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Antibodies to Squalene in Gulf War Syndrome

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Gulf War Syndrome (GWS) is a multisystemic illness afflicting many Gulf War-era veterans. The molecular pathological basis for GWS has not been established. We sought to determine whether the presence of antibodies to squalene correlates with the presence of signs and symptoms of GWS. Participants in this blinded cohort study were individuals immunized for service in Desert Shield/Desert Storm during 1990–1991. They included 144 Gulf War-era veterans or military employees (58 in the blinded study), 48 blood donors, 40 systemic lupus erythematosus patients, 34 silicone breast implant recipients, and 30 chronic fatigue syndrome patients. Serum antibodies to squalene were measured. In our small cohort, the substantial majority (95%) of overtly ill deployed GWS patients had antibodies to squalene. All (100%) GWS patients immunized for service in Desert Shield/Desert Storm who did not deploy, but had the same signs and symptoms as those who did deploy, had antibodies to squalene. In contrast, none (0%) of the deployed Persian Gulf veterans not showing signs and symptoms of GWS have antibodies to squalene. Neither patients with idiopathic autoimmune disease nor healthy controls had detectable serum antibodies to squalene. The majority of symptomatic GWS patients had serum antibodies to squalene.

INTRODUCTION

The illnesses afflicting men and women who served in the military conflict in the Persian Gulf during 1990–1991 remain ill-defined. A constellation of symptoms including fatigue, rashes, headaches, arthralgias, myalgias, lymphadenopathy, diarrhea, memory loss, autoimmune thyroid disease, increased allergies and sensitivities to environmental elements, and neurological abnormalities collectively referred to as Gulf War Syndrome (GWS) have been described in veterans from this conflict (Persian Gulf Veterans Coordinating Board, 1985; Grady *et al.*, 1998; Fukuda *et al.*, 1998; Unwin *et al.*, 1999; Coker *et al.*, 1999). A symptom-based case definition of GWS has recently been described (Fukuda *et al.*, 1998). While GWS patients in general do not suffer from classic rheumatic diseases, the signs and symptoms are reminiscent of entities, such as arthralgias, fibromyalgia, lymphadenopathy, autoimmune thyroid disease, chronic fatigue syndrome, malar rashes, and musculoskeletal signs and symptoms associated with various autoimmune conditions and exposure to silicone, an organic material developed, in part, to be used as an immunological adjuvant for vaccines (Ismail *et al.*, 1999; Straus, 1999; Hyams *et al.*, 1996). Many, if not most, of these signs and symptoms are caused, promoted, or modulated by cytokines (Dinarello, 1988; Akiro *et al.*, 1990), further details of which are beyond the scope of this paper. Serological abnormalities including hypergammaglobulinemia and abnormal serum proteins have been reported in 45% of GWS patients (Grady *et al.*, 1998). A variety of possible explanations for GWS have been proposed. The Persian Gulf Veterans Coordinating Board addressed the issues of possible chemical and biological

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weapons to account for these illnesses (Persian Gulf Veterans Coordinating Board, 1995). Haley *et al.* (1997) grouped various reported symptoms into six different syndromes based upon self-reported possible exposure to chemicals in the Persian Gulf. It has been suggested that a combination of chemical and biological weapons exposure may account for GWS illnesses. Abou-Donia *et al.* (1996) examined the acute effects of pyridostigmine bromide and organophosphate exposure in chickens and suggested that the toxicity observed may be similar to that suffered by Gulf War veterans. Another explanation for GWS is that it is posttraumatic stress syndrome (Hyams *et al.*, 1996).

It has also been suggested that GWS may be due to exposure to biological weapons, dysregulation of the immune system (Rook *et al.*, 1998), or imbalance in the TH1/TH2 ratio, either as an adverse reaction to the intense vaccination schedule or as a result of exposure to biological agents in the Persian Gulf (Rook *et al.*, 1998).

Gulf War veterans and attendant civilian personnel received a variety of immunizations in preparation for possible deployment to the Persian Gulf theater. A similar intensive vaccination regimen was also used in British troops (David *et al.*, 1997). Epidemiological studies indicate that multiple vaccinations or vaccination against biological warfare agents are the factors with the highest correlation with GWS symptomatology (Unwin *et al.*, 1999).

We have identified a group of GWS patients who served in American and British military forces or worked as civilian employees to the U.S. military or their contractors during Desert Shield/Desert Storm in the Persian Gulf, 1990–1991. These patients served in all branches of the military and received the required immunizations. They served throughout the Persian Gulf, including on ships of the U.S. Navy not in combat or exposed to environmental toxins at ground level. We have found antibodies to squalene, an experimental immunological adjuvant, in a high percentage of these GWS cases.

MATERIALS AND METHODS

Patients were admitted to the study based upon service in the United States or the United Kingdom military or as civilian employees of the U.S. military or their contractors in the Persian Gulf during 1990–1991. Patients became aware of the study via the Internet and word of mouth with other veterans and were enrolled consecutively on a voluntary basis. No fees were paid by the subjects or to the subjects who participated in this study. Included were individuals who fit the recently proposed case definition for GWS (Fukuda *et al.*, 1998) and others without GWS symptoms. Service occurred in Desert Shield/Desert Storm, Operation Provide Comfort (in northern Iraq where there were no chemical weapons), CENTCOM in Saudi Arabia, Kuwait, Camp 4 (front lines), and medical units in various locations in Saudi Arabia. Some were in theater for months. Others were evacuated due to illness after as little as 48 h after arrival and before the war commenced. We tested deployed personnel who served in various parts in theater during the war, but were and are not sick. We tested patients referred to as nondeployed veterans, those immunized for duty in the Persian Gulf, but who did not leave the United States or were deployed elsewhere. None participated in NIH experimental vaccination trials, although our positive control subjects had participated in such trials and were known to have received squalene-containing adjuvant injections. Further controls had idiopathic autoimmune disease or silicone breast implants or were healthy subjects with no stigmata of autoimmune disease.

Patient records and histories were obtained from the Gulf War-era participants. Board-certified rheumatologists, neurologists, and endocrinologists made all diagnoses. Compilation of data, including commercial lab results, was done by chart review by one investigator (P.B.A.) and was reviewed by board-certified rheumatologists.[2] Serum samples from study participants were collected by laboratory personnel via standard phlebotomy procedures using vacutainer tubes and butterfly needles and were stored at -20°C until they were shipped to Tulane University School of Medicine in New Orleans. Samples from Gulf War-era veterans were blinded. The identities or exact number of samples from each category was not made available to the Tulane laboratory until after completion of the diagnostic testing. All samples were tested twice under the same conditions. Results from all samples in both tests were consistent. At the end of the study, patient data were matched with the outcome of the anti-squalene antibody (ASA) assay and results were tabulated.

Anti-squalene Antibody Assay

The ASA assay measures the binding of serum immunoglobulin (IgG) to squalene immobilized on nitrocellulose. It is similar in format to the antipolymer antibody (APA) assay

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for partially polymerized acrylamide (Tenenbaum *et al.*, 1997). Seropositivity on the APA assay has been shown to correlate with severe musculoskeletal signs and symptoms present in a subset of silicone breast implant recipients (Tenenbaum *et al.*, 1997). For the blinded study, squalene (>99% purity) was diluted 10-, 100-, 1000-, and 10,000-fold in distilled water, applied to nitrocellulose membranes, and allowed to air-dry. The nitrocellulose membranes were then cut into 4-mm-wide strips, placed in 20-well trays, and rinsed in wash buffer (Tris-buffered saline containing 0.3% polyoxyethylene sorbitan monolaurate and 0.005% thimerosal, pH 7.4). The strips were incubated in 2 ml blocking buffer (Tris-buffered saline containing 5% powdered instant milk, 4% goat serum, and 0.008% thimerosal, pH 7.4) for 45 min prior to the addition of 5 μl of patient sera (1:400 dilution) followed by a further 90-min incubation. This dilution factor was chosen based upon the very strong antibody responses found in GWS patients. All incubations and washes were carried out at room temperature on a rocking platform. The blocking buffer was then removed and the strips were washed with washing buffer (three times for 5 min each). After the strips were washed, 2 ml of blocking buffer containing biotin conjugated to goat anti-human IgG (Kirkengaard & Perry Laboratories, Gaithersburg, MD), diluted 1:1000, was added. After a 60-min incubation, the strips were again washed as above, and 2 μl of blocking buffer containing avidin-conjugated horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), diluted 1:500, was added. Following another 60-min incubation, the strips were washed, as above, and 2 ml of detection-buffered saline containing 30% methanol, 0.6 mg/ml 4-chloro-1-napthol, 0.03% hydrogen peroxide; pH 7.4) was added. The reaction was allowed to proceed for 15 min and was stopped by rinsing the strips in distilled water. The strips were allowed to air-dry for visual scoring on a scale of 0 to +4.

Statistical Analysis

The strength of binary relationships was tested using χ^2 tests of independence. This protocol was a feasibility study. Accordingly, no power studies were performed.

RESULTS

Primary Studies

To ascertain that our assay could detect antibodies to squalene, we had positive controls who were two subjects who had volunteered to participate in a vaccine trial at the NIH involving the use of a squalene-containing adjuvant (Table 1). Subsequent to vaccination, they developed a multisystem disorder similar to that of Persian Gulf veterans. Their symptoms are listed in Table 2.

TABLE 1
Squalene Reactivity of NIH Vaccine Trial Participants

Patient	Doses of squalene	ASA reactivity
A	1	+1
B	3	+3

Patient A received a single injection and became ill within 3 weeks, with signs and symptoms including arthritis, fibromyalgia, lymphadenopathy, photosensitive rashes, fatigue, headaches, and fasciculations. This patient had lower than normal acetylcholinesterase and histological evidence of IgG-mediated demyelinization. The NIH vaccine study code was broken; only adjuvant containing squalene had been administered as a placebo. This patient was weakly positive for ASA. Patient B went through the complete experimental vaccination protocol before manifesting a similar set of signs and symptoms and was +3 for ASA.

Fukuda and co-workers (1998) have reported that individuals deployed to the Persian Gulf who became sick have a chronic multisystem disease. The cohort of GWS patients in our study have many signs and symptoms of autoimmune connective tissue and neurological disease with arthritis (94%), fibromyalgia (94%), lymphadenopathy (94%), rashes (94%), weakness (86%), fatigue (81%), chronic headaches (78%), and memory loss (72%) as the most frequent symptoms (Table 3).

It should be noted, however, that most patients did not have

TABLE 2
Symptoms Which Appeared after a Single Adjuvant Injection

Arthritis
Fibromyalgia
Lymphadenopathy
Rashes
Photosensitive rashes
Malar rashes
Chronic fatigue
Chronic headaches
Fasciculations
Lymphocytic infiltrates around vascular tissue
IgG-mediated demyelinization
Lower than normal levels of acetylcholinesterase

TABLE 3
Symptoms and Diagnostic Lab in GWS Patient Groups

	D-S (%)	D-W (%)	ND-S (%)	UK-D (%)
Arthritis	94	0	100	100
Fibromyalgia	94	8	100	100
Lymphadenopathy	94	0	100	100
Rashes	94	0	100	100
Photosensitive rashes	25	0	75	100
Malar rashes	17	0	63	100
Chronic fatigue	81	33	100	100
Chronic headaches	78	0	100	100
Abnormal body hair loss	19	0	38	33
Nonhealing skin lesions	42	0	63	66
Apthous ulcers	36	0	63	66
Dizziness	47	8	100	66
Weakness	86	17	100	66
Memory loss	72	25	100	66
Seizures	14	0	50	66
Mood changes	72	0	63	100
Neuropsychiatric problems	44	0	88	66
+FANA	20	0	50	Unknown
Anti-dsDNA	14	0	Unknown	Unknown
Low C3 and C4	14	0	Unknown	Unknown
Anti-thyroid	14	0	Unknown	Unknown
Anemia	14	0	50	Unknown
Elevated ESR &/or CRP	25	0	75	Unknown
SLE	17	0	50	Unknown
MS	3	0	Unknown	Unknown
ALS	8	0	0	0
Raynaud's phenomenon	42	0	75	66
Sjogren's syndrome	8	0	Unknown	33
Chronic diarrhea	36	0	63	66
Night sweats	36	0	88	66
Low grade fevers	39	0	88	66

Note. D-S, deployed, sick ($N = 38$); D-W, deployed, well ($N = 12$); ND-S, nondeployed, sick ($N = 8$); UK-D, deployed, sick, UK ($N = 3$).

an optimal workup for connective tissue and neurological autoimmune diseases because of the limited resources in the Veterans' Administration hospitals or military hospitals. Nevertheless, all patients reported here meet the case definition recently established (Fukuda *et al.*, 1998). In agreement with a prior study (Grady *et al.*, 1998), some of these GWS patients also had abnormal laboratory values, including positive antinuclear antibodies (ANA;17%), anti-dsDNA (14%), low C3 and C4 (14%), anemia (14%), anti-thyroid microsomal antibodies (14%), and elevated ESR and/or CRP (22%). A minority of symptomatic patients met diagnostic criteria for classical autoimmune diseases, including Sjogren's syndrome (8%), multiple sclerosis (3%), ALS (8%), and systemic lupus erythematosus (17%).

Likewise, military personnel from the United Kingdom have shown the same array of signs and symptoms as those from the United States. Their signs and symptoms included arthritis (100%), fibromyalgia (100%), lymphadenopathy (100%), rashes (100%), chronic fatigue (100%), chronic headaches (100%), and memory loss (66%). Laboratory data are not unavailable for this group. They also had malar rashes, Raynaud phenomenon, and sicca syndromes. Thus, our cohort represents a subset of veterans that displays manifestations of GWS. The severity of symptoms in our cohort can be explained by a self-selection bias in that the patients volunteered for our study.

Persons activated to deploy who were vaccinated, but did not deploy for a variety of reasons, had an array of signs and symptoms with even higher frequencies of arthritis (100%), fibromyalgia (100%), lymphadenopathy (100%), rashes (100%), weakness (100%), fatigue (100%), chronic headaches (100%), and memory loss (100%) (Table 3). The nondeployed individuals had higher rates of dizziness (100%), seizures (50%), and neuropsychiatric abnormalities (88%). The number in this group was small, and these differences were not statistically significant. Laboratory values for the nondeployed individuals with GWS were abnormal, with positive ANA (50%), anemia (50%), and elevated ESR and/or CRP (75%).

In contrast, abnormal signs, symptoms, and laboratory values were rare in the cohort of Gulf War-era veterans who considered themselves well and upon examination did not have debilitating health problems. They reported some signs and symptoms, but their illnesses were not multisystemic (Table 3). The signs and symptoms reported included fibromyalgia (8%), chronic fatigue (33%), weakness (17%), memory loss (25%), and thyroid disease (8%). None reported positive laboratory values for autoimmune processes or were so diagnosed.

Musculoskeletal signs and symptoms are more common in females than males, and autoimmune diseases are predominantly found in females in ratios ranging from 8:1 to 14:1 (Michet *et al.*, 1985; Geirsson *et al.*, 1994). We wished to determine why predominantly male military personnel, both deployed and nondeployed, initially found fit for duty during the war, would develop signs and symptoms common to autoimmune diseases. Many studies have shown that adjuvants used to enhance vaccine efficacy can induce autoimmune disease (Zamma, 1983; Lorentzen *et al.*, 1995; Madzhidov *et al.*, 1986; Kleinau *et al.*, 1995). Thus, we sought whether GWS patients who received immunizations had antibodies to an immunological adjuvant. Squalene was chosen as it has been used in many experimental vaccine adjuvant formulations since 1987. A variation of a previously

described assay, one which measures the binding of serum antibodies to low-molecular-weight polymers (Tenenbaum *et al.*, 1997), was used in the current study. This immunological assay, similar in format to Western immunoblotting, quantitates the binding of antibodies to squalene immobilized on nitrocellulose (Fig. 1). Serum samples were tested blindly. We found that GWS patients who deployed had ASA responses ranging in intensity from +1 to +4. Most of the sick Gulf War veterans had +2 and +3 reactivity to squalene at a serum dilution of 1:400. One individual had an especially strong reaction rated as +4. A high majority (95%) of symptomatic deployed individuals with GWS were positive on the ASA assay (Fig. 2A).

Interestingly, all sick veterans who did not deploy but had received immunizations as preparation for deployment also had antibody reactivity to squalene. In contrast, none of the persons deployed to the Gulf who thought of themselves as well were ASA positive.

Other Studies

Squalene is an organic polymer, with some antigenic epitopes which might be shared with other organic polymers, acting as immunostimulants. Antibodies to silicone and partially polymerized acrylamide (the antigen in the antipolymer assay) were weakly positive in fewer than 10% of the symptomatic Gulf War-era veterans. Four patients with musculoskeletal signs and symptoms and exposure to silicone breast devices were tested to see if antibodies to squalene were present; none were reactive (see below). To determine if antibodies to squalene occurred in idiopathic autoimmune diseases, samples were taken from patients who had defined autoimmune diseases, both rheumatic and neurologic, but none were reactive. To determine if healthy individuals from the general public might have antibodies to squalene, we tested members of the general public. Again, none showed antibody reactivity (Table 4).

In a broader unblinded antibody-screening study, antibodies to squalene were studied in larger groups of individuals (Fig. 2B). Blood samples of Gulf War veterans from different medical centers were tested for ASA. This group contained a high percentage of ASA-positive individuals (69%). The samples included were not segregated according to their clinical status and included healthy controls. Squalene is in some cosmetic products, so we tested to determine if antibodies were present in the general population. Samples of blood from blood banks indicated only 5% antibody reactivity to squalene and the reactions were much less intense (Fig. 1). To determine if antibody to squalene was a marker for autoimmune disease processes, tests were conducted on blood samples from patients with systemic lupus erythematosus. This group had 10% ASA weakly positive reactivity (Fig. 2B). Patients suffering from chronic fatigue syndrome have some of the signs and symptoms of GWS patients, but showed only 15% weak reactivity. Prior studies have shown that most individuals exposed to silicone breast devices with

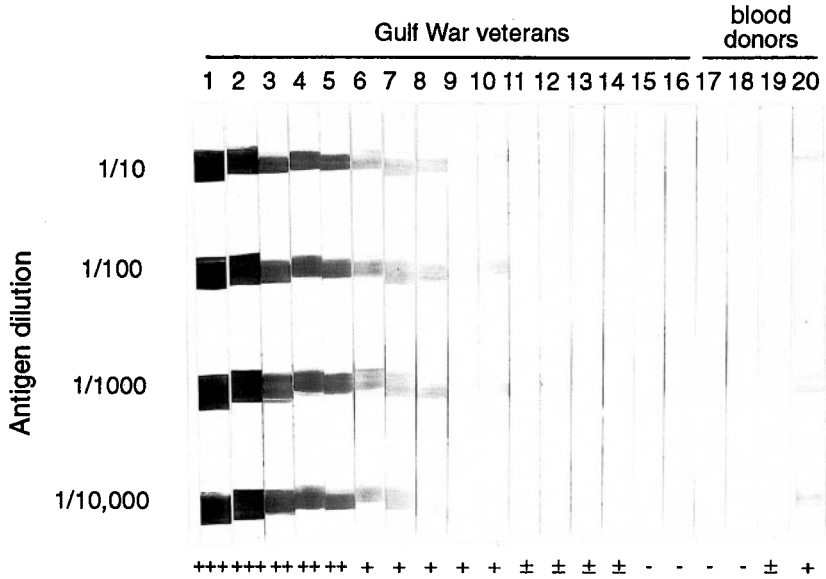

FIG. 1. Antisqualene antibody responses in representative Gulf War Syndrome patients and blood donors.

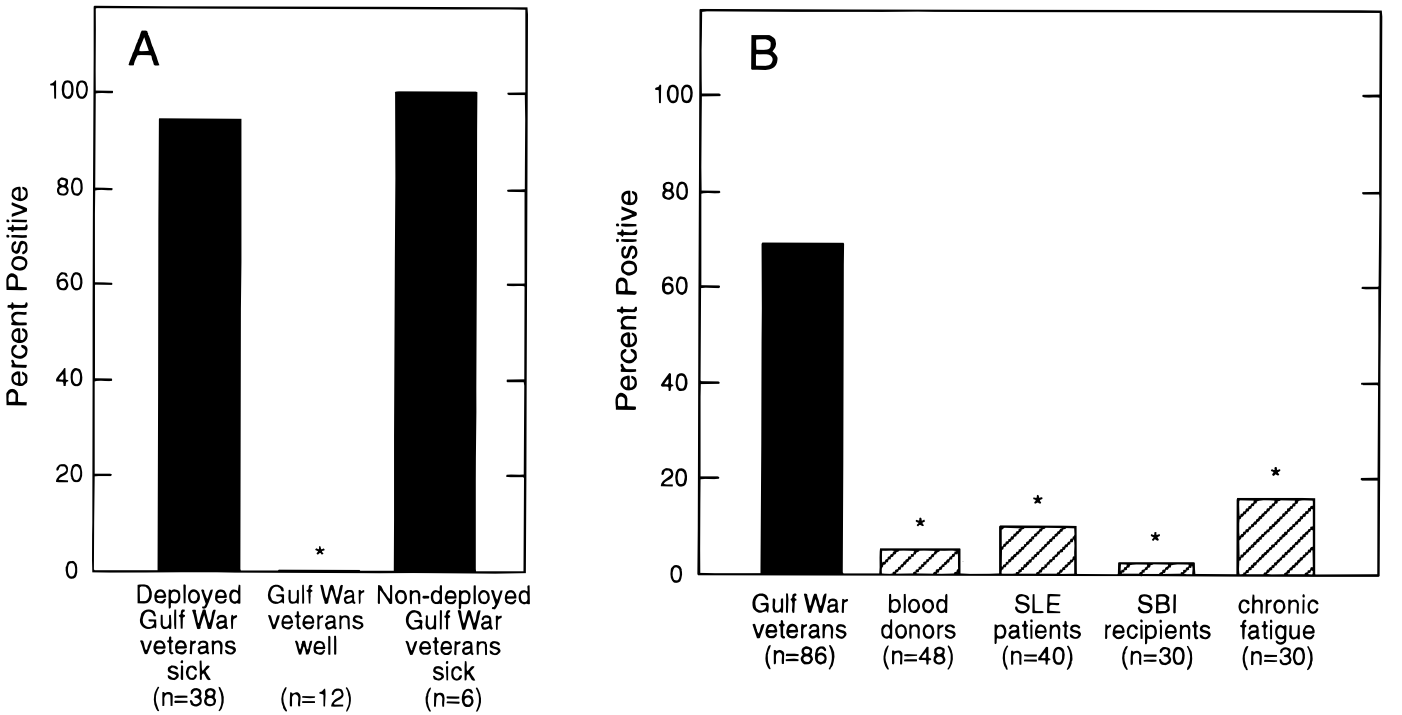

FIG. 2. Antisqualene antibody responses in Gulf War Syndrome patients, blood donors, systemic lupus erythematosus (SLE) patients, chronic fatigue syndrome patients, and symptomatic silicone breast implant (SBI) recipients. (A) Blinded samples. *, $P < 0.001$ compared to percentage positive in well Gulf War veterans by χ^2 test. (B) Unblinded samples. *, $P < 0.001$ compared to percentage positive in Gulf War Syndrome patients.

severe musculoskeletal signs and symptoms have serum antibodies reactive to a synthetic polymer (polyacrylamide) (Tenenbaum *et al.*, 1997). Both silicone and acrylamide, like squalene, are potent immunological adjuvants (Naim *et al.*, 1997; Nicholson *et al.*, 1996; Yoshida *et al.*, 1994; Sergott *et al.*, 1986). Therefore, we tested for cross-reactive antibodies to squalene in serum from patients exposed to SBI. Only 10% of this group were weakly positive for antibodies to squalene (Fig. 2B), confirming the results with the smaller sample in the blinded portion of the study.

TABLE 4
Squalene Antibodies—Blinded Study Patient Groups

Patient group	ASA reactivity (%)
D-S	95
D-W	0
ND-S	100
UK-D	100
Breastimplants	0
NIH vaccine participants	100
Idiopathic autoimmune disease	0
Healthy general public	0

Note. D-S, deployed, sick ($N = 38$); D-W, deployed, well ($N = 12$); ND-S, nondeployed, sick ($N = 8$); UK-D, UK deployed, sick ($N = 3$); NIH vaccine trial patients ($N = 2$).

DISCUSSION

The illnesses afflicting military veterans and civilians who served in the Persian Gulf in 1990–1991 have remained clouded in confusion and controversy. Several recent studies have indicated that the Gulf War-era patients are suffering from a chronic multisystemic illness, but with a continuum of signs and symptoms not within the definitions of "classic" rheumatic diseases or other specific disorders (Fukuda *et al.*, 1998; Ismail *et al.*, 1999; Straus, 1999). In some, onset of illness occurred within a few weeks after receiving immunizations. This includes personnel never deployed due to illness. It also included some who did deploy, but were in theater for as little as 48 h before being sent home before the war began because of severe joint and muscle pain and neurological problems. Other Gulf War veterans became ill

years after the war, but showed illnesses similar to those who became ill soon after vaccination. The variability of expression of symptoms and severity may be due to individual immune responses genetically regulated by the histocompatibility complex (Lorentzen *et al.*, 1995; Madzhidov *et al.*, 1986).

Our results suggest that ASA reactivity is a marker for the signs and symptoms of GWS. Finding serum antibodies to squalene in Gulf War patients is unexpected, and the basis for the presense of these antibodies remains unclear. ASA are not a general marker for autoimmune disease due to their absence in idiopathic autoimmune patients and rarity in patients with other, presumed environmentally induced, autoimmune diseases. The signs and symptoms of our Gulf War patients are similar to those of a subset of female patients following exposure to silicone. Some individuals with silicone exposure suffer from many of the multisystem symptoms, viz, arthralgias, myalgias, lymphadenopathy, and neurological disorders prevalent in GWS patients in the current study (Bridges *et al.*, 1993; Brautbar *et al.*, 1995; Wolford, 1997). Symptomatic silicone breast implant recipients also have high levels of antibodies to synthetic polymers (Tenenbaum *et al.*, 1997) and to silicone,[3] but did not have high prevalence of ASA.

It has been suggested that abnormal immune responses may be involved in GWS (Rook *et al.*, 1997). Immunological adjuvants have the generally desirable property of eliciting cell-mediated immunity and antibodies when administered with an antigen. They may also cause a more generalized and indiscriminate stimulation of the immune system and disrupt the balance of immune self-regulatory mechanisms, which may lead to autoimmune disease (Zamma, 1983; Lorentzen *et al.*, 1995; Madzhidov *et al.*, 1986; Kleinau *et al.*, 1995). Squalene has been used extensively as an adjuvant in animal models to induce autoimmune diseases (Lorentzen, 1999; Beck *et al.*, 1976; Kohashi *et al.*, 1977; Garrett *et al.*, 1985; Whitehouse *et al.*, 1974; Yoshino *et al.*, 1994). Cytokines are mediators of immunological regulation and inflammatory responses (Van der Meide *et al.*, 1996), and increased cytokine levels are associated with the development of autoimmune disease in established rodent models of autoimmunity (Fitzpatrick *et al.*, 1996). Squalene has been shown to induce increased levels of interleukin-5 (IL-5), IL-6, and interferon-γ (Valensi *et al.*, 1994). Several different adjuvants have been demonstrated to produce or exacerbate autoimmune diseases in experimental models. Adjuvant-induced arthritis is a well-characterized autoimmune disease induced in rats and other species (Zamma, 1983; Lorentzen *et al.*, 1995; Madzhidov *et al.*, 1986; Kleinau *et al.*, 1995). The disease process in adjuvant-induced arthritis is complex, affecting multiple organ systems. For example, a cachetic syndrome (Rofe *et al.*, 1994) and testicular dysfunction (Clemons *et al.*, 1989) have been associated with adjuvant-induced arthritis. Uveitis, a T-cell mediated intraocular inflammatory disease, can also be induced by adjuvants (Petty *et al.*, 1996). Neurological diseases can be the result of immunological mechanisms, including autoimmunity (Rogers *et al.*, 1996; Tebin *et al.*, 1996; Honnorat *et al.*, 1995; Wucherpfennig *et al.*, 1990; Cross *et al.*, 1991; Bansal *et al.*, 1994), and neurological symptoms are commonly seen in autoimmune diseases (McNichollet *et al.*, 1994; Zanone *et al.*, 1993; Moll *et al.*, 1993).

All pharmacology is controlled toxicology. Although not approved by the Food and Drug Administration for human use, squalene has been used as an adjuvant in experimental vaccines against a variety of pathogens, including *Bacillus anthracis* (Ivins *et al.*, 1994), *Plasmodium falciparum* (Hoffman *et al.*, 1994), and herpes simplex virus (Burke *et al.*, 1994). Effectiveness of adjuvants has been shown to parallel toxicity defined as the initiation of autoimmune disease processes (Zamma, 1983; Koga *et al.*, 1986). Adjuvants should not produce reactions at injection sites, be pyrogenic, or induce anterior uveitis, arthritis, or other protean autoimmune processes (Allison *et al.*, 1991). A study using squalene as an adjuvant in influenza vaccine reported moderate to severe local and systemic reactions in humans (Keutek *et al.*, 1993). The participants suffered induration, erythema, lymphadenopathy, fever, chills, nausea, and dizziness, symptoms which lasted for several days. Another squalene-containing adjuvant was used with gp120 in a human immunodeficiency virus vaccine, where it induced severe systemic and local reactions in 15 of 30 vaccinees (Keefer *et al.*, 1996). Similarly, in a study of simian immunodeficiency vaccine in macaques, squalene was used as an adjuvant, and the animals developed anti-human-cell antibodies and autoimmune-like symptoms (Vaslin *et al.*, 1992). Future studies should determine whether or not ASA have a role in these pathological processes.

Squalene is a naturally occurring molecule absorbed from food and synthesized as a precursor for cholesterol, myelin, and hormones. This synthesis occurs within the hepatocytes and is further processed into cholesterol in the endoplasmic reticulum (Stamellos *et al.*, 1993). Fecal analysis indicates that about 60% of dietary squalene is absorbed (Strandberg *et al.*, 1990). Dietary squalene is absorbed through lymphatic vessels after being cyclized to sterols during transit through

[3]Cao, Yan *et al.*, unpublished observations.

the intestinal wall (Tilvis *et al.*, 1983). It is processed into chylomicrons by the epithelial cells of the small intestines. It becomes a lipid droplet covered by β-lipoprotein containing triglyceride and cholesterol ester. This increases serum levels of free and esterified methyl sterol contents. About 90% of absorbed squalene is in lipoproteins, appearing in chylomicrons and VLDL, suggesting that removal of dietary squalene may indicate metabolism of intestinal lipoproteins (Gylling *et al.*, 1994).

Squalene is a nonsteroid precursor of cholesterol. Reports have indicated that high titers of autoantibodies to cholesterol, once considered to be a poorly immunogenic molecule, could be generated by immunization with liposomes containing cholesterol and lipid A as adjuvant (Swartz *et al.*, 1988; Alving *et al.*, 1991; Dijkstra *et al.*, 1996). Injection of either silicone gel or silicone oil intraperitoneally also resulted in high titers of autoantibodies to cholesterol (Alving *et al.*, 1996). The silicone component serves as an adjuvant as well as initiating the autoimmune process. The high titers were IgM with relatively low titers of IgG to cholesterol (Dijkstra *et al.*, 1996; Alving *et al.*, 1996). The specificity of these antibodies was to cholesterol and structurally similar sterols containing a 3β-hydroxyl group. Anticholesterol binding activity was significantly diminished if the 3β-hydroxyl domain was altered by oxidation, substitution, epimerization, or esterification (Dijkstra *et al.*, 1996). It has been reported that naturally occurring autoantibodies have been detected in humans (Alving *et al.*, 1989), but these were much lower in titer than those produced with either lipid A or silicone.

Several facts argue against our assay detecting cross-reactive antibodies to cholesterol instead of antibodies specific for squalene. First, squalene is neither a sterol nor does it have a 3β-hydroxyl group. The respective molecular structures, internal molecular bonding, charge distribution, and antigenic epitopes are different. Second, if high-titer autoantibodies to cholesterol that are cross-reactive with squalene are normal, we should see no difference between our various patient groups. The GWS patients and NIH positive control patients are very distinct in their strong IgG antibody reactivity to squalene. Third, if silicone alone can generate antibodies to cholesterol and these are cross-reactive to squalene, we should see high antibody reactivity to squalene in patients exposed to silicone in addition to the GWS and NIH patients. This did not occur.

In the course of these studies, we examined two volunteers for a vaccine trial at the NIH involving squalene as adjuvant. They developed a multisystem disease similar to that seen in Persian Gulf veterans subsequent to their participation in the trial. One received a single injection and became ill within a few weeks with signs and symptoms including arthritis, fibromyalgia, lymphadenopathy, photosensitive rashes, fatigue, headaches, and fasciculations. This individual had lower than normal acetylcholinesterase, histological evidence of lymphocytic infiltrates around vascular tissue, and IgG-mediated demyelinization. After this NIH vaccine study code was broken, it was found that only adjuvant squalene had been administered as placebo. This patient was weakly positive for ASA. Another patient who went through the whole experimental protocol before manifesting a similar set of signs and symptoms was 3+ positive for ASA.

Multiple vaccinations and vaccination against biological warfare agents are the factors with the highest correlation with GWS symptomatology (Unwin *et al.*, 1994). It is important to note that our laboratory-based investigations do not establish that squalene was added as adjuvant to any vaccine used in military or other personnel who served in the Persian Gulf War era. Several investigators have speculated that GWS is the result of either exposure to chemicals, chemical weapons, or to biological agents encountered in the Persian Gulf (Persian Gulf Veterans Coordinating Board, 1995; Abou-Donia *et al.*, 1996; David *et al.*, 1997; Haley,1997). However, such exposure would likely have immediate effects and many Gulf War veterans were well until months or years after the military conflict. Many of these GWS patients have improved on treatment regimens prescribed by their personal physicians, rheumatologists, and neurologists, namely the immunosuppressives used for classical rheumatological conditions.[4] These treatments have included steroids, methotrexate, hydroxychloroquine, and cytoxan. Such treatments would have no effect on subjects exposed to chemical weapons. If GWS was due to an exogenous infectious agent, the immunosuppressive regimens used would likely result in an exacerbation of the symptoms. This did not occur. The molecular pathology of GWS must be defined before its etiology can be assigned. We present here evidence of an immune factor based upon the adjuvancy of squalene. Further studies are required to define the role of ASA, if any, in the pathogenesis of GWS.

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[4]Asa, P.B. *et al.*, unpublished observations.

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