

LOW-LEVEL INHALATION EXPOSURE TO CHEMICAL NERVE AGENT VAPOR INDUCES EXPRESSION OF NEURONAL APOPTOSIS AND REGENERATION GENES

J.W. Sekowski^{*1}, M.A. Orehek¹, J. Bucher¹, M. Vahey², M. Nau², M.S. Horsmon¹,
D. Menking¹, C. Whalley¹, B. Benton¹, R. Mioduszewski¹, S. Thomson¹, and
J.J. Valdes¹

¹U. S. Army Research, Development and Engineering Command, Edgewood Chemical Biological Center,
AMSSB-RRT-BM, 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010, USA; ²Gene Array
Laboratory, Walter Reed Army Institute of Research
Bethesda, MD 20850, USA

1. ABSTRACT

For over a decade following end of the first Gulf War in 1991, there has been increasing concern to understand the immediate and persistent effects of sub-acute and low-level exposures to chemical warfare agents. Although low-level exposures may not cause obvious pathology at the time of exposure, they may cause molecular-level toxic injuries in the brain and other vital organ systems.

The work described in this manuscript was designed to measure the gene and protein expression alterations in the brains of male and female rats exposed to sub- and peri-mitotic levels of the aerosolized nerve agents sarin (GB) and cyclosarin (GF). Gene expression was assessed using DNA microarray analysis. The microarray data were verified by real-time RT-PCR and Western immunoblotting were possible.

The overall aim of this study is to measure and characterize the gene or protein level alterations that could reveal near term operational risks as well as molecular events that may predispose an individual to injury or disease later in life. To date, our results indicate that: 1) low-level inhalation exposure to GB and GF results in the differential expression of a number of neuronal genes, including a group that participate in cellular processes critical to neurological injury and regeneration and 2) there are significant gender-associated differences in the level and type of gene expression response.

2. INTRODUCTION

For more than 50 years, the acute pathological, histological, and enzymological aspects of nerve agents have been extensively examined (Wiener and Hoffman, 2004). The most common nerve agents are the “G” series agents and VX. The G agents, developed by the Germans, were synthesized initially as pesticides. Just prior to World War I, they were found to be acute inhibitors of the enzyme Acetylcholinesterase (AChE) in humans that caused death within minutes from the overwhelming accumulation of synaptic Acetylcholine. Soon after the war ended, the U.S. and its allies discovered the stockpiles, and began synthesizing and testing these and other nerve agents. While the only known battlefield use of nerve agents was in the Iran–Iraq War, many countries and non-state sponsored political groups are known to have possession of and knowledge to manufacture organophosphate nerve agent and nerve agent munitions.

At the end of the Gulf War, when the Khamisiyah chemical munitions pit was destroyed, many individuals may have been exposed to low levels of nerve agent. Soon after the end of the Gulf War, awareness was raised that diverse symptoms, collectively named Gulf War Syndrome, were being experienced by many Gulf War veterans including many of whom were working in Khamisiyah vicinity (Smith et al., 2003). In the U.S., a new research focus was begun, led primarily by the U.S. Veterans Administration, that has engendered much of the work in the area of low-level chemical agent toxicology.

After the Aum Shinrikyo cult of Japan used sarin in two terrorist attacks (Matsumoto in 1994 and Tokyo in 1995) awareness was again raised that there is a great paucity of information regarding the effects of low-level

Report Documentation Page			Form Approved OMB No. 0704-0188		
Public reporting burden for the 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 the 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. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 16 NOV 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Low-Level Inhalation Exposure To Chemical Nerve Agent Vapor Induces Expression Of Neuronal Apoptosis And Regeneration Genes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U. S. Army Research, Development and Engineering Command, Edgewood Chemical Biological Center, AMSSB-RRT-BM, 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010, USA				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004., The original document contains color images.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

chemical agent plumes. In these two horrific attacks on innocent men, women and children, 6,100 people were treated and 19 people died. Many of those treated were exposed to low-levels of sarin vapor (Sidell et al., 2002). From these tragic events, however, at least one positive outcome has been gleaned. The data from patients and “lessons learned” from physicians, emergency response planners, and others have been able to give valuable medical and operational insights toward better preparing for future low-level nerve agent exposure scenarios (Asai and Arnold, 2003; Okudera, 2002). Hopefully, the operational insight and the overall the greater awareness of the problem will lead to the development of better response procedures, as well as detection, protection, and treatment measures against future terrorist and wartime CWA exposures.

In this manuscript we describe work examining the molecular-level responses of the rat brain exposed to GB and GF vapor via whole body inhalation. The aim of this work is to model the molecular toxicological events that follow low-level exposure to two common nerve agents. The experiments illustrated herein have been designed to measure the gene and protein expression level changes in the brain of male and female Sprague-Dawley rats exposed to low-level doses (0.004-0.033 mg/m³) of the aerosolized nerve agent GB and GF via whole body inhalation for 4 hours. Control animals were exposed to air under the same conditions for the same time period. The levels of mRNA transcripts from the whole brains of these animals were analyzed using DNA microarray (Affymetrix GeneChip) analysis and were verified by real-time RT-PCR. The gene products of the altered transcripts (for which commercial antibodies exist) were measured via Western immunoblotting (data not discussed here due to space limitations).

Beyond this study, several ongoing molecular toxicological studies in the rat and other animal model systems, with other CWA and other dosing regimens, will allow us to begin to piece together a more accurate timeline of toxicologic effect (immediate vs. persistent). We also hope to begin to account for species and agent specificity. Furthermore, through parallel collaborative studies we plan to begin to correlate our molecular-level findings with behavioral, physiological, and pathological endpoints. Overall, we hope to reveal the near term operational risks as well as molecular events that may lead to injury or disease later in life.

3. MATERIALS AND METHODS

Housing and exposure of Sprague-Dawley rat to agent vapor: See Mioduszeewski R.J. et al, 2002.

Collection and Preparation of Tissue RNA: Upon resection, the brains are wrapped, labeled, and immediately frozen in liquid nitrogen. They are cataloged and stored at -135 degrees Celsius. To prepare the mRNA, whole brains are homogenized in TRIzol reagent (Gibco BRL Life Technologies) using a polytron and immediately placed on ice. The total RNA extraction and precipitation steps are carried out essentially as described by Chomczynski, P. and Sacchi, N. (1987).

Synthesis of biotin-labeled cRNA and target preparation: The *in vitro* synthesis and purification of biotin-labeled antisense cRNA (target) is carried out according to Affymetrix instructions, with modifications as described in Nau et al (2000). The biotin-labeled cRNA is fragmented using 5X fragmentation buffer (200mM Tris-acetate, pH 8.1; 500mM KOAc; 150mM MgOAc) as described in Nau et al (2000).

Hybridization, staining, washing, and scanning of the DNA microarrays: For the rat GB study, the Affymetrix Rat Neurobiology Chip (RN-U34) was used. For the GF study the Affymetrix Whole Rat Genome Chip (RG-U34) was utilized. The hybridization, washing, staining, and scanning steps are carried out according to the manufacturer’s instructions (Affymetrix) and as described in Nau et al (2000).

Analysis of Transcriptomic Data: To analyze the raw data, Data Mining Tool, Microarray Suite (Affymetrix, Santa Clara, CA), Partek Pro (Partek, St. Louis, MO), and a variety of web-based databases and statistical resources are used.

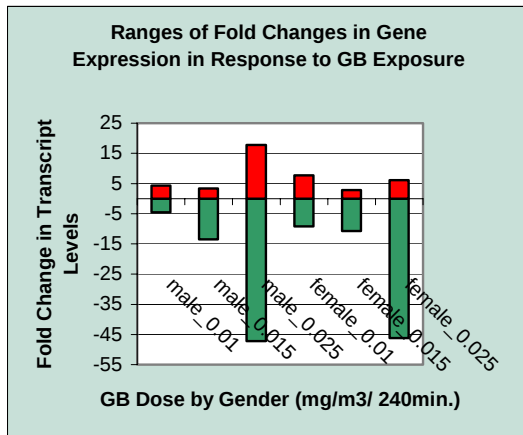
Real time RT-PCR: The AmpliTaq Gold® DNA polymerase mix and the RT enzyme mix (Applied Biosystems, Branchburg, New Jersey) are combined with the TaqMan® probes, DNA primers, and input mRNA to carry out the RT-PCR reactions. The reactions are assembled and performed according to manufacturer’s instructions. The following cycling parameters are used: reverse transcription at 48°C for 30

min; initial denaturation at 95°C for 10 minutes, and 45 cycles with 95°C for 15 s and 60°C for 1 min. Each sample assay is run in duplicate along with 2 negative control reactions.

4. RESULTS

4.1 Brain gene expression patterns differ between exposed and unexposed rats.

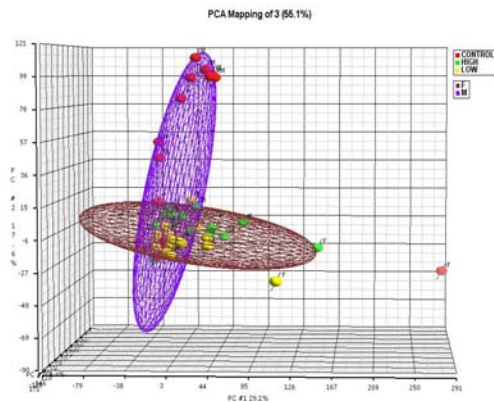
Analysis of the whole brain gene expression data reveals that there is a dose–response relationship between the level of OP agent and the increase in the range of gene expression changes (Fig. 1)



4.2 Brain gene expression patterns differ between male and female rats.

A principal components analysis (PCA) of the rat brain gene expression data with a wire mesh delineating male and female samples illustrates the lack of similarity between the male and the female gene expression response. In figure 2, the male control animals (red dots in vertical purple mesh) appear to be more varied in the levels of brain gene expression than their female counterparts (red dots in horizontal brown mesh).

Fig. 2. Principal components analysis (PCA) of the rat brain GF exposure gene expression data with wire mesh delineating male and female samples.



4.3 Real-time RT-PCR verification of verification of microarray data.

In order to verify the DNA microarray results, TaqMan RT-PCR amplification assays were used to measure the relative abundance of specific transcripts found in control and exposed animals. The RT-PCR data shown in figure 3 are the amplification profiles indicating the relative levels of transcript specific to each gene (as indicated).

These are averaged triplicate reactions using brain RNA pooled from 4 male rats exposed to 0.013 mg/m³ GF vapor for 4 hours. Cono0trl RNA was from an equal number of air-exposed males. In this example, the CT₅₀ of the real time RT-PCR reaction was 22.5 cycles for the exposed animals and 24.5 cycles for the control animals. A difference of 2 cycles translates to approximately a 4-fold difference in transcript level. This RT-PCR measurement is considered a good verification of the Gene Chip results.

Fig. 3 RT-PCR verification of many of the genes identified by microarray analysis (This is a subset of the total RT-PCR assays).

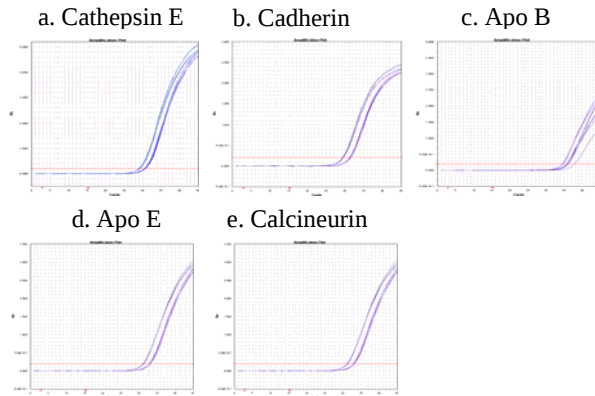


Table I. Induced neuronal regeneration genes in the male rat brain exposed to 0.013 mg/m³ GF vapor for 240 minutes.

Gene	Fold	p-value	RT-PCR fold	Known function
BDNF	2.04	0.003	TBD	Synapto-genesis
Apo E	8.1	0.009	3.8	Lipid transport
Cadherin	4.7	0.000...	3.78	Cell adhesion
Calponin	5.0	0.002	TBD	Plasticity
MBP	10.2	0.009	TBD	Myelin
Complexin II	6.4	0.010	TBD	Vesicle release

Table II. Induced neuronal degeneration/ cell death/ apoptosis-related genes in the male rat brain exposed to 0.013 mg/m³ GF vapor for 240 minutes. No miosis was observed in these animals.

Gene	Fold	p-value	RT-PCR fold	Known function
Cathepsin E	3.25	0.001	2.7	Excitatory cell death
Calcineurin	2.2	0.001	1.8	Apoptosis
Bcl-2	4.12	0.002	TBD	Apoptosis inhibitor

5. DISCUSSION

Since the end of the Gulf War, there has been increased interest in the effects of sub-acute and low-level exposure to chemical agents, including organophosphorus (OP) nerve agents. While the acute and overt effects of OP agent toxicity are well known, the more subtle and molecular level alterations of these agents are not well understood. Chemical-induced damage and alteration occurring at the molecular level has been implicated in the etiology of injury and disease that may present itself weeks, months, and even years after the initial toxicant exposure (NRC, 1997; Jamal et al., 1998; McDairmid et al, 2000). With this in mind, we carried out a series of experiments designed to examine the persistence (one week post-exposure) of the molecular level effects of low-level chemical agent vapor exposure. This time point was chosen since normal pupillary function is completely restored at this point in every animal tested, regardless of the dose. The results of this work suggest that exposure to GB and GF vapor at or below the levels that cause miosis, creates significant alterations in the expression profile of a number of genes in the brain. More specifically, many of the genes significantly altered by exposure to agent are related to processes of neuronal degeneration, apoptosis and regeneration. These data also clearly indicate that there significant gender-associated differences in the gene expression responses to these agents. The data resulting from this study open many avenues of further investigation into the toxicological events associated with these gene expression changes. Furthermore, these results will inform the design for future low-level molecular toxicological studies (e.g. to include female animals when possible) aimed at obtaining greater understanding of the effects of important industrial, agricultural, and militarily important chemicals. Future toxicogenomic and proteomic investigations promise to yield information critical for improving detection, protection, and decontamination materiel to ensure the health, safety, and operational success of the warfighter.

ACKNOWLEDGEMENTS

This work was made possible through the In-House Laboratory Initiative Research (ILIR) Program at the Edgewood Chemical Biological Center.

6. REFERENCES

- Asai Y, Arnold JL. (2003) Terrorism in Japan. *Prehospital Disaster Med.* 8(2):106-14.
- Chomczynski, P. and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Jamal, G.A. (1998) Gulf War syndrome- a model for the complexity of biological and environmental interaction with human health. *Adverse Drug React Toxicol. Rev* 17(1), 1-17.
- Mioduszewski R.J., Manthei, J.H., Way, R., Burnett, D.C., Gaviola, B.P., Muse, W.T., Sommerville, D.R., Crosier, R.B., Scotto, J.A., McCaskey, D.A., Crouse, C.L., Matson, K.L. and Thomson, S.A. (2002) Low-Level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size. U.S. Army Technical Report, ECBC-TR-235, May 2002.
- Nau, M.E, Emerson, L.R., Martin, R.K., Kyle, D.E., Wirth, D.F., and Vahey, M. (2000) Technical assessment of the affymetrix yeast expression GeneChip YE6100 platform in a heterologous model of genes that confer resistance to anti-malarial drugs in yeast. *J Clin Microbiol.* 38 (5):1901-8.
- Oudera, H. (2002) Clinical features on nerve gas terrorism in Matsumoto. *J Clin Neurosci.* Jan;9(1):17-21.
- Sidell, FR, Takafuji, ET, and Franz DR (2002) Medical Aspects of Chemical and Biological Warfare. Govt Reports Announcements & Index (GRA&I), Issue 11, 2002.
- Smith TC, Gray GC, Weir JC, Heller JM, Ryan MA (2003) Gulf War veterans and Iraqi nerve agents at Khamisiyah: postwar hospitalization data revisited. *Am J Epidemiology.* Sept. 1(158)(5): 457-67.
- The National Research Council's (NRC) Committee on Toxicology: The First 50 Years 1947-1997. (1997) Natl. Acad. Of Sciences, 1-38.

- Oudera, H. (2002) Clinical features on nerve gas terrorism in Matsumoto. J Clin Neurosci. Jan;9(1):17-21.
- Wiener R. and Hoffman, R.S. (2004) Nerve Agents: A Comprehensive Review. Journal of Intensive Care Medicine, Vol.19 (1), 22 – 37.