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

Gulf War Illness (GWI) is one of many post-war syndromes with different ostensible origins. 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 attempted 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 genetic hypothesis in the first study was not supported, with one exception. The second study, currently underway, 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 deployed or nondeployed controls.

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Principal Investigator's Signature

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Date

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[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 post-deployment 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.[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 this project team. 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 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. Based on some initial findings obtained by Dr. Oksana Lockridge[33] at the Eppley Institute at the University of Nebraska Medical Center at Omaha, we expected that there would be a strong correlation between

autonomic symptomatology and being a heterozygote carrier of the A or F variant of BChE. Dr. Lockridge 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 mutation. However, her study was conducted with a self-selected sample and self-described GWI/healthy status, without the benefit of a validated and structured symptom and history questionnaire. Dr. Lockridge is a member of this project's research team, and she has been helping us re-test the hypothesis that emerged from her preliminary findings using the methods of molecular biology on a well-defined population of GWV.

Section 2.
Body

2.1 Task 1—Preparation for Studies 1 and 2

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. An amendment, approved by the MRI IRB, changing the wording on the questionnaire was requested on May 3, 2001 and was approved by the HSRRB on May 12, 2001.

2.2 Task 2—Data Collection and Assays

2.2.1 Study 1

As noted in the introduction, 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 mutation.

Based on Lockridge's results, our 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 also hoped to clarify the incidence of the K/K homozygotes in this group, by having a larger sample size.

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 and F variants, and homozygote carriers of the K mutations.

Methods

Experimental Design and Subjects

The study compared 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. 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 served in one of the U.S. Armed Forces, and approximately 10% were women. Written informed consent was obtained from all subjects participating in the study.

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). Details of blood collection, analysis of enzyme activity, and DNA preparation and analysis were included in the previous annual report, and repeated here under Section 2.2.2.

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, thanks to the enthusiastic participation of the volunteers who were contacted.

2.2.2 Study 2

The hypotheses of Study 2, generated before the results from Study 1 became available, are:

- Veterans with GWI will show greater autonomic responsivity than nondeployed veterans; deployed veterans who do not have symptoms of GWI will show the least reactivity.

- Heterozygote carriers of the A and F variants, and homozygote carriers of the K BChE variant, will show autonomic reactivity greater than or equal to that shown by veterans with GWI. Based on the results of Study 1, showing a particularly strong association between the K/K genotype and case status, one would speculate that K/K volunteers will also have a greater autonomic reactivity than K heterozygotes, or carriers of the A and F mutations.

- Veterans with GWI will show less startle inhibition (as measured by pre-pulse inhibition [PPI]) and less habituation of the startle response than either the nondeployed or asymptomatic deployed control groups.

The technical objectives are to:

- Recruit up to 45 veterans with GWI (cases), up to 30 veterans who served in the Persian Gulf but do not have symptoms of GWI (deployed controls), up to 30 veterans who did not serve in the Persian Gulf (nondeployed controls), and up to 25 veterans identified as heterozygous for atypical (A) or fluoride (F) variants, or homozygotes for the K variant of BChE in Study 1 (identified variants). Recruitment will be terminated when complete data are available for 40 cases, 25 deployed controls, 25 nondeployed controls and 20 identified variants.

- Perform the phenotypic and genetic analyses as described for Study 1 for each subject.

- Obtain questionnaire data and measure autonomic responsivity under a variety of baseline and stressor situations, including heart rate (HR), heart rate variability (HRV) and systolic and diastolic blood pressure (SBP and DPB) under head-up tilt.

- Measure the startle response, and the extent to which it is inhibited.

- Evaluate those subjects from Study 1 identified as heterozygotes for the A or F mutations or homozygotes for the BChE K mutation using the autonomic test battery, and determine the relationship between autonomic responsivity and genetic findings.

Methods

Experimental Design and Overview

Three groups of Army veterans or soldiers, and one group consisting of over 20 veterans identified as heterozygous for atypical (A) or fluoride (F) variants, or homozygotes for the K mutation of BChE in Study 1 are participating in the laboratory study. Volunteers are being drawn from the Kansas Veterans Project (KVP), or from a list, provided by DoD, of veterans with a last known address in Missouri. The case group

(Cases, N = 40) consists of GWV who meet the KVP criteria for GWI according to the screening interview described below. One control group (deployed controls, DC, N = 25) consists of veterans who served in the Persian Gulf during the war, but do not report symptoms of GWI and do not meet Kansas or CDC criteria for GWI. Every effort will be made to include the same number of Cases and DC subjects from each unit. Selecting cases and DCs from the same unit will help to control for exposure to chemicals, smoke, and other environmental factors that were present in the Gulf War. Volunteers will be drawn from more than one unit to increase the generalizability of the results. A second control group (N = 25) is made up veterans who were in the armed services during the Gulf War, but were not deployed to the Gulf area (non-deployed controls, NDC). Control groups are proportionally matched to cases with respect to sex and age. The KVP registry contains information on 2,031 veterans, 499 of which meet the registry's criteria for GWI. We therefore have a pool that is more than adequate to recruit the number of subjects needed for the study, even without supplementation from veterans whose last known address was in Missouri.

Testing is currently being conducted onsite at Junction City, Kansas and was performed previously onsite at MRI. If required to meet the target number of volunteers in each group, testing will also be conducted in Topeka, Kansas. The field sites have been selected because they are in areas with large concentrations of veterans; this will optimize convenience for the participants. When data collection has ended, each participant will have: (1) completed an autonomic reactivity battery (ARB) designed to evaluate responses to a variety of stimuli and situations that affect the ANS; (2) provided blood samples for genetic analysis; and (3) completed a set of questionnaires selected to provide information on symptoms, military service, and personality factors that might affect autonomic function.

Subject Selection and Recruitment

Veterans who have registered with the KVP, or who have listed a last known address is in Missouri, form the pool from which potential volunteers are being selected. Based on previous studies, we plan to test 40 cases, 25 deployed controls, and 25 nondeployed controls. If no differences are found between the two control groups, they will be combined for comparison with the cases. The KVP health study found that rates of GWI varied as a function of branch of service, enlistment status, and sex. All veterans contacted have served as enlisted personnel in the U.S. Army between August 2, 1990, and July 31, 1991. Approximately 10% of the participants in the testing will be women. The risks and benefits of participating in the laboratory study are described and any questions answered before participation in the study begins. If interested the veteran is interviewed to assure that criteria for participation are met. In the KVP case definition, symptoms were counted only if the problem persisted or recurred over the preceding year, and first began in 1990 or later. The KVP criteria were selected for Study 2 because they provide better differentiation between cases and controls.

Exclusion criteria for Study 2 include all of the exclusion criteria for Study 1. The volunteers must be able to speak, read and write English, and meet the additional

inclusion and exclusion criteria associated with being in the GWI or control groups. Criteria for excluding subjects will be 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). In addition, volunteers in the case and control groups must have served in the U.S. Army as enlisted personnel, as stated above. The fourth group, the heterozygote carriers of BChE mutations identified in Study 1, may have served in any of the branches of the armed forces.

Potential case and control subjects are randomly sampled from KVP enrollees. They are contacted by telephone and screened to determine their eligibility for inclusion. When a veteran has agreed to participate in the study and an appointment has been scheduled, a package with the questionnaire is mailed to the veteran. Veterans are asked to complete their questionnaire and bring it to the testing site when they come for their appointment. They are instructed to consume caffeine and tobacco in their typical manner, to have a light meal before arriving at the testing site, but to refrain from drinking alcoholic beverages the night before testing.

No advertisements or recruitment posters are being used. Three of the four groups will be contacted by telephone based on the information already present in the Kansas Veterans Data Base. The fourth group consists of veterans who have already participated in Study 1; their phone number is known, and they are contacted by MRI staff.

Upon arrival, the study is again described, testing procedures are described in detail, written informed consent is obtained and the participant completes the Volunteer Registry data sheet. The experimenter examines the questionnaire for completeness, and clarifies any confusing answers. Of course, some subjects will forget the packet or forget to fill it out. Such individuals are given any needed materials and asked to finish a packet before testing begins.

Questionnaires

Subjects are asked to provide information on their current symptoms, as well as service-related exposures and military history. The answers are used to confirm that the subject meets criteria for participation, and for subsequent examination of exposure and symptom patterns. The questionnaire includes items from the Autonomic Symptom Profile developed at the Mayo Clinic;[34] and the veteran's version of the SF-36,[35,36] a more general symptom checklist with demonstrated reliability.

Physiological recording

Tilt testing methods are similar to those used at the Mayo Clinic and described in the consensus document from the AAS/AAN for definition of various disorders involving syncope.[37,58] Methods for other tasks follow traditional psychophysiological and autonomic evaluation procedures as described below for each task. The electrocardiogram (ECG), tonometric BP from the dominant arm, and respiration are measured continuously throughout all the tests in the battery except pre-pulse inhibition. Data is sampled at 256 Hz and stored in magnetic media for off-line data processing. Electromyographic (EMG) measures from the orbicularis oculi, sampled at 1,024 Hz, provide quantitative information for analysis of the amplitude of the startle reflex and its inhibition (PPI).

ECG

The ECG is recorded using disposable "snap" electrodes applied to prepared skin sites on the right and left clavicles and the seventh intercostal space under the left ancillary midline, corresponding to the standard ECG Lead II configuration. ECG activity is recorded using Grass Neurodata Model 15 (Grass Instrument Division, Astro-Med, Inc., Warwick, Rhode Island) multi-channel physiological recording equipment and sampled at 256 Hz for determination of mean heart rate and HRV from R-R intervals. Our laboratory has developed and validated custom software[38] for automatic detection of R-wave fiducial points, assessment of R-R intervals and computation of HRV parameters using the U.S.–European Consensus Guidelines.[39]

Tonometric measures of BP

In studies of autonomic reactivity, reproducibility over time is limited by the fact that relatively few measures can be obtained using automatic auscultation methods. Tonometric techniques[40,41] allow the continuous noninvasive measurement of the full BP waveform from the radial artery. The instrument we are using (Colin Pilot 9200, Colin Medical Instruments Corp, San Antonio, Texas) has FDA approval. This device has an array of sensors that flatten the arterial wall, and software/hardware to optimize the pressure of the sensor and the specific sensor position in the array that provides the measurement. This approach makes it much easier to position the sensor appropriately over the wrist, a technique that is very difficult when only one sensor is used. Kemmotsu et al.[40,41] have reported correlations between tonometric and invasive intra-arterial BPs of 0.94 to 0.97 during anesthesia. Weiss et al.[42] reported lower correlations; however, they conclude that tonometric measurements provide a reliable indicator of changes in pressure during induction of anesthesia, and can be appropriate when arterial cannulation is not feasible.

Movement artifact presents the greatest challenge to accurate tonometric measures of BP. Our experience during development of the protocol and testing indicated that this source of artifact is reduced by splinting the hand and wrist on which the sensor array is placed and by maintaining the arm at heart level. Nonetheless, our experience to date

with the volunteers indicates that this part of the protocol is still the one most prone to having missing data. In addition to movement artifacts that are clearly identifiable, in a small subpopulation of volunteers there is also drift in the hold-down pressure for the tonometric sensor. When the drift happens during the tilt-up procedure, the data obtained in this subset of volunteers may be of questionable validity. This is less of a problem for the tonometric data in other parts of the protocol, because if hold-down pressure drift occurs, the sensor can be reset and a new control period of data obtained.

Respiration

Respiration is recorded using a Grass Model F-RCT Piezo Trace transducer placed around the chest under the arms or at the level of the diaphragm. As the chest expands and contracts the piezoelectric sensor is deformed, and the deformation generates a voltage signal proportional to the changes. While this placement is optimal for most individuals, records from those who primarily breath abdominally are difficult to interpret. For such individuals, the gauge is placed on a level with the xyphoid process. The gauge length is adjustable to provide optimal stretch for each individual. Data is recorded using a Grass Neurodata Model 15, with the low filter set to 0.01 Hz. This time constant provides minimally attenuated waveforms, and the data obtained are adequate to describe the rate and relative amplitude of the respiratory cycle.

EMG

The startle response is measured using small biopotential electrodes attached with double sided adhesive to the orbicularis oculi muscle under the left eye. The site is cleansed first with an alcohol pad, and Grass electrode cream serves as the contact medium. The EMG is sampled at 1024 Hz.

Tilt Test Procedures

After questionnaires are completed, sensors are attached to measure ECG, BP, and respiration. A normal part of prepping and instrumenting veterans for the physiologic tests includes recording blood pressure by auscultation in both arms. Any volunteer who exhibits a systolic pressure of less than 90 mm Hg or a diastolic pressure of less than 50 mm Hg in either arm will have the blood pressure taken again. If either of the exclusionary readings listed above are present in this second recording, the volunteer participates in all the other phases of the study, lying supine in the tilt table, but the investigators skip the phase where the volunteer is tilted head-up. The subject then lies down on a standard tilt table (Colin model CM6121.TB, Colin Medical Instruments Corp, San Antonio Texas). Data is collected using the following procedures; the estimated times listed include procedures, data collection, and answering simple questions:

Resting baseline (5 min): The subject remains quietly on the tilt table with no instructions other than to relax for about 5 min while the experimenters make sure all the equipment is recording properly. During this time, the resting respiration rate is determined.

Deep breathing (6 min): Procedures are similar to those used at the Autonomic Reflex Laboratory at the Mayo Clinic[43,44,45,46,47,48]. The rate of breathing has a profound effect on the high frequency component of heart rate variability; variability is maximal at 5 to 6 breaths per minute.[49] In our version of the task, inspiration at 33% of the baseline rate is signaled for the subject by a tone presented over a speaker or by a visual signal. Practice is given to assure that the subject understands how to breathe slowly, smoothly, and deeply. After a 1 min rest period, the subject performs the deep breathing task for 8 cycles two times, separated by a 1 min rest period. Mean HR and BP are calculated over the 5 largest consecutive respiratory cycles.

Quiet rest: The subject then rests quietly for 3 min.

Sustained hand grip at 30% of maximum (4 min): The cardiovascular response to a sustained hand grip consists of an early heart rate increase due to vagal withdrawal, followed by another increase, presumably due to sympathetic activation. Ewing et al.[50] recommend sustained hand grip of 30% of maximum for up to 5 min. Low and colleagues[47,48] note that 3 min seems to be adequate, and may be preferable since many people are unable to maintain the hand grip for 5 min. A hand dynamometer (Lafayette Instruments Model 76618, Lafayette, Indiana) is used to determine maximum grip strength in the dominant hand. The dynamometer has been modified so that grip pressure is presented on a computer. The program determines 30% of the maximal grip. The subject is instructed to squeeze the dynamometer to the selected level; then increase grip strength if a tone generated by the computer goes lower or decrease it if the tone goes higher. Grip strength is sampled at 256 Hz and the average grip strength during each 30 seconds of the task recorded. When the task is completed, the subject rates his/her perceived exertion using the Perceived Exertion Scale.[51] Low and colleagues[47,48] have noted that the hand grip test has limited sensitivity and specificity; we believe that addition of a subjective measure of exertion may improve the metric properties of the task. Mean HR and mean HR adjusted for perceived exertion are used as outcome measures.

Quiet rest: The subject then rests quietly for 2 min.

Valsalva maneuver (5 min) : In evaluating the response to the Valsalva maneuver it is necessary to analyze both HR and BP response, as the HR response is typically secondary to the change in BP.[52] A modification of the method described by Denq et al.[53] is used. Subjects are taught to blow into the tube of a dial-type sphygmomanometer with a large face and maintain pressure at approximately 40 mm Hg for 15 sec. Subjects with respiratory diseases may be unable to do this, and the target pressure is adjusted downward to a minimum of 20 mm Hg. After a brief rest, the maneuver is repeated until two similar recordings of HR and BP are obtained. A maximum of four re-tests will be allowed. The maximal HR generated by the Valsalva maneuver, divided by the lowest HR occurring within 30 sec of the beginning of the test (the Valsalva ratio), provides one outcome measure. This measure takes into account both the early part of Phase II and Phase IV[52,53] of the maneuver. Sufficient data is available to examine other endpoints as

well. Blood pressure data obtained from the same time points is used to evaluate the primary BP response.

Quiet rest: The subject then rests quietly for 2 min.

Mental arithmetic (3 min): The subject is instructed to sequentially subtract 7 from a 3-digit number larger than 500. To be sure the instructions are understood, a 15-sec practice period using subtraction from a two-digit number precedes the test. Subtraction continues for 2 min. Mean HR, standard deviation of HR, and change in SBP and DBP serve as the dependent variables.

Quiet rest: The subject then rests quietly for 5 min to provide an adequate baseline for the emotional stress task described next.

Emotional Stress (8 min): The subject is instructed to spend 30 seconds to 1 min thinking about "a stressful experience you have had in your life," and to spend 5 min telling the experimenter about it (where you were, what the environment was like, what happened, how you felt about it).

These procedures are similar to those used by Cohen et al.[54] in a study comparing patients with Post-Traumatic Stress Disorder with healthy controls, but we are using shorter recording periods. Others [55] found that 5 min recording periods for measurement of HRV were consistent over time, and Sloan et al.[56] found significant correlations between HRV obtained from 5 min recordings compared to 24-hour recordings. While intervals as short as 2.5 min have been used,[57] in our experience recording periods of less than 5 min produce unacceptably noisy data. Outcome measures include changes in mean HR, spectral and time-domain measures of HRV, mean SBP, and mean DBP from the baseline period to the exposition period. In addition, during the preparation phase, changes in respiration rate and amplitude will also be outcome measures.

Quiet rest: The subject then rests quietly for 2 min.

Head-up tilt (20 min): There is an extensive literature on the use of head-up tilt to diagnose autonomic dysfunction. The procedures we use follow the Mayo Clinic[43,44,45,46,47,48] and the consensus statements prepared by the AAS/AAN.[37,58] While following the general outlines provided by the Mayo clinic investigators and the AAS/AAN consensus document, our tilt table test is a shorter, research test and not a clinical procedure. The standard clinical tilt table protocols typically last 30-40 min, and if large blood pressure drops, dizziness or fainting are not elicited, infusions of isoproterenol may be used to provide a further cardiovascular stress. The clinical test is not terminated prematurely just by systolic blood pressure drops unaccompanied by reports of dizziness or fainting. In contrast, our research test uses tilt to examine cardiovascular responses during the first twenty 20 minutes, with built-in premature termination criteria designed to make it extremely unlikely that any volunteer will experience fainting or dizziness. In addition, as noted in the methods, we record SBP and DPB by non-invasive arterial tonometry, where the blood pressure values are displayed

on the screen on a beat-by-beat basis. Thus there is always very little lag time between any significant drop in blood pressure and the investigator being able to restore the volunteer to a supine position. While those potential side effects of the test were noted to the IRB and HSRRB, and are mentioned in the informed consent form, our procedures have been designed to extract the maximum amount of research information with the absolute minimum risk of discomfort to the volunteer.

Any volunteer who exhibits a systolic pressure of less than 90 mm Hg or a diastolic pressure of less than 50 mm Hg by auscultation in both arms does not participate in the head-up tilt part of the study. The subject should be supine for at least 20 min prior to testing. Since the tests described above take at least 35 min, this requirement is fulfilled. The activities in the preceding portions of the battery are all carried out with the subject supine and should not affect the response to tilt. The tilt table, with the subject's arm supported at heart level on an arm board and feet resting comfortably on a foot support, is raised (8-9 sec) to 80 degrees, and is maintained at that angle for either 20 min or until the subject reports feeling faint, SBP exhibits a sustained drop of more than 30 mm Hg, or DBP exhibits a sustained drop of more than 15 mm Hg, whichever occurs first. If any of these occur, the table is immediately returned to the horizontal position. Both the initial response to tilt that occurs within the first minute and the sustained response are analyzed using HR, HRV, SBP, and DBP as the outcome variables.

Recovery (up to 20 min): The table is returned to the horizontal position, and the subject lies quietly on the tilt table while HR and BP are monitored. The test is terminated after 15 min or when HR and BP have returned to pre-tilt levels, whichever comes first. Time to recovery is recorded as outcome data.

Startle Response (8 min): The startle stimulus consists of a 50 msec burst of 105 dB white noise. On half the 20 trials, the startle stimulus is preceded by a 50 msec tone pip at 440 Hz and 90 dB; the rise/fall time is 25 msec. The subject is instructed to listen to the tones, and count the double tones. Electromyographic (EMG) measures from the orbicularis oculi will provide quantitative information for analysis of the amplitude of the startle reflex and its inhibition (PPI).

Blood Collection

Two 9.5 mL tubes of blood are collected from each subject after physiological measurements have been completed. The first tube contains no anticoagulant. The blood is allowed to clot, is centrifuged, and the serum supernatant is stored at ~ –20°C until assay for BChE phenotype.[33] The second tube contains citrate anticoagulant. The tube is spun down and the buffy coat harvested. The buffy coat is stored ~ –20°C until ready for assay as source of DNA for genotyping the F and K variants. After the removal of the buffy coat, the tube containing the packed red blood cells is vortexed briefly to resuspend the cells. The cells are mixed 1:1 with a citrate-phosphate buffer, pH 6.0, and stored at ~ –20°C.

Enzyme Activity

For phenotyping, enzyme activity is measured with 50 μM benzoylcholine as the substrate[59] in 0.067 M Na/K phosphate buffer, pH=7.4 at 25°C. Hydrolysis is measured spectrophotometrically at 240 nm and activity calculated from $\Delta E = 6.7\ mM^{-1}cm^{-1}$ 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 is 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.[33,60,61]

DNA Preparation and Analysis

DNA is 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 are used. A one-eighth-inch punch is used to punch out dozens of filter circles from a single IsoCode paper strip. Thawed buffy coat or leukocytes, about 5 μL, are applied to each filter circle. Several filter circles of the same sample are placed inside a closed microtube containing Drierite, and the tube covered with a KimWipe plug. Filter circles are 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 is placed in a 0.5-mL tube containing 50 μL distilled autoclaved water. The tube is 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 is 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[62,63]. PCR reactions consist of 3 to 7 μL of genomic DNA in a 50-μL reaction. Taq polymerase (Promega) and 3 mM $MgCl_2$ are used in the reaction. The annealing temperature is 57°C to 60°C. Four primers for two different PCR amplifications have been designed and used.[33] 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) is being 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.[33,64,65]

DNA of samples that phenotype as heterozygous for the F variant of BChE are amplified and sequenced to determine which of the three reported DNA mutations are responsible for fluoride resistance.[66,67] It will not be necessary to genotype samples that phenotype as heterozygous for the BChE A variant[68] (Asp 70 to Gly) because dibucaine inhibition of serum activity is extremely accurate in this determination.

Carbamate Affinity Testing

We measure carbamate affinities to AChE with a radioisotopic assay based upon the quantitation of [^{3}H]acetate produced by hydrolysis of labeled [^{3}H]acetylcholine, as described by Johnson and Russell (1975),[69] modified by Nostrandt et al. (1993),[70] and further modified in our lab to increase the extraction efficiency of the ^{3}H-labeled acetate into the fluor and reduce sample variation. This assay permits the use of essentially undiluted samples. Incubation with carbamates and no substrate for one hour achieves a plasma-like equilibrium. Total assay time after addition of substrate is no more than 30 sec. Our standard substrate is unlabelled acetylcholine iodide (0.015 M) with tracer [acetyl-H^3] acetylcholine iodide (0.23 mM). Assays are run in triplicate for each specimen, and a substrate blank is run in duplicate at least every hour once the incubations begin to determine the amount of spontaneous hydrolysis of the acetylcholine. Samples incubate with three concentrations of pyridostigmine between 0.1 and 1 μM for 1 hour before residual activity is assayed; samples with K_{app} outside our population ranges are reassayed with seven concentrations between 0.05 and 10 μM. Our internal control is a commercially available compound containing acetylcholinesterase and butyrylcholinesterase at known levels.

Site Selection and Management

Prior to setting up a field site, project staff contacted as many local resources as possible in order to optimize use of the time on-site. We identified and evaluated the adequacy of potential field office sites. During our site visits, we evaluated other local resources that may be required to support the project (e.g., couriers, ice suppliers, medical waste disposal, rental options for office furniture and equipment, accommodations for on-site staff, utilities, telephone installation) and security of the site. These site visits were conducted approximately 2 months prior to specimen and data collection at each site and took place over a three or four day period, including travel to and from the site.

MRI, with assistance from the KVP, obtained leads on facilities from a variety of resources, including veteran's groups, local health departments, realtors, and community centers. Minimum requirements for the facility were provided to local resources to assist them in identifying potential sites prior to the site visit. The following physical features were considered as necessary: a reception area; a quiet room for consent and questionnaire administration; an autonomic testing room; a restroom; and a lab processing/venipuncture room for blood specimen collection, processing, storage, and shipment preparation (i.e., refrigerated centrifuging, aliquoting, and clerical work). A refrigerator/freezer (~–20°C) is located in the specimen processing area for storage of specimens. A small bed or cot is available for use when performing difficult venipuncture or if the participant becomes ill or faints. Other considerations for field office selection include safety and security of the site, and convenience to participants.

MRI is responsible for establishment of utility services for the site to include: telephone service, electricity/gas, water, sewer, adequate lighting and electrical service to accommodate computers, office and laboratory equipment. The field office is

temperature controlled to ensure optimum functioning of electronic equipment and the comfort of participants. MRI contacted the Kansas State Health Department or health care administration agency early in the planning phase to determine if special licensing or notification requirements exist for the establishment of testing facilities in the State.

MRI is responsible for the safe handling and disposal of medical waste generated during field activities, and manages the waste in accordance with applicable federal, state, and local regulations. An arrangement for disposal of the waste was be made through a licensed medical waste hauler/disposal service that is local to the field office area. Needed medical waste supplies will be obtained through the disposal service or through a commercial vendor. MRI's Health and Safety Office in Kansas City, Missouri will maintain documentation of proper disposal of medical waste generated during field activities.

2.3 Task 3—Statistical Analysis of Results

2.3.1 Study 1

Participants

Participants in the current study were recruited from Gulf War veterans who lived in the Kansas City area. The majority of the participants were part of the Kansas Veteran's Project (KVP) database. Additional participants were identified using a database of veterans who resided in Missouri and through advertisements. The KVP database has been described previously[32]. Potential participants were screened over the phone to determine whether or not they met criteria for inclusion into the study. Participants who passed this screening were classified as case or control based on their responses to questions concerning symptoms; the Centers for Disease Control and Prevention (CDC) case definition criteria was used for the classification. Recruitment continued until approximately equal numbers of cases and controls were enrolled into the study and the total enrollment number reached at least 300. Questionnaire data and blood samples were collected from 304 individuals consisting of 144 (47%) identified as cases and 160 (53%) identified as controls at the time of screening interview.

The sample reported here was largely: male (93%), white (89%), enlisted personnel (79%), army personnel (55%), active duty military (66%) rather than reserve or national guard, and enlisted at the time of the Gulf War (79%). At the time the study data were collected (Fall, 2000), the average age of the sample was 38 yrs (range 28 to 64), 24% were still in the military, and 88% had education above a high school level.

Measures

Analyses presented in this report consist primarily of the relation between the genotype classification, self-report symptom data, and classification as case or control using both the CDC criteria and the Kansas (KVP) criteria[32]. Each participant provided a

blood sample for genetic analysis and completed a questionnaire to provide information on Gulf War experience exposures while deployed in the Gulf, symptoms that have been experienced, medical conditions, and demographics.

Self-report

Each participant completed a questionnaire concerning: military service between August, 1990 and July, 1991, including time in the Gulf area, location of deployment, exposures while in the Gulf, and military assignment and occupation; symptoms experienced during the past six months including severity (mild, moderate, severe) and timing of first occurrence of the symptom (i.e., before Gulf deployment vs after Gulf deployment); general health status; and demographics. The symptom list included items required to determine case/control status for both the CDC and Kansas classification system, as well as items that reflect various dysfunctions of the autonomic nervous system (ANS). An 8-item scale, that reflects ANS dysfunction, was developed by summing the severity response (no experience, mild, moderate, severe) to the symptoms that reflect ANS dysfunction. These items included: breathing stops for a few seconds while sleeping; loud snoring; dizziness or faintness; sweating an unusual amount; night sweats; heart racing or pounding; feeling dizzy or light-headed when standing up; and gastrointestinal (GI) symptoms such as diarrhea, nausea, or abdominal pain. The ANS scale had acceptable reliability (Cronbach's alpha = .750), and it was not necessary to perform a transformation to improve the scale's distributional properties.

Case/Control Classification

The questionnaire provided symptom self-report data that were used to classify each participant as a case or control using both the CDC criteria for GWI[24] and also the Kansas criteria for GWI[32]. Standard scoring procedures were followed to arrive at the GWI case/control classification using the CDC criteria. Participants were classified as a case if they reported symptoms in at least two of the following three categories of symptoms: fatigue; pain (muscle pain, joint pain, joint stiffness); and mood/cognitive (problems getting to sleep or staying asleep; difficulty concentrating, difficulty remembering recent information, trouble finding words when speaking, feeling moody, feeling anxious, feeling down or depressed).

The Kansas criteria for case/control classification takes into account the timing of the reported symptoms. In order to be classified as a GWI case, symptoms that are reported must have begun either during or after Gulf deployment rather than before deployment. Case classification was based on reporting either moderate or severe symptoms, or multiple mild symptoms, in at least 3 of the following symptom groupings: fatigue (fatigue, feeling unwell after physical exercise or exertion, problems getting to sleep or staying asleep, not feeling rested after sleeping); pain (joint pain, muscle pain, body pain where you hurt all over); neurological (headaches; feeling dizzy; lightheaded, or faint; eyes very sensitive to light; blurred or double vision; numbness or tingling in your extremities; tremors or shaking; low tolerance for heat or cold; night sweats; having physical or mental symptoms after breathing in certain smells or chemicals; difficulty

speaking; feeling down or depressed; feeling irritable or having angry outbursts); skin (skin rashes, other skin problems); gastrointestinal (diarrhea, nausea or upset stomach, abdominal pain or cramping); and respiratory (difficulty breathing or catching your breath, frequent coughing when you don't have a cold, wheezing in your chest).

Statistical Methods

The questionnaire data were examined for completeness and validity of responses. Distributional properties of all continuous measures were examined to determine whether transformations were necessary to meet statistical assumptions. Statistical analyses were completed after receipt of genetic data from the analysis of the blood samples. The primary statistical analysis techniques used were chi square analysis, and comparison of means using either analysis of variance (ANOVA) or independent groups t-test, using BMDP software. For each comparison of means using independent groups t-test, the equality of the variances of the groups was examined and the appropriate statistic is reported. The statistical analyses are summarized below.

Results

Comparison of Case/Control Classification Using CDC and Kansas Definitions

Participants were recruited into the study and assigned an initial case/control classification, using the CDC definition, based on their responses to the screening interview. Case/Control classification for purposes of analyses were made using responses to the questionnaire.

As expected, there was significant agreement on case/control classification between the Kansas and CDC definitions (83% agreement; kappa = .656, $p < .0001$, 95% confidence limits .574 - .736). There were discrepant classifications for only 53 of the 304 participants. As has been found previously[32], the majority of these discrepancies (48 of 53) were the result of case classification using the CDC criteria while the Kansas definition resulted in a control classification.

Given our hypotheses about potential autonomic nervous system (ANS) involvement in GWI, we examined in detail the subset of the questionnaires that dealt with ANS symptoms. Scores on the ANS scales were compared for cases and controls using both case definitions. Cases had a significantly higher report of ANS-related symptoms compared to the controls (Kansas definition: T = 12.37, df 302, $p < .0001$, 6.9 vs 2.0; CDC definition: T = 11.68, df 302, $p < .0001$, 6.0 vs 1.7).

Relationship Between Butyrylcholinesterase Genetics and Case/Control Classification

The following table presents the distribution of the genetic status of BChE by case/control classification. The top half of the table presents the case/control classification using the CDC definition, and the bottom half presents the data distribution

using the Kansas definition. The upper row gives the genetic assignment, and it should be remembered that some mutations in BChE will often appear together in one allele; for example, the A and K mutations will often appear together in one allele, accounting for the ten U/AK and the one AK/F volunteers. U refers to the "usual" or wild-type form of the enzyme.

Table 1. Distribution of Case versus Control for each genetic category

Genetics	U/U	U/K	K/K	U/AK	U/A	A/F	AK/F	Total
CDC								
Case	115	54	8	6	2	1	1	187
Control	74	33	5	4	1	0	0	117
KANSAS								
Case	89	41	7	5	1	0	1	144
Control	100	46	6	5	2	1	0	160
Total	189	87	13	10	3	1	1	304

The simple form of the hypothesis, from Dr. Lockridge's earlier work[33], that heterozygote carriers of the A and F mutations and homozygote carriers of the K mutation of BChE are more likely to report symptoms and be cases was not supported with regard to case/control classification. The overall proportion of cases was 62% using the CDC definition and 47% using the Kansas definition. This general distribution was generally maintained for each of the genetic categories that had a large enough sample size to evaluate. However, more detailed analyses uncovered strong, significant associations between the K/K genotype and specific symptom scores, as noted below.

Relationship Between BChE Genetics and Symptom Report

The genetic hypothesis was also tested by examining the relationship between genetic status of BChE and reported symptoms. While there were no significant differences in symptom report when all of the 7 genotypes present in our population were compared, there were differences when the genotypes were grouped. Participants were grouped into one or the following three genetic classifications, based on the degree of enzyme hydrolytic velocity: (1) U/U or U/K, (2) K/K, and (3) U/AK, U/A, A/F, or AK/F (to be referred to as the [AKF] group).

Participants in the K/K group reported significantly more symptoms related to gastrointestinal symptoms (i.e., diarrhea, nausea/upset stomach, abdominal pain or cramping) than did participants in either the [UU-UK] or the [AKF] group ($F = 4.00$, df 2, 301, $p < .02$; 2.46 vs 1.05 and .53). The KK group also reported more respiratory symptoms (i.e., difficulty breathing or catching breath, frequent coughing without a cold, wheezing in chest) than did either the [UU-UK] or the [AKF] group ($F = 3.29$, df 2, 301, $p < .04$; 1.85 vs .74 and .93); the difference between the K/K and the [UU-UK] groups was significant ($p < .03$). There was also a trend for the K/K group to report more fatigue symptoms (i.e., fatigue, feeling unwell after exercise or exertion, problems getting to

sleep or staying asleep, not feeling rested after sleep) than the other two groups ($F = 2.48$, df 2, 301, $p < .09$; 4.85 vs 2.97 and 2.33). The difference of the K/K group and the other groups with respect to GI and respiratory symptoms is significant, and not present if the subject is carrying only one copy of the K allele, whether in the comparisons with the [AKF] pooled group or the UK group. Subgroup analyses of the U/K volunteers indicated them to be statistically indistinguishable from the U/U group.

Relationship Between Case/Control Classification and Exposure

As would be expected from the fact that case/control classification is based on the reporting of symptoms, these two groups differed significantly from each other on all symptom items. In order to understand the possible role that exposure might play in symptom report, additional analyses were conducted to test the relationship between case/control classification and the exposures to potentially stressful factors. Cases were significantly more likely than controls to report exposure to a wide variety of agents. The table below reports the proportion of each group that reported experiencing each kind of exposure. Only those items with significant differences using both the Kansas and the CDC case/control definitions are included in the table; the percentages and p values reported are based on the Kansas definition of case/control.

Table 2. Comparison of Exposures Between Cases and Controls

Exposure	% **Cases**	% **Controls**	**P ≤**
Smoke from oil well fires	82	65	.001
Had SCUD missile explode within one mile	48	31	.002
Saw Iraqis or civilians who had been badly wounded or killed	65	40	.0001
Handled or came into contact with POWs	59	35	.0001
Saw or came into contact with dead animals	54	34	.001
Saw destroyed enemy vehicles	74	58	.003
Came into direct contact with destroyed enemy vehicles	60	36	.0001
Used pesticide cream or spray on skin	57	31	.0001
Received one or more shots in the arm while in theater	73	58	.006
Received one or more shots in the buttocks while in theater	43	29	.02
Took pyridostigmine pills	72	44	.0001
Frequently had less than four hrs of sleep in a 24-hr period	69	49	.001

Additional analyses were conducted to explore the relationship between sleep loss and case/control status separately for specific genetic groups. The relationship was significant for those participants who were in the [UU-UK] genetic group ($p < .01$ and $p < .02$ for the Kansas and CDC definitions, respectively). The relationship was stronger

for those in the K/K group ($p < .0005$ and $p < .003$ for the Kansas and CDC definitions, respectively). Regardless of which case/control definition is used, every participant with the K/K genotype who reported frequently having less than 4 hrs of sleep in a 24-hr period is a case. This clear split in the distribution was not found for either the [UU-UK] or the [AKF] genetic groups.

Other analyses explored whether these specific associations with the K/K genotype could be explained by the known differences in enzymatic hydrolytic velocity between the wild-type enzyme and the various identified mutations. As expected, there was a significant difference in activity level between the 7 genotypes in our sample ($F = 15.71$, df 6, 297, $p < .0001$).

Genotype	Mean Enzyme Activity (μmoles benzoylcholine per min per mL)
U/U	1.19
U/K	1.01
K/K	0.78
U/AK	0.76
U/A	1.03
A/F	0.92
AK/F	0.69

Thus, in terms of enzyme velocity U/U > U/K, K/K, U/AK, but genotype U/K > K/K, U/AK. K/K does not differ from any of the other mutant groups (i.e., U/AK, U/A, A/F, A/KF). The finding with respect to symptoms held up when the genotypes were grouped as: U/U vs U/K vs K/K vs (U/AK, U/A, A/F, AK/F) ($F = 30.03$, df 3, 303, $p < .0001$). Thus, the differences in symptoms reported by the K/K volunteers did not correlate with mean enzyme activity.

In summary, the original form of the hypothesis[33], that heterozygote carriers of the A and F mutations and homozygote carriers of the K/K mutation of BChE would be present in a higher frequency in the cases than in the controls, was not supported. However, the use of a questionnaire that permitted clear case-control assignment (by either the CDC or the KVP criteria) and a large sample size allowed us to uncover a significant association between the K/K genotype and case status and GI and respiratory symptom score, as well as a significant association between that same genotype and reported sleep loss. These results do not correlate with the enzyme velocity, and are not present in the volunteers that only have one copy of the K allele, regardless of whether the other copy has a normal velocity (U) or has one or more other mutations (A, F, or AK).

Other Genetic Findings

In addition to the above results, we discovered a new naturally occurring mutation, Asp70His, in human butyrylcholinesterase. As noted above, we phenotyped 304 Gulf War veterans, since some genetic assignments can be made unambiguously with appropriate phenotyping. We also examined 4 nonveteran internal controls. In addition, we genotyped all of the suspected K mutations. Serum samples were phenotyped by measuring activity with benzoylcholine, and inhibition of activity by dibucaine, sodium

fluoride, and the Roche compound RO 2-0683. In our report last year we had noted that one sample had "not worked out in two attempts." One sample, from a veteran, out of the 308 was found whose inhibition values did not match the values for any of the known genetic variants of human BChE. The serum had an activity of 0.96 μmoles benzoylcholine hydrolyzed per minute per ml, similar to the activity of 1.2 μmoles per min per ml for 191 wild-type samples in the group. However, its dibucaine number of 42, fluoride number of 24, and Roche number of 33 were a novel set. Our initial interpretation was that the genotype was A/F with one allele containing the D70G (atypical - A) mutation and the other a new, hitherto unreported Fluoride variant. However, DNA sequencing showed that this interpretation was incorrect.

A single mutation was found in one allele. Codon 70 had C in place of G, thus changing Asp 70 (GAT) to His (CAT), nucleotide 208G->C. No other mutations were found in the coding region. The presence of the mutation was confirmed by repeating the PCR and sequencing in both directions. To obtain the D70H mutant in a homozygous state, the D70H mutant was transiently expressed in 293T human embryonic kidney cells and the secreted BChE collected into serum-free medium. The dibucaine number of the homozygous D70H was 31, the fluoride number was 13, and the Roche number was zero.

The catalytic constant (kcat) value for benzoylcholine was determined by measuring maximal velocity (Vmax) and titrating the active sites with chlorpyrifos oxon. The kcat value for D70H was found to be higher than that for wild-type BChE, 18-25,000 min-1 rather than 15,000 min-1. The Km value for benzoylcholine was 46 μM for D70H, 5 μM for wild-type, and 27 μM for D70G.

The D70G atypical allele is carried by 1 out of 25 Americans and Caucasians. The D70H allele is expected to have a 50 fold lower frequency, because D70H has been found only once in 50 atypical alleles sequenced from unrelated individuals. Fifteen atypical alleles are from the present work and 35 from previous work. This newly-discovered D70H mutation brings to 40 the total number of naturally occurring BChE mutations identified in the human population.

People homozygous for the atypical (A) variant, D70G, always respond with prolonged apnea to a normal dose of succinylcholine or mivacurium. Since the D70H variant has an even poorer binding affinity than D70G, it is expected that people homozygous for D70H will also experience prolonged apnea. These results on D70H have been written up for publication and the manuscript was accepted in the Annals of Clinical Biochemistry.

2.3.2 Study 2

Study 2 is in progress, no statistical analyses have been performed as yet.

Section 3.
Key Research Accomplishments

3.1 Study 1

- Recruitment goals were successfully completed, with 160 cases and 144 controls (target values were 150 each).
- All genetic testing was completed for the subjects that took part in Study 1.
- Statistical analysis was completed.
- The original form of the hypothesis being tested, that the A, K and F mutations of BChE would be present in a higher frequency in the cases than in the controls, was not supported. However, some related, statistically-significant findings emerged.
- There was a significant association between the K/K genotype and case status.
- There was also a strong and significant association between the K/K genotype and reported sleep loss.
- These results do not correlate with the enzyme velocity, and are not present in the volunteers that only have one copy of the K allele, regardless of whether the other copy has a normal velocity (U) or has one or more mutations (A, F, or AK).

3.2 Study 2

- Testing of subjects for Study 2 is ongoing.
- We have completed testing at the first site (Kansas City) and are currently recruiting and running subjects at the first field site (Junction City).
- As of December 31, 2001, we had completed a total of 92 appointments out of the 120 appointments that were scheduled.
    - Of the 28 that have not completed, 20 subjects have chosen to withdraw from the study or were dropped by the principal investigator for repeated missed appointments, 1 subject did not come to his appointment and has not yet been rescheduled, and 7 appointments are pending.
- Of the 92 appointments completed, 23 are cases, 24 are deployed controls, and 22 are nondeployed controls. The genetic status for those three groups (69 volunteers) remains to be determined. We also completed appointments with 23 carriers of BChE mutations who were originally recruited for Study 1 and returned for autonomic testing (11 cases, 12 deployed controls). Since the protocol calls for recruitment to be terminated when complete data are available for 40 cases, 25 deployed controls, 25 nondeployed controls and at least 20 identified variants, our progress has been excellent.
- The remaining genetic analyses have not been performed yet.
- We anticipate completing subject testing by the end of February 2002.

Section 4.
Reportable Outcomes

4.1 Study 1

We discovered a new naturally occurring mutation, Asp70His, in human butyrylcholinesterase. These results on D70H have been written up for publication and the manuscript was accepted in for publication. (Boeck, A., D. L. Fry, A. Sastre, O. Lockridge, "Naturally Occurring Mutation, Asp70His, in Human Butyrylcholinesterase," *Annals of Clinical Biochemistry* [in press, 2002]).

4.2 Study 2

Data collection is in progress for study 2.

Section 5.
Conclusions

- The original, simple form of the hypothesis being tested in Study 1, that the A, K and F mutations of BChE would be present in a higher frequency in the cases than in the controls, was not supported. However, some related, statistically-significant findings emerged with respect to carriers of the K/K genotype.

- There was a significant association between the K/K genotype and case status.

- There was also a strong and significant association between the K/K genotype and reported sleep loss.

- These results do not correlate with the enzyme velocity, and are not present in the volunteers that only have one copy of the K allele, regardless of whether the other copy has a normal velocity (U) or has one or more mutations (A, F, or AK).

- Cases had a significantly higher report of ANS-related symptoms compared to the controls, regardless of which case definition was used.

- The results of our ongoing Study 2, in which genetic status and autonomic nervous system reactivity is studied in deployed cases, deployed controls and non-deployed controls, will shed light on the basis for the association between K/K genotype and symptom scores, especially ANS symptom scores.

Section 6. References

1. Holmes, R., *Acts of War: The Behavior of Men in Battle,* Macmillian, Inc., New York (1985).
2. Keegan, J., *The Face of Battle*, Viking-Penguin, Inc., London (1976).
3. Keegan, J., R. Holmes, and J. Gav, *Soldiers: A History of Men in Battle*, Biking Penquin, Inc., New York (1986).
4. Marshall, S. L. A., *Men Against Fire,* W. Marraus, Co., New York (1947).
5. Stouffer, S. A., et al., *The American Soldier*, Vol. 1, "Adjustment During Army Life;" Vol. II, "Combat and its Aftermath," J. Wiley, Princeton (1965).
6. Letz, R., "A Discussion of Issues Concerning the Role of Stress in Veterans Reporting of Symptoms Following Deployment to the Gulf War," *A Report of the Special Investigation Unit on Gulf War Illnesses of the Committee on Veterans' Affairs,* One Hundred Fifth Congress, Washington, D. C. (1998).
7. Hyams, K. C., F. S. Wignall, and R. Roswell, "War Syndromes and Their Evaluation: From the U. S. Civil War to the Persian Gulf War," *Annals of Internal Medicine,* 125(5), 398-405 (1996).
8. Rowe, P.C., I, Bou-Holaigah, J. S. Kan, and H. Calkins, "Is Neurally Medicated Hypotension an Unrecognised Cause of Chronic Fatigue?" *The Lancet*, 345, 623 (1995).
9. Bou-Holaigah, I., P. C. Rowe, J. Kan, and H. Calkins, "The Relationship Between Neurally Mediated Hypotension and Chronic Fatigue Syndrome," *Journal of the American Medical Association,* 274, 12 (1995).
10. DeBecker, P., P. Dendale, K. DeMeirleir, I. Campine, K. Vandenborne, and Y. Hagers, "Autonomic Testing in Patients with Chronic Fatigue Syndrome," *American Journal of Medicine,* 105(3A) 22S-26S (1998).
11. Kandel, B., "Gulf War Syndrome Examined," *USA Today* (September 9), 2A (1992).
12. Haley, R. W., T. L. Kurt, and J. Horn, "Is There a Gulf War Syndrome? Searching for Syndromes by Factor Analysis of Symptoms," *Journal of the American Medical Association* 277, 215-222 (1997a).
13. Haley, R. W., J. Horn, P. S. Roland, W. W. Bryan, P. C. Van Ness, F. J. Bonte, M. D. Devous, D. Mathews, J. L. Fleckenstein, F. H. Wians, G. I. Wolfe, and T. L. Kurt, "Evaluation of Neurologic Function in Gulf War Veterans," *Journal of the American Medical Association,* 277 (3), 223-230 (1997b).
14. Haley, R.W., and T. L. Kurt, "Self-reported Exposure to Neurotoxic Chemical Combinations in the Gulf War," *Journal of the American Medical Association,* 277(3), 231-237 (1997c).

15. Hyams, K. C. and R. H. Roswell, "Resolving the Gulf War Syndrome Question," *American Journal of Epidemiology*, 148, 339-342 (1998).

16. NIH Technology Assessment Workshop Panel, "The Persian Gulf Experience and Health," *Journal of the American Medical Association*, 272, 391-395 (1994).

17. Murphy, F. M., "Gulf War Syndrome: There May be No Specific Syndrome, but Troops Suffer After Most Wars," *British Medical Journal*, 318, 274-275 (1999).

18. Straus, S. E., "Bridging the Gulf in War Syndromes," *The Lancet*, 353, 162-163 (1999).

19. Persian Gulf Veterans Coordinating Board, "Unexplained Illnesses Among Desert Storm Veterans: A Search For Causes, Treatments, and Cooperation," *Archives of Internal Medicine*, 155, 262-268 (1995).

20. Department of Veterans Affairs, Veterans Health Administration, Office of Public Health and Environmental Hazards, "Consolidation and Combined Analysis of the Combined Databases of the Department of Veterans Affairs, Persian Gulf Registry, and the Department of Defense's Comprehensive Clinical Evaluation Program" (1997).

21. Goss, G., *"Health Study of Canadian Forces Involved in the 1991 Conflict in the Persian Gulf,"* Report Prepared for Gulf War Illness Advisory Committee, Canadian Department of National Defense (1998).

22. Joseph, S. C. and the Comprehensive Clinical Evaluation Program Evaluation Team, "A Comprehensive Clinical Evaluation of 20,000 Persian Gulf War Veterans," *Military Medicine,* 162, 149-155 (1997).

23. Pierce, P. F., "Physical and Emotional Health of Gulf War Veteran Women," *Aviation, Space, and Environmental Medicine,* 68, 317-321 (1997).

24. Fukuda, K., R. Nisenbaum, G. Stewart, W. W. Thompson, L. Robin, R. M. Washko, D. L. Noah, D. H. Barrett, B. Randall, B. L. Herwaldt, A. C. Mawle, and W. C. Reeves, "Chronic Multisymptom Illness Affecting Air Force Veterans of the Gulf War," *Journal of the American Medical Association,* 280, 981-988 (1998).

25. Stretch, R. H., P. D. Bliese, D. H. Marlowe, K. M. Wright, K. H. Knnudson, and C. H. Hoover, "Physical Health Symptomology of Gulf War-Era Service Personnel from the States of Pennsylvania and Hawaii," *Military Medicine*, 160, 3, 131-136 (1995).

26. Unwin, C., N. Blatchley, W. Coker, S. Ferry, M. Hotopf, L. Hull, K. Ismail, I. Palmer, A. David, and S. Wessely, "Health of UK Servicemen Who Served in the Persian Gulf War," *The Lancet*, 353, 169-78 (1999).

27. Lane, T. J., D. A. Matthews, and P. Manu, "The Low Yield of Physical Examinations and Laboratory Investigations of Patients with Chronic Fatigue," *The American Journal of Medical Sciences,* 299, 313-318 (1990).

28. Valdini, A. S., S. I. Steinhardt, and E. Feldman, "Usefulness of a Standard Battery of Laboratory Tests in Investigating Chronic Fatigue in Adults," *Family Practice*, 6, 286-291 (1989).

29. U.S. Senate Committee on Veterans' Affairs, *"Report of the Special Investigation Unit on Gulf War Illnesses,"* U.S. Government Printing Office 50-273 CC (1998).

30. Gray, G. C., A. W. Hawksworth, T. C. Smith, H. K. Kang, J. D. Knoke, and G. D. Gackstetter, "Gulf War Veterans Health Registries, Who Is Most Likely to Seek Evaluation?" *American Journal of Epidemiology*, 148, 343-349 (1998).

31. Barrett, D. H., "Study of Gulf War Vets in Iowa and Pennsylvania," presented at The National Gulf War Resource Center's 2nd Annual Conference on Gulf War Illnesses (November 7-9), Atlanta, Georgia (1997).

32. Steele, L., "Prevalence and Patterns of Gulf War Illness in Kansas Veterans: Association of Symptoms with Characteristics of Person, Place, and Time of Military Service," *American Journal of Epidemiology*, 152 (10), 991-1001, (2000).

33. Lockridge, O., "Butyrylcholinesterase genetic variants in persons with Gulf War Illness," Final Report, U.S. Army Medical Research and Materiel Command, (1999).

34. Suarez, G. A., T. L. Opfer-Gehrking, K. P. Offord, E. J. Atkinson, P. C. O'Brien, and P. A. Low, "The Autonomic Symptom Profile: A New Instrument to Assess Autonomic Symptoms," Neurology, 52, 523-528 (1999).

35. Ware, Jr., J. E., and C. D. Sherbourne, "The MOS 36-item Short-form Health Survey (SF-36), I. Conceptual Framework and Item Selection," Medical Care, 6, 473-83 (1992).

36. Kazis, L. E., X. S. Ren, A. Lee, K. Skinner, W. Rogers, J. Clark, and D. R. Miller, "Health Status in VA Patients: Results from the Veterans Health Study," American Journal of Medical Quality, 14: 28-38 (1999).

37. "Consensus Statement on the Definition of Orthostatic Hypotension, Pure Autonomic Failure, and Multiple System Atrophy," published by the Consensus Committee of the American Autonomic Society and the American Academy of Neurology, 46, 1470 (1996).

38. Sastre, A., M. R. Cook, and C. Graham, "Nocturnal Exposure to Intermittent 60-Hz Magnetic Fields Alters Human Cardiac Rhythm," Bioelectromagnetics, 19, 98-106 (1998).

39. Anonymous, "Heart Rate Variability: Standards of Measurement, Physiological Interpretation, and Clinical Use: Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology," Circulation, 93(5) 1043-65 (1996).

40. Kemmotsu, O., M. Ueda, H. Otsuka, T. Yamamura, A. Okamura, T. Ishikawa, D. C. Winter, and J. S. Eckerle, "Blood Pressure Measurement by Arterial Tonometry in Controlled Hypotension," Anesthesia and Analgesia, 73, 54-58 (1991a).

41. Kemmotsu, O., M. Ueda, H. Otsuka, T. Yamamura, D. C. Winter, and J. S. Eckerle, "Arterial Tonometry for Noninvasive, Continuous Blood Pressure Monitoring During Anesthesia," Anesthesiology, 75(2), 533-540 (1991b).

42. Weiss, B. M., D. R. Spahn, H. Rahmig, R. Rohling, and T. Pasch, "Radial Artery Tonometry: Moderately Accurate but Unpredictable Technique of Continuous Non-invasive Arterial Pressure Measurement," British Journal of Anesthesia, 76, 405-411 (1996).

43. Low, P. A., "Autonomic Nervous System Function," Journal of Clinical Neurophysiology, 10(1), 14-27 (1993a).

44. Low, P. A., "Clinical Evaluation of Autonomic Function," 2nd ed., Lippincott-Raven, Philadelphia, Pennsylvania (1993b).

45. Low, P. A., "Composite Autonomic Scoring Scale for Laboratory Quantification of Generalized Autonomic Failure," Mayo Clinic Proceedings, 68, 748-752 (1993c).

46. Low, P. A., T. L. Opfer-Gehrking, B. R. McPhee, R. D. Fealey, E. E. Benarroch, C. L. Willner, G. A. Suarea, C. J. Proper, J. A. Felten, C. A. Huck, and J. L. Corfits, "Prospective Evaluation of Clinical Characteristics of Orthostatic Hypotension," Mayo Clinic Proceedings, 70, 617-622, (1995).

47. Low, P. A., "Laboratory Evaluation of Autonomic Function," In Clinical Autonomic Disorders, 2nd ed., edited by P.A. Low, Lippincott-Raven Publishers, Philadelphia, Pennsylvania (1997a).

48. Low, P. A., J-C Denq, T. L. Opfer-Gehrking, P. J. Dyck, P. C. O'Brien, and J. M. Slezak, "Effect of Age and Gender on Sudomotor and Cardiovagal Function and Blood Pressure Response to Tilt in Normal Subjects," Muscle and Nerve, 1561-1568 (1997b).

49. Angelone, A., and N. A. Coulter, "Respiratory Sinus Arrhythmia: A Frequency Dependent Phenomenon," Journal of Applied Physiology, 19, 479-482 (1964).

50. Ewing, D. J., et al., "Cardiovascular Responses to Sustained Handgrip in Normal Subjects and in Patients with Diabetes Mellitus: A Test of Autonomic Function," Clinical Science and Molecular Medicine, 46, 295-306 (1974).

51. Borg, G., "Perceived Exertion as an Indicator of Somatic Stress," Scandinavian Journal of Rehabilitation Medicine, 2, 92-98 (1970).

52. Benarroch, E. E., et al., "Analysis of Valsalva Maneuver in Normal Subjects," Annals of Neurology, 26, 186A (Abstract) (1989).

53. Denq, J-C, P. C. O'Brien, and P. A. Low, "Normative Data on Phases of the Valsalva Maneuver," Journal of Clinical Neurophysiology, 15(6), 535-540 (1998).

54. Cohen, H., M. Kilter, M. A. Matar, A. Kaplan, U. Loewenthal, H. Miodownik, and Y. Cassuto, "Analysis of Heart Rate Variability in Posttraumatic Stress Disorder Patients in Response to a Trauma-Related Reminder," Biological Psychiatry, 44, 1054-1059. (1998).

55. Sinnreich, R., J. D. Kark, Y. Friedlander, D. Sapoznikow, and M. H. Luria, "Five Minute Recordings of Heart Rate Variability for Population Studies: Repeatability and Age—Sex Characteristics," Heart, 80, 156-162 (1998).

56. Sloan, R. P., P. A. Shapiro, E. Bagiella, M. M. Myers, J. T. Bigger, Jr., R. C. Steinman, and J. M. Gorman, "Brief Interval Heart Period Variability by Different Methods of Analysis Correlates Highly with 24 h Analyses in Normals," Biological Psychology, 38, 133-142 (1994).

57. Liao, D., J. Cai, W. D. Rosamond, R. W. Barnes, R. G. Hutchinson, E. A. Whitsel, P. Rautaharju, G. Heiss, "Cardiac Autonomic Function and Incident Coronary Heart Disease: A Population-Based Case-Cohort Study. The ARIC Study: Atherosclerosis Risk in Communities Study," American Journal of Epidemiology, 145 (8), 696-706 (1997).

58. "Assessment: Clinical Autonomic Testing Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology," American Academy of Neurology, 873 (1996).

59. Kalow, W., and H. A. Lindsay, Canadian Journal of Biochemistry and Physiology, 33, 568-574 (1955).

60. Bartels, C. F., F. S. Jensen, O. Lockridge, A. F. L. van der Spek, H. M. Rubinstein, T. Lubrano, and B. N. La Du, "DNA Mutation Associated with the Human Butyrylcholinesterase K Variant and Its Linkage to the Atypical Variant Mutation and Other Polymorphic Sites," American Journal of Human Genetics, 50, 1086-1103 (1992a).

61. Bartels, C. F., K. James, and B. N. La Du, "DNA Mutations Associated with the Human Butyrylcholinesterase J-variant," American Journal of Human Genetics, 50 (5), 1104-1114 (1992b).

62. Overstreet, D. H., D. S. Janowsky, J. C. Gillin, P. J. Shiromani, and E. L. Sutin, "Stress-Induced Immobility in Rats with Cholinergic Supersensitivity," Biological Psychiatry, (7) 657-664 (1986).

63. Markou, A., K. Matthews, D. H. Overstreet, G. F. Koob, and M. A. Geyer, "Flinders Resistant Hypocholinergic Rats Exhibit Startle Sensitization and Reduced startle Thresholds," Biological Psychiatry, 36(10), 680-688 (1994)

64. Shibuta, K., M. Abe, and T. Suzuki, "A New Detection Method for the K Variant of Butyrylcholinesterase Based on PCR Primer Introduced Restriction Analysis (PCR-PIRA)," Journal of Medical Genetics, 31(7), 576-579 (1994).

65. Jensen, F. S., L. R. Nelsen, and M. Schwartz, "Detection of the Plasma Cholinesterase K Variant by PCR Using an Amplification-created Restriction Site," Human Heredity, 46, 26-31 (1996).

66. Nogueira, C. P., C. F. Bartels, M. C. McGuire, S. Adkins, T. Lubrano, H. M. Rubinstein, H. Lightstone, A. F. L. van der Spek, and O. Lockridge, B. N. La Du, "Identification of Two Different Point Mutations Associated with the Fluoride-

Resistant Phenotype for Human Butyrylcholinesterase," American Journal of Human Genetics, 51(4), 821-828 (1992).

67. Sudo, K., M. Maekawa, S. Akizuki, T. Magara, H. Ogasawara, and T. Tanaka, "Human Butyrylcholinesterase L330I Mutation Belongs to a Fluoride-Resistant Gene, by Expression in Human Fetal Kidney Cells," Biochemical and Biophysical Research Communications, 240(2), 372-375 (1997).

68. McGuire, M. C., C. P. Nogueira, C. F. Bartels, H. Lightstone, A. Hajra, A. F. L. van der Spek, O. Lockridge, and B. N. La Du, Proceeding of the National Academy of Sciences USA, 86, 953-957 (1989).

69. Johnson, C. D., and R. L. Russell, "A Rapid, Simple Radiometric Assay for Cholinesterase, Suitable for Multiple Determinations," Analytical Biochemistry, 64, 229-238 (1975).

70. Nostrandt, A. C., J. A. Duncan, and S. Padilla, "A Modified Spectrophotometric Method Appropriate for Measuring Cholinesterase Activity in Tissue from Carbaryl-Treated Animals," Fundamental and Applied Toxicology, 21, 196-203 (1993).