Immunization of Healthy Adult Subjects in the United States with Inactivated Mycobacterium vaccae Administered in a Three-Dose Series


Heat-killed Mycobacterium vaccae vaccine was administered in a three-dose intradermal schedule to 10 healthy adult volunteers at 0, 2, and 10 months. Local and systemic side effects were monitored and vaccine site reactions were measured and photographed at visits 2 days, 14 days, and 2 months after each dose. Reactions to skin tests with purified protein derivative (PPD) and Mycobacterium avium sensitin (MAS) and titers of antibody to arabinose lipoarabinomannin were determined at baseline and after each dose of vaccine. Lymphocyte proliferation responses to MAS were determined after the final dose of vaccine. Immunization was safe and well tolerated, with maximal induration (range, 6–25 mm) at 2 days. PPD skin test conversions did not occur. Seven subjects completed the three-dose schedule; preexisting immunologic responses to mycobacteria were boosted in three, and a new response was elicited in one. M. vaccae vaccine is safe and induces measurable immunologic responses to mycobacterial antigens in some healthy adults.

Several lines of evidence suggest that natural or vaccine-induced immunity may be effective in the prevention of infections due to Mycobacterium avium complex (MAC). In experimental animals, BCG vaccine affords partial protection against M. avium infection [1–3]. Epidemiological data from Sweden and Finland indicate that BCG immunization reduces the incidence of childhood cervical adenitis due to MAC [4, 5].

Our group has recently completed an epidemiological study of disseminated MAC infection in persons with AIDS; this study demonstrated that patients with extensive occupational exposure to soil or water (environmental sources of MAC and other nontuberculous mycobacteria) before the onset of HIV infection have a reduced risk of disseminated MAC infection in the late stage of AIDS [6]. Finally, a case-control study has shown that prior tuberculosis reduces the risk of subsequent M. avium infection in patients with AIDS [7].

Protection against disseminated MAC infection in patients with AIDS would require immunization before the development of profound immunosuppression. BCG vaccine, the only mycobacterial vaccine licensed in the United States, is a live vaccine and has itself been associated with disseminated infection in patients with AIDS [8, 9]. Mycobacterium vaccae vaccine is a heat-killed vaccine prepared from a rapidly growing environmental mycobacterium that expresses antigens common to many mycobacteria [10], and studies suggest that it produces beneficial immune responses to Mycobacterium tuberculosis and Mycobacterium leprae [11].

Because M. vaccae vaccine would be safe for immunocompromised patients and because it recently became available for investigational use, we initiated studies to investigate its potential role in the prevention of infections due to M. avium in patients with AIDS and to test methods for evaluating the effects of a potential vaccine against M. avium on skin test and in vitro markers of immunologic response. Although M. vaccae immunotherapy has been noted to be safe in other studies [12, 13], detailed observations have not been published on systemic and local reactions to a multiple-dose schedule, immunogenicity of a multiple-dose schedule, or whether a multiple-dose schedule leads to conversion of PPD skin tests. This report describes the results of a phase I study of the safety and immunogenicity of a three-dose schedule of M. vaccae immunization in healthy adult volunteers in the United States.

Methods

Vaccine preparation. The M. vaccae strain for the vaccine was isolated by Dr. John Stanford (University College and Middlesex School of Medicine, London) from mud near the Nile River in Uganda in 1971 [14]. The original isolate, designated R877, contains smooth and rough colonial variants; the
Table 1. Protocol for a study of Mycobacterium vaccae vaccine.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Dose 1 (0 month)</th>
<th>Dose 2 (2 months)</th>
<th>Dose 3 (10 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day -2</td>
<td>Day 0</td>
<td>Day 2</td>
</tr>
<tr>
<td>M. vaccae immunization</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Examination of vaccine site</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skin tests</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antibody studies</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lymphocyte proliferation assay</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NOTE. + = indicates when immunization or specified test or examination was done.

smooth variant produces rough variants at a low frequency, and the rough variant is morphologically stable. A rough colony, designated R877R, was selected and deposited with the National Centre for Type Cultures in London as NCTC 11659. Inactivated M. vaccae SRL 172 is prepared by growing strain NCTC 11659 on Sauton’s medium solidified with 1.5% agar. Bacteria are harvested during the logarithmic growth phase when they are suspended in borate buffered saline (pH, 8.0) at a concentration of 10 mg of wet weight/mL. This suspension is dispensed in 0.3-mL aliquots in 3-mL amber glass ampules and then is sterilized by autoclaving at 121°C for 15 minutes. The final product contains ~10^9 organisms/0.1 mL of borate buffered saline and is a turbid suspension containing small orange-colored particles.

Subjects and immunization protocol. The immunization protocol is summarized in table 1. Ten healthy adult volunteers were given three doses of inactivated M. vaccae vaccine (lot MV001) at 0 month (dose 1), 2 months (dose 2), and 10 months (dose 3) between June 1994 and May 1995. Dose 3 was added later in the study to satisfy requirements of the Food and Drug Administration for a parallel three-dose pediatric study. The vaccine was administered as a 0.1-mL intradermal injection over the deltoid muscle. Patients were given oral thermometers to record their temperatures daily for 14 days.

Patients returned for visits 2 days, 14 days, and 2 months after each dose of vaccine and were questioned about possible side effects. At each visit, the vaccine site was examined and photographed (photographs were obtained only at the 2-month visit for dose 3), and oral temperature and induration at the vaccine site were measured. Serum samples for antibody studies were drawn at baseline and at four other times. Blood specimens for the lymphocyte proliferation assay were obtained 7 weeks after dose 3.

Skin tests. Reagents for intradermal skin tests included 0.1 mL of M. avium sensitin (MAS;^1 MAS [serovar 2] PPD RS 10/2, 0.1 µg/0.1 mL; filling lots 61 and 62, Statens Seruminstitut, Copenhagen) and 0.1 mL of M. tuberculosis PPD (Connaught Laboratories, Willowdale, Ontario, Canada). MAS is a PPD from a strain of M. avium initially isolated from a patient in Denmark [15]. Skin tests were performed at baseline and at three other times, and reactions were read at 48 hours as millimeters of induration in the transverse diameter by an investigator blinded to the placement of the two antigens (table 1).

Controls for skin tests were 24 healthy medical students with negative reactions to MAS and PPD skin tests who were tested on two additional occasions 6–15 weeks apart. The MAS skin test was considered positive if the reaction size was ≥5 mm, and conversion was considered to have occurred if there was an increase in the reaction size of ≥15 mm. The PPD skin test was considered positive if the reaction size was ≥5 mm, and seroconversion was considered to have occurred if there was an increase in the reaction size of ≥10 mm.

Lymphocyte proliferation assay. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation with use of Ficoll-Hypaque Plus (Pharmacia, Uppsala, Sweden). PBMCs were washed twice with Hank’s balanced salt solution (Biowhitaker, Walkersville, MD) and were resuspended in Iscove’s modified Dulbecco’s medium (Mediatech, Herndon, VA) supplemented with 2 mM L-glutamine (GIBCO, Grand Island, NY) and 10% pooled human male serum (Lampire Biological Laboratories, Pipersville, PA). PBMCs were cultured in U-bottom 96-well microtiter plates (Costar, Cambridge, MA) in a final volume of 200 µL of media at a density of 1 x 10^5 cells per well.

Cells were incubated in triplicate with media alone, MAS (RS 10/2, 1 mg/mL; filling lot 33, Statens Seruminstitut), or phytohemagglutinin (Sigma, St. Louis) at 37°C in a 5% CO₂ atmosphere and 95% humidity for 96 hours and then were pulsed with 1 µCi of ³H-labeled thymidine (6.7 Ci/mmol) (ICN Pharmaceuticals, Costa Mesa, CA) per well for an additional 18–24 hours. Incorporation of ³H-labeled thymidine into harvested cells was determined by liquid scintillation spectrometry. Controls for lymphocyte proliferation assay were six healthy volunteers: three with reactions to MAS skin tests of ≥10 mm (positive controls) and three with reactions to MAS skin tests of 0 mm (negative controls).

Antibody studies. Responses of serum IgG and IgA antibodies to mycobacterial antigens were measured by ELISA.
Briefly, Immulon II wells (Dynatech Laboratories, Chantilly, VA) were coated with the following antigens: arabinose lipoarabinomannan (Ara-LAM, derived from a rapidly growing *Mycobacterium* species [16]; batch 15N, 0.1 μg per well) or Erdman (mannose) lipoparabinomannan (Man-LAM, derived from the Erdman strain of *M. tuberculosis*; batch 4P, 0.1 μg per well) (both Ara-LAM and Man-LAM were obtained from Dr. Patrick Brennan, Colorado State University, Fort Collins), MAS (1.25 μg per well: RS 10/2, 1 mg/mL; filling lot 33, Statens Seruminstitut), or a sonicate of *M. vaccae* NCTC 11659 (1.25 μg per well; provided by Dr. John Stanford). After blocking and washing, wells were incubated with threefold serial dilutions of test sera (starting with either a 1/1 or 1/100 dilution).

Following incubation with alkaline phosphatase–labeled goat antibody to human IgA (α-chain specific, diluted 1/500; Sigma) or goat antibody to human IgG (α-chain specific, diluted 1/1000; Sigma), wells were developed by the addition of p-nitrophenol phosphate (Sigma) and were read with use of an MRX microplate reader (Dynatech Laboratories). Following background subtraction, results were calculated as endpoint titrations by extrapolation of the linear portion of a curve obtained by plotting log10(1/dilution) vs. log10(absorbance at 405 nm). Controls for antibody studies included an immunocompetent patient with pulmonary MAC infection (positive control) and a healthy subject with a negative reaction to an MAS skin test (negative control).

### Results

**Subjects.** The study subjects were 10 healthy volunteers (six men and four women) who were aged 23–68 years (median age, 38 years). All subjects denied a history of illness associated with immunosuppression (including HIV infection). Only subject 6 had a history of BCG immunization (given at age 15 in the United Kingdom). This subject became pregnant after dose 1 and did not receive doses 2 and 3. Seven subjects completed the three-dose schedule.

**Systemic side effects.** After dose 1, one subject reported mild malaise, and one subject noted a sensation of fever (but a normal temperature was recorded); the remaining eight subjects had no systemic side effects or fever (one patient was unable to accurately record the temperature for dose 1). After dose 2, three of nine subjects reported mild systemic symptoms (headache, 1; malaise, 1; feverishness, 1), and none recorded above normal temperatures. After dose 3, one subject reported headache, and none recorded above normal temperatures. Electronic oral temperatures of all subjects at 2-day and 14-day study visits after doses 1–3 were normal.

**Local reactions.** After dose 1, five subjects reported minor arm soreness, and two of these subjects reported superficial skin breakdown and scant drainage at the vaccine site for 5–7 days; an additional subject reported minor pruritus at the vaccine site for 3–6 days. A small white papule was present at the vaccine site of five of 10 subjects at 14 days. A visible area of erythema (usually 2–3 mm in size without induration) was apparent at the vaccine site of seven of 10 subjects at 2 months and the vaccine site of five of nine subjects at 4 months.

After dose 2, two subjects reported minor arm soreness, one reported minor pruritus, and two noted superficial skin breakdown without drainage. A small white papule was present at the vaccine site of six of nine subjects at 14 days. A visible area of erythema (usually 2–3 mm in size without induration) was apparent at the vaccine site of three of nine subjects at 2 months.

After dose 3, two subjects reported minor arm soreness, and one noted superficial skin breakdown. A visible area of erythema (usually 2–3 mm in size without induration) was apparent at the vaccine site of three of nine subjects at 2 months. Induration was accompanied by erythema of similar magnitude.

**Skin test reactions.** Skin test reactions of all 10 subjects at baseline and 7–8 weeks after the last dose of vaccine are presented in table 2. Two subjects had reactions to PPD skin tests of ≥10 mm at baseline. Conversion of PPD skin tests did not occur, although subject 2 had an initial reaction size of 0 mm and a final reaction size of 6 mm (i.e., a final reaction size that would be considered positive for a person with HIV infection).

Three subjects had baseline reactions to MAS skin tests of ≥10 mm (including both subjects with reactions to PPD skin tests of ≥10 mm), and five subjects had baseline reactions to MAS skin tests of ≥5 mm (these subjects were subsequently considered to be *Mycobacterium*-positive). The reaction of one subject (no. 4) to the MAS skin test changed from 7 mm to 22 mm. Small increases in reactions to MAS skin tests occurred in four additional subjects.

The 24 controls with baseline reactions to MAS skin tests of 0 mm had mean reactions of 1.0 mm to a second MAS skin test and 3.3 mm to a third MAS skin test; conversion of the three MAS skin tests (increases in reaction sizes of ≥15 mm) occurred for two (8.3%) of the 24 controls.

**Antibody studies.** Responses of IgG antibody to Ara-LAM, MAS, and *M. vaccae* sonicate are presented in table 3. The titer of IgG to Ara-LAM was 3,193 for the negative control and 56,081 for the high positive control. For two subjects (nos. 2 and 4), baseline titers of IgG antibody to Ara-LAM were more than two times that for the negative control; these subjects were considered to be *Mycobacterium*-positive. For four subjects, titers of IgG to Ara-LAM increased two or more times after immunization. For both subjects with titers of IgG antibody to Ara-LAM that increased two or more times and who had serum samples drawn at both 2 weeks and 7 weeks after dose 3, titers were highest at 7 weeks after immunization.

For three *Mycobacterium*-positive subjects (determined by an antibody or skin test) with titers of IgG antibody to Ara-
Table 2. Reactions to skin tests and vaccination with *Mycobacterium vaccae* by number of doses of vaccine.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Age (y)</th>
<th>No. of doses received</th>
<th>Baseline PPD</th>
<th>Final PPD</th>
<th>Baseline MAS</th>
<th>Final MAS</th>
<th>Vaccine site reaction (mm of induration)</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
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<td>0</td>
<td>0</td>
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<td>2</td>
<td>23</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>11</td>
<td>18</td>
<td>85</td>
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<tr>
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<td>3</td>
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<td>0</td>
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<td>0</td>
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<td>4</td>
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<td>7</td>
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<td>6</td>
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</tr>
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<td>69</td>
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<td>12</td>
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<td>25</td>
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<td>0</td>
<td>5</td>
<td>9</td>
<td>4</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE. MAS = *Mycobacterium avium* sensitin; NA = not applicable.
* Drainage from vaccine site.

LAM that increased two or more times, the increases occurred after two doses in two subjects (nos. 7 and 10) and after one dose in one subject (no. 2). Titers of IgG to Man-LAM (not shown) and MAS did not increase two or more times in any of the 10 subjects. Titers of IgA antibody to Ara-LAM, Man-LAM, and MAS were uniformly negative.

**Lymphocyte proliferation responses.** Three negative controls had stimulation values of 4,377, 5,555, and 8,326 counts per minute (cpm) with stimulation indices of 4.41, 2.53, and 8.41, respectively. Three positive controls had stimulation values of 13,177, 13,333, and 25,623 cpm with stimulation indices of 5.99, 3.89, and 31.28, respectively. The lymphocyte proliferation responses of the immunized subjects are shown in table 3. Three subjects (nos. 4, 7, and 10) had stimulation values that were greater than those for the negative controls (i.e., >8,500 cpm); these subjects all had baseline reactions to MAS skin tests of >5 mm and were considered to be *Mycobacterium*-positive.

**Summary of responses.** Of the seven subjects who completed the three-dose series, three (nos. 4, 7, and 10) were *Mycobacterium*-positive at baseline, and all three of these subjects had skin test or in vitro responses consistent with immunologic boosting. One *Mycobacterium*-negative subject (no. 5) developed a positive antibody response. Three of the seven subjects who completed the three-dose series had no evidence of baseline or vaccine-induced immunologic responses to mycobacteria.

Table 3. Antibody and lymphocyte proliferation responses in 10 healthy adult subjects given a multiple-dose schedule for *Mycobacterium vaccae* vaccine.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>No. of doses received</th>
<th>Titer of IgG antibody to Ara-LAM</th>
<th>Ratio</th>
<th>Titer of IgG antibody to MAS</th>
<th>Ratio</th>
<th>Titer of IgG antibody to <em>M. vaccae</em> sonicate</th>
<th>Ratio</th>
<th>Control value (cpm)</th>
<th>Stimulation value (cpm)</th>
<th>Stimulation index</th>
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<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>3,936</td>
<td>1.97</td>
<td>1,599</td>
<td>.29</td>
<td>1,411</td>
<td>1.47</td>
<td>498</td>
<td>2,691</td>
<td>5.40</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>54,521</td>
<td>5.95</td>
<td>1,122</td>
<td>1.67</td>
<td>2,580</td>
<td>1.26</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>3</td>
<td>3</td>
<td>4,838</td>
<td>0.91</td>
<td>1,425</td>
<td>0.88</td>
<td>1,269</td>
<td>0.90</td>
<td>1,249</td>
<td>4,886</td>
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<tr>
<td>4</td>
<td>3</td>
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<tr>
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<td>931</td>
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<tr>
<td>7</td>
<td>3</td>
<td>6,267</td>
<td>1.94</td>
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<td>4,116</td>
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<tr>
<td>10</td>
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<td>1.08</td>
<td>2,468</td>
<td>10,714</td>
<td>4.34</td>
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</table>

NOTE. Ara-LAM = arabinose lipopolysaccharide; cpm = counts per minute; MAS = *Mycobacterium avium* sensitin; NA = not available.
* Determination after dose 2 only (NA after dose 3).
† Determination 2 weeks after the last dose.
Discussion

This study demonstrates that a three-dose intradermal schedule of inactivated *M. vaccae* vaccine is safe and well tolerated in healthy adult subjects. Local reactions were mild, with minimal tenderness, self-limited drainage, and/or skin breakdown in a few subjects, and scarring was minimal. Local reactions were more prominent in subjects with positive baseline reactions to MAS skin tests. The four subjects with reactions to MAS skin tests of ≥5 mm at baseline were the only subjects with vaccine site indurations of ≥10 mm, and drainage at the vaccine site occurred only in the three subjects who had reactions to MAS skin tests of ≥10 mm. Systemic adverse reactions were minimal. Further, immunization with *M. vaccae* did not lead to conversion of the PPD skin test.

The side effects of *M. vaccae* vaccine compare favorably with those observed with BCG immunization of healthy adults in the United States. BCG immunization causes local ulceration and drainage in most subjects, local pain in >50% of subjects, and seroconversion of the PPD skin test for at least 1 year for all subjects [17].

Determining the immunogenicity of a mycobacterial vaccine is a more challenging task. BCG vaccine–mediated protection against *M. tuberculosis* infection has never been shown to correlate with the new development of delayed-type hypersensitivity (DTH) to *M. tuberculosis* as measured by conversion of the PPD skin test [18, 19]. Although baseline DTH to nontuberculous mycobacteria is associated with protection against *M. tuberculosis* infection [20, 21], we are not aware of studies that have attempted to correlate conversion of skin tests for nontuberculous mycobacteria with the efficacy of BCG immunization. Attempts to assess the immunogenicity of BCG immunization must be considered in the context of our current understanding that the vaccine modifies the course of primary infection with *M. tuberculosis* (and perhaps reduces the risk of reactivation) but does not prevent it [22].

The purpose of a mycobacterial vaccine providing protection against disseminated MAC infection in patients with AIDS may be different than that of BCG vaccine against tuberculosis, and, consequently, the approaches to identifying in vitro correlates of response may also be different. Skin test studies with MAS suggest that as many as 30% of healthy subjects have had prior infection with MAC or other nontuberculous mycobacteria (C. F. von Reyn, unpublished data), but reactivation has not been shown to be a mechanism of MAC disease.

Epidemiological data suggest that disseminated MAC infection in AIDS is a newly acquired infection [23, 24]. Patients with occupational exposure to or infection with MAC before the acquisition of HIV infection may be protected against disseminated MAC infection during AIDS [6]. Thus, reasonable objectives for a vaccine against MAC infection in patients with AIDS may be both to prevent primary infection by simulating the effects of natural infection and to prevent reinfection by boosting the immune response in individuals with prior infection.

Prior infection can be identified either by measuring titers of antibody to mycobacteria or by testing cellular immune response. Cellular immune response can be assessed either with DTH (MAS skin testing) or the lymphocyte proliferation response to MAS. For our three subjects with positive reactions to MAS skin tests for whom lymphocyte proliferation assays were performed, there was a correlation between the two measures of cellular immune response.

The data presented here indicate that a three-dose series of *M. vaccae* vaccine meets these objectives for some healthy subjects. Of the seven subjects who completed the three-dose series, three had either DTH or antibody responses suggesting prior mycobacterial infection; responses were boosted in all three of these *Mycobacterium*-positive subjects. One subject without evidence of prior mycobacterial infection developed a significant antibody rise. Thus, four of seven subjects had measurable responses to a three-dose series. Three *Mycobacterium*-negative subjects had no response to three doses. Of the two subjects who received two doses, one (who was *Mycobacterium*-positive) demonstrated an antibody boost.

IgG antibody to Ara-LAM proved to be the most sensitive measure of humoral immune response to the protocol of *M. vaccae* vaccine used here; antibody to Man-LAM did not develop. These observations fit with available data indicating that lipoarabinomannan is the dominant cell wall polysaccharide in mycobacteria and is highly immunogenic. Ara-LAM is present in rapidly growing strains of mycobacteria (and would be expected to be present in *M. vaccae*), while Man-LAM is present in organisms of the *M. tuberculosis* complex (e.g., *Mycobacterium bovis* and the Erdman strain of *M. tuberculosis*) [25]. Antibody to MAS, which is composed of secreted proteins that are denatured during processing, did not develop [26].

The protocol used in the present study employed three intradermal doses of *M. vaccae* vaccine and as many as five intradermal skin tests with MAS. Since repeated skin testing with tuberculin may cause an increase in the reaction size for subjects with prior mycobacterial exposure or immunization [27], it is possible that some of the boosting of cellular or humoral immune responses observed in this study was due to the MAS skin test itself. However, the fact that antibody to MAS did not develop in the study subjects suggests that the skin test itself did not contribute to the humoral immune responses.

The present results indicate that *M. vaccae* vaccine has the potential to stimulate an immunologic response to mycobacteria and suggest that future protocols should test *Mycobacterium*-negative subjects with an expanded schedule (e.g., five doses) and *Mycobacterium*-positive subjects with a reduced schedule (e.g., two doses). On the basis of the time course of the appearance of antibody in the present study and the observation that DTH to mycobacterial antigens may take 8–12 weeks to develop after sensitization, dose intervals of 2–3 months appear to be optimal.
Acknowledgments

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