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Antibodies to Squalene in Recipients of Anthrax Vaccine

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We previously reported that antibodies to squalene, an experimental vaccine adjuvant, are present in persons with symptoms consistent with Gulf War Syndrome (GWS) (P. B. Asa *et al., Exp. Mol. Pathol* **68,** 196–197, 2000). The United States Department of Defense initiated the Anthrax Vaccine Immunization Program (AVIP) in 1997 to immunize 2.4 million military personnel. Because adverse reactions in vaccinated personnel were similar to symptoms of GWS, we tested AVIP participants for anti-squalene antibodies (ASA). In a pilot study, 6 of 6 vaccine recipients with GWS-like symptoms were positive for ASA. In a larger blinded study, only 32% (8/25) of AVIP personnel compared to 15.7% (3/19) of controls were positive ($P > 0.05$). Further analysis revealed that ASA were associated with specific lots of vaccine. The incidence of ASA in personnel in the blinded study receiving these lots was 47% (8/17) compared to an incidence of 0% (0/8; $P < 0.025$) of the AVIP participants receiving other lots of vaccine. Analysis of additional personnel revealed that in all but one case (19/20; 95%), ASA were restricted to personnel immunized with lots of vaccine known to contain squalene. Except for one symptomatic individual, positive clinical findings in 17 ASA-negative personnel were restricted to 4 individuals receiving vaccine from lots containing squalene. ASA were not present prior to vaccination in preimmunization sera available from 4 AVIP personnel. Three of these individuals became ASA positive after vaccination. These results suggest that the production of ASA in GWS patients is linked to the presence of squalene in certain lots of anthrax vaccine.

Key Words: anthrax vaccines; adverse adjuvant effect; squalene toxicity; Gulf War Syndrome; multisystem disorders.

INTRODUCTION

Bioterrorism is an important domestic and international security concern (Friedlander, 2000; Henderson, 1999; Leggiadro, 2000; Mazzuchi *et al.,* 2000; Wiener, 1996; Zoon, 1999). Much of this concern has focused on *Bacillus anthracis*, the etiological agent of anthrax (Gordon, 1999; Ibrahim *et al.,* 1999; Inglesby *et al.,* 1999). The study of immunological responses to the anthrax bacillus and the development of vaccines to immunize populations against this organism have been and should continue to be pursued vigorously (Abalakin *et al.,* 1990; Baillie *et al.,* 1999; Coulson *et al.,* 1994; Ezzell *et al.,* 1988; Friedlander *et al.,* 1999; Habig, 1993; Ivins *et al.,* 1986; Ivins *et al.,* 1988; Ivins *et al.,* 1992; Ivins *et al.,* 1994; Ivins *et al.,* 1998; McBride *et al.,* 1998; Miller *et al.,* 1998; Pasechnia *et al.,* 1992; Pile *et al.,* 1998; Pittman *et al.,* 2000; Sharma *et al.,* 1996; Shlyakhov *et al.,* 1997; Singh *et al.,* 1998; Stepanov *et al.,* 1996; Turnbull *et al.,* 1986; Welkos *et al.,* 1988A; Welkos *et al.,* 1988B; Williamson *et al.,* 1999).

The United States Department of Defense (DOD) announced the Anthrax Vaccine Immunization Program (AVIP) on December 15, 1997 (Cohen, 1997), to immunize 2.4 million military personnel (Cohen, 1998a,b) at risk for exposure to the anthrax bacillus. Adverse reactions to the vaccine have been reported by Hayes and World (2000), Hotopf *et al.* (2000), and Swanson-Bierman and Krenzelok (2001). Hotopf *et al.* (2000) categorized reported signs and symptoms into four groups: (1) psychiatric morbidity, (2) fatigue, (3) health perception, and (4) physical functioning.

We here report medically more traditional, more specified signs and symptoms experienced by many of the individuals entered into our study. These included joint and muscle pain, rashes, chronic fatigue, dizziness, headaches, seizures, and possible autoimmune thyroid disease. This constellation of signs and symptoms is similar to those referred to collectively as Gulf War Syndrome (GWS) (Coker *et al.,* 1999; David *et al.,* 1997; Fukuda *et al.,* 1998; Grady *et al.,* 1998; Hotopf *et al.,* 2000; Ismail *et al.,* 1999; Persian Gulf Veterans Co-ordinating Board, 1995; Unwin *et al.,* 1999). While the illnesses reported by United States and British military personnel after the Persian Gulf War in 1991 remain ill defined, multisystemic (Hotopf *et al.,* 2000) and rheumatological (Asa *et al.,* 2000a)

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aspects constitute the core of the disorder, as these eight citations amply demonstrate. The Anthrax Vaccine Immunization Program has been the subject of vocal controversy (Alving and Grabenstein, 2000; Asa *et al.,* 2000b; Goldstein, 1999; Morris, 1999).

We previously reported the finding of antibodies to squalene, an experimental vaccine adjuvant, in persons with clinical signs and symptoms consistent with the case definition of Gulf War Syndrome (Asa *et al.,* 2000a). Antibodies were found in military personnel of the United States and United Kingdom, both deployed and nondeployed, and in civilian employees of these agencies during the Gulf War (Asa *et al.,* 2000a). This was an unexpected finding, and the basis for the antibodies was not identified by that study. Three key observations suggested the possibility of one or more autoimmune disorders in these individuals: (1) an association between vaccinations received just before and during the Gulf War and ill health (Hotopf *et al.,* 2000), (2) an unexpectedly high incidence of adverse reactions to anthrax vaccine per se (Hayes *et al.,* 2000), and (3) a similarity between the signs, symptoms, and laboratory findings we observed in AVIP personnel and those of Gulf War era veterans (Asa *et al.,* 2000a; this report). Accordingly, we have now tested for anti-squalene antibodies in several groups of AVIP personnel.

MATERIALS AND METHODS

The subjects admitted to the study were American military personnel vaccinated against anthrax through the Army program. Lot numbers of the anthrax vaccine were taken from patient immunization records issued by the DOD. The site location of each vaccination was recorded as well. Age- and sex-matched controls were 19 healthy individuals recruited by accepted institutional review board standards and practices. None had concurrent or recent military service or civilian employment by the United States military after 1988 or had been enrolled in any other vaccine trials by any agency of American government or any other health program. No fees were paid by or to participants in this study.

Patient medical records and data, including diagnostic laboratory results from commercial laboratories, were collected by one of us (P.B.A.). These were reviewed by a board certified rheumatologist.[4]

Serum samples were collected from study participants by laboratory personnel using standard phlebotomy methods with vacutainer tubes and butterfly needles and then stored at −20°C until shipped to the laboratory for assay for anti-squalene antibodies. This assay was blinded (RFG and RBW); viz., samples and controls were randomized and assigned numbers for identification during all subsequent processing. All samples were tested four times under identical conditions. At the conclusion of the assays, patient data were matched with the outcome of the anti-squalene antibody test (ASA) and the results were tabulated.

Anti-squalene Antibody Assay

The ASA method used was the same as that previously reported (Asa *et al.,* 2000a), except that a squalene dilution of 1:20,000 in water was used in test strips for this particular study. Briefly, the method involves drying progressive dilutions of squalene on nitrocellulose membranes, rinsing in wash buffer, and preincubating with a blocking buffer prior to adding a 1:400 dilution of serum from each subject. Incubation times, washing, and biotin–avidin-conjugated horseradish peroxidase marking steps were in accordance with commonly used procedures with detection by buffer containing methanol, 4-chloro-1-naphthol, and 0.03% hydrogen peroxide. The final reaction was ended after 15 min by rinsing in distilled water. Air-dried strips were scored visually on a scale of 0 to 4+. Further particulars are described in U.S. Patent 6,214,566 (2001).[5]

RESULTS

Pilot Study

After the initiation of the AVIP, verbal reports of adverse reactions came to us from some recipients of the anthrax vaccine. These reactions included extreme pain and swelling at the injection site and rashes. Then, weeks and months later, many recipients experienced joint and muscle pain, dizziness, chronic headaches, low-grade fevers, chronic fatigue, weakness, seizures, memory loss, and cognitive problems. The similarity of these clinical symptoms to the cluster of health problems reported by Gulf War era veterans (Asa *et al.,* 2000a; Coker *et al.,* 1999; David *et al.,* 1997;

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[5] Tulane University holds U.S. Patent 6,214,566 for the anti-squalene antibody assay. Autoimmune Technologies LLC, a private New Orleans, LA, start-up company, has been granted exclusive rights by Tulane University for use of the assay. Drs. Asa and Garry will receive royalties from this agreement. Dr. Wilson is Chief Scientific Officer and President of Autoimmune Technologies LLC.

TABLE 1
AVIP Participants Initially Tested for ASA

Patient	ASA[a]	Vaccine lot (number of injections)	Clinical and laboratory findings
1. 23 years, male	+	FAV020 (2)	Fatigue, joint pain, GI dysmotility
2. 36 years, female	+	FAV020 (2)	Ataxia, seizures, chronic fatigue, chronic severe headaches, weakness; being evaluated now for possible multiple sclerosis
3. 42 years, male	+	FAV030 (4)	Ataxia, cognitive problems, chronic fatigue, severe headaches, muscle weakness, joint and muscle pain
4. 47 years, male	+	FAV030 (2)	Ataxia, chronic fatigue, rashes, frequent severe headaches, memory problems, cognitive disorders, polyneuropathy; antibodies to myelin basic protein
5. 34 years, female	++	FAV030 (2)	Fatigue, joint pains
6. 38 years, male	+++	FAV030 (2)	Joint and muscle pain

[a] Intensity of anti-squalene antibody reaction.

Fukuda *et al.*, 1998; Grady *et al.*, 1998; Hotopf *et al.*, 2000; Ismail *et al.*, 1999; Persian Gulf Veterans Co-ordinating Board, 1995; Unwin *et al.*, 1999) is obvious.

We tested serum samples from six anthrax vaccine recipients for ASA; all six were positive for the anti-squalene antibodies (Table 1). We then performed a larger, blinded study to confirm and further examine the association between ASA and anthrax vaccination.

Expanded Blinded Testing of AVIP Participants

Sera from AVIP participants ($n = 25$) and controls who did not receive the vaccine ($n = 19$) were blinded and submitted for ASA analysis. After completion of the assay we found 8 of the 25 vaccinated service personnel (32%) to be positive for ASA, while only 3 of 19 controls (15.8%) were positive.

This difference is not statistically significant in this size sample.[6] The 3 positive controls had neither symptoms nor other laboratory evidence for autoimmune disorders; however, they had remote histories of major surgery with no sequelae, a finding absent from the histories of the other controls. Age, sex, and the clinical findings for ASA-positive AVIP personnel are shown in Table 2; those for ASA-negative AVIP personnel are in Table 3. Inspection of the data in Tables 2 and 3 revealed a clustering of reported sequelae and ASA reactivity with certain vaccine lot numbers. These were FAV030, FAV038, FAV041, and FAV043. When the AVIP personnel were divided into groups according to which lots they received, those vaccinated from the five lots and those who were not, a significant effect is seen in the data (Table 4). The four lots, FAV020, FAV030, FAV038, FAV041, and FAV043, were given to 17 of the 25 vaccinated individuals; 8 of these (47.06%) tested positive for ASA while none receiving other lots was positive (Table 4). Although the number of samples tested was small, the difference between the two groups was statistically significant ($P < 0.025$).

Two individuals who tested positive after vaccination had been tested prior to receiving anthrax vaccine; both earlier samples were negative for ASA. Patient No. 4 was sampled 3 months after a third inoculation using lot FAV043. Patient No. 7 became symptomatic after his third shot from lot

[6] $n = 44$, $df = 1$, $\chi^2 = 1.513$, $P = 0.2187$. However, a sample of 112 subjects with the same ratios between positive and negative results would be statistically significant, with $\chi^2 = 3.841$, $P = 0.0500$; similarly, a sample of 132 would yield $\chi^2 = 4.5389$, $P = 0.0331$. More positives in an expanding sample would, of course, mean fewer individuals were needed to reach $P \leq 0.05$.

TABLE 2
AVIP Participants Positive for Anti-Squalene Antibodies

Patient	ASA[a]	Vaccine lot (number of injections)	Clinical and laboratory findings
1. 36 years, male	+	FAV030 (2)	Arthritis; +FANA
2. 39 years, male	+	FAV030 (2)	Joint, muscle pain
3. 40 years, male	+	FAV030 (2)	Joint, muscle pain; +FANA
4. 39 years, male	+	FAV043 (3)	Urticaria, chronic fatigue, headaches; joint and muscle pain, rashes[b]
5. 52 years, male	+	FAV043 (3)	Fatigue, joint pain
6. 23 years, male	+++	FAV038 (1) FAV043 (3)	Anterior uveitis
7. 50 years, male	+++	FAV041 (3)	Autoimmune thyroid disease, polymyositis, elevated liver enzymes[b]
8. 38 years, male	++++	FAV030 (2)	Arthritis, active synovitis; +FANA 1:160

Note. FANA, Fluorescent Anti-Nuclear Antibody
[a] Intensity of anti-squalene antibody reaction.
[b] These individuals had been tested before anthrax vaccination (both were negative for ASA) and twice afterward (see also Table 5).

TABLE 3
AVIP Participants Negative for Anti-Squalene Antibodies

Patient	ASA[a]	Vaccine lot (number of injections)	Clinical and laboratory findings
1. 34 years, female	0	FAV030	Arthritis, myalgias, chronic fatigue, chronic headaches; +FANA (titer not stated, >1:40 assumed)
2. 38 years, male	0	FAV030	EEG-confirmed seizures, fatigue
3. 31 years, male	0	FAV030	None
4. 37 years, male	0	FAV030	None
5. 34 years, male	0	FAV030	None
6. 33 years, male	0	FAV030	None
7. 42 years, male	0	FAV041	Joint pain, chronic fatigue, memory loss; +FANA (titer not stated, >1:40 assumed)
8. 39 years, male	0	FAV043	Blistering rash after second shot
9. 51 years, female	0	FAV043	Seropositive rheumatoid arthritis
10. 23 years, male	0	FAV017	None
11 34 years, male	0	FAV017	None
12. 33 years, female	0	FAV031	None
13. 37 years, male	0	FAV031	None
14. 48 years, male	0	FAV031	None
15. 28 years, male	0	FAV034	None
16. 32 years, female	0	FAV036	None
17. 23 years, male	0	FAV037	None

Note. FANA, Fluorescent Anti-Nuclear Antibody; EEG, Electroencephalogram.
[a] Intensity of anti-squalene antibody reaction.

FAV041. Both had sought care for illness before the ASA results were known.

Individual reactions for those who tested negative for ASA are listed in Table 3. Five individuals who received lots FAV030, FAV038, FAV041, and FAV043 tested negative for ASA but had some of the clinical findings found in personnel positive for ASA. AVIP participants receiving lot numbers other than those seemingly associated with a positive finding of ASA reported no reactions to the shot at the time of administration, were not diagnosed with any related clinical disorders, and had no demonstrable antibodies to squalene.

TABLE 4
Anti-Squalene Antibody Reactions in AVIP Participants

Number (male:female)	ASA-positive	Vaccine lot numbers	Clinical disorders	P[a]
17 (15:2)	47% (1+ to 4+)	FAV020, 030, 038, 041, 043	Yes	—
8 (6:2)	0%	All others with known lot numbers	No	<0.025
19 (16:3)	15.8% (1+)	None	No	<0.01

[a] Compared to those receiving vaccine lot numbers 020, 030, 038, 041, or 043; Student's t test.

TABLE 5
Time-Comparative Anti-Squalene Antibodies in AVIP Participants

Patient	Antibody reaction			Lot number
	Prevaccination	2000	2001	
1. 39 years, male[a]	0	+	+	FAV043
2. 42 years, male	0	ND	+++	FAV043
3. 41 years, male	0	ND	0	FAV043
4. 50 years, male[a]	0	+++	++	FAV041[b]
5. 52 years, male	ND	+	++	FAV043
6. 51 years, male	ND	0	0	FAV043

Note. ND, not done.
[a] These two individuals are also listed in Table 2.
[b] Inoculated Dover AFB, Dover, DE. All other personnel were vaccinated at the 164th TN ANG, Memphis, TN.

Time-Related Studies

Little is known about antibody responses to squalene over time. Several additional samples became available after the completion of the blinded portion of our study. These included anthrax vaccine recipients who had developed antibodies to squalene within a few months of immunization, including personnel sampled before immunization. Prevaccination serum samples, where available, were run simultaneously. The samples were blinded as noted earlier during the ASA assay. The results are shown in Table 5. There were six such individuals with a total of 14 independent antibody tests; four were tested twice and two were tested three times. There were 10 postvaccination tests with 7 positive results (70.0 percent).

Posttrial Observations

Three additional individuals were tested after the conclusion of the main blinded sequence of this study (Table 6). All received vaccine from Lot FAV043 and all three were positive for ASA.

TABLE 6
Posttrial Observations

Patient	Antibody reaction			Lot number
	Prevaccination	2000	2001	
1. 37 years, female	ND	ND	+	FAV043
2. 27 years, male	ND	ND	++	FAV043
3. 37 years, male	ND	ND	++	FAV043

Note. ND, not done.

DISCUSSION

We previously reported persons suffering with the symptom-based case definition of Gulf War Syndrome to have serum antibodies to squalene (Asa *et al.,* 2000a). The antigen(s) inducing these antibodies in Gulf War veterans is unknown at the time, but it is possible that predeployment immunizations against various biowarfare agents is associated with induction of ASA. Our testing for anti-squalene antibodies in persons receiving anthrax immunization as part of AVIP identified many antibody-positive individuals. This contrasts with a lack of antibodies in all of the preimmunization sera so far available. In addition, we found that all of the current cohort positive for antibodies to squalene had received anthrax vaccine from a specific subset of lot numbers as part of AVIP. In all but one case (19/20; 95%), ASA were restricted to personnel immunized with lots of vaccine known to contain squalene. This suggests fairly strongly that anti-squalene antibodies are related specifically with these lots of vaccine.[7]

Investigators at the U.S. Food and Drug Administration (FDA) assayed anthrax vaccine in June 1999 for squalene content by gas/liquid chromatography (GLC). Identified as positive were certain lot numbers: FAV020, FAV030, FAV038, FAV043, and FAV047 (Committee, 2000). Squalene can be isolated and quantitated using either high-performance liquid chromatography (HPLC) or GLC, the latter yielding a more precise quantitation (Sulpice *et al.,* 1984). Lots with small amounts of squalene identified by the FDA closely match the lots associated in this study with anti-squalene antibodies. There is one exception; we identified one ASA-positive individual who received vaccine from Lot FAV041.

The source of the squalene in certain lots of anthrax vaccine is unknown; however, squalene is not found in *Bacillus anthracis* (Kaneda, 1977). *Bacillus anthracis* lipid chains are no longer than 17 carbons and are exclusively monounsaturated (Kaneda, 1977), while squalene contains 30 carbons and is highly polyunsaturated with six double bonds and iodine numbers in the range of 380–400, depending on the formulation (Whitehouse *et al.,* 1974). In addition, squalene is not present in the growth medium used to prepare cultures of *B. anthracis* (Johnson *et al.,* 1981; Lynch *et al.,* 1963; Wright *et al.,* 1954, 1957).

The amount of squalene, in four of the five lots of anthrax vaccine for which we found antibodies, was determined by the FDA to be 10–83 parts per billion (Committee, 2000). These levels have been dismissed as too low to have an immunological effect (SqualeneFacts.HTM, 2000). It is true that the precise biological significance of low levels remains to be determined, and in what context, but we suggest that they cannot be dismissed summarily. The immune system is exquisitely sensitive to small quantities of antigen. This sensitivity results from cell-to-cell priming, clonal proliferation, upregulation of MHC II molecules, and elaboration of cytokines and prostaglandins, amplifying the effect of small amounts of an antigen (Baker *et al.,* 1985; Carnaud, 1994; Grabbe *et al.,* 1996; Hodgkin *et al.,* 1998; Mudde *et al.,* 1996; Nakashima *et al.,* 1975; Volpe, 1988). Moreover, before the molecular nature of antibodies was fully appreciated, it was accepted that as little as a single molecule of antigen could stimulate antibody production (Cannon, 1942). Booster shots received in the AVIP program would enhance these effects. There is no lower safety concentration limit as yet established for squalene in vaccines with it as a supplemental adjuvant. It is possible that the quantities of squalene determined by the FDA do not accurately represent the original concentration of squalene in these vaccines. First, squalene is a nonpolar lipid which readily separates into a distinct layer from the aqueous vaccine antigen solution.[8] Secondly, squalene is subject to oxidation and peroxidation (Whitehouse *et al.,* 1974). The oxidative and peroxidative changes in chemical structure and their effect on antigenicity of squalene have been described (Whitehouse *et al.,* 1974). These changes can be detected in squalene within 4 h of atmospheric exposure (Dennis *et al.,* 1990). The breakdown products or other chemicals of the anthrax vaccine by GLC analysis were not provided by the FDA, as reported in the Congressional Record (Metcalfe, 2000). Squalene is one of a few naturally occurring lipids which function as immunological adjuvants when injected (Lorentzen *et al.,* 1995; Lorentzen, 1999; Whitehouse *et al.,* 1974). Immunological adjuvants have been sought for the past century to enhance the efficacy of vaccines. Increased resistance of bacteria to antibiotics and the human immu-

[7] The lot number for the severely ill person reported by Swanson-Bierman and Krenzelok (2000) is unknown (personal communication to the Editors).

[8] RIBI Immunochemicals, Inc., Hamilton, MT (personal communication to the authors).

nodeficiency virus epidemic are just two of the many reasons for an increased desire to find such agents.

Adjuvants have not been generally acceptable for human use, however, due to a capacity to induce the loss of self-tolerance and, often, to induce autoimmune disease. This feature has been used to study pathogenesis and treatment of many autoimmune illnesses, including inflammatory cardiomyopathies, autoimmune hepatitis, autoimmune uveoretinitis and anterior uveitis, autoimmune labyrinthitis, myositis, and peripheral neuritis (Broekhuyse *et al.,* 1993; Clemons *et al.,* 1989; Howell *et al.,* 1994; Ikezono *et al.,* 2000; McAllister *et al.,* 1995; Petty *et al.,* 1989; Roberge *et al.,* 1992; Schultheiss *et al.,* 1998; Stucky *et al.,* 1993).

More specifically, squalene, and the saturated form, squalane, have been shown to initiate autoimmune rheumatologic and neurologic disease (Beck *et al.,* 1976; Carlson *et al.,* 2000; Gajkowska *et al.,* 1999; Garrett *et al.,* 1985; Kohashi *et al.,* 1977; Lorentzen, 1999; Smialek *et al.,* 1997; Tsujimoto *et al.,* 1986; Whitehouse *et al.,* 1969, 1974; Whitehouse, 1982). Indeed, it has been shown that a single injection of squalene induces T-cell-mediated arthritis (Carlson *et al.,* 2000). Other studies have shown that adjuvant arthritis, experimental allergic encephalomyelitis, and experimental autoimmune thyroid disease, initiated by adjuvants containing squalene, could be passively transferred to syngeneic animals by thoracic duct lymphocytes (Whitehouse *et al.,* 1969; Whitehouse *et al.,* 1974). When squalene was substituted for mineral oil in Freund adjuvant, the resistance of the Buffalo and Norway strains of rats against the development of autoimmune disease was overcome, compared to treatment with only standard Freund adjuvant (Kohashi *et al.,* 1977). The RIBI adjuvant formulation, which contains squalene, is known to induce pathological changes as severe as those induced by Freund adjuvant (Leenaars *et al.,* 1994, 1998a,b; Leenaars and Hendriksen, 1998). In another study, RIBI adjuvant induced significant granulomatous lesions, but less severely than Freund adjuvant per se (Lipman *et al.,* 1992). When serial inoculations of adjuvant formulations were studied, RIBI adjuvant produced significantly lower antibody levels, and booster inoculations produced greater intradermal reactions with chronic lesions detectable at necropsy (Johnston *et al.,* 1991). TiterMax, which contains squalene, has also been shown to induce swelling and encapsulation (Zwerger *et al.,* 1998). These studies clearly demonstrate that significant problems do exist if squalene is used as an adjuvant in humans.

When squalene is administered intravenously, it disappears from the circulation within 2 to 4 min and is rapidly cyclized to methyl sterols and cholesterol, as well as biliary and fecal sterols and bile acids (Tilvis and Miettinen, 1982). However, when squalene is administered intramuscularly, as part of an adjuvant formulation, it drains into lymph nodes, where it remains for at least 48 h (Dupuis *et al.,* 1998). Effective antigen presentation by macrophages requires 60 min, and B-cells require between 6 and 8 h (Singer and Linderman, 1990). Once in the lymph nodes, squalene comes into contact with antigen-presenting cells, including dendritic cells, and lymphocytes. Dendritic cells displaying markers DEC-205 and MHC class II molecules have been shown to internalize squalene (Dupuis *et al.,* 1998). Adjuvants not only stimulate the immune system nonspecifically but may also serve as immunogens themselves. By stimulating an immune reaction, an adjuvant also comes under the definition of an immunogen. The concept of looking at adjuvants as antigens was initially suggested with Calmette-Guerin bacillus and *Vibrio cholera* neuraminidase (Seiler, 1980). The possible antigenicity of squalene was first shown in the military serving in the Persian Gulf War (Asa *et al.,* 2000a). This finding was confirmed by the induction of antibodies to squalene in an animal model, although significant levels of anti-squalene antibodies require coadministration of an adjuvant formulation (Matyas *et al.,* 2000). Also, Matyas and co-workers (2000) could not detect antibodies to squalene prior to immunization. In this study, as well as in our previous report (Asa *et al.,* 2000a), we found mostly males with rheumatological and neurological signs and symptoms. Idiopathic autoimmune diseases have been mostly in women at ratios of 8:1 to 14:1 (Michet *et al.,* 1985; Giersson *et al.,* 1994), while autoimmune disease induced by adjuvants have shown no difference between the sexes with regard to incidence or severity (Taurog *et al.,* 1988). Thus, our results are consistent with the possibility that the illness observed in GWS patients and AVIP personnel is due to an adjuvant reaction. The limits of this study, small sample size and likely a self-selection bias, constrict efforts to definitively address this issue.

We also found some personnel receiving vaccinations from squalene-positive lots to be ASA-negative, and we found some vaccinated by lots with squalene who did not develop signs or symptoms. There are several possible explanations for these observations:

(1) Adjuvants can act as superantigens and have been shown to induce immunological anergy to themselves in humans (Lamoureux *et al.,* 1974).

(2) Our test detects only IgG antibodies to squalene. Anti-squalene IgM antibodies have already been identified in mice (Matyas *et al.,* 2000), and anti-squalene IgA, IgE antibodies may also be produced.

(3) The relationship between the development of autoimmunity, the production of antibodies to squalene, and their relationship to each other is yet to be defined.

(4) Adjuvant disease has been shown to have a latency of onset in humans ranging from 2 weeks to 18 years after exposure (Brawer, 1996).

(5) It cannot be assumed that inoculations from multiple dose vials (5-ml vials programmed for 10 injections) are fully uniform in volume or degree of chemical mixing.

(6) Finally, these patients may not be genetically predisposed to develop antibodies to squalene or to other, as yet unidentified, immunogens.

These results and those of others (Asa *et al.*, 2000a; Matyas *et al.*, 2000) strongly suggest that the production of anti-squalene antibodies is linked to symptoms of Gulf War Syndrome and to the presence of squalene in certain lots of anthrax vaccine in some individuals.

A large epidemiological and biochemical study incorporating the ASA assay and a precise vaccination history, medical record review, and complete medical and physical examination of a large cohort of Gulf War Syndrome patients and AVIP personnel is justified from this evidence. The common practice of using squalene in vaccine enhancement is challenged by these data and the supportive literature. Prudence in use and redesign of the process henceforth would seem to be an appropriate recommendation.

REFERENCES

Abalakin, V. A., Ozhabirov, S. S., Kalita, V. A., Kuttugulov, V. K., Amireev, S. A., Knop, A. G., and Cherkasski, B. L. (1990). Postinfection and postvaccinal antianthrax immunity in human subjects. *Zh. Mikrobiol. Epidemiol. Immunobiol.* **6,** 71–76.

Alving, C. R., and Grabenstein, J. D. (2000). Letter to the editor. *Exp. Mol. Pathol.* **68,** 196–197.

Asa, P. B., Cao, Y., and Garry, R. F. (2000a). Antibodies to squalene in Gulf War Syndrome. *Exp. Mol. Pathol.* **68,** 55–64.

Asa, P. B., Cao, Y., and Garry, R. F. (2000b). Reply to letter to the editor. *Exp. Mol. Pathol.* **68,** 197–198.

Baillie, L. W., Fowler, K., and Turnbull, P. C. (1999). Human responses to human anthrax vaccine. *J. Appl. Microbiol.* **87,** 306–308.

Baker, P. J., Taylor, C., Fauntleroy, M. B., Stashak, P. W., and Prescott, B. (1985). The role of antigen in the activation of regulatory T cells by immune B cells. *Cell. Immunol.* **96,** 376–385.

Beck, F. W. J., and Whitehouse, M. W. (1976). Modifications in the establishment of allergic encephalomyelitis (EAE) in rats: An improved assay for immunosuppressant drugs. *Agents Actions* **6,** 460–467.

Brawer, A. E. (1996). Chronology of systemic disease development in 300 symptomatic recipients of silicone gel-filled breast implants. *J. Clean Technol. Environ. Toxicol. Occup. Med.* **5,** 223–233.

Broekhuyse, R. M., Kuhlmann, E. D., and Winkens, H. J. (1993). Experimental autoimmune anterior uveitis (EAAU). III. Induction by immunization with purified uveal and skin melanins. *Exp. Eye Res.* **56,** 575–583.

Cannon, P. R. (1942). Antibody production and the anamnestic reaction. *J. Lab. Clin. Med.* **28,** 127–139.

Carlson, B. C., Jansson, A. M., Larsson, A., Anders, B., and Lorentzen, J. C. (2000). The endogenous adjuvant squalene can induce chronic T-cell mediated arthritis in rats. *Am. J. Pathol.* **156,** 2057–2065.

Carnaud, C. (1994). Cell cooperation in immune responses. *Rev. Prat.* **44,** 13–19.

Clemons, J., and Brout, B. (1989). Testicular dysfunction in the adjuvant-induced arthritic rat. *J. Androl.* **10,** 419–421.

Cohen, W. A. (1997). Defense Department to start immunizing troops against anthrax. U.S. Department of Defense News Release No. 679-97, December 15, 1997. http://www.defenselink.mil/news/Dec1997/b12151997_bt679-97.html.

Cohen, W. A. (1998a). Accelerated anthrax vaccination program to enhance force protection announced. U.S. Department of Defense News Release No. 094-98, March 3, 1998. http://www.defenselink.mil/news/Mar1998/b03031998_bt094-98.html.

Cohen, W. A. (1998b). Total force anthrax vaccination decision announced. U.S. Department of Defense News Release No. 255-98, May 22, 1998. http://www.defenselink.mil/news/May1998/b05221998_bt255-98.html.

Coker, W. J., Bhatt, B. M., Blatchley, N., and Graham, J. T. (1999). Clinical findings for the first 1000 Gulf War veterans in the Ministry of Defence's medical assessment programme. *BMJ* **318,** 290–294.

Committee on Government Reform Hearings for the United States House of Representatives, October 3rd and 11th, 2000. "Accountability of DoD, FDA and BioPort Officials for the Anthrax Vaccine Immunization Program (AVIP)." http://www.house.gov/reform/hearings/healthcare/00.10.03/accountability.doc.

Coulson, N. M., Fulop, M., and Titball, R. W. (1994). *Bacillus anthracis* protective antigen expressed in *Salmonella typhimurium* SL 3261 affords protection against anthrax spore challenge. *Vaccine* **12,** 1395–1401.

David, A., Ferry, S., and Wesseley, S. (1997). Gulf War illness. *BMJ* **314,** 239–240.

Dennis, K. J., and Shibamoto, T. (1990). Gas chromatographic analysis of reactive carbonyl compounds formed from lipids upon UV-irradiation. *Lipids* **25,** 460–464.

Dupuis, M., Murphy, T. J., Higgins, D., Ugozoli, M., Van Nest, G., Ott, G., and McDonald, D. M. (1998). Dendritic cells internalize vaccine adjuvant after intramuscular injection. *Cell Immunol.* **186,** 18–27.

Ezzell, J. W., Jr., and Abshire, T. G. (1988). Immunological analysis of cell-associated antigens of *Bacillus anthracis*. *Infect. Immunol.* **56,** 349–356.

Friedlander, A. M., Pittman, P. R., and Parker, G. W. (1999). Anthrax vaccines: Evidence for safety and efficacy against inhalation anthrax. *J. Am. Med. Assoc.* **282,** 2104–2106.

Friedlander, A. M. (2000). Anthrax: Clinical features, pathogenesis, and potential biological warfare threat. *Curr. Clin. Top. Infect. Dis.* **20,** 335–349.

Fukuda, K., Nisenbaum, R., Stewart, G., Thompson, W. W., Robin, L., Washko, R. A., Noah, D. L., Barrett, D. H., Randall, B., Herwaldt, B. L., Mawle, A. C., and Reeves, W. C. (1998). Chronic multisystem disease affecting Air Force veterans of the Gulf War. *J. Am. Med. Assoc.* **280,** 981–988.

Gajkowska, B., Smialek, M., Ostrowski, R. P., Piotrowski, P., and Frontczak-Bariowicz, M. (1999). The experimental squalene encephaloneuropathy in the rat. *Exp. Toxicol. Pathol.* **51,** 75–80.

Garrett, I. R., Whitehouse, M. W., Vernon-Roberts, B., and Brooks, P. M.

(1985). Ambivalent properties of gold drugs in adjuvant-induced polyarthritis in rats. *J. Rheum.* **12,** 1079–1082.

Geirsson, A., Steinsson, K., Gusmuddsson, S., and Sigurosson, V. (1994). Systemic sclerosis in Iceland: A national epidemiological study. *Ann. Rheum. Dis.* **53,** 502–505.

Goldstein, R. (1999). The controversial anthrax vaccine. *Nurs. Spect. (Washington, DC)* **9,** 20–21.

Gordon, S. M. (1999). The threat of bioterrorism: A reason to learn more about anthrax and smallpox. *Cleve. Clin. J. Med.* **66,** 592–595.

Grabbe, S., and Schwarz, T. (1996). Immunoregulatory mechanisms involved in the elicitation of allergic contact sensitivity. *Am. J. Contact Dermatol.* **7,** 238–246.

Grady, E. P., Carpenter, M., Koenig, C., Older, S., and Battafarano, D. F. (1998). Rheumatic findings in Gulf War veterans. *Arch. Intern. Med.* **158,** 367–371.

Habig, W. H. (1993). Potency testing of bacterial vaccines for human use. *Vet. Microbiol.* **37,** 343–351.

Hayes, S. C., and World, M. J. (2000). Adverse reactions to anthrax immunization in a military field hospital. *J. R. Army Med. Corps* **146,** 191–195.

Henderson, D. A. (1999). The looming threat of bioterrorism. *Science* **283,** 1279–1282.

Hodgkin, P. D., Rush, J., Gett, A. V., Bartell, G., and Hasbold, J. (1998). The logic of intercellular communication in the immune system. *Immunol. Cell. Biol.* **76,** 448–453.

Hotopf, M., David, A., Hull, L., Ismail, K., Unwin, C., and Wesseley, S. (2000). Role of vaccinations as risk factors for ill health in veterans of the Gulf War: Cross sectional study. *BMJ* **320,** 1363–1367.

Howell, C. D., and Yoder, T. D. (1994). Murine experimental autoimmune hepatitis: Nonspecific inflammation due to adjuvant oil. *Clin. Immunol. Immunopathol.* **72,** 76–82.

Ibrahim, K. H., Brown, G., Wright, D. H., and Rotscafer, J. C. (1999). *Bacillus anthracis:* Medical issues of biological warfare. *Pharmacotherapy* **19,** 690–701.

Ikezono, T., Tomiyama, S., Pawankar, R., Jinnouchi, K., Suzuki, Y., and Yagi, T. (2000). Passive transfer of experimental autoimmune labyrinthitis. *Audiol. Neurootol.* **5,** 292–299.

Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Friedlander, A. M., Hauer, J., McDade, J., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Russell, P. K., and Tonat, K. (1999). Anthrax as a biological weapon: Medical and public health management: Working Group on Civil Biodefense. *J. Am. Med. Assoc.* **281,** 1735–1745.

Ismail, K., Everitt, B., Blatchley, N., Hull, L., Unwin, C., David, A., and Wesseley, S. (1999). Is there a Gulf War syndrome? *Lancet* **353,** 179–182.

Ivins, B. E., Ezzell, J. W., Jenski, J., Hedlund, K. W., Ristroph, J. D., and Leppla, S. H. (1986). Immunizations studies with attenuated strains of *Bacillus anthracis. Infect. Immunol.* **52,** 454–458.

Ivins, B. E., and Welkos, S. L. (1988). Recent advances in the development of an improved human anthrax vaccine. *Eur. J. Epidemiol.* **4,** 12–19.

Ivins, B. E., Welkos, S. L., Little, S. F., Crumrine, M. H., and Nelson, G. O. (1992). Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infect. Immunol.* **60,** 662–668.

Ivins, B. E., Fellows, P. F., and Nelson, G. O. (1994). Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine* **12,** 872–874.

Ivins, B. E., Pitt, M. L., Fellows, P. F., Farchaus, J. W., Benner, G. E., Waag, J. M., Little, S. F., Anderson, G. W., Jr., Gibbs, P. H., and Friedlander, A. M. (1998). Comparative efficacy of experimental anthrax vaccine against inhalation anthrax in rhesus macaques. *Vaccine* **16,** 1141–1148.

Johnson, A. D., and Spero, L. (1981). Comparison of growth and toxin production in two vaccine strains of *Bacillus anthracis. Appl. Environ. Microbiol.* **4,** 1479.

Johnston, B. A., Eisen, H., and Fry, D. (1991). An evaluation of several adjuvant emulsion regimens for the production of polyclonal antisera in rabbits. *Lab. Anim. Sci.* **41,** 15–21.

Kaneda, T. (1977). Fatty acids of the Genus Bacillus: An example of branched-chain preference. *Bacteriol. Rev.* **41,** 391–418.

Kohashi, O., Pearson, C. M., Beck, F. T. W., and Alexander, M. (1977). Effect of oil composition on both adjuvant-induced arthritis and delayed hypersensitivity to purified protein derivative and peptidoglycans in various rat strains. *Infect. Immunol.* **17,** 244–249.

Lamoureux, G., and Poisson, R. (1974). BCG and immunological anergy. *Lancet* **i,** 989.

Leenaars, P. P., Hendriksen, C. F., Angula, A. F., Koedam, M. A., and Claassen, E. (1994). Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. *Vet. Immunol. Immunopathol.* **40,** 225–241.

Leenaars, M., and Hendriksen, C. F. (1998). Influence of route of injection on efficacy and side effects of immunization. *ALTEX* **15,** 87.

Leenaars, M., Koedam, M. A., Hendriksen, C. F., and Claassen, E. (1998a). Immune responses and side effects of five different oil-based adjuvants in mice. *Vet. Immunol. Immunopathol.* **61,** 291–304.

Leenaars, P. P., Koedam, M. A., Wester, P. W., Baumans, V., Claassen, E., and Hendriksen, C. F. (1998b). Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. *Lab. Anim.* **32,** 387–406.

Leggidro, R. J. (2000). The threat of biological terrorism: A public health and infection control reality. *Infect. Control Hosp. Epidemiol.* **21,** 53–56.

Lipman, N. S., Trudel, L. J., Murphy, J. C., and Sahali, Y. (1992). Comparison of immune response potentiation and *in vivo* inflammatory effects of Freund's and RIBI adjuvants in mice. *Lab. Anim. Sci.* **42,** 193–197.

Lorentzen, J. C., Ollson, T., and Klareskog, L. (1995). Susceptibility to oil-induced arthritis in the DA rat is determined by MHC and non-MHC genes. *Trans. Proc.* **27,** 1532–1534.

Lorentzen, J. C., and Klareskog, L. (1997). Comparative susceptibility of DA, LEW, and LEW.1AV1 rats to arthritis induced with different arthritigens: Mineral oil, mycobacteria, muramyl dipeptide, avridine and rat collagen type II. *Trans. Proc.* **29,** 1692–1692.

Lorentzen, J. C. (1999). Identification of arthritigenic adjuvants of self and foreign origin. *Scand. J. Immunol.* **49,** 45–50.

Lynch, J. W., Barcley, C., Alahov, C., and Wright, G. G. (1963). Large-scale production of *Bacillus anthracis* in anaerobic culture. *Appl. Microbiol.* **3,** 330–334.

Matyas, G. R., Wassef, N. M., Rao, M., and Alving, C. R. (2000). Induction and detection of antibodies to squalene. *J. Immunol. Methods* **245,** 1–14.

Mazzuchi, J. F., Claypool, R. G., Hyams, K. C., Trump, D., Riddle, J., Patterson, R. E., and Bailey, S. (2000). Protecting the health of U.S. military forces: A national obligation. *Aviat. Space Environ. Med.* **71,** 260–265.

McAllister, M. M., O'Toole, D., and Griggs, K. J. (1995). Myositis, lameness, and paraparesis associated with use of an oil-adjuvant bacterin in beef cows. *J. Am. Vet. Med. Assoc.* **207,** 936–938.

McBride, B. W., Mogg, A., Telfer, J. L., Lever, M. S., Miller, J., Turnbull, P. C., and Baillie, L. (1998). Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. *Vaccine* **16,** 810–817.

Michet, C., McKenna, C., Elveback, L., Kaslow, R., and Kurland, L. (1985). Epidemiology of systemic lupus erythematosus and other con-

nective tissue diseases in Rochester, Minnesota, 1950 through 1979. *Mayo Clin. Proc.* **60,** 105–113.

Miller, J., McBride, B. W., Manchee, R. J., Moore, P., and Baillie, L. W. (1998). Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis. Lett. Appl. Microbiol.* **26,** 56–60.

Morris, K. (1999). U.S. military face punishment for refusing anthrax vaccine [News]. *Lancet* **353,** 130.

Mudde, G. C., Reischul, I. G., Corvaia, N., Hren, A., and Poellabauer, E. M. (1996). Antigen presentation in allergic sensitization. *Immunol. Cell. Biol.* **74,** 167–173.

Nakashima, I., and Kato, N. (1975). Amplification of cell-associated immunological memory by secondary antigenic stimulus: Secondary type increase in memory. *Immunology* **29,** 643–652.

Paschenia, V. A., Shone, C. C., and Hambleton, P. (1992). Purification of bacterial exotoxins: The case for botulinum, tetanus, anthrax, pertussis, and cholera toxins. *Bioseparation* **3,** 267–283.

Persian Gulf Veterans Coordinating Board. (1995). Unexplained illnesses among Desert Storm veterans. *Arch. Intern. Med.* **155,** 262–268.

Petty, R. E., Johnston, W., McCormick, A. Q., Hunt, D. W. C., Rootman, J., and Rollins, D. F. (1989). Uveitis and arthritis induced by adjuvant: Clinical, immunological, and histological characteristics. *J. Rheumatol.* **16,** 499–505.

Pile, J. C., Malone, J. D., Eitzen, E. M., and Friedlander, A. M. (1998). Anthrax as a potential biological warfare agent. *Arch. Intern. Med.* **158,** 429–434.

Pittman, P. R., Mangiofico, J. A., Rossi, C. A., Cannon, T. L., Gibbs, P. H., Parker, G. W., and Friedlander, A. M. (2000). Anthrax vaccine: Increasing intervals between the first two doses enhances antibody responses in humans. *Vaccine* **19,** 213–216.

Roberge, F. G., Xu, D., and Chann, C. C. (1992). A new effective and non-harmful chemical adjuvant for the induction of experimental autoimmune uveoretinitis. *Curr. Eye Res.* **11,** 371–376.

Schultheiss, H. P., Pauschinger, M., and Kuhl, U. (1998). Pathogenesis of inflammatory cardiomyopathies. *Med. Klin.* **93,** 229–235.

Seiler, F. R., and Sedlacek, H. H. (1980). BCG versus VCN: The antigenicity and the adjuvant effect of both compounds. *Recent Results Can. Res.* **75,** 53–60.

Sharma, M., Swain, P. K., Chopra, A. P., Chaudhary, V. F., and Singh, Y. (1996). Expression and purification of anthrax toxin protective antigen from *Escherichia coli. Protein Exp. Purif.* **7,** 33–38.

Shlyaklov, E., Rubenstein, E., and Novikov, L. (1997). Anthrax postvaccinal cell-mediated immunity in humans: Kinetic pattern. *Vaccine* **15,** 631–636.

Singer, D. F., and Linderman, J. J. (1990). The relationship between antigen concentration, antigen internalization, and antigenic complexes: Modeling insights into antigen processing and presentation. *J. Cell Biol.* **111,** 55–68.

Singh, Y., Ivins, B. E., and Leppla, S. H. (1998). Study of immunization against anthrax with purified recombinant protective antigen of *Bacillus anthracis. Infect. Immunol.* **66,** 3447–3448.

Smialek, M., Gajkowska, B., Ostroski, R. P., and Piotrovski, P. (1997). Experimental squalene encephaloneuropathy in the rat. *Folia Neuropathol.* **35,** 262–264.

Squalenefacts.HTM. (2000). The facts on squalene. Pp. 1–7. URL: http://www.anthrax.osd.mil/Site_Files/qna/SQUALENEFACTS.HTM.

Stepanov, A. V., Marinin, L. I., Pomerantev, A. P., and Staritsin, N. A. (1996). Development of novel vaccines against anthrax in man. *J. Biotechnol.* **44,** 155–160.

Stucky, C. L., Galeazza, M. T., and Seybold, V. S. (1993). Time-dependent changes in Bolton–Hunter-labeled ^{125}I-substance P binding in rat spinal cord following unilateral adjuvant-induced peripheral inflammation. *Neuroscience* **57,** 397–409.

Sulpice, J. C., and Ferezou, J. (1984). Squalene isolation by HPLC and quantitative comparison by HPLC and GLC. *Lipids* **19,** 631–635.

Swanson-Biearman, B., and Krenzelok, E. P. (2001). Delayed life-threatening reaction to anthrax vaccine. *J. Toxicol. Clin. Toxicol.* **39,** 81–84.

Taurog, J. D., Argentieri, D. C., and McReynolds, R. A. (1988). Adjuvant arthritis. *Methods Enzymol.* **162,** 339–355.

Tilvis, R. S., and Miettinen, T. A. (1982). Fate of intravenously administered squalene in the rat. *Biochim. Biophys. Acta* **712,** 374–381.

Tsujimoto, M., Kotani, S., Shiba, T., and Kusemoto, S. (1986). Adjuvant activity of 6-*O*-acyl-muramyl-dipeptides to enhance primary cellular and humoral immune responses in the guinea pig: Dose response and local reactions observed with selected compounds. *Infect. Immunol.* **53,** 517–521.

Turnbull, P. C., Broster, M. G., Carmon, J. A., Manchee, R. J., and Melling, J. (1986). Development of antibodies to protective antigen and lethal factor components of anthrax toxin in human and guinea pigs and their relevance to protective immunity. *Infect. Immunol.* **52,** 356–363.

Unwin, C., Blatchley, N., Coker, W., Ferry, S., Hotopf, M., Hull, L., Ismail, K., Palmer, I., David, A., and Wesseley, S. (1999). Health of UK servicemen who served in (the) Persian Gulf War. *Lancet* **353,** 169–178.

Volpe, R. (1988). The immunoregulatory disturbance in autoimmune thyroid disease. *Autoimmunity* **2,** 55–72.

Welkos, S. L., and Friedlander, A. M. (1988a). Pathogenesis and genetic control of resistance to the Sterne strains of *Bacillus anthracis. Microbiol. Pathol.* **4,** 53–69.

Welkos, S. L., and Friedlander, A. M. (1988b). Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microbiol. Pathol.* **5,** 127–139.

Whitehouse, M. W., Whitehouse, D. J., Vande Sande, B., and Pearson, C. M. (1969). Inhibition of rat adjuvant-induced arthritis, EAE and EAT without drugs: Further observations illuminating the development of these disorders (Abstract). *Arthritis Rheum.* **14,** 191.

Whitehouse, M. W., Orr, K. J., Beck, F. W. J., and Pearson, C. M. (1974). Freund's adjuvant: Relationship of arthrogenicity and adjuvanticity in rats to vehicle composition. *Immunology* **27,** 311–330.

Whitehouse, M. W. (1982). Rat polyarthritis: Induction with adjuvants constituted with mycobacteria (and oils) from the environment. *J. Rheum.* **9,** 494–501.

Wiener, S. L. (1996). Strategies for the prevention of a successful biological warfare aerosol attack. *Mil. Med.* **161,** 251–256.

Williamson, E. D., Beedham, R. J., Bennett, A. M., Perkins, S. D., Miller, J., and Baillie, L. W. (1999). Presentation of protective antigen to the mouse immune system: Immune sequelae. *J. Appl. Microbiol.* **87,** 315–317.

Wright, G. G., Hedberg, M. A., and Stein, J. B. (1954). Studies on immunity to anthrax. II. Elaboration of protective antigen in a chemically-defined nonprotein medium. *J. Immunol.* **72,** 263–269.

Wright, G. G., and Puziss, M. (1957). Elaboration of protective antigen of *Bacillus anthracis* under anaerobic conditions. *Nature* **179,** 916–917.

Zoon, K. C. (1999). Vaccines, pharmaceutical products, and bioterrorism: Challenge for the U.S. Food and Drug Administration. *Emerg. Infect. Dis.* **5,** 534–536.

Zwerger, C., Plesker, R., Papadopulos, K., Cubetaler, K., and Hartinger, J. (1998). A comparison of commercially available adjuvants in BALB/c-mice immunized with a weakly immunogenic peptide. *ALTEX* **15,** 83–86.