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13. ABSTRACT ***(Maximum 200 Words)***

Gulf War Illness (GWI) is one of many post-war syndromes with different ostensible origins. Several investigators have pointed out that GWI symptoms are similar to the symptoms of such disorders as chronic fatigue syndrome (CFS), post-traumatic stress disorder (PTSD), fibromyalgia, and neurally mediated hypotension (NMH), all of which are thought to arise from disorders of the autonomic nervous system. This project tests the hypothesis that autonomically-mediated GWI is not synonymous with PTSD, CFS or NMH, but shares a number of autonomic symptoms that are common to these conditions. Our hypothesis is that individuals who are autonomically hyperresponsive are more likely to develop GWI when exposed to the stresses of war, and that alteration in the function of neural pathways that use acetylcholine is an important element in such autonomic dysfunction. We are testing our hypotheses via two interconnected studies. The first will attempt to confirm the findings of Lockridge (1999) who found a predominance (10:1) of carriers of specific butyrylcholinesterase (BChE) variants among symptomatic Gulf War veterans compared to veterans without symptoms. The second study will examine autonomic reactivity, BChE genotype, and ex vivo carbamate affinity for BChE and acetylcholinesterase. We expect to find a higher incidence of autonomic hyperresponsiveness in the cases and in the heterozygote BChE variant carriers compared to either of the controls.

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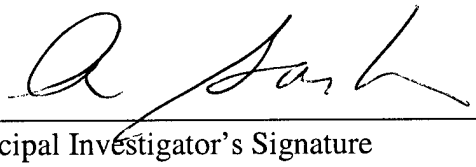

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Contents

Section 1. Introduction

For military personnel, the sequellae of war include the immediate dangers of combat and the potential for long-term medical and psychological disability. Factors such as fatigue, hunger, lack of sleep, and exposure to weather extremes combine to present the soldier with cumulative physical stress, and with the physiological stress that is an unavoidable consequence of physical stress. The valid fear of dying or of being wounded and the emotional effects of seeing others injured or dead, combine with physical stressors to define the actualities of past and present day battlefield experience for many men and women.[1,2,3,4,5] Reports indicate that the overwhelming majority of soldiers experience fear during or before battle with physical symptoms that have been well-documented. Over time, prolonged exposure to such combined physical and psychological stressors can result in individual exhaustion and decreased combat effectiveness.

Historically, a number of war syndromes (WS) with different labels and different ostensible origins have been described. Dual physiological and psychological syndromes have been reported previously; as discussed by Letz (1998),[6] the duality of these syndromes perpetuates the misconception of "stress" as a dismissible, subjective weakness, and not as a physiological reality with objective, measurable consequences. In the American Civil War, the dual syndromes were known as "irritable heart syndrome" and "nostalgia," in WWI as "soldier's heart" and "trench neurosis" or "shell shock" in WWII as "battle fatigue" and "war neurosis."[7] Similar effects have been observed in other American wars and in the Arab-Israeli war of 1973. Hyams et al.[7] have reviewed WS since the U.S. Civil War. WS have been attributed to malingering, accumulated stress, pre-disposing psychiatric problems, and exposure to unique environmental factors (e.g., trench conditions and lewisite mustard gas in WWI, stress of guerrilla warfare and Agent Orange during the Vietnam War, desert conditions and the coexistence of potential exposures to battlefield toxicants, pyridostigmine and unusual immunizations during the Gulf War). These authors note that these syndromes appear to share a common set of symptoms (fatigue, shortness of breath, headache, gastrointestinal disturbances, sleep disturbances, forgetfulness, and impaired concentration). Several investigators have pointed out that these symptoms are similar to the symptoms of chronic fatigue syndrome (CFS), post-traumatic stress disorder (PTSD), fibromyalgia, and neurally mediated hypotension (NMH),[8,9,10] all of which are thought to arise, at least in part, from disorders of the autonomic nervous system.

The unexplained symptoms and conditions reported by Gulf War Veterans (GWV) have been, dubbed "Gulf War Syndrome" (GWS) by media reports.[11] But researchers and government review panels have questioned whether a "GWS" actually exists, that is, whether some illness or group of symptoms is uniquely a result of deployment to the Gulf War.[12,13,14,15,16] Most have concluded, based on available evidence, that there does not appear to be a single, unique syndrome associated with Gulf War service,[17,18,19] suggesting that what has been referred to as "GWS" represents a range of different

problems that do not fit neatly into any one category. At the same time, government registries and research studies, using different designs and study populations, have described a fairly consistent group of symptom types and illness categories in different groups of GWV.[20,21,22,23] Population-based studies have invariably found these symptoms and illnesses to occur at significantly higher rates among veterans who deployed to the Gulf War, than among military personnel serving elsewhere.[24,25,26]

Despite a growing body of research on health problems reported by GWV, relatively little is known about basic epidemiologic parameters of these conditions. Questions regarding the prevalence, onset, and duration of health problems among GWV, as well as differential risk associated with military, demographic, and exposure subgroups remain to be answered. The lack of progress in identifying these parameters may be due in no small part to the difficulty of investigating symptom-based health problems that lack objective clinical signs and diagnostic tests, and for which no accepted case definition exists.

1.1 Prevalence of Health Problems

Most of what is known about these health problems is based on veterans' own reports of their symptoms. As with similar conditions associated with fatigue and nonspecific symptoms,[27,28] standard clinical evaluations and diagnostic tests have been of limited value in characterizing the problems reported by GWV,[24] and sophisticated neurological tests, such as those recently developed for CFS, have not yet been applied to GWV.

In the absence of a clearly defined "syndrome," estimates of the number of affected veterans have been quite variable. American government reports[29] have suggested that as many as 12% of veterans who served during Desert Storm are having health problems. These figures are based on participation in voluntary registries offered by the U.S. Departments of Defense and Veterans Affairs. However, registry information can provide only a preliminary indication of the number or characteristics of ill veterans, since participation is affected by a wide range of factors.[30]

Population-based studies, using samples that include both ill and healthy veterans, suggest that a substantially higher proportion of GWV may be experiencing health problems. A study of Iowa Gulf War-era veterans found that 48% of veterans who served in Desert Storm reported symptoms indicative of one or more chronic conditions, compared to 32% of era veterans who did not deploy to southwest Asia. A study of four Air National Guard units[31] by the Centers for Disease Control and Prevention (CDC) derived a case definition for a "multisymptom illness" among Gulf-era veterans, and found that 45% of deployed veterans report symptoms that meet criteria for that case definition, compared to 15% of a comparison group of nondeployed veterans.[24] Recently, Unwin et al. (1999)[26] reported that 62% of British veterans who served in the Gulf War met the CDC multisymptom illness criteria, compared to 36% of era veterans who did not serve in the Gulf and 37% of veterans who had served in Bosnia.

The Kansas Persian Gulf War Veterans Health Project (KVP) is directed by Dr. Lea Steele, who is a member of the project team for this project. It is a state-supported program initiated in 1997 and charged with conducting research to answer basic questions about the health status of the state's GWV. The research effort was specifically tasked to determine whether these veterans have excessive health problems associated with their wartime service and if so, the nature and magnitude of those problems. A second objective was to evaluate the impact of war-related health problems on veterans, their family members, and the state. The Kansas Gulf Veterans Health Study, an epidemiologic survey of 2,031 Gulf War-era veterans residing in Kansas, was conducted in 1998 by Dr. Steele. Dr Steele's[32] results (2000) are compatible with those of other population-based studies in finding that Desert Storm veterans report symptoms at a significantly higher rate than their nondeployed counterparts. For example, the prevalence of the CDC-defined "multisymptom illness" is 47% in Kansas GWV, compared to 20% among nondeployed era veterans.

The Kansas study also identified a number of interrelated symptom groupings, or illness subtypes, which individually and collectively, occur at significantly higher rates among deployed than nondeployed veterans. Based on these overlapping subtypes, a symptom-based definition of "Gulf War Illness" (GWI) was derived. This definition has been useful in identifying a nonrandom distribution of illness among Kansas veterans; i.e., in distinguishing subgroups of veterans who appear to be experiencing health problems at an elevated rate. Preliminary results indicate that veterans' self-reported symptoms occur in clearly identifiable patterns, and are associated with areas of deployment in the Kuwaiti Theater of Operations. Taken together, 30% of Desert Storm veterans in Kansas report one or more of the symptom patterns of GWI, compared to 8% of nondeployed era veterans.

1.2 Hypothesized Mechanisms of GWI

The overall hypothesis is that individuals with hyperresponsive autonomic nervous system (ANS) activity for developmental and/or genetic reasons are more likely to develop GWI when exposed to the physiological and psychological stresses of war. We do not claim that GWI is a single syndrome. We expect that with more refined analyses, veterans reporting GWI symptoms can be classified into a small group of well defined symptom clusters, one of which will have an autonomic etiology with prominent autonomic symptoms. It is our contention that ANS mediated GWI is not synonymous with PTSD, CFS, or NMH, but shares a number of autonomic attributes and symptoms that are common to these conditions. We also hypothesize that alteration in the function of central and peripheral neural pathways that use acetylcholine as a transmitter is an important element in autonomic dysfunction. Further, we believe that dysfunction in cholinergic metabolic pathways (including tissue acetylcholinesterase [AChE; EC 3.1.1.7] and circulating butyrylcholinesterase [BChE; EC 3.1.1.8]) can lead to functional alterations in end-organ responses. We expect that there will be a strong correlation between autonomic symptomatology and being a heterozygote carrier of the

A or F variant of BChE. The correlation should be weaker for homozygote carriers of the K variant of BChE.

Section 2.
Body

2.1 Task 1

A detailed protocol for study 1 was prepared and approved by the MRI IRB. This protocol was then submitted to the HSRRB on May 23, 2000. The revised protocol was approved on August 30, 2000, and the contract modification approving the use of human subjects was received on September 14, 2000. While awaiting approval, checklists were prepared for all aspects of the study procedures.

A detailed protocol for study 2 was prepared and approved by the MRI IRB. This protocol was then submitted to the HSRRB on September 20, 2000. A revised protocol was submitted on November 21, 2000. Preliminary approval was granted on December 26, 2000. The requested changes were made to the protocol and submitted to the HSRRB. Final approval was given on January 9, 2001. Checklists are under preparation.

2.2 Task 2

2.2.1 Study 1

The goal of Study 1 was to partially replicate and extend the work of Lockridge.[33] She found that heterozygote carriers for the A and F variants of BChE were found in a greater proportion (9:1-10:1) of veterans with symptoms of GWI than in veterans without such symptoms. There was also a much weaker association with homozygous carriers for the K variant. Study 1 focused on deployed GWV, and compared approximately 150 veterans who report GWI with approximately 150 who do not. We expected to find a higher rate of heterozygotes carrying the above genetic variants in the veterans with GWI. We will also clarify the incidence of the K/K homozygotes in this group.

The technical objectives were to:

- Obtain blood samples and questionnaire data from approximately 150 veterans with GWI and approximately 150 veterans who served in the Persian Gulf, but do not have GWI symptoms.
- Perform phenotypic and genetic analysis of BChE to identify heterozygote carriers of the A, F, and homozygote carriers of the K variants.

2.2.2 Methods

2.2.2.1 Experimental Design and Subjects

The study will compare two groups of veterans: approximately 150 Gulf War veterans who meet the CDC criteria for GWI and approximately 150 who were deployed to the Gulf and who do not meet symptom criteria for GWI. We estimated sample size requirements using Epi Info (V.6 StatCalc), a software package developed by the Epidemiology Program Office of the U.S. Centers for Disease Control and Prevention. Calculations indicate that a sample of 300 will allow us to detect a significant difference between cases and controls (at alpha = .05, power = .80), even if the difference is somewhat less pronounced than that described by Lockridge.[33] Specifically, our sample will have the power to detect a significant association if the proportion of variants among controls is as described by Lockridge (0.7%), but the proportion among cases is as low as 8%. On the other hand, if the proportion among controls is as high as 2%, our sample has the power to detect a significant association if the proportion among cases is at least 10.5%.

Study subjects were recruited from individuals included in the Kansas Veteran's Project (KVP) database who live in the Kansas City area. Study subjects were also recruited, using advertisements, from individuals not included in the KVP database who live in the Kansas City area. All veterans will have served in one of the U.S. Armed Forces, and approximately 10% will be women. Veterans will be contacted by telephone and asked to complete a short screening interview. Veterans who consented to the screening interview and were found to be eligible were asked to complete a more detailed questionnaire and come to MRI to provide a blood sample. Consenting veterans were mailed information about the appointment and a copy of the study questionnaire. When the volunteers arrived at MRI, the risks and benefits of participating were described to each potential volunteer, and any questions were answered. Written informed consent was obtained.

2.2.2.2 Inclusion and Exclusion Criteria

The volunteers for Study 1 were required to speak, read and write English, and meet the additional inclusion and exclusion criteria associated with being in the GWI or Healthy group. Criteria for excluding subjects were the same as those used by the KVP: cancer (other than skin cancer, excepting melanoma), diabetes, heart disease (other than high blood pressure), stroke, multiple sclerosis, lupus, long-term problems from serious injuries, chronic infections lasting over 6 months (e.g., tuberculosis, hepatitis, HIV), history of serious psychiatric disorders (schizophrenia, bipolar disorder) or any current psychiatric disorder that required hospitalization since 1991 (depression, PTSD, alcoholism, drug dependence).

2.2.2.3 Blood Collection

Two 9.5-mL tubes of blood (about four teaspoons) were collected from each subject. The first tube contained no anticoagulant. The blood was allowed to clot, spun down, and the serum supernatant was stored at ~ –20°C until ready for assay for BChE phenotype.[33] The second tube contained citrate anticoagulant. The tube was spun down and the buffy coat harvested. The buffy coat was stored ≈ –20°C until ready for assay as source of DNA for genotyping the F and K variants.

2.2.2.4 Enzyme Activity

For phenotyping, enzyme activity was measured with 50 μM benzoylcholine as the substrate[34] in 0.067 M Na/K phosphate buffer, pH=7.4 at 25°C. Hydrolysis was measured spectrophotometrically at 240 nm and activity calculated from ΔE = 6.7 $mM^{-1}cm^{-1}$ and expressed as micromoles benzoylcholine hydrolyzed per min per mL of serum, defined as units per mL (U/mL) at 25°C. Inhibition of activity by 10 μM dibucaine will be used to identify the "atypical" and fluoride-resistant phenotypes. In cases of unusual dibucaine inhibition, degree of inhibition obtained with 50 μM NaF is measured to distinguish between the UA, UF, AF, FF, and FS phenotypes.[33,35,36]

2.2.2.5 DNA Preparation and Analysis

DNA was isolated from the buffy coat layer using the IsoCode PRC DNA Sample Isolation Device (Schleicher & Schuell); established procedures to reduce possible contamination of DNA samples by other DNA were used. A one-eighth-inch punch was used to punch out dozens of filter circles from a single IsoCode paper strip. Thawed buffy coat or leukocytes, about 5 μL, were applied to each filter circle. Several filter circles of the same sample were placed inside a closed microtube containing Drierite, and the tube covered with a KimWipe plug. Filter circles were dried overnight at 37°C, and rinsed in 500 μL distilled autoclaved water with 5 sec pulse-vortexing. To elute genomic DNA, one filter circle of blood was placed in a 0.5-mL tube containing 50 μL distilled autoclaved water. The tube was heated at 95°C for 30 min, pulse-vortexed 15 times after 15 min, and then 60 times after 30 min PCR amplification of genomic DNA followed by restriction enzyme digestion was used to genotype DNA at the polymorphic site for BChE located at Ala/Thr 539. Wild-type BChE has Ala 539, whereas the K-variant has Thr 539 in this position.[35, 36] PCR reactions consisted of 3 to 7 μL of genomic DNA in a 50-μL reaction. Taq polymerase (Promega) and 3 mM $MgCl_2$ will be used in the reaction. The annealing temperature was 57°C to 60°C. Four primers for two different PCR amplifications have been designed and used.[37,38] The A amplification creates a Mae III restriction site when the K-variant ACA codon (Thr 539) is present. The B amplification creates a Bgl I restriction site when the GCA codon (Ala 539) is present. Because of previous disappointing work using a primer that created a Dra I site, the more expensive but more reliable Mae III (Roche Molecular Biochemicals) was used along

with a new amplification primer that creates a Mae III site in K-variant alleles. Mae III has been used to detect the K-variant mutation.[33,37,38]

DNA of samples that phenotype as heterozygous for the F variant of BChE will be amplified and sequenced to determine which of the three reported DNA mutations was responsible for fluoride resistance.[39,40] It will not be necessary to genotype samples that phenotype as heterozygous for the BChE A variant[41] (Asp 70 to Gly) because dibucaine inhibition of serum activity is extremely accurate in this determination.

2.2.2.6 Volunteer Recruitment and Data Collection

Subject recruitment for study 1 was commenced on September 15, 2000, and completed on December 6, 2000. Subject appointments started on September 25, 2000, and were completed on December 20, 2000. Data collection for study 1 was anticipated to require 12 months, but was completed in 4 months, ahead of schedule. Analysis of blood samples is currently underway.

During study 1, telephone interviewers made contact attempts to a total of 1401 veterans. Of these veterans: 1228 (88%) had received their final disposition, 134 (9%) were pending final disposition at the time that recruitment was halted (i.e. no answer, message left on answering machine, etc), and 39 (3%) had indicated they would call back.

Of the 1228 with final dispositions: 614 (50%) completed the screening interview, 300 (24%) were incorrect or non-working numbers, 140 (11%) were refusals, 98 (8%) were ineligible for the study because they had not been in the Persian Gulf during the index year, and 76 (6%) were unable to participate (moved out of area, disabled, deceased, etc).

Of the 614 who completed the screening interview: 163 (27%) were ineligible for the study due to exclusionary conditions or case/control status and 451 (73%) were invited to participate.

Of the 451 invited to participate: 385 (85%) accepted and 66 (15%) declined.

Of the 385 scheduled to participate: 183 (48%) were cases and 202 (52%) were controls. Eleven (6%) of the cases were women; 15 (7%) of the controls were women.

Of the 385 scheduled appointments: 304 (79%) completed appointments.

Of the 304 completed appointments: 143 (47%) were cases and 161 (53%) were controls. Six percent of the cases were female and 8% of the controls were female.

Section 3.
Key Research Accomplishments

3.1 Study 1

We have completed data collection for study 1 well ahead of schedule. We recruited 304 subjects for study 1 as we had originally planned to. Of these subjects 143 were cases. This is within 10% of our originally proposed number. Additionally, 9% of the subjects were female, which is also close to our originally proposed study numbers.

Assays for phenotype and molecular genetics are currently underway. Out of 308 samples (samples from 4 employees were also analyzed so that possible contamination sources could be ruled out), 228 have been phenotyped to date. Twelve of these appear to have the A variant, possibly as U/KA, and one F. Work has begun to genotype the K variant and to confirm A and F variants. To date, 14 are KK, 99 UK, 194 UU, and one has not worked in two attempts. Work is progressing at a rate sufficient to meet our original estimates. Entry of data from the questionnaires is complete, and statistical analysis can begin when all results of genetic testing have been received.

3.2 Study 2

The protocol for study 2 was approved on January 9, 2001. Work is ongoing to implement the stress testing protocols.

Section 4.
Reportable Outcomes

4.1 Study 1

Data collection for study 1 has concluded. We have received phenotype information for 228 of the subjects.

Section 5.
Conclusions

We have completed data collection for study 1 ahead of schedule. After sample analysis has been finished, we will start statistical analysis of the data. We have received approval and are preparing to start study 2.

Section 6.
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Administrative Review*

☑ RJ ☐ MP

Award Agreement#:

☐ Distribution changed to Limited

PI: ☐ PI Credential added

PI Addition(s)

COVER ☒ Cover Created

Title

Org ☒ State Abbreviation sp out

☒ Street address removed ☐ Zip code corrected per award agreement

Report Type

Report Type changed to

☐ Annual ☐ Final ☐ Phase ☐ I ☐ II

☐ Final Proceedings ☐ Annual Summary

Report Date:

☐ Day removed

Prepared For:

SF298 ☒ SF298 Created

Date Changed to:

Report Type changed to

☐ Annual ☐ Final ☐ Phase ☐ I ☐ II

☐ Final Proceedings ☐ Annual Summary

SF298

Subject Terms:

Sec Class:

☐ Page Count Added ☐ SF298, Field 11, color graphs/photos

Page Count Changed from 0 to 19

Lab:

☐ Foreword-Category changes

Selected	De-Selected
☐ Humans	☒ Humans
☐ Animals	☐ Animals
☐ DNA	☐ DNA
☐ Hazardous organism	☐ Hazardous organism

☐ Table of Contents created ☐ Table of Contents Incomplete

☐ Foreword Spell-Corrected

☐ Renumbered

☐ Removed page numbers

Appendices

☐ Removed double numbering of appendices

☐ Handwritten label(s) typed

Figures/Tables:

☐ "Confidential" removed from all pages, report limited

☐ Fax Header/Footer removed

☐ Extraneous header/footer info removed ☐ Financial Report removed

Edit Codes

1 - Re-formatted

2 - Changed to reflect award agreemen

3 - Spelling, grammar correction

Notes:

* Changes made to original report as indicated

WEB FORM

☒ WEB FORM - COVER

☒ WEB FORM - SF298

Exit Review: Administrative Review

If Final: Write completion date on file, retire, remove reference in acceptance letter to future reports, requests for contact update

15 2 2 ✓