



The Facts on Squalene

- 1) Executive Summary.
- 2) What is squalene?
- 3) Does the anthrax vaccine use squalene as an adjuvant?
- 4) Does the anthrax vaccine contain squalene?
- 5) Should we be concerned about the presence of trace quantities of squalene in tetanus, diphtheria, and anthrax vaccines?
- 6) Can squalene cause harm?
- 7) If you wanted to use squalene as an adjuvant, what form would it take?
- 8) What do we know about the European influenza vaccine that uses MF59 (an adjuvant containing squalene).
- 9) What testing has been done?
- 10) What did SRI find the first time?
- 11) What did the FDA find?
- 12) What did SRI find after it revised its test procedures?
- 13) Did DoD mislead or lie to anybody about the squalene tests conducted by SRI?
- 14) Has anyone, anywhere found squalene added as an adjuvant to any US-licensed vaccine?
- 15) Where did the squalene FDA found in its anthrax vaccine tests come from?
- 16) What did the U.S. Senate say about squalene?
- 17) Did the British government test its anthrax vaccine for squalene?
- 18) What are the claims about anti-squalene antibodies?
- 19) Have any independent panels evaluated the claims of researchers to find anti-squalene antibodies in the blood of ill Gulf War veterans?
- 20) Are these panels really independent?
- 21) What did the GAO say about squalene testing and what are DoD researchers doing?
- 22) What did the competitively funded research project find regarding squalene antibodies?
- 23) Has DoD ever tested squalene-adjuvanted vaccines in humans against any disease?
- 24) Could squalene concerns have anything to do with various reported clusters of illnesses among people given anthrax vaccine?
- 25) Bottom line, is there any reason for alarm here?
- 26) References:

The Facts on Squalene

1) Executive Summary.

A few people claim the Department of Defense (DoD) added squalene to anthrax vaccine to stretch the vaccine supply. Four civilian panels have looked into these allegations since 1999 and repeatedly found them groundless. Neither DoD nor anybody else added squalene to anthrax vaccine for our troops. DoD does not conduct illegal experiments. Details and links to independent sources of data appear below.

2) What is squalene?

Squalene is a naturally occurring substance found in plants, animals, and humans. Squalene is manufactured in the liver of every human body and circulates in our bloodstreams. Squalene is present in the oil left by human fingerprints (Asano et al, 2002). Humans cannot live without squalene, because we use squalene as an essential building block to make hormones and other substances in our bodies.

Squalene is also found in a variety of foods (for example: eggs, olive oil (0.7%), cookies, yeast, meat), cosmetics (for example: eye makeup, lipstick, baby powder), over-the-counter medications, and health supplements. Squalene in olive oil may contribute to the low cholesterol levels of people who consume Mediterranean-style diets (Smith, 2000). People can purchase squalene at health food stores. It is more commonly known as "shark liver oil." [Click here](#) to view some commercial squalene resources.

3) Does the anthrax vaccine use squalene as an adjuvant?

An adjuvant is a substance to improve the body's immune response to a vaccine (Vogel et al, 1998; Burdin et al, 2004).

No, the adjuvant in the anthrax vaccine is aluminum hydroxide.

4) Does the anthrax vaccine contain squalene?

Maybe. Some lab tests come up positive for squalene.

Because of the difficulty of removing squalene-containing fingerprint oils from laboratory glassware, it is hard to know whether the squalene is truly present in some lots of the vaccine or is introduced by the testing process itself. DoD, the Food & Drug Administration (FDA), and several civilian advisory committees agree that squalene at such low levels has no adverse health consequences.

In September 2000, DoD became aware of FDA test results finding trace amounts of squalene in three out of three US vaccines tested: tetanus, diphtheria, and anthrax. The level of squalene identified by the FDA test is so minute that it is likely the result of squalene in the oil of a fingerprint not completely cleaned from lab glassware.

It is hard to completely remove fingerprint oils from glassware. Before they go looking for squalene, lab workers have to use a chemical solvent such as hexane to completely remove their own fingerprint oils from lab glassware. When lab workers intentionally tested an extract of fingerprint oil, the squalene reading went off the chart.

Before the FDA test results became known, Stanford Research International (SRI), under DoD contract, looked for squalene in anthrax vaccine. At the limit of detection of its test, 140 parts per billion, SRI found no squalene in several lots of anthrax vaccine. The FDA's test, which was developed later, is more sensitive. It is able to detect as little as 10 parts per billion. The FDA found squalene at 10 to 83 parts per billion in diphtheria toxoid, tetanus toxoid, and anthrax vaccine. The trace level of squalene found by the FDA in anthrax vaccine is less than the concentration naturally present in human blood (250 parts per billion) (Miettinen, 1982; Nikkila et al, 1992).

After the FDA reported its results, DoD asked SRI to refine its assay. Using an improved method that could detect as little as 1 part per billion, SRI found no squalene in 32 out of 33 lots of anthrax vaccine tested (including lots in which FDA found low levels of squalene). In one lot, they found up to 9 parts per billion. The details appear below.

5) Should we be concerned about the presence of trace quantities of squalene in tetanus, diphtheria, and anthrax vaccines?

No. The trace level of squalene found by the FDA and SRI in diphtheria, tetanus, and anthrax vaccines is well below the concentration naturally present in human blood (250 parts per billion). Injecting trace amounts of squalene are unlikely to have any biological effect, given that it is already present in the body. In fact, without squalene in the body to manufacture hormones and other substances in our bodies, we would die.

In Congressional testimony on 3 October 2000, FDA official Mark Elengold said that the trace quantities of squalene detected were "both naturally occurring and safe."

6) Can squalene cause harm?

Some animal research to study arthritis used injections of tuberculosis-like bacteria (mycobacteria) dissolved in squalene (e.g., arthritis-prone rats, mice). Other studies assessed 100% squalene injected into rat tails or injected directly into joints. (Yoshino & Yoshino, 1994; Lorentzen, 1999; Kuroda et al, 2004)

The relevance of findings in susceptible animal species to humans is unclear (IOM/Sox, 1999; Kuroda et al, 2004).

Based on other research, it is clear that whether squalene causes harm or not is related to selected conditions of concentration, dose, route of application, and other factors (Benisek et al, 2004).

7) If you wanted to use squalene as an adjuvant, what form would it take?

If you wanted to use squalene as an adjuvant (to boost immune responses) you would have to multiply the amount of squalene found by the FDA about 1 million times, as well as change it from a simple liquid (its natural state) to an emulsion. An emulsion is a stable suspension of tiny droplets, like an oil-and-vinegar mixture that doesn't separate. This double difference is like the difference between a teaspoon of oil and 2,000 pounds of mayonnaise. [If you emulsify oil with eggs, you get mayonnaise.]

Squalene in the form of an emulsion (emulsified squalene, such as an adjuvant called MF59) has been added as an adjuvant to some investigational vaccines in the U.S. (Burdin et al., 2004)

There is no squalene adjuvant in any US-licensed vaccine.

Whatever the arguments for or against squalene as a vaccine adjuvant, the fact is that none of the anthrax vaccine administered to U.S. troops contained squalene as an adjuvant. Based on manufacturing records, FDA can verify that no squalene was added to any vaccine formulation used during the Gulf War. This includes the anthrax vaccine. To date, the FDA has licensed, and US manufacturers have used, only aluminum salts (for example, aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate) as adjuvants.

8) What do we know about the European influenza vaccine that uses MF59 (an adjuvant containing squalene).

In 1997, European health agencies approved emulsified squalene (with influenza virus in the center of each droplet) for use as an adjuvant in an influenza vaccine (Fluad, Chiron Corporation, Marburg, Germany, and Siena, Italy, www.forum-impfen.de/impfnavigator/packungsbeilage/5205fluad.pdf; Sesardic & Dobbelaer, 2004). Some clinicians consider influenza vaccine with MF59 adjuvant to be better able to induce immunity in elderly people (Banzhoff et al, 2003).

To make this influenza vaccine work, researchers needed a squalene concentration of 1.95% (about 2 parts per hundred or 20 million parts per billion) to boost the immune response. This squalene had to be in the form of an emulsion (a mixture of tiny droplets) to be recognized by the immune system. Squalene in its oily state is naturally present inside the human body.

Tens of millions of doses of this European influenza vaccine have been administered safely since 1997.

9) What testing has been done?

Three sets of US tests have been performed: Initial tests by SRI, tests by FDA, and improved tests by SRI. Each is described below.

10) What did SRI find the first time?

To determine whether squalene was present in anthrax vaccine, the DoD contracted with an independent civilian laboratory, Stanford Research Institute (SRI) International of Menlo Park, California www.sri.com, to test for the presence of squalene in anthrax vaccine. SRI developed a laboratory method to detect squalene as dilute as 140 parts per billion (ppb). At this level of detection, extraordinary measures must be taken to avoid contaminating samples, glassware, and equipment with squalene from the skin, because squalene is a natural component of the oils in our skin. The SRI test used a technique called high-pressure liquid chromatography (HPLC) with ultraviolet detection at a wavelength of 203 nanometers. SRI tested 17 lots of anthrax vaccine: FAV008, FAV017, FAV019, FAV020, FAV024, FAV030, FAV031, FAV033, FAV034, FAV036, FAV037, FAV038, FAV041, FAV043, FAV044, FAV047, and FAV048B. SRI reported "based on triplicate analysis, no squalene was detected in the sample. The limit of detection is 70 nanograms per 0.5 milliliter dose (140 ppb)." (Spangord et al., 2002)

11) What did the FDA find?

Using a more sensitive test, developed after the initial SRI test, the Food & Drug Administration (FDA) found trace amounts of squalene in three out of three US vaccines tested in Jun 1999: diphtheria toxoid, tetanus toxoid, and anthrax vaccine (http://www.anthrax.mil/media/pdf/squalene/FDA_squalene1.pdf). The FDA test used a technique called gas chromatography with flame-ionization detection. The FDA method could detect squalene as dilute as 10 parts per billion (ppb). Testing five lots of anthrax vaccine and two lots each of diphtheria and tetanus vaccines, FDA concluded, "there were only trace amounts of squalene in the lots tested." Based on manufacturing records, FDA verified that no squalene was added to any vaccine formulation used during the Gulf War.

The amounts of squalene identified in the specific lots were:

Anthrax lot FAV020 11.3 ppb
Anthrax lot FAV030 10.1 ppb
Anthrax lot FAV038 27.1 ppb
Anthrax lot FAV043 40.0 ppb
Anthrax lot FAV047 82.9 ppb
Diphtheria lot 3710 22.5 ppb
Tetanus lot 7271 28.7 ppb

Squalene is constantly present in the human blood stream at 250 ppb (250 nanograms per milliliter), a concentration 3 to 25 times higher than the level detected in the FDA test. The amount of squalene added as an adjuvant to a European-approved influenza vaccine is 4 grams per 100 ml (4 parts per hundred), which is about 1,000,000 times more than the concentration of squalene detected in the FDA test. This European influenza vaccine has been administered safely to hundreds of thousands of people.

12) What did SRI find after it revised its test procedures?

After the FDA released its findings in September 2000, SRI revised its squalene test, lowering its limit of detection of 1 ppb or 0.5 nanograms per 0.5 ml. With this more sensitive test, SRI found no squalene in 32 out of 33 lots tested. SRI found squalene in each of three vials of lot FAV008, at 1, 7, and 9 ppb.

SRI found no squalene in lots 12, 13, 18, FAV001, FAV002, FAV003, FAV004, FAV005, FAV006, FAV007, FAV009, FAV012, FAV016, FAV017, FAV018, FAV019, FAV020, FAV022, FAV024, FAV030, FAV031, FAV032, FAV033, FAV034, FAV036, FAV037, FAV038, FAV041, FAV043, FAV044, FAV047, and FAV048B.

SRI also tested some non-vaccine injectable pharmaceuticals. SRI found no squalene in human insulin regular U-100, human insulin isophane (NPH) U-100, lidocaine 2% solution, sodium chloride 0.9% solution, or potassium chloride 2 mEq/ml solution.

13) Did DoD mislead or lie to anybody about the squalene tests conducted by SRI?

No. DoD truthfully and fully reported its findings at each step since May 1999, when SRI first developed its squalene test. DoD did not know of FDA's findings until they were publicly released.

At the initial limit of detection of its test, 140 parts per billion, SRI found no squalene in anthrax vaccine (Spangord et al., 2002). It was scientifically proper to say 'no squalene was found to the limit of detection of the assay,' which DoD officials sometimes oversimplified to say 'there is no squalene present.'

14) Has anyone, anywhere found squalene added as an adjuvant to any US-licensed vaccine?

No.

15) Where did the squalene FDA found in its anthrax vaccine tests come from?

The most likely source of the trace squalene in the FDA tests is the result of squalene in the oil of a fingerprint not cleaned from lab glassware. Squalene is not added to anthrax vaccine or any US-licensed vaccine. It is hard to completely remove fingerprint oils from glassware. Lab workers have to use a chemical solvent such as hexane to completely remove fingerprint oils from lab glassware.

16) What did the U.S. Senate say about squalene?

In its investigations of illnesses among Gulf War veterans, the Senate Special Investigations Unit (SIU) found no credible information indicating that vaccines used during the Gulf War contained squalene (1998, page 123)

<http://veterans.senate.gov/Reports/chapt3.pdf> (chapter 3, page 23 of 55)

In its report, the SIU stated that according to the Food and Drug Administration (FDA), squalene can be contained in a vaccine due to two different processes: 1) as an adjuvant, which is an agent to enhance the immune response; or 2) in minute quantities in certain vaccines manufactured using eggs, since eggs are rich in squalene and cholesterol. The FDA verified that none of the vaccines used during the Gulf War contained squalene as an adjuvant.

17) Did the British government test its anthrax vaccine for squalene?

Yes, The United Kingdom's Ministry of Defence arranged for an independent laboratory to test 11 lots of the British anthrax vaccine manufactured at Porton Down, as well as other vaccines. No squalene was detected in those lots of vaccine, with a limit of detection of 0.1 microgram/ml (100 parts per billion). See: <http://www.mod.uk/issues/gulfwar/info/medical/squalene.htm>

18) What are the claims about anti-squalene antibodies?

In an effort to explain the health problems of some Gulf War veterans, a few people have theorized that a vaccine adjuvant may have caused an autoimmune disease in veterans. A *Vanity Fair* article by Gary Matsumoto, "The Pentagon's Toxic Secret" (May 1999), alleges that the DoD possibly used "an illicit and secret anthrax vaccine" on its own soldiers. The writer's interpretation and presentation of the facts regarding the Department's use of anthrax vaccine are speculative, inflammatory, and wrong. His allegations and the reported "clinical evidence" are not new. Since 1997, reports in the *Washington Times*, its magazine *Insight on the News*, and the (Wilmington) *Delaware News Journal*, have made similar allegations regarding "secret medical experiments" and the like.

Investigators cited in these articles (Pamela Asa, Ph.D., Memphis, TN, and Robert Garry, Ph.D., Tulane University School of Medicine, New Orleans, LA) report they developed in 1997 and patented a test for anti-squalene antibodies (ASA). Autoimmune Technologies, LLC, of New Orleans, has an exclusive license on the use of this test. The investigators report that they detected anti-squalene antibodies in the blood of ill Gulf War veterans. Their methods were published in the February 2000 and August 2002 issues of the journal *Experimental and Molecular Pathology*.

In the February 2000 article, the authors themselves conclude: "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." Asa and colleagues published a second article in the August 2002 issue of *Experimental and Molecular Pathology*, but it also provides no validation of the original assay. As a result, the findings of the second article are also in question. The authors' comment that the Matyas article of Nov 2000 supports their findings is mistaken.

19) Have any independent panels evaluated the claims of researchers to find anti-squalene antibodies in the blood of ill Gulf War veterans?

Yes, four independent civilian panels considered the February 2000 article by Asa and colleagues and other allegations related to squalene and anti-squalene antibodies.

When the Institute of Medicine (part of the National Academy of Sciences) Committee on Gulf War and Health (the "Sox committee") evaluated the 2000 Asa claims of anti-squalene antibodies in the blood of ill Gulf War veterans, it concluded that the paper contains shortcomings, some serious, that combine to invalidate the authors' conclusions. The report says: "The committee does not regard this study as providing evidence that the investigators have successfully measured antibodies to squalene." See <http://www.nap.edu/books/030907178X/html>, pages 311-312.

The civilian experts on the Armed Forces Epidemiological Board (AFEB) said in July 2000, "the research reported in this paper does not support this claim; ... it remains unclear if the assay actually measures antibodies to squalene, as the authors assert..." <http://www.ha.osd.mil/afeb/reports/squalene.pdf>

Regarding assertions that Service Members who received anthrax vaccination from the five lots cited in the FDA squalene tests experienced more or more severe adverse events after vaccination, the civilian physicians

on the Anthrax Vaccine Expert Committee (AVEC) evaluated adverse events by lot and geographic location. They found no meaningful differences based on lot or on geographic location. (Sever et al. 2002 http://www.anthrax.mil/media/pdf/AVEC_ms.pdf, especially pages 198-200, and Sever et al, 2004 <http://www.anthrax.mil/media/pdf/SeverArticle.pdf>, especially pages 13-15)

Of note, the five lots cited in the FDA squalene tests were shipped to multiple DoD installations. In addition, Dover AFB received lots other than the five lots mentioned above.

After the comprehensive review of anthrax vaccine safety by the National Academy of Sciences (the "Strom committee," March 2002, www.nap.edu/catalog/10310.html), which included hearing from personnel from Dover AFB and elsewhere concerned that they suffered adverse events after anthrax vaccination, the civilian physicians and scientists concluded that "The [SRI] study report, dated August 14, 2001, found that 1 lot of over 30 lots tested contained measurable levels of squalene. Three samples from that lot [FAV008] contained squalene at 7, 9, and approximately 1 parts per billion, respectively. Use of vaccine from that lot has not been associated with elevated rates of adverse events. ... Because the available data ... demonstrate that the presence of trace amounts of squalene is not associated with an increase in the rates of adverse events following vaccination with AVA, the committee concludes that further investigation of possible AVA contamination is not warranted at this time."

20) Are these panels really independent?

The IOM committee members were selected by the National Academy of Sciences to be fully independent of both the Department of Defense and the Department of Veterans Affairs.

The AVEC committee members were selected by the Department of Health & Human Services to be fully independent of the Department of Defense.

The AFEB is appointed by the Secretary of the Army to advise the Surgeons General of the military services. These civilians constitute a highly accomplished and widely respected scientific advisory board. These civilians are free to render whatever opinions they wish, and their candidness is important to ensuring that DoD is using the best possible medical information.

21) What did the GAO say about squalene testing and what are DoD researchers doing?

In March 1999, the U.S. General Accounting Office (GAO, now the Government Accountability Office) released a report "Gulf War Illnesses: Questions about the Presence of Squalene Antibodies in Veterans Can be Resolved" (GAO/NSIAD-99-5). The Department of Defense disagreed with the GAO's opinion that "the first step is to determine the extent to which they [antibodies to squalene] are present in a larger group of sick Gulf War-era veterans." The proper first step is to show that the test for squalene antibodies measures what it claims to measure. Further, the medical significance and the origin of antibodies to squalene, even if their existence is corroborated, remain unknown. Without such information, Gulf War veterans get only speculation about the meaning of the test result and its implication for their health. Gulf War veterans deserve objective evidence and recommendations based on sound science.

To investigate the anti-squalene antibody theory, a scientifically proven test for squalene antibodies is needed to assess whether Gulf War veterans have antibodies to squalene. In response to a DoD solicitation for research on illnesses among Gulf War veterans, a DoD investigator and nationally recognized expert on antibodies to cholesterol and other lipids submitted a research proposal to determine the feasibility of developing a test for antibodies to squalene. The competitively funded research project to determine whether antibodies to squalene exist has five main objectives: 1) Development and validation of an enzyme-linked immunosorbant assay (ELISA) for antibodies against squalene. 2) Evaluation and potential development of other assays for antibodies to squalene. 3) Development of a positive control antibody to squalene. 4) Production of the positive control antibody to squalene for use in the assays. 5) Testing of normal human serum for antibodies to squalene by ELISA and other methods.

22) What did the competitively funded research project find regarding squalene antibodies?

In April 2000, the research project published its first peer-reviewed report, describing an enzyme-linked immunosorbent assay (ELISA) that could detect antibodies to squalene induced in mice. Use of squalene alone did not produce a significant amount of anti-squalene antibodies. A special chemical was needed to induce the antibodies against squalene in mice. After injecting mice with liposomes (fat globules) containing 71% squalene (710 million parts per billion), plus a second chemical called lipid A, antibodies to squalene were readily induced in mice. The validity of the method was established using positive and negative controls to preclude false-positive and false-negative test results. The investigators concluded that squalene is a weak antigen (a weak inducer of antibodies). (Matyas et al., 2000).

By September 2001, researchers reported improving the assay and ensuring these tests were reproducible and sensitive enough to detect 80 ng/ml of anti-squalene antibody. The test was also reproducible from experiment to experiment (Matyas et al., 2001).

The third study from this research effort, published in 2004, adapts the test described above so that it could detect anti-squalene antibodies if present in human serum. Serum from three groups of people were tested: retired employees of the US Army Medical Research Institute of Infectious Diseases (average 68 years of age, 88% of whom received anthrax vaccine, mean = 26 doses per person), civilian volunteers of similar age from Frederick, Maryland (none of whom received anthrax vaccine), and random blood donors from Fort Knox, Kentucky (vaccination status unknown). This next study indicates that anti-squalene antibodies are found in 7.5% of the vaccinated USAMRIID alumni, 15% of the unvaccinated Frederick civilians, and in 0% of the Fort Knox blood donors. The antibodies described in the previous sentence were a type of antibody called IgG. Researchers found another type of anti-squalene antibody called IgM in all three groups (37%, 32%, 19%). The researchers found that anti-squalene antibodies are more common with increasing age (a characteristic also found in mice). The presence of anti-squalene antibodies was unrelated to anthrax vaccination status. They concluded that anti-squalene antibodies occur naturally in humans (Matyas et al., 2004).

23) Has DoD ever tested squalene-adjuvanted vaccines in humans against any disease?

Yes. The DoD conducted several human clinical trials exploring the value of investigational vaccines containing squalene-based adjuvants to prevent malaria and HIV infection. The squalene-containing adjuvants principally involved products known as MF59 (licensed from Chiron Corporation) and AS02A (licensed from GlaxoSmithKline). Each of these studies involved an FDA-approved scientific plan in human volunteers told the contents of the vaccine.

Malaria: Hoffman et al, 1994; Epstein et al, 2004; Wang et al, 2004.

HIV: Nitayaphan et al, 2000; Pitisuttithum et al, 2003.

The Department of Defense (DoD) has never exposed any military member or civilian to any squalene-adjuvanted investigational product without the person's informed consent, abiding by FDA regulations.

Civilian researchers, including some funded by the National Institutes of Health, have conducted clinical trials of these and other squalene-adjuvanted vaccines on human volunteers, ranging from infants to the elderly.

24) Could squalene concerns have anything to do with various reported clusters of illnesses among people given anthrax vaccine?

A panel of civilian physicians selected by the Department of Health & Human Services reviewed all reports of adverse events after anthrax vaccination from 1998 to 2001 (Sever et al, 2002; Sever et al, 2004). This panel was known as the Anthrax Vaccine Expert Committee (AVEC)

To evaluate assertions that Service Members who received anthrax vaccination from the five lots cited in the FDA squalene tests experienced more or more severe adverse events after vaccination, these civilian physicians evaluated adverse events by lot and geographic location. They found no meaningful differences based on lot or on geographic location.

Of note, the five lots cited in the FDA squalene tests were shipped to multiple DoD installations. In addition, Dover AFB received lots seven lots other than the five test-positive lots mentioned above.

25) Bottom line, is there any reason for alarm here?

No. Squalene is not added to any US-licensed vaccine, including anthrax vaccine. The background level of squalene found by the FDA is less than the concentration normally present in human blood. The FDA confirms that these trace levels are "naturally occurring and safe." Improved tests found no squalene in the lots where FDA found it.

Nonetheless, DoD continues to compile additional knowledge about squalene and anti-squalene antibodies.

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Food and Drug Administration
CBER/OCBQ/DMPQ/LAC
HFM-673
1401 Rockville Pike
Rockville, MD 20852

Date: June 25, 1999

From: Joan C. May, Ph.D., Chief, LAC, DMPQ HFM-673
Alfred V. Del Grosso, Ph.D.
Laura Swartz, Ph.D.
Joseph J. Progar

Subject: Chemical Test Results for Michigan Department of Public Health, Anthrax Vaccine Adsorbed, Lots FAV020 and FAV030

To: Neil Goldman, Ph.D. HFM-20

Aluminum was measured by flame atomic absorption spectrophotometry on June 17, 1999. CBER's results are as follows:

<u>Lot #</u>	<u>mg Al/mL</u>
FAV020	1.30
FAV030	1.33

The limit for aluminum as stated in Title 21, Sec. 610.15 of the Code of Federal Regulations is no more than 0.85 mg of aluminum in the recommended individual dose when determined by assay or no more than 1.14 mg of aluminum by calculation on the basis of the amount of aluminum added. The dose for this product is 0.5 mL. The above lots meet this requirement.

BioPort Corporation has set limits of 0.8-1.5 mg/mL of aluminum (0.4-0.75 mg/0.5mL dose). The above lots meet this requirement.

Formaldehyde concentration was measured by colorimetry (Hantzsch method) on June 17, 1999. CBER's results are as follows:

<u>Lot #</u>	<u>Percent Formaldehyde</u>
FAV020	0.009
FAV030	0.009

CBER's requirement specifies that the free formaldehyde in the finished product be less than 0.02 percent free formaldehyde (200 ug formaldehyde per mL). The above lots meet this requirement.

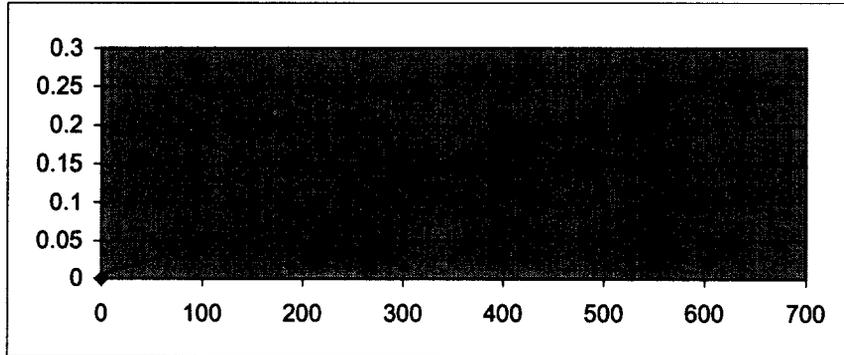
BioPort Corporation has set a limit of less than 0.02 percent formaldehyde for this product. The above lots meet this requirement.

Squalene GC

6/24/1999

File:Sq06249A

ppb Sq.	ISTD	Squalene	Squalene/ISTD
0	2329	0	0
90	2066	84	0.040658277
300	2672	370	0.138473054
600	1915	500	0.261096606
900	1720	650	0.377906977



SUMMARY OUTPUT

<i>Regression Statistics</i>	
Multiple R	0.999346296
R Square	0.99869302
Adjusted R Square	0.99825736
Standard Error	0.006532251
Observations	5

<i>ANOVA</i>					
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	0.097816045	0.097816045	2292.367986	2.00615E-05
Residual	3	0.000128011	4.26703E-05		
Total	4	0.097944055			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>
Intercept	0.004747641	0.004421048	1.073872457	0.361569842	-0.009322119	0.018817402	-0.009322119
X Variable 1	0.000420316	8.77877E-06	47.87867987	2.00615E-05	0.000392378	0.000448254	0.000392378

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
MDPH FAV020		4488	244 0.054367201	118.1	11.3
		4632	231 0.049870466	<u>107.4</u> 112.7	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
MDPH FAV030		3780	163 0.043121693	91.3	10.1
		3386	175 0.051683402	<u>111.7</u> 101.5	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
Wyeth Diphtheria 3710		3382	340 0.100532229	227.9	22.5
		3047	299 0.098129308	<u>222.2</u> 225.0	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
PM Connaught Tetanus 7271		6401	792 0.123730667	283.1	28.7
		8315	1054 0.12675887	<u>290.3</u> 286.7	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
MDPH FAV038		2860	304 0.106293706	241.6	27.1
		2284	299 0.130910683	<u>300.2</u> 270.9	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
MDPH FAV043		3160	557 0.176265823	408.1	40.0
		3631	614 0.169099422	<u>391.0</u> 399.5	

Sample	ISTD	Squalene	Squalene/ISTD	ppb Squalene Prep.	Sample
MDPH FAV047		3187	1043 0.327267022	767.3	82.9
		4560	1728 0.378947368	<u>890.3</u> 828.8	

Benzethonium chloride, an antimicrobial preservative, was measured using an adaptation of the colorimetric titration procedure originally specified by Michigan Department of Public Health and currently used by BioPort Corporation for this product. CBER testing was performed on June 25, 1999. Results for the two subject lots along with results obtained from three other lots of anthrax vaccine are as follows:

<u>Lot #</u>	<u>Percent Benzethonium Chloride</u>
FAV020	0.0020
FAV030	0.0015
FAV008-2	0.0017
FAV031-1	0.0019
FAV038	0.0020

Limits for benzethonium chloride content of this product were specified by Michigan Department of Public Health as 0.0015 – 0.0030 %. The above lots meet this requirement.

Squalene was determined by gas chromatography with flame ionization detection following solvent extraction and concentration. Verification of the characteristic mass spectrometric fragmentation pattern obtained from the chromatographic peak was used as part of the validation of the analytical procedure. Three other lots of anthrax vaccine were tested for comparative purposes along with two lots of other bacterial vaccines containing alum adjuvants. CBER testing was performed on 6/23 and 6/24/99. Results are as follows:

<u>Lot #</u>	<u>ppb (parts-per-billion) Squalene</u>
FAV020	11
FAV030	10
FAV038	27
FAV043	40
FAV047	83
Wyeth Diphtheria Lot 3710	22
Connaught Tetanus Lot 7271	29

Squalene content of the subject lots was determined to be in the level of low parts-per-billion and was comparable to levels determined in other lots of anthrax vaccine and in the other bacterial vaccines that were tested.

Ministry of
DEFENCE

Gulf Veterans' Illnesses

DETECTION OF POTENTIAL SQUALENE IN VARIOUS VACCINES

Defence Issues

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Introduction

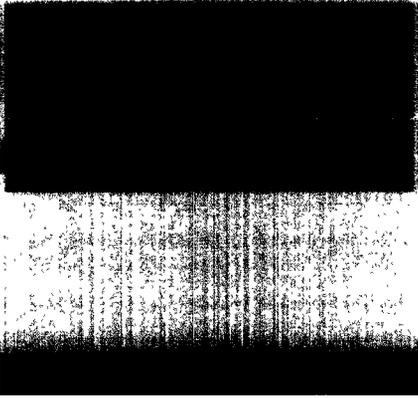
1. Following reports in the media and concerns raised in Parliament and by Gulf veterans that vaccines used at the time of the 1990/1991 Gulf conflict to protect UK Forces may have contained squalene, the Ministry of Defence contracted an independent laboratory to carry out an analysis of vaccines used for the presence of squalene using gas chromatography.

Results

2. No squalene was detected in any of the vaccine samples tested. The limit of detection used was 0.1 mg/ml. Three of the samples vaccines required the limit of detection to be compensated due to low recovery values. No squalene above a limit of detection of 0.2 mg/ml was detected in those vaccines.

3. The Ministry of Defence has stated on a number of occasions that none of the vaccines used for the medical countermeasures programme contained squalene, nor was squalene used as an adjuvant. The Ministry of Defence has also stated that, as far as has been ascertained, none of the public health vaccines given to service personnel during the Gulf conflict contained squalene. The independent laboratory's findings, based on the samples provided, confirm that none of the vaccines tested positive for squalene.

4. The independent laboratory produced a **final report in June**



2001 (33Kb) (ADOBE pdf format).

Ministry of Defence
May 2002

Last Updated: 22 Jul 02



DEPARTMENT OF DEFENSE
ARMED FORCES EPIDEMIOLOGICAL BOARD
5100 LEEBSBURG PIKE
FALLS CHURCH VA 22041-3258



AFEB (15-1a) 00-6

11 July 2000

MEMORANDUM FOR THE ASSISTANT SECRETARY OF DEFENSE (HEALTH AFFAIRS)
THE SURGEON GENERAL, DEPARTMENT OF THE ARMY
THE SURGEON GENERAL, DEPARTMENT OF THE NAVY
THE SURGEON GENERAL, DEPARTMENT OF THE AIR FORCE

SUBJECT: Armed Forces Epidemiology Board (AFEB) Recommendations
Regarding Review of the Paper, "Antibodies to Squalene in Gulf
War Syndrome by P. B. Asa, Y. Cao and R. F. Garry."

1. The AFEB was tasked by the Department of Defense (Health Affairs) to conduct an objective analysis of the above paper following a request by Congressman Jack Metcalf to Health Affairs.
2. A Special Subcommittee was formed to review the paper. Results of the review and the paper were distributed to the rest of the Board prior to the AFEB meeting. The Subcommittee's findings were presented to the whole Board at the AFEB Meeting held 28-29 February 2000 at Fort Sam Houston, Texas. After discussions and several additional reviews, the report was finalized.
3. The AFEB has thoroughly reviewed the paper by Dr. Asa and colleagues who describe a laboratory test they feel may identify individuals ill with "Gulf War Syndrome." The following is a summary of the findings:
 - a. THE RESEARCH REPORTED IN THIS PAPER DOES NOT SUPPORT THIS CLAIM.
 - b. THE PAPER CONTAINS NUMEROUS SHORTCOMINGS, SEVERAL OF THEM SERIOUS, THAT COMBINE TO INVALIDATE THE AUTHORS' CONCLUSIONS.
 - c. IT REMAINS UNCLEAR IF THE ASSAY ACTUALLY MEASURES ANTIBODIES TO SQUALENE, AS THE AUTHORS ASSERT; THE ASSAY MAY MEASURE SOMETHING ELSE OR THEIR FINDINGS MAY BE A NON-SPECIFIC CHEMICAL REACTION.

AFEB (15-1a) 00-6

11 July 2000

SUBJECT: Armed Forces Epidemiology Board (AFEB) Recommendations Regarding Review of the Paper, "Antibodies to Squalene in Gulf War Syndrome by P. B. Asa, Y. Cao and R. F. Garry."

4. The Board unanimously endorses and approves the above findings and the enclosed report. Details of their findings can be found in the enclosed report.

FOR THE ARMED FORCES EPIDEMIOLOGICAL BOARD:

F. Marc LaForce

F. MARC LAFORCE, M.D.
AFEB President

Benedict M. Diniega

BENEDICT M. DINIEGA
Colonel, USA, MC
AFEB Executive Secretary

- 3 Encls
- 1. Report
- 2. Tasking Letter
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- CDR Mark Tedesco, USPHS
- COL Andrew S. Warde,
BvetMed Msc MRCVA
- LCol Maureen Fensom, CFMS

REVIEW OF THE PAPER

ANTIBODIES TO SQUALENE IN GULF WAR SYNDROME
by PB Asa, YCao and RF Garry

published in
Experimental and Molecular Pathology, Volume 68, pp 55-64 (2000)

A REPORT FROM
THE ARMED FORCES EPIDEMIOLOGICAL BOARD
JUNE 22, 2000

SUMMARY OF FINDINGS

The Armed Forces Epidemiological Board has thoroughly reviewed the paper by Dr. Asa and colleagues who describe a laboratory test they feel may identify persons ill with "Gulf War Syndrome." The AFEB has concluded unanimously that the research reported in this paper does not support this claim. The paper contains numerous shortcomings, several of them serious, that combine to invalidate the authors' conclusions. It remains unclear if the assay actually measures antibodies to squalene, as the authors assert; the assay may measure something else, or their findings may be a non-specific chemical reaction.

BACKGROUND

The Armed Forces Epidemiological Board (AFEB) was tasked by the Department of Defense (Health Affairs) to conduct an objective analysis of the above captioned paper by Asa *et al.* The tasking letter is enclosed.

A special subcommittee¹ of the AFEB was formed to initiate the task. The Special Subcommittee read the above captioned paper by Asa *et al.* The subcommittee fully discussed its impressions, questions and concerns, and developed a consensus document. The chair of the subcommittee then formally presented the subcommittee's findings to the entire AFEB² which had been supplied with the paper and the consensus document in advance of the meeting. After input from the entire AFEB, this final report is offered to the requester by the AFEB president.

FINDINGS

The AFEB reviewed the paper with great interest. However, the AFEB found the paper to contain a large number of scientific flaws, some of which are extremely grave. These flaws invalidate to an almost complete degree the conclusions regarding squalene and the implications that proceed from them. The major flaws include the following:

Controls: Despite assertions and disclaimers in the paper, there are no valid controls.

- For a valid positive control, one needs serum previously proven to contain antibodies to squalene; only this can validate that the assay can detect antibodies to squalene. What the authors use as and assert is a positive control are two sera from individuals reportedly vaccinated (either once or three times) with an NIH trial vaccine containing squalene. The authors provide no pre-vaccination data to demonstrate that the activity detected in their assay was not present before vaccination with a squalene adjuvant.
- Negative controls are essential to prove that the assay is not detecting something other than anti-squalene antibodies. Missing are controls which omit serum containing the presumed antibodies or which omit the avidin-conjugated horse radish peroxidase. Also missing is a negative specificity control to rule out non-specific binding of normal IgG molecules to squalene.

Blinding: It is unclear if the researchers were blind as to illness/wellness status of study participants.

- The paper asserts at several points that this is a blinded study, but it remains possible that the critical element of knowing the illness/wellness status or category may have been known, even if, as the paper states, "...The identities or exact number of samples from each category were not made available..."

¹ S Music, Chair, E Barrett-Connor, P Landrigan, Members; *curricula vitae* attached per written request of Congressman Metcalf to Defense Secretary Cohen, as "...objective analysis...including identification of those who are providing the analysis and their professional credentials."

² During the 30-31 May 2000 meeting of the AFEB at Ft. Detrick, MD.

- Thus, the authors' assertions, that they did not know which subjects had "Gulf War Syndrome" and which did not, are not convincing. If the authors knew which blood samples came from Gulf War veterans, this could have biased their interpretation of their test findings.

Specificity: Does the ASA Assay actually measure antibodies to squalene?

- In this type of blotting experiment, one normally demonstrates specificity of the reaction by blocking (or adsorbing) the antibody with the antigen (in solution). This is not demonstrated.
- Hence, it is not possible to know what the ASA assay detects. It is a Western-blot type assay, and is either positive (+) or negative (-). Since the paper describes it being used in only one dilution of patient serum (1:400), it seems the assay can determine only whether "something" was detectable or not, and this "something" is not presently definable.
- Antibodies to squalene, or to any other substance for that matter, should be detectable across a range of concentrations, so antibody assays are normally constructed to demonstrate this, the most common form today being an enzyme-linked immunoassay (ELISA). The actual level or concentration of antibody, ranging from undetectable to just detectable through high concentration, should have medical/biological correlations and implications, with some threshold point that correlates with the development of symptoms or disease.
- Nitrocellulose is a highly reactive substance that binds many materials. The paper does not show that the squalene deposited on the membrane is actually still there at the end of the assay. For example, one could imagine that squalene could "block" the nitrocellulose membrane long enough to protect the "dot" from the milk treatment and then be washed out, as polyoxyethylene sorbitan laurate is a detergent that could remove a lipid like squalene. This could leave a naked spot of nitrocellulose to react with some other protein.
- If this were a valid assay it should work with another substrate (other nylon membranes, like Immobilon).
- Given the relationship between squalene and cholesterol, do these sera react with cholesterol? The authors raise the question but don't answer it.
- Can one actually raise antibodies, deliberately, to squalene? It is a common component of cells and should be present in amounts that would swamp out any squalene-specific antibodies.

Dose response: None is apparent.

- In the figures of the Aza *et al* paper, there is no obvious dose response in relation to the amount of antigen (squalene) deposited on the nitrocellulose membrane.
- A dose-response should be seen with respect to antigen and antibody concentration; neither is shown.

CONCLUSIONS

In summary, the clear failure to provide positive controls and negative controls as well as unambiguous blinding, invalidates the authors' ability to argue for the meaningfulness of their test and any conclusions they might draw from these results. This is true even before one gets to the more technical issue of the specificity of the ASA assay.

Therefore, the AFEB has little confidence that the patent-pending ASA assay actually measures antibodies to squalene, though we cannot entirely eliminate this possibility.

Whatever the paper's flaws and since the AFEB cannot exclude the remote possibility that the authors have identified a laboratory means of distinguishing persons with possible Gulf War Syndrome (GWS) from all others, replicability becomes the major unresolved issue. The AFEB recognizes the difficulties inherent in defining a possible case of GWS since there is no standardized case definition. However, the AFEB feels that the symptom list in the *Asa et al* paper is a good potential starting point, and that, for example, cases might be selected from tertiary referral centers for GWS such as the one at Walter Reed, with controls from a civilian, non-exposed workforce. Therefore we recommend that a suitable test of replicability be done in cooperation with the authors and with attention to the following design elements:

- selection of participants - cases and control subjects - by an independent *ad hoc* body or committee, chaired by a tenured academic from a well-known medical research institution
- establishing clear *a priori* selection and exclusion criteria for cases and for controls
- serological testing done in a secure and absolutely blind manner with strict chain of custody rules and documentation in place
- a sufficient number of subjects to have statistical power to detect a true difference, if one exists, with 80% likelihood and with a 5% chance or less of finding a difference due to random chance alone.
- a study design with at least two arms - testing done as in the paper by the people who have licensed this patent-pending technique, versus testing done by one or more lipid laboratories using more standard antibody techniques such as enzyme-linked immunoassay to detect antilipid antigens

We wish to be clear that we are not discussing a study to validate whether the ASA assay can detect antibodies to squalene. Rather, we are trying to leap over this intermediate obstacle and get quickly and inexpensively to a more meaningful bottom line: does the ASA assay clearly, reliably and unequivocally distinguish people with GWS from all others, and, if so, with what specificity and sensitivity? Many caveats and qualifiers would have to be in place to assure meaningfulness, and the preceding bulleted list can (and probably should) be usefully expanded and further refined to help assure that any ensuing serological study be definitive.

The AFEB is extremely doubtful that the assay reported by *Asa et al* is a valid or accurate test for illness among Gulf War veterans. However in an effort to leave no stone unturned in evaluating veterans' complaints, the AFEB feels it may be worthwhile to repeat the study, using appropriate scientific methods as outlined above. This recommendation should definitely not be considered an endorsement of the paper by *Asa et al* that we have herewith reviewed.



HEALTH AFFAIRS

OFFICE OF THE ASSISTANT SECRETARY OF DEFENSE
1200 DEFENSE PENTAGON
WASHINGTON, DC 20301-1200

09 MAR 2000

MEMORANDUM FOR EXECUTIVE SECRETARY, ARMED FORCES EPIDEMIOLOGICAL
BOARD

SUBJECT: Objective Analysis of Article "Antibodies to Squalene in Gulf War Syndrome"

I request that the Armed Forces Epidemiological Board (AFEB) convene a subcommittee and review and provide OASD(HA) with an objective analysis of the attached article, "Antibodies to Squalene in Gulf War Syndrome" published in the February 2000 issue of *Experimental and Molecular Pathology*. Congressman Jack Metcalf requested this objective analysis. Congressman Metcalf would also like the curriculum vitas of the reviewers.

OASD(HA) will provide Congressman Metcalf with this critique and the curriculum vitas of the reviewers when complete. Please provide this review NLT 15 May 2000. To assist in this review, I have attached an extensive review of the work on squalene as a cause of illnesses among Gulf War veterans by the interagency Research Working Group of the Persian Gulf Veterans Coordinating Board prior to publication of the article and previous correspondence with Congressman Metcalf's office on this topic.

My point of contact is James R. Riddle, LtCol, USAF, BSC, (703) 681-1703, fax (703) 681-3655, or email james.riddle@ha.osd.mil.

John F. Mazzuchi, Ph.D.
Deputy Assistant Secretary of Defense
Clinical and Program Policy

Attachments:
As Stated

