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LETTERS TO THE EDITOR

To the Editor:

A recent article in this journal by Asa *et al.* (2000) purports to measure serum antibodies to squalene. The paper fails to establish the validity of the test. The essential flaws involve selection of proper positive controls and proper negative controls, quantitative methods, and selection of study populations.

The authors hypothesize that antibodies are induced by "the adjuvancy of squalene," such that injection of squalene could elicit antibodies to squalene. One approach might be to inject squalene into an experimental animal to determine *first* whether the injection can induce the purported antibodies and *second* whether the assay can detect the induced antibodies. Antibodies induced by injection, if they exist, could then serve as a positive control for the unvalidated assay.

The assay describes no positive controls that actually validate the assertion of detecting antibodies to squalene. Such positive controls would consist of comparable serum samples demonstrated to contain anti-squalene antibodies after injection with squalene.

The authors assert that they have positive controls, in the form of two human subjects previously injected with a squalene-containing placebo during a clinical trial at the National Institutes of Health. However, the authors provide no preinjection results to establish that intentional injection of squalene led to antibodies to a substance already present in the body.

The assay also lacks elementary negative controls routinely run in enzyme-linked immunoassays. Such negative controls are required to prove that the assay is not detecting cross-reacting substances. In a new, unproven assay that claims to detect a novel antibody, one must prove specificity. There were no negative controls in which the human serum containing the presumed antibodies was omitted or in which the avidin-conjugated horseradish peroxidase was omitted. There is no evidence that the assay was not simply measuring other IgG molecules with nonspecific binding to squalene. This could be easily accomplished by substituting an oil molecule similar to squalene. An excellent negative control would be squalane, the fully hydrogenated form of squalene.

The unknown human serum samples were tested only at a single dilution (1:400). Most assays for naturally occurring antibodies, particularly antibodies to lipids, start at a higher concentration of serum, typically a dilution of 1:50. Thus, the method of Asa *et al.* could miss the presence of antibodies detectable at a higher concentration of serum. It is possible that normal blood donors could give positive results at a higher concentration of serum.

A further drawback of using only a single dilution of serum, rather than a series of dilutions, is that there is no way to obtain a quantitative measure of the degree of activity in the sample. Titers are routinely obtained when antibody levels are measured. The absence of quantitation in this assay weakens meaningful comparisons between unknown serum samples from subjects accrued in a nonrandom manner.

Figure 1, said to show "antisqualene antibody responses," is particularly flawed. In this figure, unspecified quantities of squalene were added as aqueous dilutions of 1:10, 1:100, 1:1000 and 1:10,000 for impregnation of nitrocellulose. No explanation is provided for how an oil such as squalene, not soluble in water, could be diluted in water by the published methods. Further, a washing solution containing polyoxyethylene sorbitan monolaurate could have detergent-like qualities that could remove squalene. Despite the extensive dilutions of the squalene, there is no evidence of a dilution curve (assessing each strip vertically), regardless of whether the antibody reactions were rated as 3+, 2+, or 1+. This suggests that nonspecific binding of serum immunoglobulin may have occurred.

The conclusions of Asa and colleagues, purporting to correlate anti-squalene with Gulf War illnesses, in our opinion, rely on circular logic. Positive results with an assay not previously validated to detect antibodies cannot be used as scientific proof that antibodies to the antigen exist in samples of unknowns. It is premature to proceed directly to testing

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serum samples from healthy people and sick people before conducting the fundamental validation steps.

The critique offered here is not meant to imply that antibodies to squalene do not or cannot exist. As pointed out by the authors, extensive work demonstrates that antibodies to cholesterol, a molecule for which squalene serves as a precursor, are found in virtually all normal human sera. A recent report proposes that naturally occurring antibodies to cholesterol may serve a vital physiologic function in helping regulate low-density lipoprotein metabolism in humans (Alving and Wassef, 1999).

REFERENCES

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Reply

To the Editor:

Alving and Grabenstein declare that our methods "do not establish the validity of the test." They are mistaken and have made a number of false assumptions about our methods and about which experiments were and were not performed to validate the anti-squalene antibody (ASA) assay. We also strongly disagree that animal work must precede human studies.

Our study (1) is the first description of anti-squalene antibodies in humans. Replicating our results in an animal model may well be useful for studying the possible role of ASA in Gulf War Syndrome (GWS), but is not a prerequisite by any standards we (or the peer reviewers of our manuscript) are aware of for establishing the validity of an immunoassay. For example, it was not essential to demonstrate antinuclear antibodies (ANA) in animals to develop a useful ANA assay for human autoimmune disease. Moreover, there is no assurance that small animals or even primates would respond immunologically to a squalene challenge. Production of ASA may require coinjection with or coexposure to additional substances or an autoimmune process not readily reproduced in an animal model.

It would also be unethical to inject squalene, a substance that has a 25-year history of causing both autoimmune rheumatological disease and neurological disease (Lorentzen, 1999; Grajkowska *et al.*, 1999), into humans to see if we could raise antibodies to it.

The ASA assay, a variation on the well-characterized Western blot assay, was validated by standard approaches used in immunoassay development. Alving and Grabenstein assert that "the assay lacks negative controls." However, each of the "elementary" negative controls they suggested, as well as many other contols, was in fact performed. The descriptions of these simple tests were not included in our paper for brevity. Assays in which either human serum or avidin-conjugated horseradish peroxidase was omitted gave no reaction. It should be noted that the reagents we used are precisely the same stringently validated reagents used to detect human antibodies to human immunodeficiency virus in commercially available Western blot assays. Squalane, a molecule similar to squalene, also gave no reaction in this assay. Furthermore, preincubation of positive human sera with squalene (but not squalane or other oils) blocked the assay in a dose-dependent manner. Squalene did not block another immunoassay, the HIV Western blot, further confirming the validity of the ASA assay.

Alving and Grabenstein are incorrect in their assumption that "the samples were tested at only a single dilution." In the process of optimizing the ASA assay, samples were tested at varying dilutions between 1:25 and 1:4000. 1:400 was determined to be the optimal dilution.

We did not indicate that squalene was soluble in water. Squalene, like many oils, can be finely dispersed in water and diluted as indicated. Western blot-style immunoassays differ from other types of immunoassays. Titers are not routinely obtained in Western blot-style immunoassays. At lower serum dilutions, some normal donors do react on the

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