



DEPARTMENT OF DEFENSE
ARMED FORCES EPIDEMIOLOGICAL BOARD
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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.

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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

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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 Asa *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
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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

