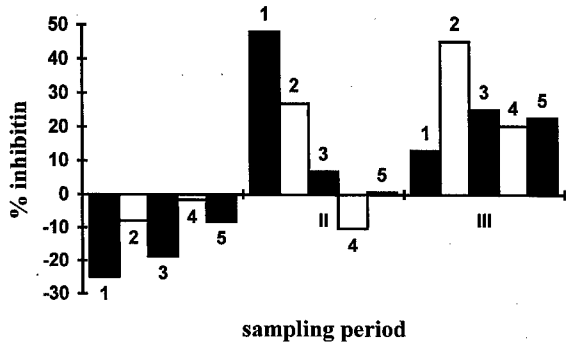

Fig. 6. Effects of the samples from the river Tisza on *Lemna* growth rate. Arabic numbers: sampling points; Roman numbers: sampling periods.

3.1.3. Veszprém Séd

Samples taken from eight points in March and April of year 2001 were tested. When comparing the two series of samples (presented in Figs. 3 and 4) it can be seen that the Thamnotox test indicated toxic effect at different sampling points.

3.2. Daphnia heart rate

The efficacy of the test was checked with pyrethroid type pesticides. We found nice dose–response functions for deltamethrin (Fig. 8).

3.2.1. River Tisza

Significant inhibition was caused by the water samples taken at some of the sampling points. The highest value of inhibition was observed at point 3 in the year 2000 and at point 2 in the year 2001, as seen in Fig. 5.

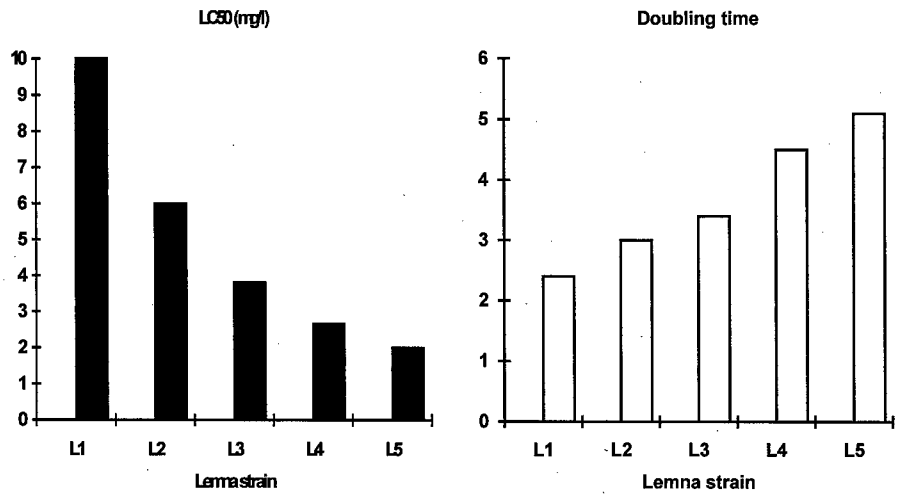

Fig. 7. T_D and LC_{50} values of various *Lemna* populations.

3.2.2. Veszprém Séd

No significant inhibition could be demonstrated except point 3. This latter effect showed a clear correlation with the Thamnotox results (Fig. 3).

3.3. Lemna test

3.3.1. Lake Balaton

No considerable changes in the growth rate were caused by any of the water samples.

3.3.2. River Tisza

In the case of the first sampling period it was found that instead of growth inhibition growth stimulation could be detected. (Fig. 6). This stimulation may point to the eutrophication potential of the analyzed sample. It is to be noted (as seen in the same figure) that in the 2nd and 3rd sampling periods an increasing growth inhibition was shown.

For *Lemna* test it seems to be very important to know the T_D value for the given strain, because an inverse relation between the sensitivity to toxic effect and doubling time (T_D) of the strains has been demonstrated.

T_D and LC_{50} values of various *Lemna* cultures collected from different habitats are summarized in Fig. 7. It seems that there is an inverse relation between the sensitivity and T_D of the strains: the more the T_D is, the less is the LC_{50} value: the sensitivity to toxic effect increased.

3.3.3. Veszprém Séd

No considerable changes in the growth rate were caused by any of the water samples.

4. Discussion

The results generally revealed that the alternative tests used are sensitive to indicate the combined effect of various pollutants in aquatic ecosystems. It has to be noted that in this series of examinations the samples have been taken from surface waters classified into classes of "no or slight toxicity" based on both conventional bioassays and chemical analysis. It is true even in the case of river Tisza, where no acute toxicity has remained, as a consequence of the cyanide and heavy metal pollution. Therefore, it is evident that any acute effects indicated a special type of sublethal toxicity unlike those tested by standardized bioassays (e.g., *Daphnia* acute immobilization). This may be one of the reasons of the heterogeneous results in different places.

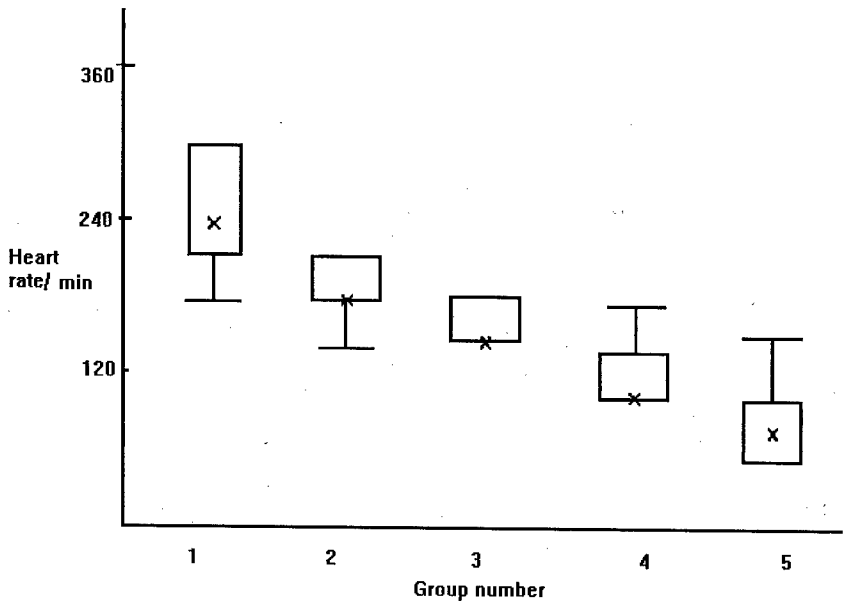

Fig. 8. Concentration dependence of the effect of Chinetrin on *Daphnia* heart rate *Y*-axis: heart rate/min, *X*-axis: Group No. 1, control; 2, Chinetrin 0.01 ml/l; 3, Chinetrin 0.05 ml/l; 4, Chinetrin 0.1 ml/l; 5, Chinetrin 0.5 ml/l.

In the two main river tests (Tisza and Danube[1]) the mortality rate in Thamnotox test was between 5 and 40%. The latter, relatively high value was detected in the most industrialized sites with a number of wastewater discharges. Here, the differences among the various tests may be caused by the selective toxicity of some compounds, mainly pesticides, heavy metals etc. It seems to be that the tests using crustacean species (also the cyst-based ones) would give realistic results in the case of well-defined point sources, but they can be accepted somewhat more skeptic, when comparing samples taken form different habitats. These results model well an "early warning" situation, when an acute, sudden concentration increase occurs like in a specific point of river Tisza (caused probably by the ferry boat), or in some sites of Veszprém Séd. The same explanation is suggested in case of the inflows of Lake Balaton, where an acute pollution may occur, probably of agricultural origin.

As to the *Daphnia* heart rate, it seems to be more specific only to a limited number of compounds. However, in case of an effect, it is one of the most powerful test methods. We found nice dose–response functions for pyrethroid type pesticides (Fig. 8).

For some mixtures of pollutants there was a correlation between Thamnotox and *Daphnia* heart data. It has been observed earlier in the Veszprém Séd (in the year 2000), probably caused by an illegal discharge of municipal waste (Kiss, 2001).

In our examinations, *L. minor* showed the most controversy results. At some sites of the river Tisza growth stimulation could be detected instead of growth inhibition. The possible reason of this behaviour of *L. minor* has been investigated. According to the results, T_D shows a strict correlation with the original environmental conditions of the test system. Various types of environment have been included in our tests: unstable environment characterized as low N and higher P content of water; favourable environment with optimal essential nutrient factors. Populations living originally in the first environment will show lower relative growth rate, as a final result

[1] Data was kindly given by Dr. G. Ecsedi, Charles River Hungary Ltd.

of nutrient availability. They are more vulnerable to toxic chemicals. In another experiment (Szalay et al., 2000), it has been shown that there is a nutrient optimum for *L. minor*. Either low or excess N is not favourable for the duckweed.

As a final conclusion, *Lemna* test may not give data suitable for any general environmental assessment. The toxicity estimation with *Lemna* strains will give us a habitat-specific indication of the pollution.

An important conclusion of the results that there is no universal bioassay available for detection of all types of pollution. The best results can be obtained with employment of a battery of bioassays. A similar approach has been proposed also by other authors (Persoon and Jansen, 1998; Persoon, 2000).

Acknowledgements

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Short communication

Harmonization of exposure assessment for food chemicals: the international perspective

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Abstract

The assessment of human exposure to chemicals present in the diet is a rapidly developing discipline. The formulation of the "risk analysis paradigm" by the Codex Alimentarius Commission in 1994 defined the exposure assessment as an essential step of the risk assessment process. This has re-enforced demands to those joint FAO/WHO scientific bodies who evaluate the safety of chemicals in foods to estimate routinely intakes for food additives, flavors, contaminants, and residues of pesticides and veterinary drugs as part of their safety assessments. The approaches chosen by the Joint FAO/WHO Expert Committee for Food Additives (JECFA) and the Joint FAO/WHO Expert Meeting on Pesticide Residues (JMPR) for these compounds are considerably different. These differences can only be understood when considering the different risk policies of the Codex Alimentarius Committees involved. Specific problems emerge if global intake assessments are requested; lack of representative regional data for consumption patterns and insufficient knowledge about levels of chemicals occurring in foods in many countries bear the risk that exposure assessments do not provide risk managers with a true global picture. There is a need to improve the collection and dissemination of such data.
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Keywords: Exposure assessment; Intake assessment; Food additives; Contaminants; Residues of veterinary drugs; Residues of pesticides; Food safety

1. Introduction

In this age of globalization, the use of the word global often leads to some undesired reflections by readers, therefore, international is used in the title of this essay. It deals with issues related to the application of the risk analysis paradigm to food chemicals within the framework of the Codex Alimentarius, which is essentially a set of quantitative and qualitative food standards intended to be applied and implemented globally. De facto, we will speak therefore about global exposure assessment, the concept, the strengths, and weaknesses of exercise. The intention of this paper is to contribute to a discussion which is starting to address this essential activity of risk assessment searching for improvements and a better and more coherent understanding of what we, the scientists

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involved, can and cannot achieve when assessing exposures of food chemicals.

2. Background

The basic question how much an individual or a population ingests of a certain food chemical is answered in a very simple way (Douglass and Tennant, 1997): intake from food = food chemical concentration × food consumption; total intake = sum of intake from all foods containing the compound. The answers to more specific questions for different classes of food chemicals like pesticides or food additives, however, are quite different, differences which are caused by the use patterns of the chemicals and by the human factor, i.e. by the different scientific and regulatory committees involved in the work. Furthermore, the endeavor for a more consistent approach in risk analysis increased notably on an international level only after the methodology for the various classes of food chemicals had already been developed and applied, in some cases for many years. Before discussing these methodologies it is necessary to understand the framework of risk analysis for food, how it is structured and working today.

International regulatory standards for food are developed and adopted mainly by the Codex Alimentarius Commission and its affiliated committees, which discuss and draft specific texts. More than 160 member countries of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) and an increasing number of international non-governmental organizations participate and contribute to the Joint FAO/WHO Food Standard Programme (http://www.codexalimentarius.net). Whereas this activity falls under the definition of risk management, i.e. "the process of weighing policy alternatives to accept, minimize, or reduce assessed risks" (WHO, 1995), the scientific assessments of risks associated with food are undertaken separately under the joint responsibility of FAO and WHO. Activities of risk assessment related to food are usually carried out by Joint FAO/WHO Expert Committees, joint meetings of experts panels, or joint ad hoc consultations. As risk management body, the Codex Alimentarius Commission, does not undertake risk assessments, it will, however, develop risk assessment policies and determine priorities for the scientific bodies responsible.

As an answer to the changes of rules for international trade, a Joint FAO/WHO Expert Consultation discussed and suggested in 1995 the ways how risk analysis for food-related issues should be addressed in future (WHO, 1995). Following previously developed approaches risk analysis was understood to consist of three components: risk assessment, risk management, and risk communication; the process of risk assessment was further subdivided into hazard identification, hazard characterization, exposure assessment, and risk characterization. According to this consultation, exposure assessment is "the qualitative and/or quantitative evaluation of the degree of intake likely to occur". Due to the interplay between the Codex Alimentarius Commission, its Committees, and the scientific bodies this rather short and broad definition does not provide specific guidance how exposure assessment for food chemicals shall be undertaken. After this top-down view it is advisable to study the approaches which have been developed by the different expert bodies for individual classes of food chemicals.

3. Food additives

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) met for the first time in 1956 to develop general principles governing the use of food additives (FAO, 1957). It was proposed that the intake of an intentional food additive should be substantially below any level, which could be harmful to consumers. Knowledge on the estimated potential intake was deemed to be necessary for establishing such a safe level of an additive. During the following decade, the Committee would establish normally a numerical acceptable daily intake (ADI) which is the estimate of the amount of a substance in food or drinking water that can be ingested daily over a lifetime without appreciable risk. This ADI would usually be based on data derived from animal or human

data. Expressing a safe level of intake, a special intake assessment was not performed. However, the Committee decided to accept the use of a food additive or a processing aid without expressing any numerically specified ADI value, if the very low toxicity and the total dietary intake of the substance (arising from its use at the level necessary to achieve the desired effect or from its acceptable background in food) do not represent a hazard to health. Such an ADI "not specified" intrinsically contains an intake component, which was based on data submitted by sponsors and found in literature. This approach was restricted to compounds of low toxicity. In the early times of the Committee a sophisticated quantitative intake assessment for food additives was usually not performed. Increasingly, also intakes were estimated using food consumption data by applying levels of food additives if used according to good technological practice (FAO/WHO, 1967).

At the request of the Codex Committee on Food Additives and Contaminants (CCFAC), JECFA discussed at its 14th meeting in 1970 a methodology proposed by WHO that used average food consumption data (FAO/WHO, 1971). In the absence of reliable other data this was considered to be the best approach although various assumptions were necessary. Prioritization of further studies could be done with the results of this method. It was noted that such estimates would very likely exceed the actual average intakes.

The guidance document which summarized the working principles of the Committee (WHO, 1987) mentioned three approaches for estimating exposure: per capita estimates, estimates from dietary surveys, and analytical values from market basket/total diet surveys. All of these procedures were considered to be useful for estimating food additive exposure in individual countries. On an international, global level, these methods did not provide accurate estimates. Such a task was characterized as "an extremely ambitious exercise that would require extensive resources". Under the title comparing ADI with potential exposure it was stated that "collateral exposure information is often useful" when establishing ADI, specifically when assigning "an ADI not specified". It was emphasized that for substances, which are also naturally present in food exposure information is indispensable. It was stressed that a comparison of exposure estimates and acceptable intakes should be based on similar assumptions, e.g. using data, which reflect lifetime exposure for both values.

CCFAC triggered a change when it started to work on the General Standard for Food Additives, a comprehensive document that lists all food additives accepted within the Codex Alimentarius and the conditions of use which apply to them. While doing so, it screened the lists under discussion using the budget method (Hansen, 1979). Four additives were identified which had a low numerical ADI and high proposed use levels. JECFA was asked to perform an intake assessment, a task undertaken by the 53rd meeting of the Committee in 1999 (WHO, 2000a). Whereas this meeting received considerable data for the compounds in question (e.g. canthaxanthin, benzoates), later intake assessments for food additives performed at subsequent meetings were hampered by scarce and disparate almost arbitrary collection of data that did not permit to develop a consistent approach. At its 57th meeting in 2001 the Committee decided to perform intake assessments only for new food additives or for others if from their last evaluation 3–5 years have passed. Questions of methodology were not addressed (WHO, 2002). At its most recent meeting the Committee suggested that intake from dietary sources other than food additives and exposure to non-dietary sources might also be of relevance and should be considered. It recommended that terms for intake assessment used in working papers should be standardized within the Committee and harmonized with those developed by OECD/IPCS. Revised guidelines for submission of national intake data were discussed (WHO, 2003).

4. Contaminants and natural toxins

The estimation of intakes of contaminants and toxins, whether they occur naturally or are formed during storage and processing, was always considered to be more important due to the fact that their ingestion very often cannot be avoided but at

best be reduced. Furthermore, the study of these compounds involves regular analytical surveys, which create useful data that can be used for calculating intakes of populations. Due to the health concerns caused by many of these compounds WHO very early initiated together with FAO and the United Nations Environment Programme (UNEP) the development of comprehensive guideline documents under the auspices of the Global Environmental Monitoring System (GEMS, 1985). These guidelines addressed all aspects relevant to establish comprehensive and accurate sets of data for food consumption and levels of contamination for certain contaminants. The GEMS/Food—originally developed after chernobyl accident for radionuclides—uses five regional diets to predict intake of contaminants (WHO, 1998). These model diets are derived from the food balance sheets of FAO which are based on agricultural data (production, import, and export). The five regional diets are Middle Eastern, Far Eastern, African, Latin American, and European (which includes North America). For each food commodity per capita consumption per day is calculated from these data. It is obvious that food consumption data for these regions represent rather highly aggregated hypothetical diets than truly representative diets, which very often cannot even be defined on a national level.

Within the international risk analysis framework JECFA is responsible for intake assessments of contaminants and toxins. When undertaking this work the Committee considers all information available and examines national survey data but also studies of other kind, e.g. undertaken with sub-populations. The Committee's work has regularly been hindered by the skewed geographical distribution of data. At the 57th meeting for dioxins and related compounds only data from some European Union member states, Canada, Japan, New Zealand, Poland, and the US were available (WHO, 2002). Nevertheless, there is an expectation from the Codex Alimentarius Commission to receive from JECFA a truly global picture. The limitation of data leads easily to estimates that cannot be validated sufficiently. This is specifically caused by the use of analytical data and food consumption from different regions, countries, or markets. At the 57th meeting, for example, analytical data on the concentrations of 3-chloro-1,2-propanediol in foods especially soya sauce were available from the UK and the US; consumption figures for possibly contaminated foods were submitted by Australia, Japan, and the US. The Committee estimated with these data potential high intakes of chloropropanols in the Japanese population (WHO, 2002). However, no information was available whether those sauces consumed in Japan contain the contaminant at such high levels (higher levels are associated with sauces which involve hydrolyzation of protein with hydrochloric acid using high temperatures; traditional fermentation does not lead to the formation of chloropropanols).

One specific issue which has slowed down the evaluation of contaminants and toxins is the decision of CCFAC that any discussion of possible maximum levels in foods was a question of risk management a topic that shall not be addressed by JECFA. In extremism, this approach leads to repetitive exposure assessments by JECFA, first of the best data available, then after CCFAC has established maximum limits for food commodities, the impact of these levels need to be reassessed by JECFA (WHO, 2000b). The elaboration of international food standards is significantly delayed by this stepwise interaction between CCFAC and JECFA.

4.1. Residues of veterinary drugs

Whereas JECFA will not discuss or propose maximum limits for contaminants and food additives, it will do so for residues of veterinary drugs. Since 1987 the Committee has evaluated more than 80 veterinary drugs and proposed maximum residue limits (MRLs) accordingly. Most of these MRLs have been adopted by the Codex Alimentarius Commission after thorough discussions within the relevant Codex Committee on Residues of Veterinary in Foods (CCRVF). The exposure assessment performed by JECFA is an integral part of the evaluation process. When discussing possible maximum residue levels the Committee uses a basket of food items with

hypothetical consumption figures thought to reflect the upper limit of the range of these foods (WHO, 1989). Daily food intakes applied are meat: 300 g, liver: 100 g, kidney: 50 g, fat: 50 g, eggs: 100 g, and milk: 1.5 l. These food consumption factors are used both by the US Food and Drug Administration and, with small amendments, by the EU Committee on Veterinary Medical Products. The potential intake is estimated by multiplication of MRLs and the food consumption factors. The intake figure from this food basket is then compared with ADI of the compound.

The approach chosen by JECFA for residues of veterinary drugs was justified by the difficulties to obtain reliable data on consumption of animal products. Furthermore, reliable data on the distribution and fate of such residues during cooking were considered to be rarely available. However, these aspects, which lead to gross overestimate of possible intakes and adds a further safety factor to ADI which is derived itself by applying safety factors, are rapidly forgotten when these MRLs are discussed by risk managers afterwards. The intake estimate based on MRL and the hypothetical food basket is considered as a realistic picture of then true exposure. The experts of JECFA themselves discussed several times regarding changes to these food consumption factors but considered them to be "realistic yet conservative values for consumption of edible animal products and, therefore, protective of human health" (FAO, 2000).

4.2. Pesticide residues

Dealing very often with a far larger basket of foods that potentially may carry residues of pesticides, the prediction of intakes is undertaken differently. It was recognized that the use of MRLs for the calculation of potential intake estimates would lead to an overestimation of the true pesticide residue intake. A joint FAO/WHO consultation was developed therefore in 1987 (FAO/WHO, 1988), an approach which is used with some modifications (GEMS, 1997) by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and the Codex Committee on Pesticide Residues (CCPR).

In a first run based on the commodity MRLs adopted by Codex and average food consumption figures (from above-mentioned GEMS/Food), a theoretical maximum daily intake (TMDI) is calculated. This TMDI is an overestimate of the true intake due to a variety of factors: not all lots of a specific crop are treated; levels will vary and will very often be significantly lower than MRL; processing of food items will affect the residue concentration; and consumers will not consume a specific item over lifetime always contaminated with this pesticide. If TMDI exceeds ADI no conclusion is permissible that consumers are at risk. Should TMDI be lower than ADI, however, it may be concluded that MRLs provide sufficient protection for the health of the consumers (GEMS, 1997).

The second value incorporates several correction factors, and it is called the international estimated daily intake (IEDI). The correction factors employed are addressing the following major shortcomings of TMDI:

- Median residue levels measured in supervised trials (STMR).
- Edible portion of a food (MRL include non-edible parts).
- Residue processing factor (decrease and increase caused by processing methods throughout the food chain).

For food consumption figures again data from the five regional diets of GEMS/Food are used.

5. Conclusions and outlook

Estimates of intakes of food chemicals are performed differently depending on the regulatory status of the compound in question. In case of residues of pesticides and veterinary drugs even for the same compound different figures are derived (which are then added up subsequently like apples and pears). Although the latter issue has been discussed at an informal harmonization meeting

between JECFA and JMPR (FAO, 1999), no satisfactory solution is in sight.

The shortcomings of the generation of meaningful food consumption data and subsequent exposure assessments were as well a topic of another recent FAO/WHO consultation (WHO, 1997). An expansion of the GEMS/Food regional diets approach to include more realistic food consumption patterns for at least 13 regions was proposed. Furthermore, dietary exposure assessments should be done in a more consistent and transparent approach across different food chemicals. It was noted that such harmonized approaches would also require the development of joint procedures across different committees (JECFA, JMPR, and Codex Committees). Special consideration should be given to developing countries by providing them with assistance and resources for generating meaningful data.

It is anticipated that the efforts to harmonize international exposure assessments will be addressed by the Joint FAO/WHO Project to Update Principles and Methods for the Risk Assessment of Food Chemicals. This project shall review principles and procedures used by JECFA and JMPR and reaffirm those that remain valid in view of current scientific knowledge. It shall as well facilitate the incorporation of new scientific tools, approaches, and knowledge in the implementation of risk assessment of food chemicals (e.g. regional diets, dose–response modeling, and biomarkers including genomics, proteomics). The major objective is to harmonize, to the extent possible, risk assessment procedures for different classes of chemicals in food (e.g. additives, contaminants, pesticide residues, veterinary drug residues, and natural toxicants). Within the framework of this project a consultation will discuss a variety of questions related to intake assessments. Topics will comprise use of food consumption data (including sampling, analytical methods, uncertainty, and variability), estimation of acute and chronic intake, biomarkers, deterministic and probabilistic approaches, and cumulative and aggregate exposure. This ambitious project aims to improve current guidelines for risk assessments undertaken by JECFA and JMPR. It may therefore be the most suitable initiative to promote harmonization where it makes sense.[1]

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Review

Methodological aspects related to aggregate and cumulative exposures to contaminants with common mechanisms of toxicity

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Abstract

Consumers can be simultaneously exposed to more than one substance having a similar mechanism of toxicity. In those cases the total exposure should be computed (cumulative exposure). Computing aggregate and cumulative exposure requires the introduction of data about when the exposures occur and how much occurs simultaneously or within a given time frame for each substance. For example, in evaluating food exposures, ideally residues of all substances of interest will be measured in the same sample of food. Estimates of the decline in residues will be useful in circumstances where exposures do not begin and end at the same time. Typically, 'worst case' assumptions and models are too blunt to provide useful information about cumulative exposures. Therefore, data and algorithms that allow more realistic (if still conservative) assessment of aggregate and cumulative exposures are required. Several approaches, including Monte Carlo assessment methods are presented along with an evaluation of the strengths and limitations of each using a case study to illustrate the methodology and the data requirements. Understanding the major contributors to the estimated exposures is complicated and available tools and techniques will be demonstrated.
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Keywords: Aggregate; Cumulative exposure; Risk assessment

1. Introduction

Foods are complex substances composed of many biologically active substances. Most of the natural components have not been systematically studied. Rather, safety of foods has been concluded based on the historical consumption of foods. As diets change and as food composition change (through selective breeding, through modern biotechnology or through subsequent processing) the ability to rapidly evaluate potential safety issues has become critical.

Evaluations of the intake of single substances have been undertaken for a broad range of food substances—including nutrients, contaminants including pesticide residues, heavy metals, and food additives (directly added or indirectly added through packaging/contact). These evaluations have been conducted assuming that the consumer

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consumes large mounts (using conservative 'case assumptions') as well as assuming more typical levels of intake (Kroes et al., 2002; Edler et al., 2002).

Foods can contain more than one substance that has the same mechanism of toxicity and in those cases it is appropriate to estimate the cumulative exposure to the total toxic residue. Some examples include different forms of the same nutrient, dioxins, and the organophosphate class of pesticides. Cumulative exposure assessments are fundamentally similar to single substance exposure assessments. However, the analyst must adjust the assessment to reflect differences in the mode of action of each chemical within the class as well as differences in the target tissue exposure. Within a class, not all chemicals have similar potency and not all exposure profiles are comparable. This paper presents key aspects to be addressed in assessing cumulative exposure along with examples to facilitate the discussion.

The framework for estimating cumulative exposure is described in this paper. A case study, using the organophosphate class of pesticides is included in order to facilitate the understanding of the approach. The method applies to any group of chemicals with a common mechanism of toxicity.

2. The exposure algorithm

The first step in selection of the most appropriate algorithms and input data is to define the goals of the model, including the population to be evaluated, the likely source of residues, the impact of processing/cooking and whether the period of exposure to be evaluated is short (acute) or long term (chronic).

Methods developed for single chemical analysis can be readily modified to provide models for cumulative exposure assessment. The issue of acute dietary exposure and risk has arisen in the UK and elsewhere in the EU. Beginning with the 'Geneva Consultation' a Joint FAO/WHO Expert Consultation on Food Consumption and Chemicals Risk Assessment was held in 1997 (WHO, 1997), several workshops and conferences have been held to investigate both the scientific rationale of acute dietary exposure assessment and the correct methodology to use in the EU. The 1998 'York Conference' was another important meeting in the development of thinking within the EU regarding acute dietary exposure and how to estimate it (Harris et al., 2000). The 'York Conference' brought together scientists from the US and Europe to exchange ideas and discuss how the data that are available within Europe might be used for acute dietary risk assessments.

Regardless of whether chronic or acute dietary exposure is being estimated, and regardless of whether the model used is deterministic or probabilistic, dietary exposure is a simple function of the amount of food consumed and the residue concentration on the food:

$$\text{Dietaryexposure} = \text{amountoffoodconsumed} \times \text{residueconcentration} \quad (1)$$

In a deterministic assessment, point estimates of the parameters are used, whereas in distributional analyses, distributions represent the amount of food consumed, and distributions of residue values may also be used. A significant advantage of the deterministic analysis is that few resources are required. A deterministic assessment typically is conducted using summary data and may be calculated relatively quickly. In contrast, a distributional analysis, in particular a probabilistic or Monte Carlo analysis requires relatively large datasets, experienced exposure assessors, and sophisticated computational algorithms. Additionally, science policies often are necessary to guide the use of data for probabilistic exposure analyses because the way in which data are used can have a significant impact upon the outcome of the model. Distributional analyses may be conducted based upon variation in either the food consumption data or the residue data or both. An assessment is not probabilistic unless model input variables are sampled in proportion to their probability of occurrence in nature. When calculating chronic dietary exposure, the deterministic models are typically used since average exposure is estimated; that is the analysis will use a single value for both food consumption and residue concentration,

thereby yielding a point estimate of dietary exposure. Similarly, non-probabilistic distributional analyses can be used to estimate acute dietary exposure (US EPA, 1996), however, difficulties are encountered in trying to sum across foods and generally a probabilistic approach is more useful.

Cumulative exposure to more than one chemical for either chronic or acute time periods can be estimated by adapting Eq. (1) to reflect differences in the toxicity of individual chemicals. This can be accomplished by determining the appropriate toxicity measure for each chemical and then estimating the relative potency of each chemical in the group. The relative potency is used to compute comparable estimates of exposure.

3. Selection of chemicals for inclusion in the assessment

A cumulative assessment should be conducted only for those chemicals acting by a common mechanism of toxicity. The criteria for determining a common mechanism should be determined prior to selection of the chemicals. Typically, the criteria will limit the assessment to those chemicals that cause the same critical effect, act on the same molecular target at the same target tissue and act by the same biochemical mechanism of action. In some situations the chemicals may have a common mechanism of toxicity because they produce a common toxic intermediate. It may be necessary to judge compounds as acting by a common mechanism of action based on structural similarity or based on clinical observations without firm knowledge of the underlying mechanisms (e.g. common toxic effect in humans or experimental animals).

The organosphosphates provide a useful example for this paper. Three initial criteria can be applied:

- Structural: these compounds are all phosphoric acid derivatives.
- Most have pesticidal action through inhibition of cholinesterase.
- Increased acetylcholine levels can be measured at cholinergic synapses which is known to cause clinical symptoms.

Applying these criteria, some phosphoric acid derivatives, such as benomyl, would not be included since they do not inhibit cholinesterase. The UK government, the US Science Advisory Panel and the FAO/WHO Joint Meeting on Pesticide Residue (JMPR) have identified more than 30 organophosphates that do inhibit cholinesterase and that are registered as pesticides in the United States (US EPA, 2002). Each of these compounds has been tested for toxicity. The characteristics of that toxicity have been found to vary. The differences are reflected in different ADIs and acute reference doses established by JMPR and other regulatory bodies for members of this class of chemicals.

4. Determination of relative potency of the chemicals to be assessed

Cumulative exposure assessments must take into account the differences in toxicity. In particular it is important to consider toxicodynamics, toxicokinetics and dose response in determining the relative potency of each compounds and in designing the exposure assessment. Again the organophosphates provide a useful example. Two toxicodynamic measures can be included: reactivation rates of the cholinesterase following inhibition and a related phenomenon called 'aging'. Toxicokinetics of organophosphates vary in that some require P450 activation, the elimination times also vary and the metabolic pathways can be different or shared.

Dose response is particularly important in evaluating exposure. Dose response has been extensively studied for the organophosphates and found to vary by several orders of magnitude between the least and most toxic members of the class. The dose response even varies for different tissues and different endpoints for the same compound. It may also be different when basic biochemistry is evaluated as opposed to clinical signs. In the case of the organophosphates the correlation between acetyl cholinesterase and clinical symptoms varies.

In conclusion, the analyst must select the endpoints that are to be evaluated and determine the

dose response and the time course of the reaction for each chemical prior to determining the corresponding exposure.

5. Estimating cumulative exposure

In selecting the most appropriate methodology to estimate cumulative exposure is useful to understood how and when exposures will occur. Fig. 1 represents a theoretical projection of exposures to two chemicals and the sum of those two chemicals. Although the underlying algorithms and data are complicated by the need to consider the presence of more than one chemical, the fundamental exposure algorithm remains:

$$\text{Exposure} = \text{amountoffoodconsumed} \times \text{totalresidueinthefood} \quad (2)$$

Toxicokinetic data (half-life, volume of distribution, clearance, and metabolic profiles) can be used in combination with estimates of residues in foods consumed in order to estimate the contribution of previous days exposure to the total exposure as shown in Fig. 1. Different subgroups of the population may metabolize/excrete a compound differently. Recovery times for many compounds are different and even for the same compound may be different at different stages in development (Ginsberg et al., 2002). In those cases different patterns will be required for each population.

In selecting methodology, it is also important, to determine what toxicity endpoint will be used to compare the results and whether a different endpoint is needed when considering short-term exposures versus long-term exposure. Fig. 2 is an example of comparing the exposure over time with different toxicity endpoints. Fig. 2 displays the exposure over time to a single individual. Once the exposure over time has been computed for each individual, decisions must be made as to how to combine those, e.g. do we estimate.

6. Selection of residue data and determination of co-occurrence of residues of the compounds of interest

Estimates of the levels of each chemical in foods as they are consumed will be most useful. Total diet studies and other types of market based studies that provide estimates in foods at or near the point of consumption provide more realistic estimates of consumer exposure than will 'worst case' control trial studies. Where only controlled studies are available, it will be important to

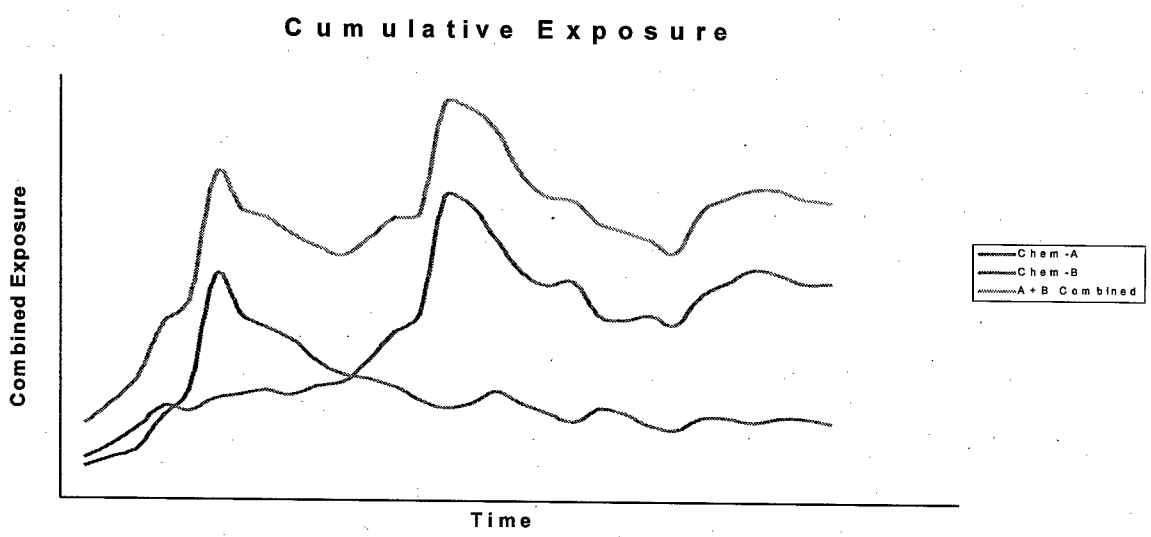

Fig. 1.

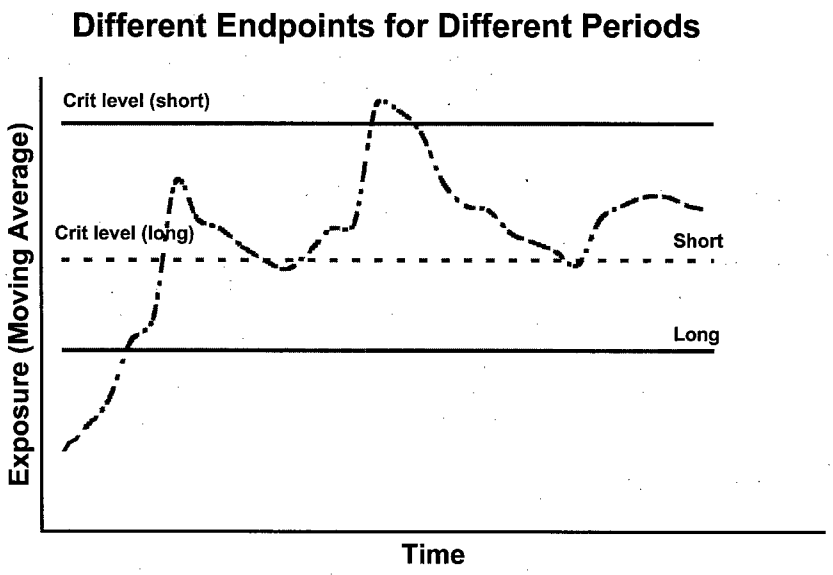

Fig. 2.

include estimates of the impact of processing and/or market share information in order to provide a meaningful estimate of exposure.

Typically many samples will not contain detectable residues of one or more of the chemicals. In the case of the organophosphate compounds, the US Department of Agriculture found that more than half of the samples of most foods did not contain any detectable organophosphate compounds (Table 1). Therefore, assumptions must be made about whether the sample contains any residues. When evaluating the exposure of one compound standard conventions have been to assume that the sample contained either or no residues, 1/2 LOD or the full LOD. These same conventions can be applied to estimating cumulative exposure. However, the conservative use of the 1/2 LOD or the full LOD can lead to inflated exposure assessments in the cumulative assessment, e.g. a sample with no detectable residues could be predicted to contain 29 times the LOD when evaluating a cumulative assessment for the organophosphate chemicals. One alternative is to review the usage data and assign a '0' residue to any crop not likely to have been treated with the particular compound.

Information about the simultaneous presence in a food of the chemicals under evaluation is critical to predicting cumulative exposure. In the case of pesticides, compounds with similar mechanisms of toxicity may also have similar mode of pesticidal action. Therefore, a farmer would likely select one from the class to use to control the pest. In some cases different compounds may have different spectrums of action so that more than one compound might be applied during the growing season at different stages of growth, as a mix to reduce resistance or for other pest control strategies. Although the vast majority of samples do not contain multiple residues of organophosphates, more than one residue are sometimes found as can be seen from Table 1.

7. Use of the residue data

The most straightforward approach will be to select one of the chemicals of the group to serve as the 'reference' compound. The potency of other chemicals will be estimated in relation to the 'reference' compound potency, e.g. compound 'A' is twice as toxic as the reference compound

and is assigned a potency factor of two. The residue data is then adjusted by the potency factor and a distribution of residues levels compiled. Each residue can also be adjusted by factors reflecting the impact of processing and/or market share as appropriate.

Table 1
Co-occurrence of Organophosphate pesticide residues in samples collected by the US Department of Agriculture Pesticide Data Program (PDP) (US EPA, 2002)

Commodity	Samples analyzed	% Samples w/one or more OPs	Number of samples with indicated detects/sample							
			0	1	2	3	4	5	>5	Total
Apple juice	1554	28.1	1117	405	28	4	0	0	0	437
Apples	2289	43.6	1290	506	419	67	7	0	0	999
Bananas	1126	0.0	1126	0	0	0	0	0	0	0
Broccoli	679	3.7	654	24	1	0	0	0	0	25
Cantaloupe	1234	9.3	1119	105	10	0	0	0	0	115
Carrots	1888	6.1	1772	101	14	1	0	0	0	116
Corn syrup	454	0.0	454	0	0	0	0	0	0	0
Celery	178	52.3	84	51	39	2	0	0	0	92
Cucumbers	730	15.2	619	102	8	1	0	0	0	111
Grape juice	1379	1.5	1358	20	1	0	0	0	0	21
Grapes	1884	24.3	1427	329	120	7	1	0	0	457
Green beans	1178	26.7	863	84	194	35	2	0	0	315
Green beans, canned	854	44.3	476	36	336	6	0	0	0	378
Green beans, frozen	743	45.1	408	51	208	73	3	0	0	335
Lettuce	876	31.5	600	186	72	18	0	0	0	276
Milk	1892	0.1	1891	1	0	0	0	0	0	1
Oats, bran	45	4.4	43	1	1	0	0	0	0	2
Oats, rolled	287	5.6	271	16	0	0	0	0	0	16
Orange juice	1392	10.6	1245	138	9	0	0	0	0	147
Oranges	1892	14.4	1619	252	20	1	0	0	0	273
Peaches	1087	66.2	367	492	188	37	3	0	0	720
Peaches, canned	756	1.3	746	8	2	0	0	0	0	10
Pears	1779	56.2	780	721	244	28	5	1	0	999
Pears, canned	371	0.5	369	2	0	0	0	0	0	2
Potatoes	1401	3.1	1358	40	3	0	0	0	0	43
Soybean grain	749	55.0	337	337	74	1	0	0	0	412
Spinach	1639	23.2	1259	301	56	14	8	1	0	380
Spinach, canned	863	2.0	846	17	0	0	0	0	0	17
Spinach, frozen	715	16.1	600	102	13	0	0	0	0	115
Strawberries	1250	17.1	1036	201	12	1	0	0	0	214
Strawberries, frozen	118	24.6	89	25	4	0	0	0	0	29
Sweet bell peppers	716	44.8	395	72	179	54	13	3	0	321
Sweet corn	19	0.0	19	0	0	0	0	0	0	0
Sweet corn, canned	652	0.0	652	0	0	0	0	0	0	0
Sweet corn, frozen	653	0.0	635	0	0	0	0	0	0	0
Sweet peas	9	11.1	8	1	0	0	0	0	0	1
Sweet peas, canned	746	0.0	746	0	0	0	0	0	0	0
Sweet peas, frozen	703	24.6	529	165	9	1	0	0	0	174
Sweet potatoes	1559	16.2	1307	241	10	1	0	0	0	252
Tomatoes	1977	37.7	1231	571	149	25	1	0	0	746
Tomatoes, canned	368	17.7	303	59	6	0	0	0	0	65
W Squash	1216	4.1	1166	38	12	0	0	0	0	50
W Squash, frozen	470	2.6	458	9	2	1	0	0	0	12
Wheat grain	1563	63.9	584	192	619	177	11	0	0	999

8. Determining the amount of foods that are eaten (quantity and frequency)

Ideally, studies would be conducted that would measure residues of the chemicals of interest in the foods that are actually consumed. This can be accomplished by conducting a study in which subjects are asked to prepare twice as much food as will be consumed; half of the food is saved for analysis. This type of study is called a duplicate diet study. Duplicate diet studies are expensive and place a large burden on respondents. It is typically more feasible to rely on food consumption/nutrition surveys to estimate foods that are eaten and then combine those estimates with the estimates of residues in similar foods. This analysis can be repeated for different subgroups and applying different assumptions about the impact of processing, distributions of residues among the foods (e.g. collecting information about regional differences).

The types of data that are available vary from country to country. The most appropriate data for each analysis should be determined by a review of the characteristics of a survey and comparison to the information required for the assessment. Although many large national surveys are of very high quality and provide a great deal of solid information, they may not capture information about the specific parameters required in a given analysis, e.g. infrequently consumed foods.

In the UK, the Ministry of Agriculture, Fisheries, and Food and the Department of Health have conducted four sets of food consumption surveys between 1983 and 1997. These surveys make up the UK's National Diet and Nutrition Survey (NDNS) program. The UK has conducted surveys for infants (Gregory et al., 1995), school children (Gregory et al., 1990), adults (Mills and Tyler, 1992), and most recently, young people (NDNS, 1997). The food consumption information from these surveys is used for the various UK dietary exposure models, including the TMDI and the NESTI calculations and by other governments (Kroes et al., 2002; Edler et al., 2002). Elsewhere in the EU, dietary risk assessments may be based upon national food consumption surveys, such as the German data (BBA, 1993), or upon the regional diets developed by the FAO/WHO GEMS/FOOD program (WHO, 1997).

In the US, the food consumption component for dietary risk assessment is provided by data from the Continuing Survey of Food Intakes by Individuals (USDA, 1992, 1993, 1994, 1995, 1996, 1997, 2000). Unlike the UK surveys, the US surveys are designed as an integrated whole that samples the entire population during the same sampling period. The data may be sorted into categories according to gender, age, region, ethnicity, and so on. The current design of the CSFII collects 2 days of data from each survey participant, with the days of collection being separated by 3–10 days. In contrast, the UK surveys sample consumption for either 4 or 7 consecutive days.

The UK and US surveys differ in their general approach to sampling. In the US, the household is the sampling unit, and data are collected for all members of the selected households. In the UK, however, census and other records are used to identify the types of individuals that are targeted, for example, 6-year-old boys. Households are queried, and if a 6-year-old boy resides in the household, consumption data are collected, but only for that individual. If a 6-year-old boy does not reside in the household, data are not collected for anyone in the household.

The US food consumption database encompasses the entire country. Different consumption patterns may be distinguished for different population groups, defined according to gender, age, region of the country, ethnicity, and so on. There is not, however, a single food consumption survey that encompasses all of the national cuisines within the EU. It does seem, however, that if detailed recipe translation schemes could be developed, it should be possible to consider EU-wide consumption patterns on the basis of raw agricultural commodities.

9. Pesticide residue data

Unlike the food consumption value, which often is available from only one source (a government survey), the value of the residue portion of the equation may be provided from a wide variety of

sources. These values range from the tolerance or MRL value, to data obtained from residue monitoring programs. The most representative data should be used whenever possible.

10. Case study: cumulative exposure to selected organophosphate chemicals

In June 2002, the US EPA released an exposure assessment for OP pesticides on food commodities for seven age groups (US EPA, 2002). The cumulative distribution of food exposure for children 1–2 years of age was computed. Exposures were <0.001 mg/kg for $>90\%$ of the children. The highest estimated exposures are shown in Table 2. EPA also evaluated the data to determine the foods that contributed most to the exposures in the upper end of the distribution. For this age group a few foods contributed most of the exposure (grape, pear, apple, apple juice, tomato, grape, snap beans and bell peppers) for those individuals who were in the highly exposed fraction of the population (US EPA, 2002).

11. Verification of modeling results

The methods discussed in this paper require the use of mathematical models. Ideally, the usefulness of the models should be confirmed using direct measurements of exposure.

Biomarkers can serve as a useful measure of direct exposure. Urinary biomarkers of OP pesticides and their metabolites have been used to characterize reference body burden levels for adult and children populations in the US and Europe (Murphy et al., 1983; Kutz, et al., 1992; Hill et al., 1995; Aprea et al., 1996, 1999, 2000; MacIntosh et al., 1999; Fenske et al., 2000; Quackenboss et al., 2000; Adgate et al., 2001; Heudorf and Angerer 2001; Krieger et al., 2001). There are at present difficulties in translating the results of measurements of substances in blood and urine to the initial exposure. Additional research is needed in this critical area.

Table 2
Upper percentiles of the cumulative distribution of food exposure for children 1–2 years of age (US EPA, 2002)

Estimated exposure (mg/kg bw)	Percent of population
0.0018	99.4
0.0016	99.77
0.0024	99.95
0.0048	99.99

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Short communication

Application of uncertainty analysis in assessing dietary exposure

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Abstract

Conventional approaches for assessing dietary exposure to contaminants and additives in food are deterministic, using point estimates for consumption and contamination. In reality, both consumption and contamination are variable. Furthermore our knowledge of them is uncertain, e.g. due to measurement uncertainty. Conventional approaches attempt to allow for this by using worst-case assumptions or safety factors, but these are often subjective and may result either in overestimation or underestimation of the true range of exposures. Probabilistic approaches take account of variability and uncertainty by using distributions rather than point estimates for consumption and contamination. The outputs are distributions for exposure, which provide a more complete and balanced description of risk for the decision-maker. These approaches also facilitate the use of sensitivity analysis to identify those factors that impact most on exposure, and to identify areas of uncertainty where additional data will improve exposure estimates. This paper reviews examples of the application of these methods to the assessment of dietary exposure to food contaminants, including dioxins in seafood, where it was found that the greatest uncertainties relate to toxicity rather than exposure. Further work required to implement probabilistic approaches for dietary exposure assessment is discussed.
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Keywords: Dietary exposure assessment; Uncertainty analysis; Probabilistic methods; Dioxins

1. Introduction

Assessment of dietary exposure is an essential element in the risk assessment process for contaminants and additives in food (Kroes et al., 2002). Conventional approaches to exposure assessment are deterministic—they use point estimates (single fixed values) for contamination of food and food consumption, and produce point estimates of exposure.

In reality, both contamination and consumption are variable. For example, in its opinion on dioxins and dioxin-like PCBs in food in November 2000, the European Commission's Scientific Committee on Food found that levels of these compounds vary over 3–4 orders of magnitude between different types of food. The levels also

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differ between countries and over time (SCF, 2000). Similarly, consumption of different food types varies widely between individuals, population subgroups (e.g. according to age), populations (e.g. regionally and nationally) and over time.

Furthermore, our knowledge of contamination and consumption is uncertain. For example, there may be significant uncertainty in the analytical measurement of contamination levels (Ellison et al., 2000). In addition, there may be substantial sampling uncertainty, if only a small number of samples are analysed for each type of food. Similarly, food consumption survey data are affected by many potentially important sources of uncertainty (Table 3 in Kroes et al., 2002).

Conventional approaches to risk assessment may attempt to allow for variability and uncertainty by using worst-case assumptions or safety factors. For example, the theoretical added maximum daily intake (TAMDI) and the maximised survey-derived daily intake (MSDI) or 'per capita $\times 10$' approach are point estimates of exposure that were designed to incorporate some degrees of conservatism into the assessment of food flavourings. However, while such approaches may appear reasonable, their relation to the true range of exposures is often very uncertain (Lambe et al., 2002).

Probabilistic approaches to risk assessment take account of variability and uncertainty by using distributions rather than point estimates (Vose, 2000). They can be used to estimate distributions for exposure (e.g. Lambe et al., 2002; Smith et al., 2002), which provide a more complete and balanced description of risk for the decision-maker. Probabilistic methods offer substantial advantages over deterministic methods for dietary exposure assessment, although there are potential disadvantages also including the increased complexity of probabilistic methods, the lack of encouragement for them in regulatory guidelines and the difficulty of accounting for interdependent variables (Kroes et al., 2002).

This paper reviews some examples of the application of probabilistic methods to the assessment of dietary exposure to food contaminants and discusses what further work is required to implement these methods more widely.

2. Examples of uncertainty analysis in exposure assessment

Risk assessment methodologies vary within and between different areas of analysis due to variety of historical, legal and policy approaches taken. Even within one government organisation (United States Food and Drug Administration: FDA) risk assessments are very diverse (Gaylor et al., 1997). Risk assessments for dietary exposure are usually performed by using single point estimates (deterministic) rather than by including variability or uncertainty explicitly (probabilistic or stochastic).

Probabilistic risk analyses are more commonly performed in other diverse areas (e.g. radioactive contamination of food pathways following a nuclear reactor accident: Helton et al., 1995; mercury contamination: Seigneur et al., 1999) and in some aspects of dietary intake assessments (e.g. mycotoxins: Pieters et al., 2002; dioxins: Smith et al., 2002, unpublished data). This approach can be applied to multiple exposure pathways, as illustrated by Moschandreas et al. (2001) for inhalation, ingestion and dermal exposure to two pesticides. These authors showed that the relative uncertainty for dermal exposure was greater than for inhalation and ingestion. However, the number of papers describing the results of specific dietary quantitative risk analyses (QRA) is very limited (Vose, 1998) and appears to be outnumbered by those papers proposing QRA (e.g. McNab, 1998; Vose, 1998; Walker, 2002; van Gerwen et al., 2000).

General introductions and reviews of probabilistic risk assessment can be found in Jaykus (1996) and Lammerding (1997) for microbial food safety. Quantitative risk analysis frameworks have been proposed to illustrate an approach to microbial food safety assessments (McNab, 1998; Vose, 1998). Microbial risk assessment is complicated by the differential growth of organisms at different storage temperatures and for different time spans. Therefore, models are required to estimate bacterial growth (e.g. Walls and Scott, 1997). A probabilistic assessment for the mycotoxin deoxynivalenol has illustrated that health effects may occur to young children, but the effect is uncertain due to large confidence limits (Pieters et al., 2002).

There is an increasing interest and pressure to perform risk analyses on chemical cocktails rather than on a single chemical, where they have a similar mechanism of action (Lefferts, 2000), and this will lead to increased uncertainty about the model specification: chemical interactions may be additive or synergistic. A prime example is the combined assessment of dioxin-like substances (PCBs, PCDDs and PCDFs). In this case, a number of congeners have been assigned to a relative toxicity factor (TEF) and the model is additive to produce a total toxicity estimate (TEQ), for which there is a tolerable daily intake (TDI; van den Berg et al., 2000). Probabilistic analyses have demonstrated that the uncertainty associated with the TEF values (toxicity) is much more important than analytical uncertainty (Smith et al., 2002, unpublished data).

A number of papers have outlined potential problems and pitfalls using QRA in dietary intake assessments. A distribution of dietary exposure is often a required input for probabilistic risk models. A specified probability distribution function may be used, or the distribution may be taken from observed data. All measurements are associated with errors and this may lead to a bias when using observed data to estimate the tails of the input distribution (Stanek, 1996). Models of dietary exposure are often based on short-term food diaries of limited numbers of people. The extreme upper tail of intake distribution is always poorly defined in relation to estimates closer to the mean, and this uncertainty should somehow be included in the risk analyses. Diary databases have limitations based on sample size. In a sample of 1000 individuals, only one person is used to define the 99.9 percentile. Such limitations may skew the exposure estimate and overestimate exposure (Wolt, 1999). If individual dietary inputs are used to estimate long-term exposure, dramatic overestimation may occur where dietary intake is right skewed (e.g. log-normal; Stanek et al., 1998) and this needs to be corrected.

Deterministic estimates of risk will not always agree with probabilistic methods as deterministic analyses are often performed by assuming a high percentile for all inputs, giving an unrealistic result (see, e.g. Whitmyre et al., 1992). There is thus a need for more published comparisons of deterministic and probabilistic risk analyses (e.g. Wenning, 2002; Whitmyre et al., 1992).

Measurement uncertainty can be estimated by performing duplicated sampling and analysis. This approach has been used to balance measurement uncertainty against the costs of analysis to produce an optimised uncertainty analysis to reduce costs (Ramsey et al., 2001). There is a clear need to harmonise the definitions of analytical measurement uncertainty (Willetts and Wood, 1998), particularly if such uncertainty is being used to generate probability distribution functions for probabilistic risk assessment.

3. Uncertainty and the limits of detection

Frequently the contaminants and additives for which dietary exposure is of interest are present at trace concentrations. Surveys for the analyte will often produce measurement results that are below the limit of detection (LOD). If the measurements of the concentration of an analyte are used to calculate an estimate of the exposure of consumers, the method used to calculate the LOD, and the way how the results recorded 'below the LOD' are interpreted will affect the outcome of the assessment.

During an ongoing collaborative trial (measurement of sweeteners in food) carried out by the authors, participants have been asked to estimate the LOD of the method and to describe the method used to estimate it. The LOD reported by the participants varied by a factor of 15. Among the methods used to calculate the LOD were:

1/10 the lowest calibration standard;
1/20 the lowest calibration standard;
3 × height of baseline noise;
visual assessment;
2.35 × the repeatability standard deviation of the lowest calibration standard;
2.95 × the sum of repeatability standard deviation of a 'blank' sample and the repeatability standard deviation of a 'near blank' sample.

Thus the meaning of LOD, in practice, is not well defined and its value can be unpredictable.

Results reported as less than an LOD are usually treated in three different ways for the purposes of deterministic intake estimates:

as being equal to the LOD (produces positive bias),
as being equal to zero (produces negative bias),
as being equal to half the LOD (may produce positive or negative bias).

Thus, for the same measurement result below the LOD, the point estimate of the concentration of sweetener in the sample could be zero or a number that varies by a factor of 30.

For the purposes of a probabilistic intake estimate, a result that is reported as less than the LOD is usually equivalent to being equal to half of the LOD with a dispersion described by a flat distribution covering the range from zero to the LOD. This, like the analogous point estimate procedure, can lead to bias although the range of resultant values will include the 'real' result. Given the wide range of practice regarding LOD for many trace analyses, intake estimates that differ from survey to survey are likely to be a reflection of different practice in estimating and recording LOD rather than a reflection of changes in concentration of analyte in samples.

The use of detection limits (and other related limits) in analytical chemistry has been argued against by Thompson (1998), and alternative methods of recording the measurement of near zero concentration have been discussed by the Analytical Methods Committee (2001) of the Royal Society of Chemistry. They conclude that interpreting results for non-expert clients by applying reporting limits of some kind is acceptable, even necessary. But a result that is reported to a knowledgeable user, or a result produced for an unknown purpose (e.g. when published) should be reported exactly as it occurs alongside an estimate of the uncertainty associated with it.

If the recommendation is followed that all results should be reported alongside an estimate of their uncertainty, the measurements that are currently reported as 'below the LOD' can be combined with other 'below the LOD results' to extract useful information, such as the dispersion associated with results below the LOD. Also, it will be possible to use the results directly in exposure estimates without applying assumptions that will lead to an avoidable bias in a deterministic exposure estimate or increase the range of uncertainty of a probabilistic estimate.

4. Discussion and conclusions

The examples reviewed above illustrate the two main features of probabilistic approaches to exposure assessment: they provide quantitative measures of variability and uncertainty and they can identify which sources of the variability and uncertainty are most important. These features offer substantial advantages to scientists and decision-makers. Probabilistic assessments provide a more complete description of risk than deterministic methods and avoid the difficulties associated with using worst-case assumptions. Identifying key sources of variability (e.g. consumption of oily fish) can help target risk reduction measures, while identifying key sources of uncertainty (e.g. number of samples analysed for dioxins) can help target additional studies if the assessment needs to be refined.

Further work is required to facilitate wider implementation and acceptance of probabilistic approaches for food risks. Some general recommendations concerning exposure assessment have recently been made by Kroes et al. (2002). Here we focus on some specific requirements suggested by our own experience.

The importance of accounting for dependencies amongst the parameters in probabilistic models is frequently emphasised (e.g. US EPA, 1997), and omitting significant dependencies can cause very misleading results (Ferson, 1996). In practice, existing assessments rarely do more than discuss possible dependencies. If probabilistic methods are to become accepted for regulatory use, it will be important to identify and incorporate significant dependencies explicitly.

The most obvious form of dependency in consumption data is between intakes of different

food types for the same individual. Most of these dependencies are likely to be negative because total consumption is limited, and so increased consumption of one type of food will often be associated with decreases in consumption of other types. However, some dependencies may be positive, at least in short-term data sets, because some food types tend to be eaten together. It would be difficult to quantify and incorporate all these interdependencies in exposure modelling. This difficulty was reduced in the model of exposure to dioxins in salmon (Smith et al., 2002) because it estimated exposure for a hypothetical average consumer and did not simulate individual variation. It can also be avoided in models of individual exposure, if the consumption of different food types by the same individual is treated as a fixed vector and kept together in simulation modelling, thus preserving any dependencies that may be present.

Several types of dependencies may be important when modelling dioxin levels in food. First, levels of different dioxin congeners in the same individual sample are likely to be positively correlated, if they derive from the same source. The models described by Smith et al. (2002) partly incorporate this by treating the concentrations in a single sample as a fixed vector. However, concentrations below the LOD were simulated by independent sampling from uniform distributions, whereas in reality they are likely to show dependencies similar to those that occur above the LOD. Similarly, measurement errors for different congeners within a single sample are likely to be correlated. Future models should attempt to include both these forms of dependencies.

Another form of dependency, temporal autocorrelation, generally affects estimation of long-term exposure. Using estimates of consumption from short-term survey data when estimating long-term exposure assumes in effect that each individual retains exactly the same pattern of consumption indefinitely (i.e. perfect auto-correlation), which is unrealistic. Ferson (2001) shows that this leads to gross overestimation of between-individual variation in long-term exposure. Various methods have been suggested for extrapolating appropriately from short- to long-term consumption, and should be considered for inclusion in future models (Lambe et al., 2002).

To date, probabilistic models have more often been used for exposure assessment than for toxicity or effects assessment. The model for dioxins in salmon described by Smith et al. (2002) used simple assumptions to represent uncertainty in TEFs, and showed that this is potentially more important than the uncertainties affecting exposure. Future studies should therefore give more attention to uncertainty in the toxicity assessment.

Finally, most studies to date have used non-hierarchical Monte Carlo simulation. These methods are relatively simple to understand and are convenient due to the availability of user-friendly software. This software also permits hierarchical or 'two-dimensional' Monte Carlo, which allows variability and uncertainty to be propagated separately in the model and their effects to be shown separately in the results (Burmaster and Wilson, 1996; Vose, 2000). Given their different implications for decision-makers (see earlier), this is desirable. On the other hand, Monte Carlo methods suffer from some important disadvantages, including the need for precise information about dependencies between variables (Ferson, 1996). Future studies should therefore explore the use of hierarchical Monte Carlo, and also alternative approaches such as probability bounds analysis (Ferson, 1996). Further examination of the advantages and disadvantages of different approaches in practical examples is needed, before general recommendations can be made for regulatory use of probabilistic methods. Until a consensus on method selection is achieved, authors of probabilistic assessments should be especially careful to describe and justify their methods in detail.

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Review

Stochastic modelling of human exposure to food chemicals and nutrients within the "Montecarlo" project An exploration of the influence of brand loyalty and market share on intake estimates of intense sweeteners from sugar-free soft drinks

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Abstract

To get a more realistic view of exposure to food chemicals, risk managers are getting more interested in stochastic modelling as an alternative to deterministic approaches based on conservative assumptions. It allows to take into account all the available information in the concentration of the chemical present in foods and in food consumption patterns. Within the EC-funded "Montecarlo" project, a comprehensive set of mathematical algorithms was developed to take into account all the necessary components for stochastic modelling of a variety of food chemicals, nutrients and ingredients. An appropriate computer software is being developed. Since the concentration of food chemicals may vary among different brands of the same product, consumer behaviour with respect to brands may have an impact on exposure assessments. Numeric experiments were carried out on different ways of incorporating indicators of market share and brand loyalty in the mathematical algorithms developed within the stochastic model of exposure to intense sweeteners from sugar-free beverages. The 95th percentiles of intake were shown to vary according to the inclusion/exclusion of these indicators. The market share should be included in the model especially if the market is not equitably distributed between brands. If brand loyalty data are not available, the model may be run under theoretical scenarios.
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Keywords: Stochastic modelling; Montecarlo; Food additives; Brand loyalty; Market share; Intense sweeteners

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1. Introduction

1.1. Stochastic modelling

Ideally, the intake of a food chemical can be assessed by combining data on concentration in all food products with data on their consumption. However, the number of substances that should be monitored is huge and the market of food products changes very rapidly in relation to both product formulation and consumer preferences. Therefore, it is considered to be neither cost-effective nor necessary to collect detailed data for every substance (Lawrie and Rees, 1996). For the assessment of exposure to food-borne chemicals, the most commonly used methods in EU follow a deterministic approach based on conservative assumptions, in order to prevent consumers from being exposed to unexpectedly high intakes of certain food chemicals. Moreover, the study of exposure from diet must take into special consideration non-average individuals, and in particular those who consume relatively large quantities of foods containing higher concentrations of a substance. "Conservative" approaches are commonly adopted by assuming that the chemical is present at maximum permitted level (MPL) in all the products in which it is legally permitted. But estimates calculated under such a scenario are often implausible. To get a more realistic view of exposure to food chemicals, risk managers are getting more interested in stochastic modelling (European Commission, 1995). Stochastic modelling gives the opportunity to take into account all sources of variability in estimates of exposure. It allows the utilisation of all the available information on variability in the proportion of foods containing the chemical, in the concentration of the chemical present, and in food consumption patterns. A limited number of commercially available programmes exist for stochastic modelling and they have largely been designed for being used in other risk-laden areas such as financial services, mining, environmental impact analysis, etc., and there was therefore the need of an appropriate computer software.

The main objectives of the EC-funded project "development, validation and application of stochastic modelling of human exposure to food chemicals and nutrients" (acronym "Montecarlo") are two. The first is to develop a comprehensive set of mathematical algorithms, purpose built to take into account all the necessary components for stochastic modelling of a variety of food chemicals, nutrients and ingredients, and the second is to create an appropriate computer software. In order to engage in and validate stochastic modelling, databases of "true" intakes of food additives (based on brand-level food consumption records and ingredient composition data), pesticide residues (based on duplicate diets) and nutrients (based on biomarker studies) have been generated within a multicentre study (Montecarlo Project, 2001). Within this project, INRAN researchers were responsible for creating a "true intake" databank of exposure to intense sweeteners in a sample of teenagers and for exploring the impact of market share and brand loyalty in terms of intake exposure (INRAN, 2001).

1.2. Brand loyalty and market share

The concentration of micronutrients/additives/contaminants may vary between the different brands of the same product. It can be anticipated that the levels of concentration between the different brands of a food category will be widely different in the following cases: (1) additives that are not strictly necessary for the process that will be added in some brands and not in others, (2) micronutrients contained in categories of food products for which some brands are fortified and others are not, and (3) pesticide residues in brands of food containing organic products versus traditional ones. Consumer behaviour with respect to brands may therefore have an impact on intake exposure from a category of food. In this case, indicators of brand loyalty and market share may need to be included in a stochastic model of intake exposure.

In the marketing literature, consumers' tendency to repeat the purchase of a brand is usually referred to as brand loyalty. Increasing brand loyalty of customers is a priority objective of the marketing strategy of many food producers (Iasevoli, 2000; Hinterhuber et al., 1997). It could be a

very important factor to include in a stochastic model aimed at assessing long-term exposure to some food chemicals. Loyalty to a brand is responsible for a positive dependency between repeated events. Failure to recognise it will underestimate the risk for high intakes: the hypothesis that a consumer may always consume the brand containing the highest concentration of a food chemical needs to be considered and the probability of this situation assessed. Theoretically, brand loyalty could be one of the most important anticipated dependencies in the model. It may be included in the model under the form of conditional probabilities; in loyal subjects, the probability of consuming a specific brand is higher if this same brand has already been consumed.

In order to assess long-term consumers' exposure to brand-specific chemicals, estimates of repeat purchasing are necessary. Longitudinal data are required, such as the household purchase data, which are typically collected by the marketing research companies for various months in panels. Households' tendencies to repurchase brands can thus be estimated. Short-term brand loyalty may be assessed from nutritional surveys or consumer surveys that usually last from 1 to 14 days.

Several approaches to deal with loyalty are available from the marketing literature. Guadagni and Little (1983) introduced an approach for capturing time dependence into this model, based on the construction of a loyalty variable through exponential smoothing of past purchases. A second way to capture time dependence can be performed through purchase event feedback formulations (Jones and Landwehr, 1988), in which past purchase indicators are included among the predictor variables in the choice model. From a behavioural perspective, consumers are psychologically rewarded by the outcome of the choice decision, and this reward determines the next choice. In marketing literature, various marketing variables, such as place of purchase, price and advertising, are assessed (Wedel and Kamakura, 1997; Cuomo et al., 1998; Wedel et al., 1995). Within marketing research, quantitative analysis leads to the construction of algorithms that allow to describe, explain and predict consumer choice between different options. The final aim is to influence such choice. In the case of intake exposure assessment, only the outcome (intake) is of interest. Therefore, it should be sufficient to include behavioural variables in the model.

An indicator of brand loyalty at individual level may be the number of purchases of a single brand in proportion to the number of purchases of the food category of interest. Different cut-off points for this proportion have been used in order to categorise subjects in loyal (proportion higher than the cut-off point)/not loyal (proportion lower than the cut-off point). Indicators of brand loyalty at population level may be very simple: percentage of consumers of a specific brand that did not consume another brand of the same product, within a day, a week or any other time period of interest.

Exposure to chemicals will not depend only on brand loyalty, but also on purchase timing and purchase quantity. For example, subjects may be very loyal to a brand, but purchase the category very infrequently or in very small quantities, leading to a low exposure to the brand-specific chemical. Therefore, the model needs to consider these variables. Another important aspect is that loyalty may be correlated to the level of consumption, for example, faithful subjects could also be high consumers. Moreover, the relationship between brand loyalty and demographic variables such as age, socio-economic status and region deserves attention.

On the other hand, the market share of a product is a very simple indicator. Market share of brand A is the proportion between brand A sales and sales of the whole market, expressed in quantity. The market share of a brand depends on its "marketing mix": price, advertising and distribution. It is also a function of the "marketing mix" of the other brands. The typology of the distribution of market shares between brands varies considerably according to the categories of food products. The number of brands for a food product may be very small or extremely large. Some markets are equitably distributed between brands, whereas other markets are concentrated in a few large brands.

Market share and brand loyalty are often closely related to each other. Brand loyalty may be higher for brands which have a larger market share. There is also the possibility that market share may be inversely related to the concentration levels of additives (with producers of small brands using more additives).

1.3. Brand loyalty and market share seen in the broader context of intake exposure

Brand loyalty is usually defined as the tendency to repurchase the same brand of a product. Within an intake exposure model, the concept of brand loyalty could be seen in the broader context of product loyalty, i.e. tendency to repeat the consumption of a category of product that may contain a specific concentration of additives/pesticides/nutrients different from that of the other categories that can be consumed in alternative to it, for example,

- loyalty to a specific brand of cola (containing cyclamate) among the group of light Italian colas;
- loyalty to light soft drinks (containing sweeteners) among the group of soft drinks;
- loyalty to a specific apple species (containing a specific pesticide) among the group of apples;
- loyalty to apples (containing a specific category of pesticides) among the group of fruit;
- loyalty to organic products (with no pesticides) among the group of vegetable products; and
- loyalty to fortified candies (with added vitamins) among the group of candies.

1.4. How can brand loyalty and market share influence intake exposure? The case of intense sweeteners in sugar-free soft drinks

It is rather intuitive that when presence/absence and concentration of a chemical substance show a wide variation between brands, brand loyalty and market share may have a significant impact on intake exposure at individual level, especially in high consumers. The case of intense sweetener allows to easily illustrate this phenomenon. Intense sweeteners are allowed in specific categories of products, and different sweeteners are available to the producers. The sweeteners used and their concentration vary between brands. Considering that a product is available in three brands (A, B and C) with differing market shares (10, 30 and 60%, respectively) and that each brand contains a different sweetener (X, Y and Z, respectively), two extreme theoretical scenarios of brand loyalty can be thought up. With a scenario of total indifference of consumers towards brands (no loyalty), in the long term, each individual would consume every brand in proportion to their market share and thus ingest a "mixture" of sweeteners with 10% of X, 30% of Y and 60% of Z. In the opposite scenario of total loyalty to brands, if each individual would consume exclusively either A, B or C and therefore ingest either X, Y or Z reaching higher intakes of these substances. If no market share data are available, with the first scenario, brands A, B and C would be given the same weight (33%) and thus data would be biased in terms of intake (intake of Z would be underestimated, whereas intake of X and Y would be overestimated), whereas with the second scenario the percent of consumers of Y would be underestimated and the percent of consumers of X would be overestimated.

Point estimates of the intake of intense sweeteners are usually obtained through ad hoc codification of sugar-free products within a consumer survey. In particular, sugar-free beverages are one of the main sources of intense sweeteners in Italy as in other industrialised countries (Leclercq et al., 1999). Such consumer surveys are time consuming and expensive. Since the market of sugar-free products is continuously changing, they should be repeated in time. For this reason, developing a stochastic model to assess exposure to intense sweeteners would be particularly useful.

The importance of brand loyalty for exposure to intense sweeteners through sugar-free beverages was made clear from the observation of the Italian food ingredient database for processed foods. This database was developed in INRAN and is continuously upgraded by retrieving labels of new products and reporting additives usage levels provided by the industry. In the most common sugar-free soft drinks, large differences were found

in relation to the presence and concentration of these additives. Moreover, market share distribution is not homogeneous between brands and furthermore, the most common sugar-free soft drink is the only one containing cyclamate.

The aim of this study was to perform numeric experiments to explore tools for the inclusion of indicators of market share and brand loyalty in a stochastic model of exposure. The main objective was to investigate the differences due to the different hypothesis adopted regarding market share and brand loyalty more than to accurately estimate the intake of intense sweeteners. Even though in a limited context, this application could give useful suggestions for the appropriate application of stochastic modelling for food chemical intake studies.

2. Methods

A draft model was developed for assessing the intake of intense sweeteners from sugar-free soft drinks in teenagers.

The adopted mathematical algorithms basically follow the subsequent model:

$$E_i = \frac{[GC(1 - P/365) + GDP/365]O_i}{W},$$

where $i \in$(acesulfame K, aspartame, cyclamate, saccharin), E is exposure to the ith intense sweeteners from sugar-free soft drinks (mg/kg of body weight per day), G the number of glasses of sugar-free soft drinks per occasion of consumption (glasses per occasion), C the frequency of consumption of sugar-free soft drinks during non-dieting periods (occasions per day), D the frequency of consumption of sugar-free soft drinks during dieting periods (occasions per day), P the length of dieting periods (days per year), O_i the average occurrence of the ith intense sweeteners (mg per glass), and W the body weight (kg).

Starting from this crude model, probability distributions for each variable were derived from the data collected within the frame of the food survey INRAN-RM-2001 (Leclercq et al., 2002; Piccinelli et al., 2002). In May 2000, a questionnaire was administered to 3685 teenagers (15–19 years old) living in the county of Rome (Italy). Ten schools were randomly selected out of 256 present in the county of Rome (163 in the city of Rome and 93 in the other towns). Only one school did not participate. All students belonging to the first, second and third year were asked to fill a questionnaire related to the duration of dieting periods in the previous year and to the frequency of intake of tabletop sweeteners and sugar-free beverages during both dieting and non-dieting periods. The questionnaire was aimed at identifying females who are high consumers of sugar-free beverages and tabletop sweeteners. Questions were related to the duration of dieting periods in the previous year and to the frequency of intake of fresh fruit, tabletop sweeteners, vegetables, meal substitutes and sugar-free beverages during both dieting and non-dieting periods. Personal data such as age, sex, rural/urban residence area and education level of parents were also recorded.

The answers to six questions present in the screening questionnaire have been used to fit the distributions introduced in the model:

- with/without an attitude to diet
- prone/not prone to diet;
- dieting period in days per year: four categories;
- consumer/non-consumer of sugar-free beverages;
- number of glasses of sugar-free soft drinks per occasion: three categories;
- frequency of consumption of sugar-free soft drinks in non-dieting periods: five categories; and
- frequency of consumption of sugar-free soft drinks in dieting periods: five categories.

BestFit (version 2.0d, Palisade Corp., Newfield, NY) was used to determine the top ranking accepted distributions for the selected combinations. BestFit is a decision tool, which can be linked to Excel to fit more than 21 different distributions to data. It performs statistical tests to compare quality of fit and rank distributions by three goodness-of-fit statistics. Since data from the screening questionnaire were structured in classes, the "cumulative" option was used to input data. In

this case, the Anderson–Darling test cannot be calculated. In this study, to evaluate the quality of fit, we used the Chi-square test (Cullen and Frey, 1999). Since both test statistics and graphs should be used in interpreting the results, graphs were used to assess visually how well distributions agree with the input data (IEFS, 2001). An important criterion adopted in selecting the curves was, for each observed distribution, the minimisation of the error at the right tail. Different probability distributions were used when subgroups of the population displayed significantly different patterns. In particular, each variable was analysed according to the gender and the tendency to be on diet.

When a subject resulted to be a consumer, in order to estimate intake of intense sweeteners in the long term, our model simulated an average daily consumption of sugar-free soft drinks and also an average concentration level for each sweetener. Loyal consumers were those subjects who consume exclusively one brand; in this case, the additive average concentration level is simply the level in the consumed brand. For non-loyal consumers, who consume a mixture of brands, the average concentration level adopted was a weighted mean of the levels of the consumed brand. In this case, the weights assigned to the different brands were also simulated.

Sweetener concentration in sugar-free soft drinks was retrieved from the food ingredient database for processed foods available at INRAN. This database was recently upgraded by retrieving labels of all sugar-free soft drinks reported by teenagers in the screening questionnaire. Three brands of sugar-free soft drinks were identified; we will call them Alfa, Beta and Gamma. For each of them, additive usage levels were provided by the producer.

For each subject, a body weight was also simulated since data on intake exposure are aimed at being compared with the corresponding ADI which is expressed per kilogram body weight. To this aim, data from a random sub-sample of 207 teenagers who self-reported their body weight in a successive phase of the survey were used (Leclercq et al., 2002).

Indicators of market share and brand loyalty to sugar-free beverages were derived through specific elaboration of data relative to food purchases of 4793 Italian households collected by the market research company IHA in 1998. This database is organised according to purchase occasions and has already been used for the sulphite intake assessment (Leclercq et al., 2000). Each occasion is distinguished by family code, year, month, product weight, number of products, brand name and producer name. These data were analysed according to purchase occasions. Since not all households present in the IHA panel registered their purchases for the whole year, in order to assess brand loyalty, we excluded 536 households (11% of the sample) who registered their purchases for less than 6 months. Our sample was therefore reduced to 4257 households. For each household, the frequency of purchases of sugar-free soft drinks and of each brand of sugar-free soft drink was calculated. In order to study the tendency to buy almost always the same brand, for each single household, the number of purchases of each single brand was assessed in proportion of the number of sugar-free soft drinks bought. This allows to estimate the percentage of households who consume a mixture of brands versus exclusive consumers of a brand. The quantity of products purchased and the frequency of purchase were not taken into account in absolute terms because these variables strongly depend on the number of consumers within the family, which is not exactly known. Occasional consumers, defined as households who purchased only once a sugar-free soft drink, were excluded from the analysis. Loyalty to a brand was then assessed on the remaining households as the percentage of households who consumed more than once this brand and never consumed another brand.

Fig. 1 shows how the model allows to simulate the intake of sugar-free soft drinks, whereas Figs. 2–5 illustrate the algorithms used to simulate sweeteners' concentration. Four hypotheses regarding the availability of information about market share and brand loyalty were tested.

The first hypothesis (Fig. 2) assumes a total lack of data regarding brand loyalty and market share. In this case, the three brands are randomly

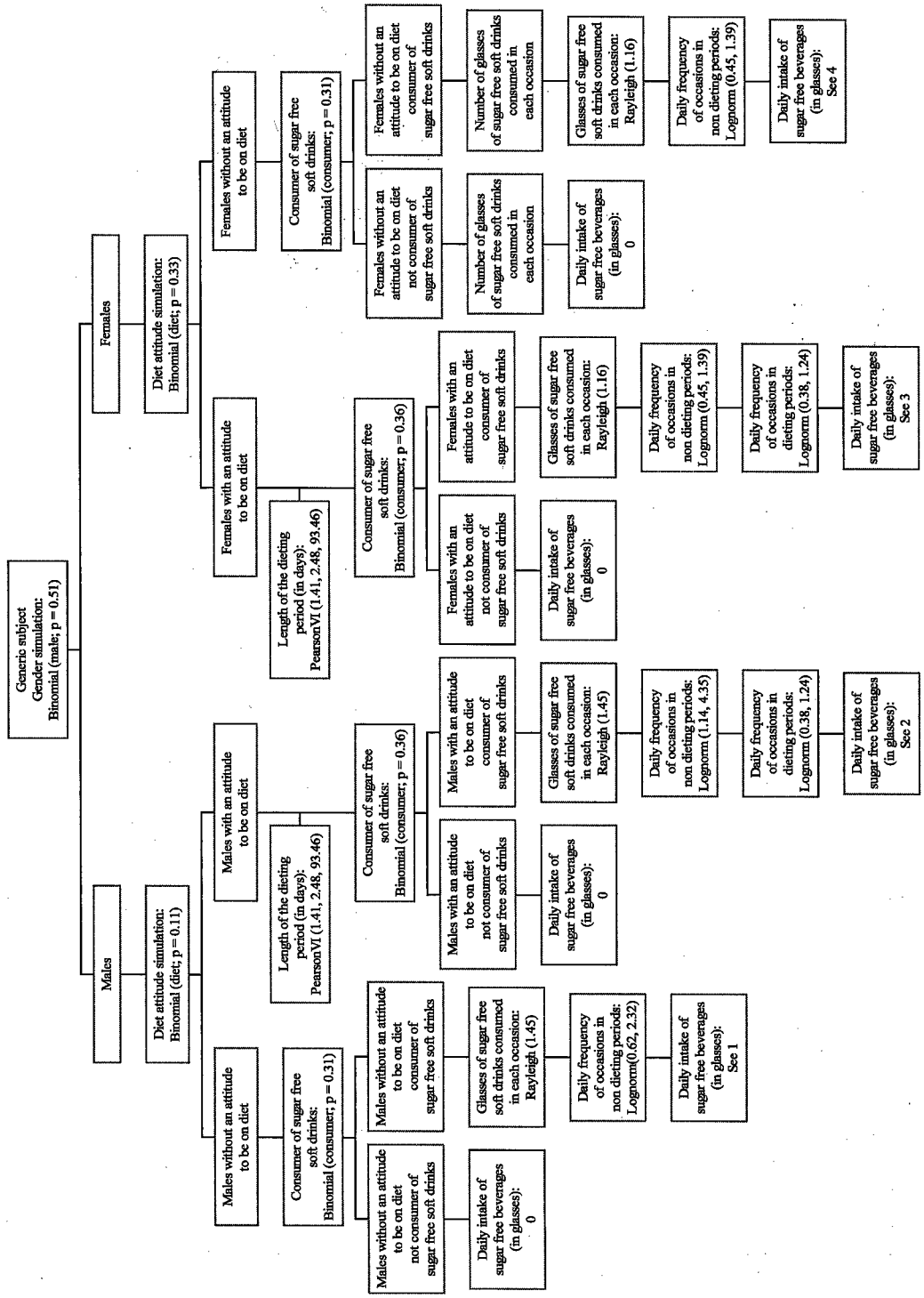

Fig. 1. Summary scheme for the simulation of sugar-free soft drinks consumption. (1) Rayleigh(1.45) × lognormal(0.62, 2.32); (2) [(365 − PearsonVI(1.41, 2.48, 93.46)) × (Rayleigh(1.45) × lognormal(1.14, 4.35))/365] + [PearsonVI(1.41, 2.48, 93.46) × (Rayleigh(1.45) × lognormal(0.38, 1.24))/365]; (3) [(365 − PearsonVI(1.41, 2.48, 93.46)) × (Rayleigh(1.16) × lognormal(0.45, 1.39))/365] + [PearsonVI(1.41, 2.48, 93.46) × (Rayleigh(1.16) × lognormal(0.38, 1.24))/365]; and (4) Rayleigh(1.16) × lognormal(0.45, 1.39).

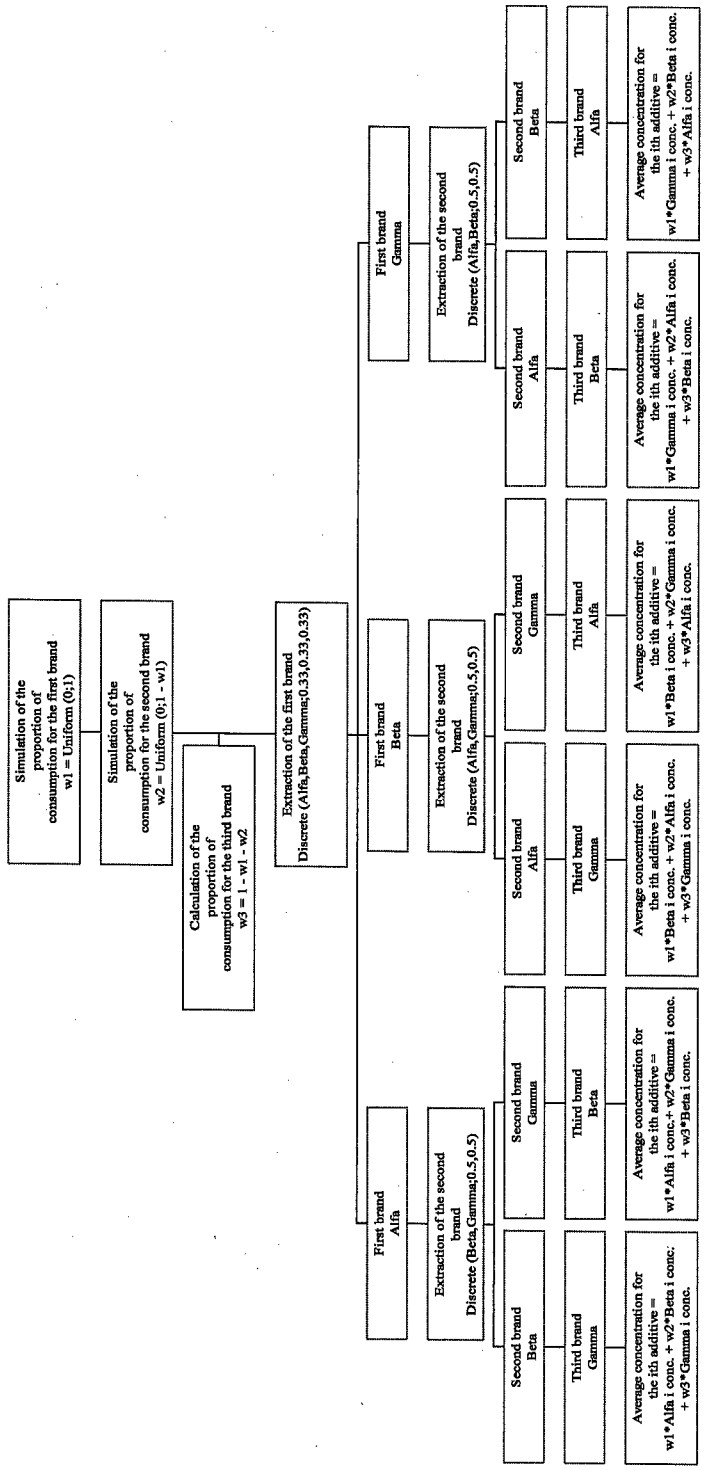

Fig. 2. Summary scheme for assessing sweeteners' concentration in sugar-free soft drinks (first hypothesis).

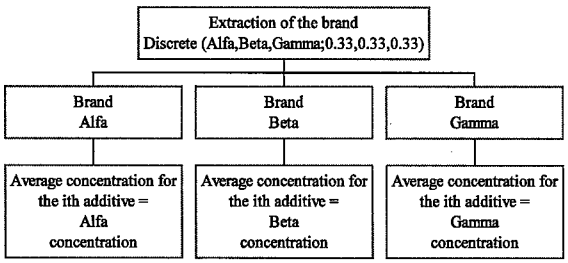

Fig. 3. Summary scheme for assessing sweeteners' concentration in sugar-free soft drinks (second hypothesis).

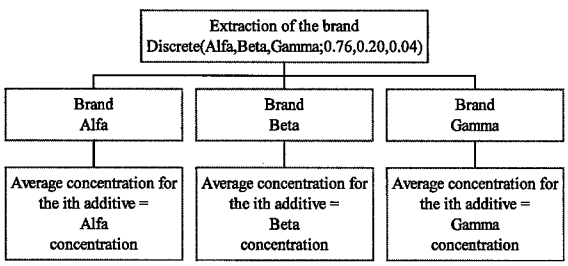

Fig. 4. Summary scheme for assessing sweeteners' concentration in sugar-free soft drinks (third hypothesis).

selected, with all the brands having the same probability to be on first, second and third position. We defined c_{i1}, c_{i2} and c_{i3} as the concentration level for the ith sweetener of the first, second and third brand, respectively extracted. Three numbers are then randomly selected: w_1, w_2 and w_3, where $w_1 = \text{uniform}(0;\ 1)$, $w_2 = \text{uniform}(0;\ 1-w_1)$ and $w_3 = 1-w_1-w_2$. In this case, the concentration level adopted (c_{im}) is the average of the three concentration levels weighted with the previously extracted random numbers such as $c_{\text{im}} = w_1c_{i1} + w_2c_{i2} + w_3c_{i3}$.

The second hypothesis (Fig. 3) assumes the total loyalty to brands by all the subjects and the absence of information regarding market share. In this case, a brand is randomly selected, with all the brands having the same probability to be extracted. Because total loyalty is assumed, the level of sweeteners' concentration of the selected brand is applied without further steps. Following the previous notation, $c_{\text{im}} = c_{i1}$.

The third hypothesis (Fig. 4) assumes the total loyalty to brands by all the subjects and the availability of data regarding market share. In this case, a brand is randomly selected, with each brand having a probability to be extracted equal to its market share: $c_{\text{im}} = \text{discrete}(c_{i\text{Alfa}}, c_{i\text{Beta}}, c_{i\text{Gamma}};\ 0.76,\ 0.20,\ 0.04)$. Because total loyalty is assumed, the level of sweeteners' concentration of the selected brand is applied to all subjects without further steps.

The fourth hypothesis (Fig. 5) assumes the availability of data regarding market share at both population and individual level and of data regarding brand loyalty for each brand. Intakes of both loyal and not loyal consumers are simulated. As in the above case a brand is randomly selected, with each brand having a probability to be extracted equal to its market share: $c_{\text{im}} = \text{discrete}(c_{i\text{Alfa}}, c_{i\text{Beta}}, c_{i\text{Gamma}};\ 0.76,\ 0.20,\ 0.04)$. Once the first brand is known, for each subject, the model simulates his loyalty. The probability of being loyal changes according to the first brand extracted; it is equal to 0.68, 0.42 and 0.42, respectively, for a subject who is a consumer of Alfa, Beta or Gamma. These figures derive from IHA purchase data; 0.68 for example is the frequency of households who bought at least once Alfa and did not purchase any other brand (after exclusion of occasional consumers). If the subject is defined as loyal, the concentration of the selected brand is applied without further steps. On the other hand, if a consumer is not loyal, an individual market share is simulated using the bootstrap method (Efron and Tibshirani, 1991); the data used in this case are the individual market shares of the households who were not loyal and have consumed at least once the brand previously extracted. In this case, the concentration level adopted (c_{im}) is the average of the three levels weighted with the market share: $c_{\text{im}} = w_{\text{Alfa}}c_{i\text{Alfa}} + w_{\text{Beta}}c_{i\text{Beta}} + w_{\text{Gamma}}c_{i\text{Gamma}}$.

The analyses were carried out using a software package designed for generalised probabilistic analyses (@RISK, Palisade Corporation, 1977). The settings used for running the model with @RISK software were as follows: number of simulations—1; number of iterations—50,000; sampling type—Montecarlo; random number generator seed of −1972; standard recalc.—expected value; convergence—left as default: monitor con-

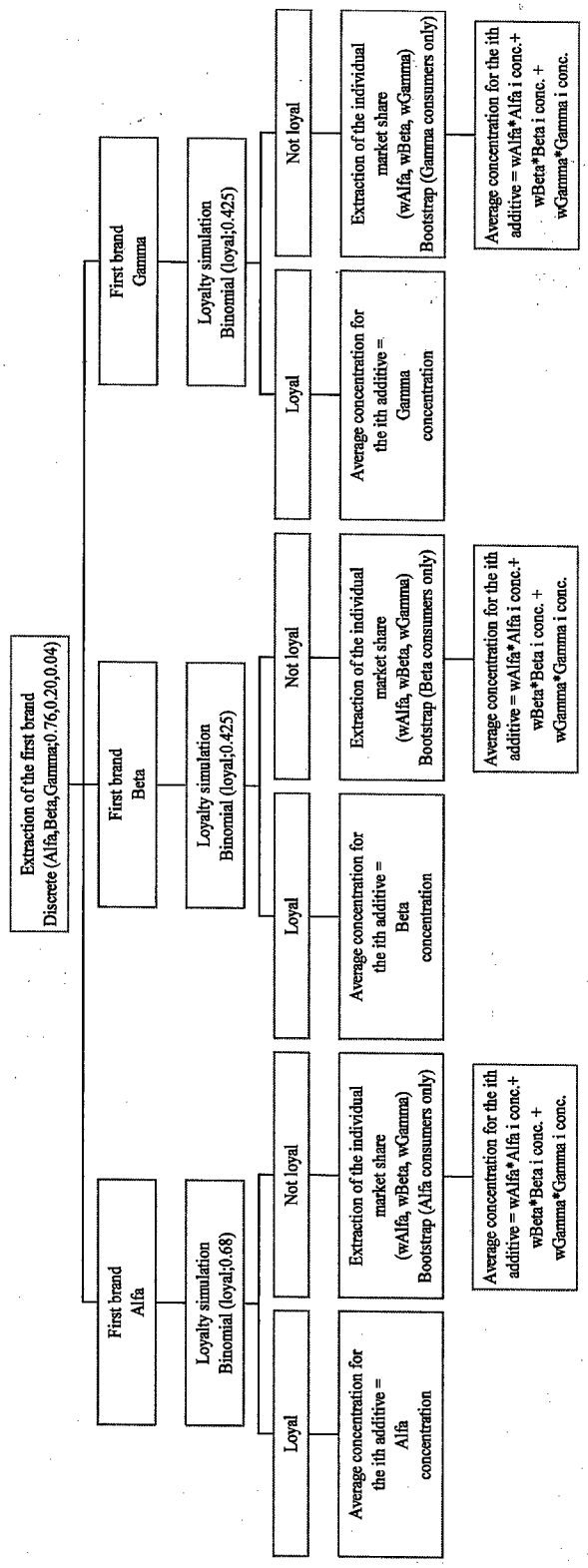

Fig. 5. Summary scheme for assessing sweeteners' concentration in sugar-free soft drinks (fourth hypothesis).

vergence, check every 100 iterations, auto-stop not ticked.

3. Results

3.1. Choice of distributions

The first simulated variable was the subject's gender. In 1998, in the county of Rome, males belonging to this age class were 101,042 (51%), whereas females accounted for 96,183 (49%) (ISTAT, 2000). The probability distribution used to simulate "being male" was binomial (male; $P = 0.51$). During the survey a higher percentage of females declared to have undertaken a diet in the previous year. The two probability distributions used to simulate the tendency to be on diet were binomial (prone to diet; $P = 0.33$) for females and binomial (prone to diet; $P = 0.11$) for males. Males and females did not differ according to the duration of the dieting period. A unique probability distribution was therefore used to simulate the length of the dieting period: PearsonVI (1.41, 2.48, 93.46).

The percentage of consumers of sugar-free soft drinks was significantly higher among those who had the tendency to be on diet. No differences were found according to the gender (Chi-square d.f. = 1; value = 0.0636; $P = 0.8009$), only among those who were prone to diet a higher proportion of males than females were consumers of sugar-free soft drinks but the difference was not statistically significant. Two probability distributions were therefore used to simulate "being a consumer of sugar-free soft drinks": binomial (consumer; $P = 0.31$) for those who are not prone to diet and binomial (consumer; $P = 0.36$) for those who are prone to diet. The distribution of the number of glasses of sugar-free soft drinks consumed in each occasion differed significantly according to the gender but not according to the tendency to be on diet. Two probability distributions were therefore used to simulate the number of glasses of sugar-free soft drinks consumed in each occasion: Rayleigh(1.16) for females and Rayleigh(1.45) for males. Among consumers of sugar-free soft drinks prone to diet, 60% reported a different daily frequency of consumption during their dieting period (41 and 19%, respectively, decreased and increased their frequency of consumption). In this case, no significant differences were found between males and females. During non-dieting periods, males and females showed a different distribution of daily frequency of consumption. The different distribution between males and females resulted also among the subgroup of those who were prone to diet and in the subgroup of those who were not prone to diet. Only among males, those who were prone to diet had a significantly different daily frequency of consumption from those who were not. During their non-dieting period, females who were prone to diet showed the same daily frequency of consumption of those who were not prone to diet. Three probability distributions were therefore used to simulate the daily frequency of sugar-free soft drinks consumption in non-dieting periods: lognormal(0.62, 2.32) for males who are not prone to diet, lognormal(1.14, 4.35) for males who are prone to diet and lognormal(0.45, 1.39) for females. During their dieting period, males and females did not show a significantly different distribution of daily frequency of consumption. A unique probability distribution was used to simulate the daily frequency of sugar-free soft drinks consumption in dieting periods of males and females: lognormal(0.38, 1.24).

The body weight differed according to gender and to the tendency to be on diet. The simulation was therefore performed by randomly selecting a weight among those reported in the appropriate category of gender and tendency to be on diet.

3.2. Market share and brand loyalty

Among those 4257 households of the IHA panel who registered their purchases for more than 6 months, 380 households (8.9%) never consumed soft drinks with sugar, whereas 3745 households (88.0% of the sample) never consumed sugar-free beverages.

These data suggest that in Italy, sugar-free beverage is a small market. The market is concentrated in three brands (Alfa, Beta and Gamma) of which one has a far larger share than the others. In fact, sugar-free beverages represented only 1.4%

Table 1
Summary statistics for the intake of acesulfame K, aspartame and cyclamate among consumers of sugar-free soft drinks only (n = 16297)

	Sweeteners' concentration in sugar-free soft drinks[a] (mg/kg bw)		
	Average intake	Standard deviation	95th percentile of intake
Acesulfame K			
First hypothesis	0.282	1.082	1.128
Second hypothesis	0.284	1.249	1.144
Third hypothesis	0.207	0.974	0.841
Fourth hypothesis	0.220	0.951	0.897
Aspartame			
First hypothesis	0.503	2.468	1.915
Second hypothesis	0.499	3.074	1.878
Third hypothesis	0.392	1.724	1.544
Fourth hypothesis	0.402	1.931	1.598
Cyclamate			
First hypothesis	0.219	1.118	0.895
Second hypothesis	0.220	1.397	0.933
Third hypothesis	0.499	2.439	2.054
Fourth hypothesis	0.462	2.060	1.890

Source: simulated data.

[a] The first hypothesis assumes a total lack of data regarding brand loyalty and market share. The second hypothesis assumes total loyalty to brands by all the subjects and absence of information regarding market share. The third hypothesis assumes total loyalty to brands by all the subjects but makes use of IHA data regarding market share. The fourth hypothesis simulates both loyal and not loyal consumers making use of IHA data on market share and on brand loyalty.

of the whole amount of soft drink purchased in 1998 by the panel. The percentage of consumers was not homogeneous among brands; 365 households (8.6%) bought Alfa at least once versus 144 (3.4%) for Beta and only 65 (1.5%) for Gamma. Brand Alfa was found to own 76.3% of the market (percentage in weight of all purchases) of sugar-free beverages. The most consumed brand, Alfa, was also the one with the higher proportion of exclusive consumers (68.3%), 57.5% of both Beta and Gamma consumers purchased also other brands of sugar-free beverages.

3.3. Simulated sample

Descriptive statistical analyses were performed to check that values of the main parameters of the simulated sample were in line with those used as input in the stochastic model. In the generated population, the number of consumers did not differ according to the gender, 8073 consumers versus 17,370 non-consumers among males, and 8224 consumers versus 16,333 non-consumers among females. A higher proportion of females had the tendency to be on diet than in males, 33% versus 11%. When only consumers of sugar-free soft drinks were considered, the percentage of males with the tendency to be on diet increased (14%), whereas in females this proportion was 38%. Simulated intakes of acesulfame K, aspartame and cyclamate are presented in Table 1. Saccharin was not taken into account because no brand of sugar-free soft drinks contained this additive.

It is important to highlight that the estimated distribution of intake varied according to the hypothesis made regarding market share and brand loyalty. The 95th percentile of intake, which is an important parameter commonly used to quantify the risk of excessive intakes of additives, significantly varied according to the four hypotheses adopted. When taking into account the whole sample, the 95th percentile of intake of acesulfame K under the third hypothesis (which assumes total

loyalty to brands by all the subjects and uses IHA data regarding market share) was 26% lower (0.84 mg/kg bw versus 1.14 mg/kg bw) than that calculated under the second hypothesis (which assumes total loyalty to brands by all the subjects and the absence of information regarding market share). On the other hand, in the case of cyclamate, always under the third hypothesis, the 95th percentile of intake was more than double respect to that calculated under the first hypothesis which assumes a total lack of data regarding brand loyalty and market share (2.05 mg/kg bw versus 0.89 mg/kg bw). These differences are due to the fact that brands of sugar-free soft drink on the Italian market present a different mixture of sweeteners and have a significantly different weight in terms of market share. For example, as already stated, Alfa is the only brand containing cyclamate and it is also the most consumed brand according to IHA data. The intake of cyclamate is therefore higher when data on market share are used (third hypothesis) and the probability of consuming exclusively Alfa is set higher. On the contrary, when these data are not taken into account, all the brands are supposed to be consumed with the same probability and a higher proportion of subjects appears to consume brands Beta and/or Gamma. In this case, the intake of cyclamate, not present in these brands, is reduced, whereas the intake of acesulfame K and aspartame, present at higher levels in these brands, is increased.

Moreover, it can be noticed that the 95th percentile of intake did not significantly differ between the third and the fourth hypothesis. In both hypotheses, IHA data on market share are used but, under the third hypothesis, a total loyalty to brands is assumed, whereas under the fourth hypothesis also not loyal consumers were simulated using individual data on market share. This result is due to the fact that in the market of sugar-free soft drinks, consumers are prevalently loyal to brands: 68% of Alfa consumers never bought other brands. This tendency was included in the model generating a large prevalence of loyal consumers also under the fourth hypothesis. For this reason, the largest differences in intake estimates can be noticed between the first two hypotheses, which do not take into account market share, and the other two which use IHA data regarding market share.

4. Discussion

It is important to highlight that the model illustrated in this paper is not yet validated and was developed primarily for the purpose of exploring how market share and brand loyalty may influence the output of stochastic analysis. For this reason, the intakes of each intense sweetener have not been compared with their respective ADI. A validation would be needed to check if the model is able to represent the intake of intense sweeteners.

The present numeric experiments suggest how to include indicators of market share and brand loyalty in a stochastic model of exposure to a chemical substance present in processed food. Results show that the distributions of intake may be fairly different according to the hypothesis formed in relation to the availability of data for market share and brand loyalty. In particular, intakes at the right tail of the distribution were shown to vary. These are particularly important in exposure assessment since they are needed to assess intakes in high consumers. The market share of food products should be included in the model, especially if most of the food items sold are produced by few companies, i.e. if the market is not equitably distributed between brands.

There is a problem of availability of data related to market share and brand loyalty. Brand-level information is routinely recorded in food consumption surveys but only to the aim of assigning a suitable food code for the assessment of nutrient intakes (IEFS, 1998). The rate at which new brands enter the market is such as to preclude the assignment of a specific code to each brand. Therefore, food consumption databases almost never include the brand information. The Dietary and Nutritional Survey of British Adults is a rare case of a large nutritional survey in which food intake data have been not only recorded but also codified at brand level (Office of Population Consensus and Surveys, 1991). More frequently,

food intake data are codified at brand level only for a specific category of products. This is the case of sugar-free products which may be coded at brand level within consumer surveys aimed at assessing the intake of intense sweeteners (Leclercq et al., 1999). This information can also be obtained by recoding specific categories of products, which were recorded at brand level in food diaries but not codified at electronic level. The only source of consumption data at brand level largely available in industrialised countries is that of household purchases collected for marketing research purposes in panel surveys. However, within such panel surveys, purchases are registered at household level, whereas one would need consumption data at the individual level. Since household data are aggregates of individual within household purchases, the heterogeneity of individual brand loyalty within households could be simulated to further refine the model. On the other hand, when no data regarding market share or brand loyalty are available it could be interesting to run the model under different theoretical scenarios and use the worse case scenario to obtain conservative intake distributions.

The model described above could be improved by including the possible dependencies between levels of consumption and brands. In fact, there is the possibility that higher consumers are those who consume exclusively or prevalently certain brands.

5. Conclusion

It is apparent that before developing a stochastic model for chemical substances in processed foods the following factors should be taken into account: (1) variability in the concentration of chemical substances between brands, (2) distribution of the market between brands, (3) level of brand loyalty according to brands, and (4) relationship between market share, brand loyalty and concentration of chemical substances. If the concentration of chemical substances does not vary between brands, there will be no need to include either brand loyalty or market share in the model. If subjects are not loyal to brands, only market shares should be included in the model, with all subjects consuming a mixture of products in proportion of their market share. If consumers are faithful to products, an indicator of brand loyalty is needed. The relationship between market share and concentration of substances deserves to be studied in depth. It is particularly important to assess if the concentration of chemical substances that could present a health risk is higher in small brands and if consumers are faithful to these brands. In such cases, the intake exposure model could not be implemented without data at brand level and indicators of brand loyalty.

On the basis of the experiments described here, indicators of brand loyalty and market share have been included in the software developed within the Montecarlo project and which is currently being validated.

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Short communication

Antinutritional effects of fumonisin B_1 and pathophysiological consequences

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Abstract

Due to its structural similarity with sphingosine, fumonisin B_1 (FB_1) inhibits ceramide synthase (a key enzyme of sphingolipid biosynthesis) leading to an intracellular accumulation of sphingoid bases with a consequent increase of sphinganine/sphingosine (SA/SO) ratio. In adult male rats, dietary exposure to fumonisin induces a significant increase in both SA concentrations and SA/SO ratio in kidney, but not in liver and brain, as well as a significant reduction of body weight gain. Regarding the brain, the developing rat is more sensitive to FB_1 than the adult rat. FB_1 treatment produces in the forebrain and brainstem: (i) an increase in SA levels and SA/SO ratio, (ii) a reduction in myelin deposition, and (iii) an impairment of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) activity. FB_1 effects on myelin are similar to those produced by starvation (temporary removal of pups from dam during postnatal period), thus suggesting that hypomyelination could be due, at least partly, to a nutritional deficiency. Finally, FB_1 reduces the uptake of folate in different cell lines. The resulting folate deficiency could explain the association of FB_1 exposure with neural tube defects.

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Keywords: Fumonisin; Ceramide synthase; 2′,3′-Cyclic nucleotide 3′-phosphohydrolase; Folate uptake; Pathophysiology

1. Introduction

Fumonisins are mycotoxins produced by *Fusarium verticillioides* (= *F. moniliforme*) and *F. proliferatum* which are found in corn crops worldwide (Bezuidenhout et al., 1988; Hopmans and Murphy, 1993). Ingestion of fumonisin B_1, which contaminates food and feed (Shephard et al., 1990; Ueno et al., 1993), has been associated with leucoencephalomalacia in both horses (Marasas et al., 1988) and rabbits (Bucci et al., 1996), pulmonary oedema in pigs (Harrison et al., 1990), and nephrotoxicity and liver cancer in rats (Gelderblom et al., 1996). Although the effects of this mycotoxin on human are difficult to evaluate,

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epidemiological studies show a high incidence of oesophageal cancer in certain areas of Transkei (South Africa) and also in China (Yang, 1980; Rheeder et al., 1992; Chu and Li, 1994).

Due to its structural similarity with sphingosine (Bezuidenhout et al., 1988; Laurent et al., 1989), FB_1 inhibits the ceramide synthase activity leading to an intracellular accumulation of sphingoid bases (mainly sphinganine relative to sphingosine), which mediate several key biological processes such as cell proliferation and DNA replication. Inhibition of ceramide synthase results in an increase of SA/SO ratio, which is considered a useful biomarker to assess fumonisin exposure. This ratio can be evaluated in tissues (such as kidney and liver) and biological fluids of both human and animals (Riley et al., 1993; Qui and Liu, 2001). Further mechanisms responsible for FB_1 toxicity include inhibition of protein and DNA synthesis (Abado-Bécognée et al., 1998) and lipid peroxidation (prevented by vitamin E), as shown in both primary rat hepatocytes (Abel and Gelderblom, 1998) and C6 glioma cells (Mobio et al., 2000).

This paper briefly reviews the results of our recent studies as well as literature data dealing with FB_1-induced disruption of sphingolipid metabolism and its consequences on folate deficiency and brain development.

2. Effects of dietary fumonisin exposure on tissue levels of sphingoid bases

In agreement with the literature data (Merrill et al., 1996; Riley et al., 1993), our recent studies (Solfrizzo et al., 2001) have shown that dietary exposure to fumonisin induces, in the adult rat, an increase in SA concentration and SA/SO ratio in kidney. In particular, mean kidney SA/SO ratios are found to be 5.8-fold higher in rats fed FB_1-contaminated diet than in rats fed control diet. Dietary exposure to fumonisin does not alter SA/SO ratio and SA concentrations in liver and brain, thus suggesting that kidney could be considered the main target of this mycotoxin. Data relative to body weight gain, feed consumption, and organ weights are reported in Table 1. Exposure to FB_1-contaminated diets produces a significant reduction of body weight gain and a significant increase of liver weight.

Table 1
Body weight, feed consumption, and organ weight data of male Wistar rats fed control diet and fumonisin-contaminated diets for 1 week

Parameters	Control	FB_1 (4 ppm)
Initial body weight (g)	268.7±5.5	277.5±5.9
Final body weight (g)	308.0±6.8	282.0±4.3
Body weight gain (g)	39.2±9.4	4.5±5.7*
Feed consumption (g)	15.0±1.3	15.6±1.6
Absolute liver weight (g)	7.27±0.30	9.45±0.45*
Relative liver weight (mg/g)[a]	23.62±1.04	33.54±1.77*
Absolute kidney weight (g)	1.92±0.05	2.08±0.12
Relative kidney weight (mg/g)[a]	6.25±0.28	7.4±0.53

[a] Organ weight (mg) to body weight (g) ratio.
* $P < 0.05$ (one-way ANOVA; $n = 4$).

3. FB_1 and nutritional deficiency outcomes on developing brain

As far as the brain is concerned, the developing rat is a more sensitive model to study FB_1 effects than the adult rat since this mycotoxin is a hydrophilic, lipid-insoluble compound with a relatively large molecular weight. In this regard, it has been shown that FB_1 administration from postnatal day (PND) 2–12 produces the following effects in the forebrain and brainstem: (i) increase in SA levels and SA/SO ratio, (ii) reduction in myelin deposition, and (iii) impairment of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) activity (Kwon et al., 1997). Moreover, a significant decrease in body weight gain is observed in FB_1-treated animals. The effects produced by FB_1 are similar to those induced by nutritional deficiencies. Temporary removal of pups from dam during postnatal period (6–7 h per day from PND 3 to 12) significantly decreases body weight gain as well as myelin deposition and CNP activity in forebrain and brainstem. However, unlike FB_1, nutritional deficiencies during postnatal period do not affect sphingoid base levels and SA/SO ratios in the brain. These data, summarized in Table 2, show that FB_1 exposure affects sphingolipid metabolism

Table 2
Effects of nutritional deficiency (ND) and FB_1 exposure in the developing rat

Parameters	FB_1	ND
Body weight gain	↓	↓
Myelin deposition (forebrain and brainstem)	↓	↓
CNP activity (forebrain and brainstem)	↓	↓
SA concentration	↑	NE
SA/SO ratio	↑	NE

↓ = reduction; ↑ = increase; NE = no effect.

in the central nervous system of developing rats by specifically impairing ceramide synthase activity as indicated by the increased SA levels. Furthermore, these findings point out that, unlike FB_1, nutritional deficiency resulting in hypomyelination and impairment of CNP activity, does not alter sphingolipid biosynthesis since no change in brain SA levels are observed after starvation. Therefore, nutritional deficiency has no effect on ceramide synthase activity.

4. Is there any link between FB_1-induced sphingolipid depletion and folate deficiency?

Sphingolipids seem to play an important role in folate receptor function. This high-affinity receptor, a glycosylphosphatidylinositol (GPI)-anchored protein, is responsible for the transport of folate into cells of several tissues with elevated requirements for this vitamin. The folate vitamins play an essential role as cofactors in many biochemical reactions including the biosynthesis of purines and thymidine, the regeneration of methionine from homocysteine, and histidine metabolism. Cellular processes dependent upon folate can be compromised if dietary levels of this vitamin are insufficient or its transport into cells is affected. The GPI-anchored folate receptor is associated with membrane domains that are enriched in cholesterol and phospholipids. Depletion of cellular cholesterol has been shown to inhibit vitamin uptake by this receptor (Chang et al., 1992). Moreover, the importance of sphingolipids for folate receptor function has been demonstrated in CaCo-2 cells treated with FB_1 that inhibits the biosynthesis of these lipids (Stevens and Tang, 1997). In FB_1-treated cells, the folate receptor-mediated transport of 5-methyltetrahydrofolate is inhibited in a concentration- and time-dependent manner. The sphingolipid levels of these cells also decrease in a concentration- and time-dependent manner, thus suggesting that the inhibition of 5-methyltetrahydrofolate uptake in FB_1-treated cells is mediated by changes in the sphingolipid composition. Moreover, a concurrent loss in the total amount of folate binding capacity in the cells is observed, as sphingolipids are depleted, thus suggesting a causal relationship between folate receptor density and vitamin uptake. Similarly, an inhibition of folate uptake is also observed in cultured primary embryonic cells treated with fumonisin. Collectively, these findings suggest that dietary exposure to FB_1 could adversely affect folate uptake and potentially compromise cellular processes dependent on this vitamin. Finally, it should be pointed out that, since folate deficiency causes neural tube defects, some birth defects unexplained by other known risk factors may be caused by exposure to FB_1.

5. Conclusions

Inhibition of sphingolipid biosynthesis, leading to an intracellular accumulation of sphingoid bases, still seems to be the main mechanism of fumonisin toxicity. The consequent increase in SA/SO ratio provides a useful biomarker to assess exposure to this mycotoxin in both human and experimental animals.

Investigations into the consequences of fetal exposure to this mycotoxin have also shown relevant developmental toxicity. The mouse fetuses surviving to birth have gross skeletal and tissutal abnormalities (Floss et al., 1994; Gross et al., 1994). Similarly to nutritional deficiency, FB_1 exposure induces hypomyelination and it impairs CNP-specific activity. Factors affecting myelin deposition after FB_1 exposure may include (i) antinutritional effects, (ii) consequences of increased brain SA levels, and/or (iii) SA-independent FB_1-induced toxicity. Since postnatal nutritional deprivation also reduces myelin synth-

esis (Wiggins et al., 1976), hypomyelination associated with FB_1 treatment is supposed to be due, at least partly, to a nutritional deficiency.

Finally, FB_1-induced depletion of sphingolipids inhibits folate uptake leading to an intracellular deficiency in this vitamin. Folate deficiency during the first trimester of pregnancy is associated with an increased risk of neural tube defects (Hibbard, 1993; Butterworth, 1993; Dansky et al., 1992). Therefore, it is possible that some birth defects unexplained by the known risk factors might be linked to dietary FB_1 exposure. For instance, high rates of neural tube defects have been observed among Hispanic (Canfield et al., 1996), for whom corn and corn products represent a sizeable portion of their diet. In conclusion, the experimental evidence that FB_1-induced depletion of cellular sphingolipids inhibits folate uptake suggests that further investigations are needed to explore the possibility that this mycotoxin may contribute to some birth defects not accounted for by other known risk factors.

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Interaction of ochratoxin A with human intestinal Caco-2 cells: possible implication of a multidrug resistance-associated protein (MRP2)

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Abstract

Ochratoxin A (OTA), a nephrotoxic mycotoxin, is absorbed from small intestine and, in plasma, binds to serum albumin. Prolonged half-live results from reabsorption by proximal tubules and enterohepatic circulation. The mechanism whereby OTA crosses intestine was investigated by means of a cell culture system consisting of Caco-2 cells, as in vitro model of human intestinal epithelium. Cytotoxicity assays on proliferating Caco-2 cells showed that 0.4 μM OTA inhibits MTT reduction by 50%. Transepithelial transport and intracellular accumulation of OTA were studied in Caco-2 cells, differentiated in bicameral inserts. At pH 7.4, OTA is transported preferentially in basolateral (BL) to apical (AP) direction, suggesting a net secretion. Conditions closer to in vivo situation in duodenum (AP pH 6.0, BL pH 7.4) increase intracellular accumulation and transepithelial transport. AP to BL transport becomes higher than BL to AP transport, suggesting OTA absorption. Addition of serum albumin in BL compartment further increases OTA absorption across Caco-2 cells and suggests that in vivo OTA transport from serosal to luminal side of enterocytes is prevented, due to its binding to plasma proteins. Competition experiments showed that carrier systems for large neutral amino acids, H^+/dipeptides cotransporter, organic anion (*p*-aminohippurate) carrier and organic anion transporter (oatp) are not implicated in OTA transport across Caco-2 cells, in contrast to what was reported in kidney and liver. AP and BL transport and intracellular accumulation of OTA are increased in the presence of non specific inhibitors of MRPs (indomethacin, genistein and probenecid) and of 1-chloro-2,4-dinitrobenzene (biotransformed into 2,4-dinitrophenyl-gluthatione, a specific inhibitor of MRPs), but are affected by verapamil, an inhibitor of P-gp. This suggests that the multidrug resistance-associated protein (MRP2) could be implicated in transepithelial transport. Therefore, absorption of OTA across the intestinal mucosa would be limited thanks to its excretion through MRP2 at the apical pole of enterocytes.

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1. Introduction

Ochratoxin A (OTA), a mycotoxin mainly produced by two ubiquitous species of fungi, *Aspergillus alutaceus* and *Penicillium verrucosum*, contains a dihydroisocoumarin moiety linked to a L-phenylalanine residue. OTA is a frequent contaminant in various plant products and animal feeds and is also found in human and animal fluids and tissues (Pohland et al., 1992). OTA has a number of toxic effects in mammals, the most notable being nephrotoxicity. It has been implicated in the human *Balkan Endemic Nephropathy* and in the porcine nephropathy and is also immunosuppressive, teratogenic, genotoxic and carcinogenic (Steyn, 1993). Molecular toxicity would result from competition with phenylalanine for protein synthesis, promotion of lipid peroxidation, inhibition of mitochondrial ATP production, as well as production of DNA adducts (Dirheimer and Creppy, 1991).

Kumagai (1998) showed that OTA is absorbed from the jejunum of rats, even when its level is higher in plasma than in jejunal lumen and that OTA uptake increases when its pH is decreased. In different species including humans, 97–100% of the toxin is bound to plasma proteins (Hagelberg et al., 1989), which facilitates the passive diffusion of OTA from the digestive tract and, furthermore, retards its elimination, by limiting its transfer from the bloodstream to the hepatic and renal cells (Marquardt and Frohlich, 1992).

Since the kidney plays a key role in OTA toxicity (Steyn, 1993), the interaction of OTA with renal cells has been extensively investigated. Uptake of OTA across the apical membrane of different renal cells was shown to be partially mediated by the L-phenylalanine carrier and the H^+-driven dipeptide carrier (Gekle et al., 1993; Schwerdt et al., 1997, 1998; Zingerle et al., 1997) and by the organic anion carrier (Bahnemann et al., 1997; Welborn et al., 1998; Groves et al., 1999).

The 170 kDa P-glycoprotein (Pgp) and the 190 kDa multidrug resistance-associated protein (MRP) are two membrane proteins implicated in the active efflux of drugs and xenobiotics (Loe et al., 1996). They belong to the ABC superfamily of transport proteins and function as energy-dependent efflux pumps, decreasing free cellular concentrations of drugs and xenobiotics. Pgp transports mainly cationic compounds whereas MRP transports amphipatic anionic conjugates (GSH, glucuronide and sulfate conjugates) as well as unaltered lipophilic substances (Paul et al., 1996). Pgp as well as MRP2 are expressed at the apical pole in Caco-2 cells, while MRP1 would not be expressed and MRP3 would be present at the basolateral pole (Hirohashi et al., 2000; Walgren et al., 2000). These efflux pumps also show overlapping substrate specificity.

Absorption within the gastrointestinal tract is the first step governing the entry of OTA in bloodstream and, eventually, its tissue distribution. The objective of this study is to investigate the mechanism(s) implicated in the transport of OTA across the human intestinal mucosa. For this purpose, we used an in vitro model of the intestinal barrier, based on the cultivation of the Caco-2 cell line on microporous membranes (Halleux and Schneider, 1991). Our results show that OTA is absorbed across the Caco-2 cells monolayers and that this absorption would be limited thanks to its excretion through MRP, probably the MRP2 isoform, at the apical pole of Caco-2 cells.

2. Materials and methods

2.1. Chemicals

Culture reagents were purchased from Invitrogen (Merelbeke, Belgium). Biochemicals were purchased from VWR Int. (Leuven, Belgium) or Sigma-Aldrich (Bornem, Belgium). [^{3}H(G)]ochra-

toxin A (14.8 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and D-[1-^{14}C]mannitol (56 mCi/mmol) from Amersham Life Sciences (Little Chalfont, UK).

2.2. Cell culture

Caco-2 cells (ATCC, Rockville, MD), between passages 36 and 55, were routinely grown as in Halleux and Schneider (1991) and Sergent-Engelen et al. (1993) in a serum-free medium (5:5:1, v:v:v) mixture of Iscove's modified Dubelcco's, Ham's F12 and NCTC 135 media (Schneider, 1989) supplemented with glucose to 16 mM, 1 μg/ml insulin, 1 ng/ml EGF, 10 μg/ml albumin-linoleic acid, 2 nM T3 and 100 nM hydrocortisone.

Cells were seeded on collagen-precoated polyethylene terephtalate microporous membranes (1 μm pore diameter, Whatman SA, Louvain-la-Neuve, Belgium) in bicameral inserts (24 mm diameter, 5 cm^2 growing area) at 160 000 cells/cm^2. The monolayers integrity was checked by measurement of the [^{14}C]D-mannitol flux and transepithelial electrical resistance (TEER; Endohm 24, World Precision Instruments, Sarasota, FL). Only monolayers exhibiting TEER values of ca. 400 Ω·cm^2 and [^{14}C]D-mannitol clearances lower than 30 μl per insert per hour were used.

2.3. Cytotoxicity assays

Cells, seeded in 96-well plates (Greiner Labortechnik, Frickenhaussen, Germany) at a density of 5×10^4 cells/cm^2, were incubated, 24 h (MTT) or 48 h (LDH) after seeding, with OTA for 48 h. MTT assay was carried out as in Mosmann (1983), using 100 μl of MTT (0.5 mg/ml in PBS), 2 h incubation at 37 °C, solubilization in 100 μl DMSO and reading at 540 nm (Titertek Multiskan MCC/340 P, Labsystems, Vantaa, Finland). LDH assay was purchased as a kit (Cytotoxicity Detection Kit, Boeringher Mannhein GmbH, Mannheim, Germany). Maximal LDH release was determined by exposing the cells to 1% Triton X-100. The reduced formazan reaction product was measured at 492 nm on a microplate reader.

2.4. Biotransformation

Cells were incubated for 3 h at 37 °C with 10 μM OTA in HBSS at either pH 7.4 or 6.0 in the donor compartment and HBSS at pH 7.4 in the acceptor compartment. Culture media were collected and cells were scraped in PBS and sonicated. After filtration on 0.45 μm filters (Millex-HV, Millipore Corporation, Bedford, MA), HPLC separation was achieved on a 3 μm Hypersil BDS reversed-phase C18 150 × 4 mm^2 ID column (Sercolab, Mechelen, Belgium) with acetonitrile–water–acetic acid (450:540:10, v:v:v) as mobile phase. Ochratoxin α was produced by hydrolysis of OTA with carboxypeptidase A. A sample containing the various hydroxylated derivatives of OTA was kindly provided by Prof. E.E. Creppy (University of Bordeaux II, France).

2.5. Transport studies

The transport medium was made of HBSS containing 5 mM glucose and 10 mM Hepes (pH 7.4) or 10 mM Mes (pH 6.0). Transepithelial passage was assayed by adding [^{14}C]D-mannitol and [^{3}H]OTA in either the upper or lower compartment, containing 1.8 or 2.8 ml of transport medium, respectively. The donor solution contained ca. 0.5×10^6 dpm/ml of both [^{3}H]OTA and [^{14}C]D-mannitol in HBSS in the presence of 10 μM OTA. At each sampling time, 100 μl of transport medium were withdrawn from the acceptor compartment and replaced by fresh buffer. The rates of transport were calculated from the slope of label appearance curves.

The effect of acidification was studied by adding HBSS containing either 10 mM Mes pH 6.0 (donor compartment) or 10 mM Hepes pH 7.4 (acceptor compartment). For competition experiments, 10 mM L-phenylalanine, 10 mM glycylsarcosine, 2 mM *p*-aminohippurate or 2 mM taurocholate were added. For energy depletion, the transport medium was depleted in glucose and supplemented with 1 mM NaN_3 and 50 mM 2-deoxyglucose in both compartments; cells were preincubated in this transport buffer for 30 min. For the experiments at 4 °C, the cells were preincubated for 30 min at 4 °C. To assess the

implication of an efflux pump, either 200 μM verapamil, 200 μM indomethacin, 100 μM genistein, 2 mM probenecid or 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) were added. The absence of toxicity of these experimental conditions was checked by optical phase contrast microscopy examination, TEER measurement and LDH release.

2.6. Intracellular accumulation

At the end of the transport experiment, cell monolayers were washed six times with PBS and then resuspended in 1 ml of 1% (w/v) Na deoxycholate (pH 11.3). After 10 min at room temperature, cells were scraped and sonicated. The radioactivity of cell homogenates was measured by liquid scintillation spectrometry (Packard Tri-Carb 1600 TR, Packard Instrument Company, Meriden, CT) after dispersion in 2 ml of Aqualuma cocktail (Lumac LSC, Groningen, The Netherlands). The amount of cell protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.7. Efflux experiments

Cells monolayers were loaded from either the AP (apical pole) or BL (basolateral) side with 10 μM [^{3}H]OTA dissolved in HBSS at pH 6.0 for 1 h at 37 °C and then washed three times with ice-cold PBS. The intracellular accumulation of [^{3}H]OTA was determined on three samples as above. For the other inserts, loaded [^{3}H]OTA was allowed to efflux into the media bathing the apical and basolateral sides. The medium was either at pH 7.4 or 6.0. After various durations (10–90 min), the amounts of [^{3}H]OTA released were determined as above.

2.8. RNA isolation and reverse transcription-PCR

Total RNA was isolated from ca. 15×10^6 Caco-2 cells cultivated for 18 days in bicameral inserts, scraped and recovered in 1 ml TRIzol. RNA was extracted with 200 μl chloroform and precipitated with 500 μl isopropanol. The precipitate was rinsed with 1 ml ethanol, dried and resuspended in 50 μl water. Total RNA (2 μg) was then reverse transcribed in 20 μl transcription buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each deoxynucleoside triphosphate, 150 ng of random primers) with 200 U of Superscript RT reverse transcriptase at 42 °C for 1 h, followed by heating to 70 °C for 15 min and immediate cooling on ice.

The reverse transcription (RT) mixture (1 μl) was amplified by PCR in 40 μl of buffer (75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate) with 0.2 μM of sense and anti-sense primers and 0.8 U of Gold-Star DNA polymerase. The PCR cycles started with a 4 min period at 94 °C. PCR was then run at 94 °C for 1 min, at 54 °C for 1 min and at 72 °C for 1 min; 30 cycles were carried out. Amplification was ended by incubation at 72 °C for 5 min. The PCR products (20 μl) were separated on 1% agarose gels and visualized by ethidium bromide staining. PCR amplification of RNA not reverse transcribed was used as negative control to exclude amplification of genomic DNA. PCR amplification of RNA isolated from human liver was used as a negative control for MRP1 and as a positive control for MRP2.

Primers for MRP1 were: sense primer, CTGTTTTGTTTTCGGGTTCC (bases 4326–4345) and anti-sense primer, CAGTTCTGACCA-GATATGCC (bases 4804–4823). They led to the amplification of a 498 bp fragment. Primers for MRP2 were: sense primer, ACACCAACCA-GAAATGTGTC (bases 3946–3965) and anti-sense primer, CCAAGGCCTTCCAAATCTC (bases 4586–4604). They led to the amplification of a 659 bp fragment. As control, the RT mixture (1 μl) was treated with primers amplifying a 574 bp fragment of human β-actin (sense primer: CCATCCTGCGTCTGGACCTG, anti-sense primer: CTCGTCATACTCCTGCTTGC). Amplification proceeded through 20 cycles at 94 °C for 1 min, at 56 °C for 1 min and 72 °C for 1 min.

2.9. Immunodetection of MRP1 and MRP2

MRP1 and MRP2 were detected by Western blot analysis using rat anti-human monoclonal

antibody MRPr1 (Alexis Biochemicals, San Diego, CA) and mouse anti-human monoclonal antibody M_2III-6 (Alexis Biochemicals), respectively. Caco-2 cells, cultivated for 18 days on microporous membranes, homogenized in 0.25 M sucrose containing 10 mM Tris–HCl and 1 mM EDTA at pH 7.4, were centrifuged for 30 min at 145 000 × *g* and resuspended in 100 mM potassium phosphate, 1 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, pH 7.4. Fifty μg of proteins in 162 mM Tris–HCl, 177 mM SDS, 0.07 mM bromophenol blue, 20% (v/v) glycerol, dithiothreitol, pH 6.5, heated at 94 °C for 4 min, were loaded onto a 7.5% polyacrylamide gel, electrophoresed and transferred to a PVDF membrane. The membranes, treated for 1 h with PBS containing 3% (w/v) milk powder and 0.01% Tween 20 and subsequently washed with PBS containing 0.1% (v/v) Tween 20, were then incubated with monoclonal antibody MRPr1 (2.5 μg/ml) for MRP1 or monoclonal antibody M_2 III-6 (2.5 μg/ml) for MRP2 in PBS containing 1% milk powder for 1 h. After washing with PBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG or rabbit anti-rat IgG (dilution 1:2000) (Dako-Prosan, Merelbeke, Belgium) in PBS containing 1% milk powder. MRP1 and MRP2 were visualized using enhanced chemiluminescence system (ECL-plus) and exposed on Kodak film.

2.10. Data analysis

Results are expressed as means ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post test for multiple comparison. The computer program was Systat 5.2.1 (Systat Inc., Evanston, IL).

3. Results

3.1. MRP expression in Caco-2 cells

Reverse transcription-PCR (RT-PCR) and agarose gel electrophoresis indicate that the expected 498 bp (MRP1 gene product) and 659 bp (MRP2) fragments are expressed in Caco-2 cells (Fig. 1A). Human liver was used as positive (MRP2) and negative (MRP1) controls. cDNA was treated with primers amplifying a 574 bp fragment of human β-actin, to control the integrity of the isolated RNA as well as the amount of cDNA used for each amplification. These results are in agreement with the low expression of MRP1 previously reported in the liver (Loe et al., 1996) as well as in Caco-2 cells (Walgren et al., 2000). In Caco-2 cells, the expression of the MRP2 gene is higher than that of the MRP1 gene.

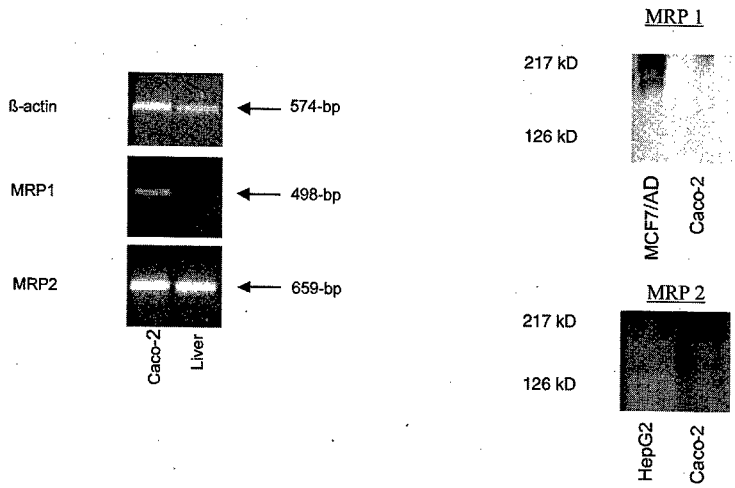
Western blot (Fig. 1B) suggests the absence of MRP1 protein in Caco-2 cells, although detected in MCF7/ADR cells used as positive control. The M_2 III-6 anti-MRP2 antibody shows an immunoreaction in the molecular weight range of 190 kDa, both for HepG2 cells (positive control) and Caco-2 cells. These results indicate the expression of MRP2 in our serum-free culture conditions.

3.2. Cytotoxicity

The cytotoxicity of OTA was determined by measuring MTT reduction and LDH release. OTA inhibits MTT reduction in a concentration-dependent manner, with an IC_{50} of 0.4 μM (data not illustrated). LDH activity released from Caco-2 cells treated with OTA up to 100 μM is not significantly different from that released from control cells. Toxicity of 10 μM OTA for differentiated Caco-2 cell monolayers cultivated in bicameral inserts was investigated by measuring the transepithelial flux of [^{14}C]D-mannitol and TEER values. Whatever the side on which OTA was applied (AP or BL), after 3 h of contact, neither a significant increase in the D-mannitol flux was observed, nor a significant decrease in TEER values (data not illustrated). It indicates that, in these experimental conditions, 10 μM OTA does not cause damages to the integrity of the cell monolayers.

3.3. Metabolization of OTA by Caco-2 cells

HPLC analysis did not reveal detectable amounts of OTα or hydroxylated derivatives at



A. : RT-PCR of mRNAs. **B. : Immunodetection of MRP1 and MRP2**

Fig. 1. Expression of MRP1 and MRP2 in Caco-2 cells. (A) RT-PCR of mRNAs. RT and PCR were performed on total RNA, isolated from Caco-2 cells cultivated on microporous membranes within bicameral inserts for 18 days, with specific primers for MRP1 and MRP2, yielding, respectively, a 498 bp fragment and a 659 bp fragment. Human liver was used as a negative control for MRP1 and as a positive one for MRP2. PCR amplification of RNA not reverse transcribed was negative. The integrity of the isolated mRNAs was checked by using specific primers for β-actin yielding a 574 bp fragment. (B) Immunodetection of MRP1 and MRP2. Western blot detection of MRP1 (upper panel) was performed with MRPr1 on a MRP1 expressing cell line (MCF7/ADR, left lane) and on Caco-2 cells (right lane). MRPr1 recognizes a 190 kDa band in MCF7/ADR but not in Caco-2 cells. Western blot detection of MRP2 (lower panel) was performed with M_2III-6 on a MRP2 expressing cell line (HepG2, left lane) and on Caco-2 cells (right lane). M_2III-6 recognizes a 190 kDa band in HepG2 and in Caco-2 cells. The 150 kDa band most likely corresponds to a degradation product.

either the AP or BL poles as well as in cell lysates from Caco-2 cells incubated for 3 h with 10 μM OTA, suggesting that OTA is not appreciably metabolized by the Caco-2 cells (data not illustrated). Similar results were obtained with samples that were methylesterified to improve separation upon HPLC.

3.4. Transepithelial transport and intracellular accumulation

The transepithelial passage of OTA was studied on Caco-2 cells cultivated for 21 days in different experimental conditions and compared with that of D-mannitol, as marker of the paracellular route. Values for OTA, expressed in pmol/(mg cell protein·min), are illustrated in Fig. 2. At pH 7.4 in both AP and BL compartments, the passage of OTA in the AP to BL direction is lower than from BL to AP, but not significantly different from AP to BL passage of D-mannitol (data not illustrated). In pH conditions better mimicking the in vivo situation, i.e., pH 6.0 on the AP side and pH 7.4 on the BL side, the passage of OTA in the AP to BL direction is increased proportionally to the duration of the experiment and becomes higher than that of D-mannitol (not shown). In these pH conditions, it reaches 23.12 pmol/(mg cell protein·min) from AP to BL, as compared with 12.09 in the opposite direction. The presence of human serum albumin (5 mg/ml) in the BL compartment, further increases the AP to BL passage of OTA (130±9% of the control value), while the BL to AP transport is markedly decreased to 19±8% of the control and becomes similar to the passage of [^{14}C]D-mannitol (not shown). The increase of AP to BL transport could be explained by a stimulation of OTA transport across the BL membrane as a result of the decrease in free OTA concentration in the presence of albumin.

Caco-2 cells incubated for 3 h with 10 μM [^{3}H]OTA accumulate 161.4±28.2 pmol/mg cell protein from AP (pH = 6.0) and 92.6±11.7

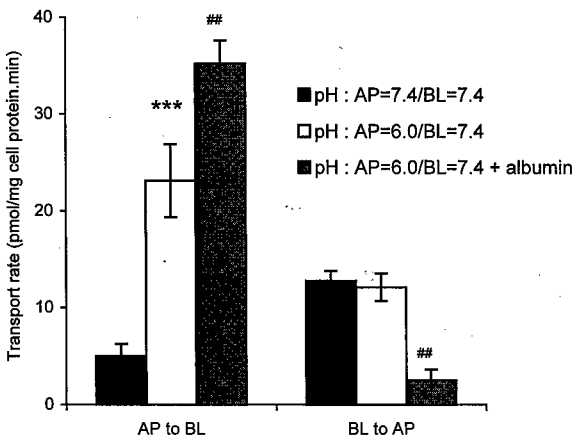


Fig. 2. Effect of pH on transepithelial transport and intracellular accumulation of OTA from AP and BL side of Caco-2 cells. Transport of 10 μM [^{3}H]OTA was measured in both AP to BL and BL to AP directions at either AP and BL pH of 7.4 or at an AP pH of 6.0, and a BL pH of 7.4 or at an AP pH of 6.0, and a BL pH of 7.4 in the presence of human serum albumin (5 mg/ml) in the BL compartment. The monolayers were incubated at 37 °C for different durations up to 3 h and the rate of transport were calculated from the slope of the appearance curves with time points taken every hour. Each data point represents the mean of four values ± standard deviation. *** indicates $P < 0.001$, compared with pH 7.4 on both sides. ## indicates $P < 0.01$, compared with AP pH 6.0 and BL pH 7.4.

pmol/mg cell protein from BL (pH = 7.4) as compared with, respectively, 10.2 ± 0.73 and 3.6 ± 2.9 pmol/mg cell protein for D-mannitol. Accumulation of OTA from AP side of the monolayers is thus higher than from BL side. Accumulation of OTA by the Caco-2 cells appears also very low as compared with the transport. Similar observations were made in the other experimental conditions.

3.5. Implication of carrier systems

To evaluate a role of the large neutral amino acids or the H^+-driven peptide transporter (OTA contains a L-phenylalanine moiety and an amide bond), which are expressed in Caco-2 cells (unpublished results), transport and accumulation of 10 μM [^{3}H]OTA were measured in the presence of an excess of L-phenylalanine (10 mM) or of hydrolysis resistant dipeptide GlySar (10 mM). Whatever the pH on the donor side (pH 7.4 or 6.0), neither transepithelial transport nor intracellular accumulation of OTA were significantly affected (data not shown). To investigate the involvement of the organic anion (PAH) transport system or of the organic anion transporter (oatp), transport and accumulation of 10 μM [^{3}H]OTA were, respectively, measured in the presence of PAH (2 mM) or taurocholate (2 mM). Neither of these substances affects the transport and accumulation of OTA (data not shown).

3.6. Effect of inhibitors on transport and accumulation

Transport experiments were performed with AP pH at 6.0 and BL pH at 7.4, on monolayers incubated at 4 °C, in the presence of metabolic inhibitors (NaN_3 and 2-DOG) or in the presence of inhibitors of the efflux pumps. Fig. 3 shows that AP to BL transport of OTA is almost completely inhibited at 4 °C, but significantly increased by metabolic inhibitors. BL to AP transport is significantly decreased at 4 °C and in the presence of metabolic inhibitors. Accumulations from AP and BL sides are unaffected by these experimental conditions (data not illustrated).

Verapamil, an inhibitor of Pgp, does not affect the transport of OTA in both AP to BL and BL to AP directions (data not illustrated) whereas it increases accumulation from AP side (to 137 ± 17%; $P < 0.05$). Indomethacin, genistein and probenecid, three non-specific inhibitors of MRPs, significantly increase the AP to BL transport of OTA whereas only indomethacin and genistein decrease the BL to AP transport (Fig. 4A). CDNB, which is transformed into 2,4-dinitrophenyl-*S*-glutathione by Caco-2 cells (Sergent-Engelen et al., 1993) a specific inhibitor of MRPs (Gotoh et al., 2000), significantly increases the AP to BL passage of OTA, while decreasing it from BL to AP. AP and BL accumulations of OTA are significantly enhanced by indomethacin, genistein and probenecid (Fig. 4B). As MRP is involved in transport of GSH conjugates, the effect of BSO, an inhibitor of γ-glutamylcysteine synthetase, has been studied on OTA transport and accumulation. When Caco-2 cells are incubated for 24 h with 100 μM BSO, neither the transport nor the intracel-

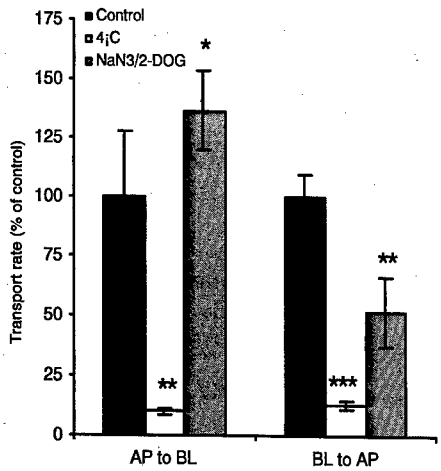


Fig. 3. Energy-dependence of the transport of OTA across Caco-2 cell monolayers in AP to BL and BL to AP directions at pH 6.0 on AP side and pH 7.4 on BL side. Caco-2 cell monolayers were preincubated (30 min) with either transport medium at 37 °C (control, solid column), transport medium at 4 °C (open column) or transport medium containing 1 mM NaN_3 plus 50 mM 2-DOG (hatched column). Subsequently, the monolayers were incubated for different durations up to 3 h with 10 μM [^{3}H]OTA. The control values for AP to BL and BL to AP transports of [^{3}H]OTA were, respectively, 19.4±5.3 and 8.4±0.8 pmol/(mg cell protein/min). Experimental values were normalized against control values and expressed as percent. Data are means±standard deviation of three values. ***, ** and * indicate, respectively, $P < 0.001$, $P < 0.01$ and $P < 0.05$ compared with the control condition.

lular accumulation of OTA are affected (data not shown).

3.7. Efflux of OTA by Caco-2 cell monolayers

Caco-2 monolayers, preincubated from either AP or BL side with 10 μM [^{3}H]OTA at pH 6.0 for 1 h were reincubated at either pH 7.4 or 6.0 in both the AP and BL compartments. To investigate the implication of MRP in the transport of OTA, the effect of indomethacin was tested. The results show that OTA is extensively excreted by Caco-2 cells at both poles and that only ca. 5% of the preloaded amount remain in the cells after 90 min of reincubation. Fig. 5 shows the efflux when OTA was preloaded from the BL side. Whatever the pH of the medium is and the side from which OTA is preloaded, indomethacin inhibits efflux towards the AP compartment and increases efflux towards the BL compartment.

4. Discussion

The transepithelial transport of OTA across the human intestinal barrier has been investigated by using an in vitro model based on the cultivation of Caco-2 cells in a serum-free nutritive medium, on a microporous membrane, separating the apical pole from the basolateral one.

RT-PCR analysis indicates that, in our serum-free culture conditions, Caco-2 cells express the MRP1 and MRP2 genes, at the mRNA level, MRP2 expression being higher than that of MRP1, as already reported by Hirohashi et al. (2000) for other culture conditions. Western blot analysis does not reveal a detectable level of MRP1 protein in Caco-2 cells. Makhey et al. (1998) and Gutmann et al. (1999) reported that MRP1 is expressed by Caco-2 cells at both the mRNA and protein levels, but differences could be related to the absence of serum for our experiments. MRP2 protein, also termed canalicular multispecific organic anion transporter (cMOAT), is detected in Caco-2 cells. It has been predominantly localized in the AP domain of hepatocytes and kidney proximal tubule cells (Keppler and König, 1997; Keppler et al., 1998). Hirohashi et al. (2000) have suggested that MRP2 is probably expressed at the AP side of the Caco-2 cells, which has been confirmed by immunochemical staining (Bock et al., 2000).

Cytotoxicity assays on proliferating Caco-2 cells show that OTA inhibits MTT reduction with an IC_{50} of 0.4 μM, lower that those reported for two kidney cell lines (Bondy and Armstrong, 1998), i.e., LLC-PK_1 (13.1 μM) and OK (34.4 μM) cells. The absence of LDH release from the cells treated with OTA indicates that, after 48 h, OTA does not induce cell necrosis.

In mammalian cells, OTA is metabolized into OTα and phenylalanine and, in the liver, in other hydroxylated metabolites by various cytochrome P450 isoforms. HPLC analysis shows that, in our experimental conditions, OTA is not appreciably metabolized by Caco-2 cells.

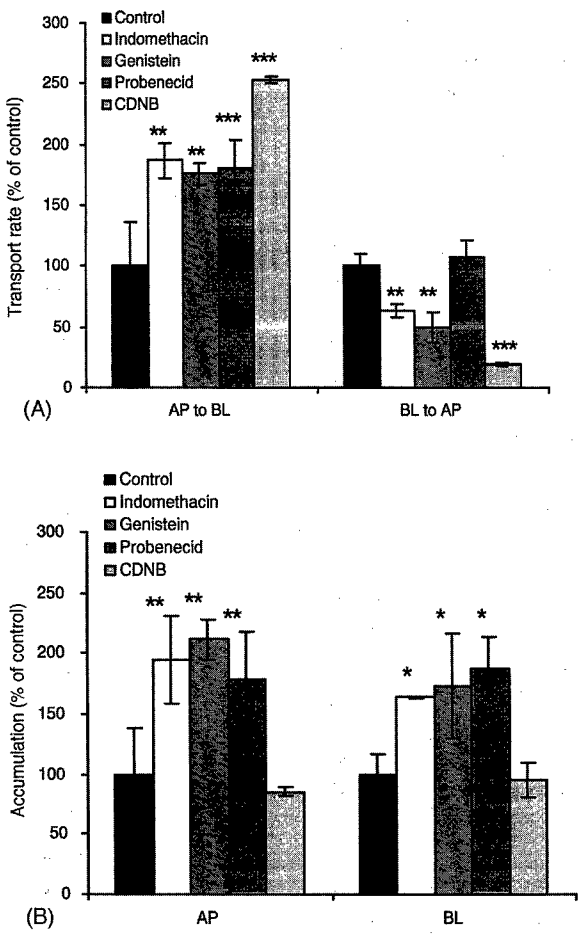


Fig. 4.

In pH conditions mimicking the in vivo situation (AP pH 6.0, BL pH 7.4), AP to BL transport of OTA is higher than BL to AP transport, indicating that OTA is absorbed across Caco-2 cell monolayers. In the presence of albumin in the BL compartment, OTA is prevented from entering Caco-2 cells via the BL membrane. This suggests that, in vivo, OTA would not be transported from the serosal to the luminal side of the enterocytes, as a result of its binding to plasma proteins.

Acidification to pH 6.0 of AP increases transepithelial transport and intracellular accumulation of OTA. Because OTA is a weak organic acid ($pK_a = 7.1$), a decrease in pH from 7.4 to 6.0 increases the fraction of uncharged OTA from 33 to 93%, making the diffusion across biological membranes easier. This could explain the stimulation of OTA transport and accumulation by acidification of the apical pole of Caco-2 cells.

If the pH is maintained at 7.4 at both cellular poles, transport of OTA in the BL to AP direction is faster than in the AP to BL direction. This strongly suggests that, in the Caco-2 cells, one or more specific polarized systems participate to the transepithelial transport of OTA.

The results failed to show that carrier systems for large neutral amino acids and H^+-driven peptide transporter, although expressed in Caco-2 cells are implicated in the transport of OTA as reported in kidney cells (Gekle et al., 1993; Zingerle et al., 1997; Schwerdt et al., 1997, 1998). Similarly, OTA transport in Caco-2 cells is not mediated by the PAH carrier or by oatp as reported, respectively, in kidney and liver cells (Gekle et al., 1993; Kontaxi et al., 1996). Nevertheless, we did not check whether these two carrier systems are expressed by Caco-2 cells.

Experiments performed at 4 °C or in the presence of metabolic inhibitors indicate that AP to BL transport is almost totally inhibited at 4 °C

Fig. 4. Effect of MRP inhibitors on the transport and accumulation of OTA across Caco-2 cell monolayers in AP to BL and BL to AP directions at pH 6.0 on AP side and pH 7.4 on BL side. (A) Transport. Caco-2 cell monolayers were incubated for different durations up to 3 h with 10 μM [^{3}H]OTA in transport medium alone (control) or in transport medium containing either 200 μM indomethacin, 100 μM genistein, 2 mM probenecid or 1 mM CDNB in both compartments. The control values for AP to BL and BL to AP transports of [^{3}H]OTA were, respectively, 17.4±6.4 and 8.4±0.8 pmol/(mg cell protein/min). Experimental values were normalized against control values and expressed as percent. Data are means±standard deviation of 3–6 values. *** and ** indicate, respectively, $P < 0.001$ and $P < 0.01$ compared with the control condition. (B) Accumulation. After 3 h of incubation of the monolayers, the intracellular accumulation of [^{3}H]OTA was recorded. The control values for AP and BL accumulations of [^{3}H]OTA were, respectively, 121.9±46.7 and 80.3±13.4 pmol/mg cell protein. Experimental values were normalized against control values and expressed as percent. Data are means±standard deviation of 3–10 values. ** and * indicate, respectively, $P < 0.01$ and $P < 0.05$ compared with the control condition.

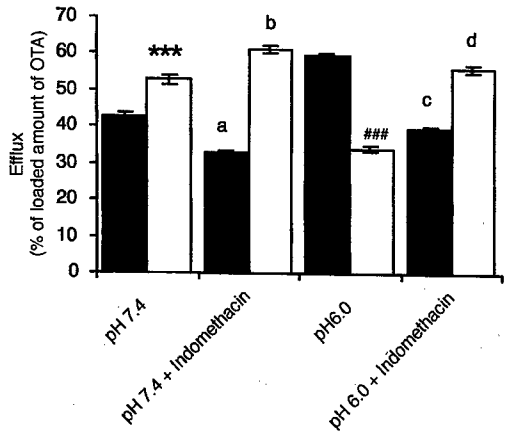


Fig. 5. Effect of indomethacin on efflux of OTA towards the AP and BL poles of the Caco-2 cells at either pH 7.4 or 6.0 on both sides of the cells. The monolayers were preloaded from BL side with 10 μM [^{3}H]OTA dissolved in transport medium at pH 6.0 for 1 h at 37 °C. After washing of the monolayers with ice-cold PBS, efflux was measured towards the AP (solid column) or BL (open column) side at 37 °C at either pH 7.4 or 6.0 on both sides of the cells in the presence or not of 200 μM indomethacin. Efflux is expressed as percentage of the preloaded amount of OTA and bars represent total cumulative amounts after 90 min. Each data point represents the mean of 3–6 values ± standard deviation. *** indicates $P < 0.001$ compared with AP efflux at pH 7.4. ### indicates $P < 0.001$ compared with AP efflux at pH 6.0. 'a' and 'b' indicate $P < 0.001$ compared with, respectively, AP and BL efflux at pH 7.4. 'c' and 'd' indicate $P < 0.001$ compared with, respectively, AP and BL efflux at pH 6.0.

but stimulated in the presence of metabolic inhibitors. BL to AP transport is significantly decreased at 4 °C and by metabolic inhibitors. These results are in agreement with inhibition of an ATP-dependent efflux pump localized at the AP pole of the Caco-2 cells, whereas the decrease of transport at 4 °C could further result from a change in membrane fluidity.

Pgp and, at least MRP2, are expressed at AP of Caco-2 cells (see above; unpublished results). Indomethacin, genistein and probenecid, non-specific inhibitors of MRP, as well as CDNB, which is biotransformed into a specific inhibitor of MRPs, but not verapamil, a Pgp inhibitor, increase AP to BL transport of OTA whereas BL to AP transport is only inhibited by indomethacin, genistein and CDNB. The MRP inhibitors increase accumulation from both AP and BL sides, whereas verapamil increases it only from AP. These results are in agreement with the inhibition of MRP, mediating the efflux of OTA at the AP side of the Caco-2 cells. Because MRP2 has been shown to be expressed at the AP side of the Caco-2 cells, the transport activity associated with OTA probably results from the function of MRP2. Furthermore, OTA has been recently reported to be a substrate of MRP2 (König et al., 1999). The effect of verapamil on OTA accumulation could be explained by the fact that verapamil, in addition to be a strong modulator of Pgp, is also a moderate inhibitor of MRP (Aszalos and Ross, 1998). In contrast, GSH, reported to play a role in drug transport by MRP (Cole and Deeley, 1998), does not seem to be involved, since BSO, an inhibitor of GSH biosynthesis, does not affect OTA transport and accumulation.

At pH 7.4 in both compartments, AP to BL transport of OTA could result only from paracellular passage whereas from BL to AP, OTA would also be transported through transcellular pathway. This would suggest the presence of a carrier system at the BL pole of the Caco-2 cells, which would mediate OTA transport.

Efflux experiments show that OTA is extensively excreted by Caco-2 cells. In addition, whatever the pH of the medium and the side from which OTA is preloaded, indomethacin inhibits efflux towards the AP compartment and increases efflux towards the BL compartment. These results confirm that MRP would be involved in the secretion of OTA at the AP pole. The indomethacin-induced increase of efflux towards the BL compartment also suggests that a carrier system would be implicated in the excretion from the BL pole. According to our hypothesis, this carrier would be a facilitated carrier system, mediating uptake as well as efflux of OTA.

In conclusion, these results suggest that, in conditions mimicking the in vivo situation, OTA is absorbed across the human intestinal mucosa. Transport across the AP membrane would result from passive diffusion of the undissociated form of OTA, whereas efflux across the BL membrane would be mediated by a facilitated carrier system. Nevertheless, the intestinal absorption would be

limited thanks to the presence of the MRP2 at the AP pole of the enterocytes. OTA reaching the bloodstream would bind to plasma proteins, preventing it to be reabsorbed by the enterocytes across their BL membrane.

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