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Award Number: DAMD17-00-1-0073

TITLE: Role of Respirable Saudi Arabian Sand and Pyridostigmine
in the Gulf War Syndrome: An Autoimmune Adjuvant Disease?

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REPORT DATE: March 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20010531 024

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE March 2001	3. REPORT TYPE AND DATES COVERED Annual (10 Feb 00 - 10 Feb 01)	
4. TITLE AND SUBTITLE Role of Respirable Saudi Arabian Sand and Pyridostigmine in the Gulf War Syndrome: An Autoimmune Adjuvant Disease?			5. FUNDING NUMBERS DAMD17-00-1-0073	
6. AUTHOR(S) Mohan L. Sopori, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Lovelace Biomedical and Environmental Research Institute Albuquerque, New Mexico 87185 E-Mail: msopori@lrri.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) We postulated that the Gulf War syndrome (GWS) might result from an aberrant immunological response by susceptible individuals to the environmental factors in the Persian Gulf [i.e., inhalation of the sand rich in silica, exposure to mycobacterium, and response to the anti-nerve gas agent, pyridostigmine bromide (PB)], leading to an autoimmune-like disease. To evaluate this possibility we proposed to develop a rat model for autoimmune adjuvant disease (AAD) using <i>Mycobacterium smegmatis</i> (<i>M. smegmatis</i>). The development of AAD was to be ascertained by the serum levels of α -2 macroglobulin and inflammation of paws. After establishing the dose of <i>M. smegmatis</i> sufficient to induce a moderate AAD response, the effects of silica inhalation \square PB on AAD response were to be determined. So far, we have completed the following tasks: (a) grown <i>M. smegmatis</i> and extracted and its antigenic material; (b) standardized the assay for α -2-macroglobulin; and (c) determined that subcutaneously administered PB does not affect the immune response. Rats are scheduled for silica inhalation starting on March 19, 2001.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 10	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

We postulated that the Gulf War syndrome might result from an aberrant immunological response by susceptible individuals to the environmental factors in the Persian Gulf [i.e., inhalation of the sand rich in silica, exposure to mycobacterium, and response to the anti-nerve gas agent, pyridostigmine bromide (PB)], leading to an autoimmune-like disease. To evaluate this possibility we proposed to develop a rat model for autoimmune adjuvant disease (AAD) using the non-pathogenic mycobacterial strain, *Mycobacterium smegmatis* (*M. smegmatis*). The development of AAD was to be ascertained by the serum levels of α -2 macroglobulin (α -2M) and inflammation of paws. After establishing the dose of *M. smegmatis* sufficient to induce a moderate AAD response, the effects of silica inhalation \pm PB on AAD response were to be determined. So far, we have completed the following segments of the proposed project:

BODY

1. Mycobacterium Cultures

M. smegmatis was purchased from ATCC, grown in special DIFCO medium according to ATCC instructions. Briefly, the bacterium was rehydrated in enriched Middlebrook 7H9 broth, inoculated on Lowenstein-Jensen agar slants, and incubated at 37°C for 7 days. The bacteria were collected from the slants, washed 3X with PBS, and heat-inactivated at 80°C for 1 h. The antigen material from this bacterium was extracted and frozen at -80°C in aliquots in quantities sufficient for all the experiments proposed in the project.

2. Assay for α -2 Macroglobulin (α -2M)

The serum α -2M concentrations were determined by the method of Ganrot (1966). This assay is based on the principle that α -2M forms a cage around trypsin, which inhibits the access of larger molecules (e.g., trypsin inhibitor) to reach the enzyme. Higher the concentration of α -2M, lower the probability of the trypsin inhibitor to affect the enzyme activity. Therefore, in the presence of α -2M, trypsin can hydrolyze a small molecular weight substrate, such as N α -benzoyl-DL-arginine p-nitroanilide (BapNa), even in the presence of soybean trypsin inhibitor. The assay was standardized with the human plasma α -2M. Briefly, various concentrations of α -2M were incubated with a known concentration of trypsin for 30

s before the addition of the soybean trypsin inhibitor and BapNa to the reaction mixture. The reaction was stopped after 20 min by 4% acetic acid the hydrolysis of BapNa determined by measuring OD at 410 μm spectrophotometrically. The OD was linear up to 1 mg/ml of α -2M. Similar standardizations were made with rat serum. Subsequently, α -2M levels were determined in serum samples from rats challenged with two inflammatory stimuli [i.e., turpentine-induced sterile abscess (Sopori et al., 1998), endotoxin (Kozak et al., 1995), and adjuvant arthritis model using *M. smegmatis* extracts (Whitehouse, 1988)]. *M. smegmatis* extracts (1 mg/rat) caused an increase of 70% in the serum α -2M level above the control. On the other hand, 50 μl of turpentine produced 2.5- to 3-fold increase in the serum α -2M level. To ascertain the effects of silica inhalation, future experiments will use *M. smegmatis* extracts at the concentration of 1 mg/rat.

3. Adjuvant Arthritis Model

To determine whether *M. smegmatis* causes adjuvant arthritis disease (AAD), LEW rats were injected in the tail vein with various concentrations of freeze dried sample of *M. smegmatis* extract suspended and emulsified in mineral oil (20 mg/ml). These animals were scored for increases in paw volume (inflammation) and the serum α -2M levels. Paw volumes were determined by measuring the thick before and after inoculation by a caliper (Oditest gauge). A moderate AAD was detected by inoculation of 1 mg/rat (70% increase in α -2M and 30% increase in paw thickness). These conditions for a low-grade inflammatory response to by *M. smegmatis* alone should allow us to quantitate the exacerbation of the inflammatory response by silica exposure. In addition, Oditest measurement is not a precise method for quantitating paw volumes. We have recently purchased the BUXCO paw plethysmometer to better quantitate this response.

4. Pyridostigmine bromide and immunity

The anti-nerve gas agent, pyridostigmine bromide (PB), was prophylactically used by the Gulf War veterans during the operation Desert Storm. PB reversibly inhibits cholinesterases and competes with the irreversible inhibitors of cholinesterases, such as the nerve gas agent, sarin. Because the brain and the immune system communicates bidirectionally, cholinergic agents may affect the neuroimmune communication by increasing the synaptic levels of acetylcholine. To evaluate whether PB, given peripherally via the subcutaneously implanted miniosmotic pumps, affected the immune response, PB-treated (4 wk, 0.8 mg of PB/rat/day) were examined for the antibody-forming cell response, T cell mitogenesis, and

intracellular Ca^{2+} levels in response to anti-T cell receptor antibodies. Under these conditions, PB had no significant effect on the immune response. However, normally PB does not cross the blood-brain-barrier, and it is possible that PB given directly (intracerebroventricularly) into the brain may affect the immune response. This possibility is being currently examined.

5. Silica exposure

- a) Silica particles: The US-Silica (Berkeley Springs, WV) has kindly donated silica for these experiments. A sample of silica was impacted in the Lovelace Multijet Impactor to obtain the particle size of approximately 2 μm . This test produced the particle size of 1.6 μm ; this size would be excellent for the inhalation studies.

- b) Silica inhalation protocol: LEW rats were purchased, and are currently in the 2-wk quarantine period prescribed by the Lovelace Animal Care and Use committee for incoming animals. Exposure of these animals to silica for 6 weeks will start on 19th March, 2001 according to the following protocol.

Animals: Male Lewis Rats from Charles River (5-6 weeks old)

1. 72 animals exposed to $5\text{mg}/\text{m}^3$ silicate sand for 6 weeks.
 - a. Exp. # 6695 (silica sand) B001-B042. (42 animals)
 - b. Exp. # 6696 (silica sand and *M. smegmatis*) C001-C030. (30 animals)
2. 72 animals exposed to air (sham controls).
 - a. Exp. # 6700 (Sham controls) D011-D052. (42 animals)
 - b. Exp. # 6694 (*M. smegmatis*) A021-A051. (30 animals)

Schedule:

1. March 5 – Animals arrived and placed in conditioning chambers for two weeks.
2. March 19 – Begin silica exposure ($5\text{mg}/\text{m}^3$ silicate sand – 1.5 micron particles) for six weeks.
3. April 27 – exposure ends (Animals should not be removed from chambers).
4. May1 – Experiment 1: Animals removed from chambers and placed in Dr. Mohan Sopori's animal room.

5. May 3 – Experiment 2a: Inject 1 μg *Mycobacterium smegmatis*/50 μl mineral oil in tail I.D. (Ray Langley).
6. May 4 – Experiment 2b: inject 1 μg *M. smeg* T.I.D. (R. Langley)
7. May 24 – Sacrifice experiment 2.
8. May 25 – Sac. Exp. 2b.
9. June 19 – Experiment 3a – Inject *M. smeg*. (R. Langley)
10. June 21 – Experiment 3b – Inject *M. smeg*. (R. Langley)
11. July 10 – Sac. Exp. 3a.
12. July 12 – Sax Exp. 3b.
13. Aug. 28 – Experiment 4a – Inject *M. smeg*. (R. Langley).
14. Aug. 30 – Experiment 4a – Inject *M. smeg*. (R. Langley).
15. Sept. 18 – Sac. Exp. 4a.
16. Sept. 20 – Sac. Exp. 4b.

Oxolozone experiment

Extra animals (D046-D052 and B036-B042) will be given to Juan Carlos Phillippides-Pena to test the effects of oxolozone.

Experiment 1:

1. Animals:

- a. Silica Animals B001-B005.
- b. Control Animals D011-D015.

2. Necropsy:

- a. Spleen: Weigh under sterile conditions.
 1. 1/3 to Ray Langley (RPMI)
 2. 1/3 to Dr. Tom March for histopathology
 3. 1/3 to Dr. Janet Benson. (weigh then cryofreeze)
- b. Lungs
 1. 1/2 Lavage to R. Langley. (Lavage calculations determined by Katie Bennett.)
 2. 1/2 fix and send to histopathology.
- c. Liver: Weigh under sterile conditions.
 1. Lobe for R. Langley (cryofreeze).
 2. Lobe to Dr. Benson (weigh then cryofreeze).
- d. Lymphnodes – send to histopathology.

e. *Brain:*

1. 1/2 to R. Langley (cryofreeze)
2. 1/2 to Dr. Benson (cryofreeze)

f. *Blood:* A. 3 ml for Serum. B. 3 ml for plasma

3. *Experiments to be performed:*

- a. *Silica content in brain, spleen and liver. (Dr. Benson)*
- b. *Histopathology for spleen, lungs and lymphnodes (Dr. March)*
- c. *Lavage: cell counts, total protein, LDH. (Dr. Shashi Singh and Juan Phillippides).*
- d. *Spleens: Con A, LPS, M. smeg. response (Dr. Roma Kalra and Dr. S. Razani.)*
- e. *Brains: RT-PCR for IL-1, IL-6, TNF- α . (R. Langley and Dr. Kalra)*
- f. *Serum: α -2 Macroglobulin determination (R. Langley)*
- g. *Liver: TBD (R. Langley)*
- h. *Paw Volume: Will be taken by R. Langley before sacrifice.*
- i. *Flow cytometry: CD-45r and CD-11b (R. Langley).*

Experiment 2a and 2b

1. *Animals: (split into two groups of 20 – five animals from each)*
 - a. *Sham controls (10 animals) D016-D025.*
 - b. *Silica only (10 animals) B006-B015*
 - c. *M. smeg. only (10 animals) A021-A030*
 - d. *Silica and M. smeg (10 Animals) C001-C010*
2. *Necropsy: repeat everything in exp. 1, except paws also to be fixed and sent to Histopathology.*

Experiment 3a and 3b

1. *Animals: (split into two groups of 20 – five animals from each)*
 - a. *Sham controls (10 animals) D026-D035.*
 - b. *Silica only (10 animals) B016-B025*
 - c. *M. smeg. only (10 animals) A031-A040*
 - d. *Silica and M. smeg (10 Animals) C011-C020*
2. *Necropsy: repeat everything in exp. 1, except paws also to be fixed and sent to Histopathology.*

Experiment 4a and 4b

1. *Animals: (split into two groups of 20 – five animals from each)*
 - a. *Sham controls (10 animals) D036-D045.*
 - b. *Silica only (10 animals) B026-B035*
 - c. *M. smeg. only (10 animals) A041-A050*
 - d. *Silica and M. smeg (10 Animals) C021-C030*
2. *Necropsy: repeat everything in exp. 1, except paws also to be fixed and sent to Histopathology.*

KEY RESEARCH ACCOMPLISHMENTS

- Inoculation of *M. smegmatis* emulsions in mineral oil produce a moderate level of AAD.
- AAD can be quantitated by paw volume and serum α -2M levels.
- Chronic subcutaneously administration of PB does not affect the immune system.

REPORTABLE OUTCOMES

Because, experiments related to the major aim of the study have not been yet completed, there are no significant reportable outcomes at this time. However, we expect to finish these studies in the next 8-10 weeks, when we would be able to determine whether inhalation of silica affects the immune system.

CONCLUSIONS

Although experiments related to the major aim of the study (i.e., role of silica inhalation on the immune system) have just been started, essentially all the assays proposed in the grant application have been standardized. PB, given peripherally, does not appear to significantly alter the immune response. However, if given directly into the brain, PB may affect the immune response through neuroimmune modulation.

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