INFECTION AND IMMUNITY, Oct. 1978. p. 87-93
0019-9567/78/0022-0087$02.00/0

Vol. 22, No. 1
Printed in U.S.A.

Heat Stability of *Mycobacterium leprae* Immunogenicity

CHARLES C. SHEPARD,* LAURA L. WALKER, AND ROSALIND VAN LANDINGHAM
Center for Disease Control, Atlanta, Georgia 30333

Received for publication 10 April 1978

The protection provided to mice by vaccines administered intradermally was measured after footpad challenge with *Mycobacterium leprae*. The protection offered by *M. leprae* suspensions was not decreased when the vaccines were killed by 60°C heat or at the higher temperatures tested, which included 215°C (autoclave). Even highly purified suspensions retained their immunogenicity. In contrast, the vaccine protection provided by intradermal *M. bovis* (strain BCG) was markedly reduced when heated to 60°C. The enlargement of the lymph nodes regional to the intradermal vaccines was measured and found generally to parallel the vaccine protection provided by *M. leprae* and by BCG.

In an earlier experiment, we found that the vaccine protection provided by *Mycobacterium leprae* underwent a slight but statistically significant increase when its viability was reduced by freezing and thawing (8). This finding was contrary to expectation; it has been found repeatedly that the immunogenicity of attenuated tubercle bacilli, including the BCG strain of *M. bovis*, is much reduced when the organisms lose viability. In fact, the general experience with bacteria has been that living organisms are much more capable of stimulating cell-mediated immunity than are an equal number of dead organisms. The question of the immunogenicity of dead *M. leprae* is an important one, because the development of an avirulent mutant, analogous to the BCG strains of *M. bovis* used for vaccine against tuberculosis, appears to be possible only if *M. leprae* can be grown on artificial medium, or perhaps in tissue culture. Indeed, the general strategy in recent efforts to develop an antileprosy vaccine is based on two other approaches: one, the use of (nonliving) *M. leprae* antigen in combination with an adjuvant, perhaps BCG, and the other, the use of a cultivable mycobacterium, probably still to be found, with the appropriate antigenic relatedness to *M. leprae* (1).

Accordingly, we have followed up the lead with freeze-thawed *M. leprae* in further experiments and have found that the immunogenicity of *M. leprae* is not decreased during heating, even by autoclaving. A condensed version of the findings has been presented (7).

MATERIALS AND METHODS

Groups of 30 mice were vaccinated 28 days before footpad challenge with 5,000 *M. leprae* in mouse passage. After challenge, the growth curve was monitored in unvaccinated controls, and when it reached plateau levels (usually above $10^{6.0}$ *M. leprae* per mouse), eight mice were taken from each group for counts of acid-fast bacteria in the challenged footpads of individual animals. The harvests were repeated 90 days later. In each experiment there were two unvaccinated control groups, one at the beginning and one at the end of the experiment, but in the figures and statistical analyses, the controls were treated as a single group of 16. The complete methods have been described (5, 6, 11).

The mice were of the CFW strain raised at the Center for Disease Control. The *M. leprae* for the vaccines came from infected armadillo livers (2). Some feral armadillos have been found to be infected with a noncultivable mycobacterium which cannot be differentiated from *M. leprae* from human tissues by any procedure yet tried (3). This noncultivable mycobacterium from feral armadillos appears to be completely identical with *M. leprae* from human tissues in lepromin tests in leprosy patients in that it gives negative Mitsuda reactions in lepromatous patients and positive Mitsuda reactions in tuberculoid patients (3); no other mycobacterium has been found that will give these results in spite of extensive search. Because it has not been possible to establish a line of armadillo (*Dasypus novemcinctus*) breeding in captivity, experimental inoculations must be made into wild-caught armadillos. The following measures were followed for the present supply. The armadillos were collected from areas not known to have natural infections. Initially, ear snips and buffy coats of peripheral blood were examined for acid-fast bacteria; suspicious lesions were also examined. Such examinations were continued after inoculation, and if any animals had shown signs of infection in the first few months after receipt they would have been discarded. When the animal developed a full-blown infection typical of the experimental disease, it was killed and the tissues were harvested aseptically. Cultures on mycobacterial media revealed no cultivable mycobacteria. Inoculations into the footpads of mice were carried out, and the resulting infections were typical of those produced by *M. leprae* from human tissues; no other mycobacterium has been found that behaves in this fashion. Some have suggested a quarantine period for wild-caught armadillos; the incubation period of the natural

infection is unknown, however. It may be very long, because some armadillos with large doses of *M. leprae* from human tissue have taken several years to develop detectable infections. One must recognize that there is no procedure that can be followed that will absolutely rule out a preexisting natural infection in experimentally inoculated, wild-caught armadillos. Probably the predominant view now is that the organism causing the infection in wild armadillos is *M. leprae*; we believe, however, that we should continue to follow procedures that will permit us to define as much as possible the source of the organisms harvested from the armadillo.

Unless otherwise stated, the *M. leprae* were partially purified by centrifugal washing and mild trypsin treatment (0.125% trypsin of a 1:250 purity for 5 min at 37°C at pH 7.6; 10). The BCG vaccine was a suspension prepared by centrifugal washing of young (12-day) cultures grown in Tween-albumin medium; the final suspension, in phosphate-buffered saline and 0.05% Tween 80 (PBS-Tw), was preserved at −60°C. The dose of *M. leprae* or BCG vaccine was usually 10^7 acid-fast bacilli per mouse. The duration of the heat treatment was 30 min except for autoclaving, which was limited to 15 min. Treatment with 2.0% phenol lasted for 16 h at 37°C, followed by washing and resuspension two times in PBS-Tw. The vaccines were administered intradermally in the right flank (12) or the footpad. After intradermal vaccination, the size of the regional (inguinal) lymph node in the intact animal was measured, usually at 28-day intervals (12). These measurements were carried out on the first 10 animals in the group. The two diameters recorded were averaged for presentation in the figures and for statistical analysis. The statistical significance of the differences between groups, both for harvests of *M. leprae* and for measurements of lymph node size, was assessed by the two-sample rank test.

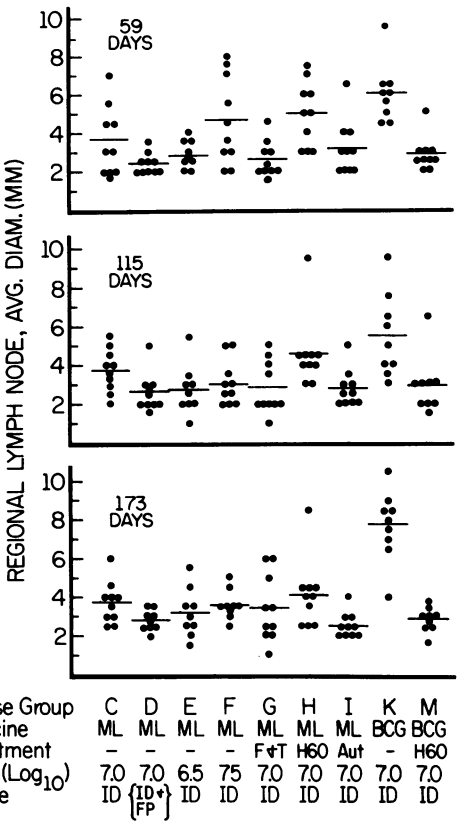

FIG. 1. *Size of regional (inguinal) lymph nodes in experiment 1 at different times after intradermal vaccinations. In each column of individual values, the horizontal bar indicates the arithmetic mean. Abbreviations for this and succeeding figures: ML, M. leprae; F&T frozen and thawed; H60, heated to 60°C for 30 min; Aut, autoclaved at 15 lb/in² for 15 min; ID, intradermal; FP, footpad.*

RESULTS

Experiment 1. The *M. leprae* for the vaccine came from an infected armadillo that had been recently killed. Bacillary viability was tested on the day of vaccination by standard footpad inoculation of mice with 5,000 *M. leprae*. The observed 5-month incubation period and average generation time of 29.7 days from incubation to harvest indicated that the proportion of viable organisms was in the range observed with human skin biopsy and mouse passage material. Groups were included to study the effects of route of vaccine administration, vaccine dosage, and inactivation of the bacilli. The diluent in all cases was PBS-Tw.

The size of the inguinal lymph nodes draining the vaccine site is shown for representative intervals in Fig. 1. On the basis of the calculated *P* values, the following conclusions could be drawn. There was a suggestion that the simultaneous footpad injection reduced the intradermal response to *M. leprae* (C versus D; $P > 0.05$, 0.02, and 0.05 at the three consecutive intervals, respectively). The variation with dosage of vaccine was not significant (C versus E versus F). The change in *M. leprae* after being frozen and thawed was not significant (G versus C). Heating to 60°C did not decrease the response to *M. leprae* (H versus C), but it did decrease that to BCG (M versus K; *P*, <0.001, 0.001 to 0.01, and <0.001). The lymph node response to the autoclaved *M. leprae* was less than that to the *M. leprae* heated to 60°C (I versus H; *P*, 0.02 to 0.05, 0.02 to 0.002, and 0.02 to 0.002).

The harvests of *M. leprae* from the various groups are shown in Fig. 2. The following conclusions could be drawn. (i) Comparison with the control group (A) showed that all vaccines afforded significant protection, although that provided by the frozen and thawed *M. leprae* (group G) was of questionable significance at the first harvest ($P = 0.05$ to 0.25). The *P* values were less than 0.001 in all other cases except for

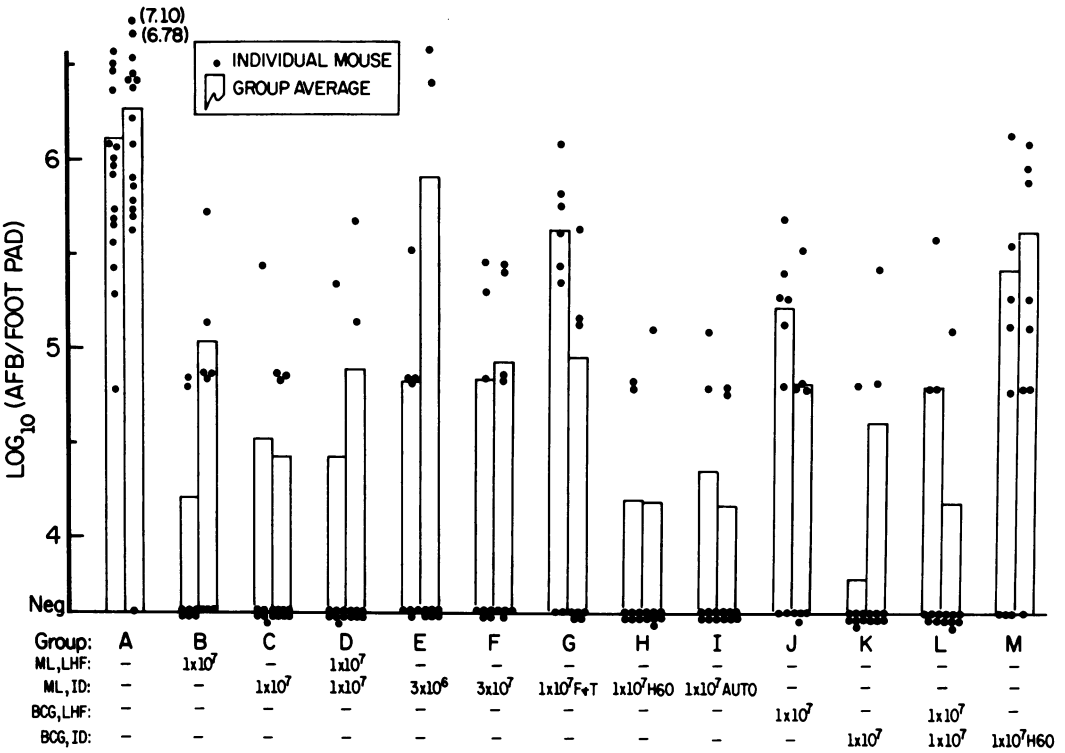

FIG. 2. *Harvests of M. leprae in experiment 1. For each vaccine, the left column gives the results at 151 days and the right gives the results at 241 days. The growth curve of M. leprae (in $\log_{10}$) in the controls was as follows: 59 days, <4.18; 81 days, 4.90; 118 days, 5.68; 137 days, 6.05. LHF, Left hind footpad.*

heat-killed BCG (group M) in the first harvest ($P = 0.001$ to 0.01) and second harvest ($P = 0.01$ to 0.025) and for the lowest dose of *M. leprae* (group E) in the second harvest ($P = 0.01$ to 0.025). (ii) A comparison of routes of administration showed no superiority of the intradermal over the footpad route for *M. leprae* (B versus C) or for BCG (J versus K) in this experiment. (iii) The groups receiving vaccine by both routes were included to learn whether the lesser effectiveness of the footpad route that was seen earlier was the result of an inhibition. This time, however, the protection provided by the individual routes was comparable, and that afforded by the combined routes was comparable to that provided by the individual routes for *M. leprae* (D versus B or C) or for BCG (L versus J or K). (iv) There was no evidence of a dose response with the three doses of *M. leprae* chosen (E versus C versus F). (v) This time, freezing and thawing *M. leprae* reduced its protection somewhat (G versus C; $P = 0.012$ in the first harvest and $P > 0.10$ in the second harvest). (vi) The vaccine effectiveness of *M. leprae* was not reduced, however, when it was killed by heat at 60°C for 30 min (H versus C) or by autoclave (I versus C). (vii) In contrast, the effectiveness of BCG was significantly reduced when it was killed by heat (M versus K; $P = 0.005$ in the second harvest).

In general, there was agreement between the lymph node enlargements and vaccine protection tests, with the possible exception of the two heated *M. leprae* preparations (H and I). Although the lymph node enlargements were less in group I, the protection was the same in groups H and I.

Experiment 2. This experiment was a follow-up of experiment 1. Some groups were included to clarify the reasons for the minor discrepancies between that experiment and the one previously reported (8); specifically there had been increased immunogenicity of *M. leprae* after freezing and thawing in the earlier experiment, in which the diluent was Hanks balanced salt solution with 0.1% bovine serum albumin (Hanks-BSA), but not in experiment 1 of this paper, in which the diluent was PBS-Tw. The *M. leprae* for the vaccine again came from an infected armadillo liver, the mice being vaccinated 4 days after the armadillo was killed. Mouse footpad inoculations were carried out to test for the

presence of viable *M. leprae* in the various vaccines. The starting material in PBS-Tw was fully viable, but no viable *M. leprae* could be detected in the vaccines with the possible exception of that frozen and thawed in Hanks-BSA (group D), in which the result indicated the possible presence of very vew viable *M. leprae*.

The lymph node measurements (Fig. 3) indicate the following. All the vaccines caused highly significant lymph node enlargement. The immunogenicity of *M. leprae* in PBS-Tw was not changed by freezing and thawing or by heating at 60, 80, or 100°C or at autoclave temperatures (recorded as 122 to 127°C). Hanks-BSA was a more favorable medium than PBS-Tw for *M. leprae* heated to 60°C (E versus F; *P*, 0.002 to 0.02, 0.002 to 0.02, and 0.002 for the three consecutive intervals shown, respectively). The influence of the medium on the frozen and thawed *M. leprae* was not significant (C versus D). Phenol inactivation did not reduce the activity of *M. leprae* (B versus J). With BCG, the immunogenicity was much reduced by 60°C heat (L versus M; *P*, 0.001 to 0.01, 0.01 to 0.025, and 0.001 to 0.01).

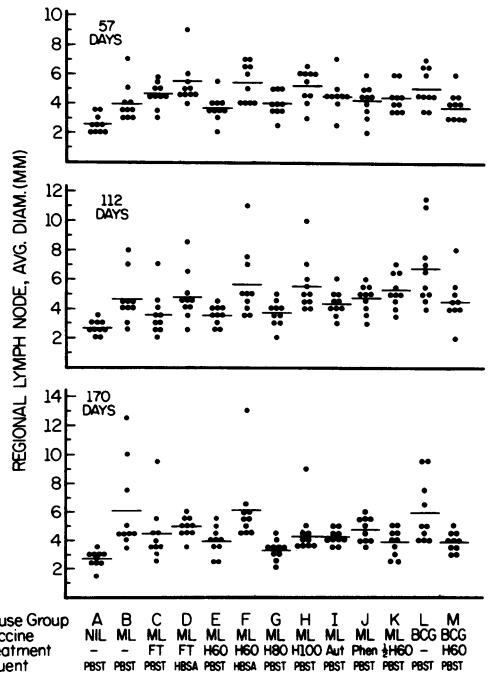

FIG. 3. *Size of regional (inguinal) lymph nodes in experiment 2 at different times after vaccination. Abbreviations: H80, Heated to 80°C for 30 min; H100, heated to 100°C for 30 min; Phen, treated with phenol; 1/2 H60, 1 volume of the suspension heated to 60°C plus 1 volume of unheated suspension; PBST, PBS with 0.05% Tween 80; HBSA, Hanks balanced salt solution with 0.1% bovine serum albumin.*

The results with the harvest of *M. leprae* (Fig. 4) lead to the following conclusions. Most of the vaccines provided highly significant protection ($P < 0.001$). Less significant protection was provided by the *M. leprae* frozen and thawed in PBS-Tw (C versus A; $P = 0.001$ to 0.01) and *M. leprae* autoclaved in PBS-Tw (I versus A; $P = 0.001$ to 0.01), however, and no protection was afforded by the heat-inactivated BCG (M versus A). The immunogenicity of *M. leprae* in PBS-Tw was not changed by freezing and thawing, by heat at 60, 80, or 100°C, by autoclaving, or by phenol treatment. The apparently increased protection provided as a result of suspension in Hanks-BSA was not statistically significant for freezing and thawing (C versus D) or for 60°C heat (E versus F). The apparently reduced protection with *M. leprae* heated to 80°C was not statistically significant (G versus E and H), nor was the apparently poorer result with autoclaving in PBS-Tw (I versus F and H). Heat inactivation of BCG caused a significant loss of vaccine protection (M versus L; $P = 0.007$).

Experiment 3. The *M. leprae* came from the same preparation as that used in experiment 2, but the mice were vaccinated 12 days after the armadillo was killed. As stated, the *M. leprae* used for vaccine were partially purified by the trypsin procedure; one portion was then more highly purified by the two-phase polyethylene glycol-dextran system, applied to *M. leprae* suspensions by Draper (4). A portion of each preparation was heated to 60°C. The diluent was PBS-Tw in all preparations.

The lymph node measurements are shown in Fig. 5. All the vaccines produced lymph node enlargements. Differences among the vaccines were no more than questionably significant.

The results with the harvests appear in Fig. 6. All the vaccines gave highly significant protection ($P < 0.001$), and there was no significant difference among the vaccines. The more highly purified preparation was fully protective after heating.

DISCUSSION

The observed lymph node enlargements presumably depend on (i) the stimulation and continued presence of cellular immunity and (ii) the focal persistence of antigen either in the lymph node or at the vaccine site with continued feeding of antigen to the lymph node. These two factors, continued cellular immunity and focal persistence of antigen, are the essential requirements for hypersensitivity granulomas. Preliminary histological observations showed that the increase in the lymph node size was attributable to increased numbers of lymphocytes, chiefly in

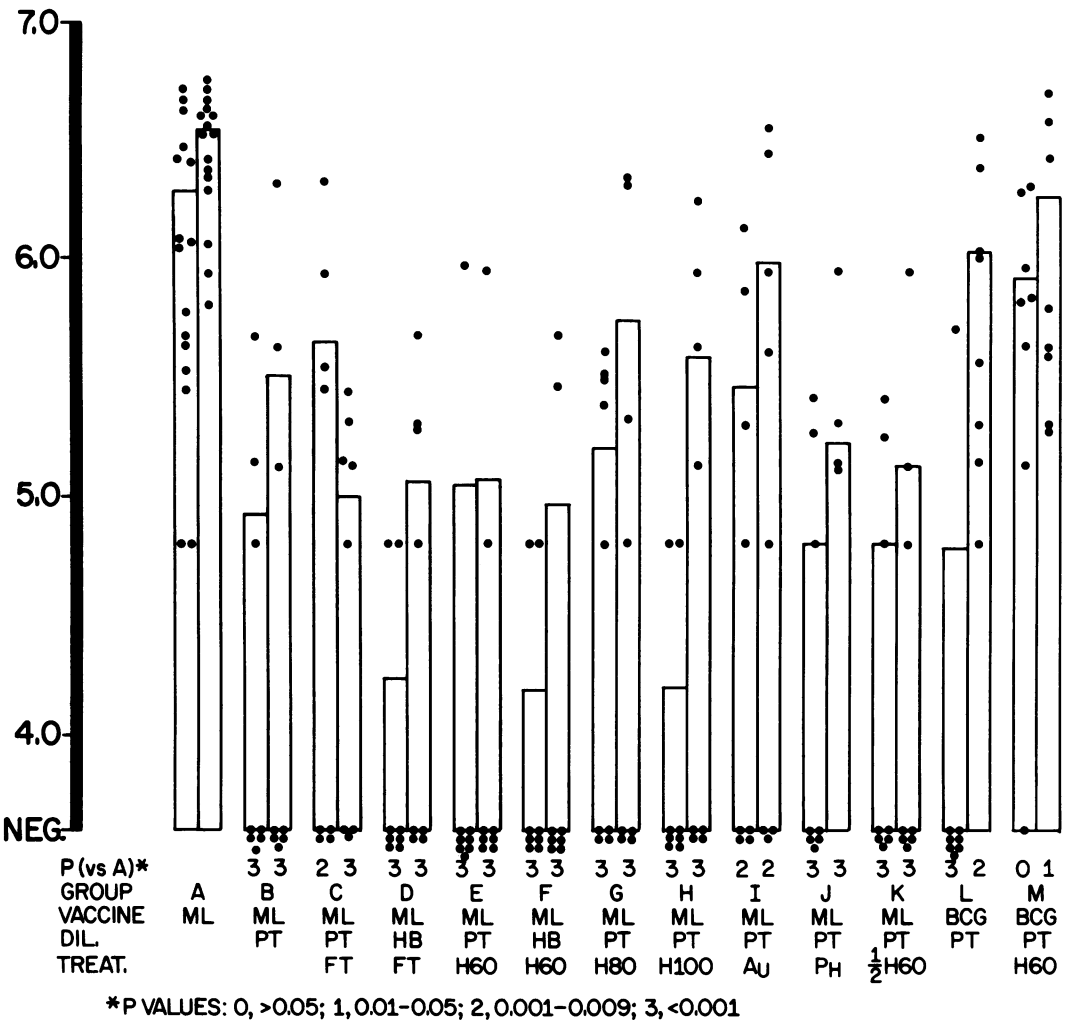

FIG. 4. *Harvests of M. leprae in experiment 2. For each vaccine the left column gives the results at 238 days and the right gives the results at 328 days. The growth curve of M. leprae (in $\log_{10}$) in the controls was as follows: 56 days, <4.11; 96 days, 4.65; 112 days, <4.18; 161 days, 5.46; 189 days, 5.06; 227 days, 6.01. Abbreviations: PT, PBS with 0.05% Tween; HB, Hanks balanced salt solution with 0.1% bovine serum albumin.*

the paracortical areas, with occasional islands of macrophages. There was no necrosis and no increase in cortical follicular activity.

Concerning the specificity of the sensitivity, one can say only that it was directed toward the persisting antigen. There is considerable cross-reactivity among mycobacteria, however, and it may be that other mycobacteria capable of causing such chronic lymph node enlargements can also provide protection against *M. leprae* challenge. In our searches among cultivable mycobacteria, we have not yet encountered, after intradermal injection, a culture other than BCG that is capable of causing distinct lymph node enlargement or of providing protection against *M. leprae* challenge. This relationship is shown in Table 1.

This parallelism with lymph node enlargement supports the tenet that the vaccine protection is based on cellular immunity rather than on some nonspecific effect of BCG. This supposition is also supported by observations of footpad enlargements occurring after footpad injections of suspensions of *M. leprae*; *M. leprae*'s activity both as stimulating and as eliciting antigen is maintained or even increased by heating (9). After mechanical disruption, however, *M. leprae* loses its immunizing activity but maintains its activity as an eliciting antigen (9).

It seems unlikely that contaminating host tis-

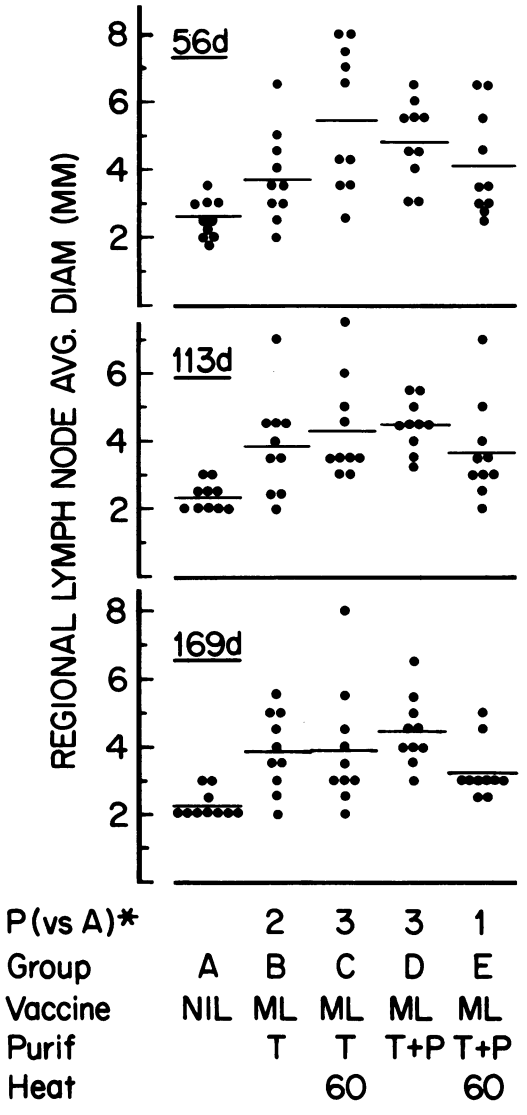

FIG. 5. *Size of regional (inguinal) lymph nodes in experiment 3 at different times after vaccination. Abbreviations: T, Partially purified by the trypsin procedure; T+P, partially purified by the trypsin procedure and then further purified by the two-phase polymer procedure; 60, heated to 60°C for 30 min. P values indicated as in Fig. 4.*

sue is responsible for the antigenic activity of the suspensions of *M. leprae*. Suspensions of normal armadillo tissue are not active as immunizing or eliciting antigens in footpad enlargement tests, and as immunizing antigens they do not produce detectable regional lymph node enlargement. Moreover, the antigenic activity of the suspensions of *M. leprae* is not decreased by high degrees of purification. As mentioned, procedures achieving disruption of *M. leprae* destroy its immunogenicity.

The results show that, unlike BCG and most bacterial pathogens, *M. leprae*, when killed, does

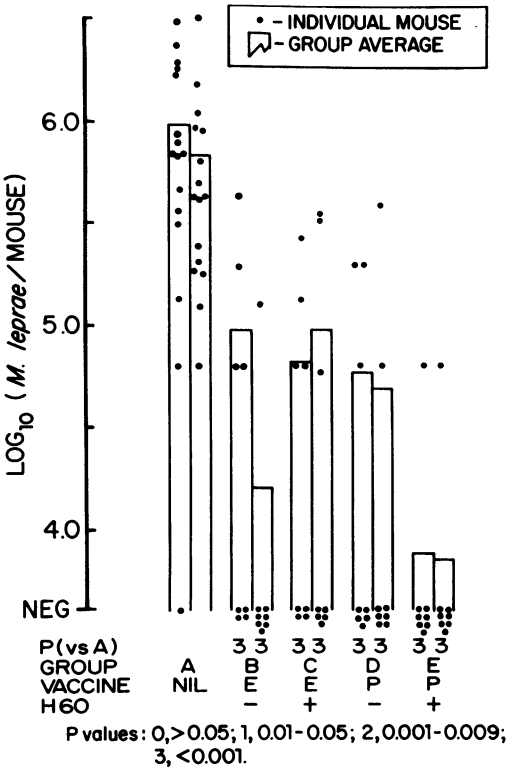

FIG. 6. *Harvests of M. leprae in experiment 3. For each vaccine, the left column gives the results at 189 days and the right gives the results at 279 days. The growth curve of M. leprae (in $\log_{10}$) in the controls was as follows: 43 days, <4.11; 58 days, 4.11; 90 days, 4.18; 113 days, 5.24; 154 days, 5.79; 182 days, 5.88. Abbreviations: E, Partially purified by the trypsin procedure; P, partially purified by the trypsin procedure and then further purified by the two-phase polymer procedure.*

TABLE 1. *Regional lymph node enlargement and vaccine protection stimulated by intradermal injection of mycobacterial suspensions*

Mycobacterial suspension	Regional lymph node enlargement	Vaccine protection
M. leprae, living	+	+
M. leprae, heat killed	+	+
BCG, living	+	+
BCG, heat killed	−	−
11 other mycobacterial cultures, living[a]	−	−

[a] From reference 11 and unpublished data.

not suffer a loss in ability to stimulate cell-mediated immunity. Three factors appear to be involved. (i) Heat stability of the antigens and the adjuvant activity of *M. leprae*. (ii) Durability in the tissues of dead *M. leprae*. In unpublished work, we have observed in mice that the regional lymph node enlargement that follows intradermal injection of *M. leprae* lasts at least a year. This factor is also reflected in the long persistence of *M. leprae* in the skin of leprosy patients, even those on the tuberculoid end of the scale, after effective chemotherapy (collaborative effort of the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program and the Leonard Wood Memorial; unpublished results of a chemotherapeutic trial in paucibacillary leprosy). All this indicates that *M. leprae* remains immunogenic after it is killed because it can persist in the tissues and that other bacteria and mycobacteria, including BCG, lose immunogenicity when they are killed because they lose the ability to persist in the tissues. Although RNA preparations from tubercle bacilli provide protection against *M. tuberculosis*, they do not provide protection against *M. leprae* challenge (13). (iii) An additional factor might be the agglutination of *M. leprae* that occurs consistently when a suspension is heated. The moderate increase in immunogenicity usually seen after heating may be the result of greater local retention of antigen.

The relevance of the results to possible *M. leprae* vaccines for man is obvious. *M. leprae* maintains its immunogenicity well even when heated at temperatures high enough to ensure that no *M. leprae*, or other living agent that might contaminate the suspensions, can survive. Moreover, a high degree of purification seems not to be detrimental to the immunogenicity.

Considerable experience with the intradermal injection of autoclaved suspensions of *M. leprae* is available because of the widespread use of integral lepromin (lepromin containing intact *M. leprae*) in patients and in normal persons. This material consists of a filtered suspension of *M. leprae*-containing autoclaved tissues from lepromatous patients, and the human skin test dose contains about one-fifth that used here to immunize mice (11). The dose of lepromin used in man is selected to show a high rate of Mitsuda reactivity in tuberculoid patients and normal persons and an absence of reactivity in lepromatous patients. Because the proportion of positive Mitusda reactions increases somewhat on repeated testing, it is widely accepted that lepromin tests are sometimes immunizing. It is possible, however, to consider the positive reaction itself as a visualization of a subject's primary or secondary immune response, that is, that the positive reactors consist of two groups: those who had no previous cellular immunity to *M. leprae* and are developing it for the first time, and those who had previous cellular immunity to *M. leprae* and are undergoing a secondary immune response. By this view, negative reactors would be those who are incapable of developing cellular immunity by 4-weeks, the time the test is usually read.

ACKNOWLEDGMENTS

This work was partly supported by the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases (NIAID), by means of an interagency agreement between NIAID and the Center for Disease Control, and by the World Health Organization through its IMMLEP Program. The infected armadillo livers were kindly supplied by W. F. Kirchheimer through the IMMLEP Program.

LITERATURE CITED

1. **IMMLEP Project Group.** 1974. Report of the first meeting of the IMMLEP Project Group (4–8 November 1974). WHO Document. IMM/74.3. World Health Organization, Geneva, Switzerland.
2. **Kirchheimer, W. F., and E. E. Storrs.** 1971. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39**:693–702.
3. **Meyers, W. M., G. P. Walsh, E. E. Storrs, and C. H. Binford.** 1976. Indigenous leprosy-like diseases in armadillos in Louisiana. Int. J. Lepr. **44**:540–542.
4. **Rees, R. J. W.** 1976. The WHO program for research on immunology of leprosy (IMMLEP). B. Some areas of scientific progress. Int. J. Lepr. **44**:280–283.
5. **Shepard, C. C.** 1960. The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J. Exp. Med. **112**:445–454.
6. **Shepard, C. C.** 1964. Vaccination against experimental infection with *Mycobacterium leprae*. Am. J. Epidemiol. **81**:150–163.
7. **Shepard, C. C.** 1976. Heat stability of *M. leprae's* immunogenicity. Int. J. Lepr. **44**:554.
8. **Shepard, C. C.** 1976. Vaccination of mice against *M. leprae* infection. Int. J. Lepr. **44**:222–226.
9. **Shepard, C. C.** 1978. The immunogenic competence of killed *M. leprae*. Int. J. Lepr. **46**:118–119.
10. **Shepard, C. C., and R. S. Guinto.** 1963. Immunological identification of foot-pad isolates as *Mycobacterium leprae* by lepromin reactivity in leprosy patients. J. Exp. Med. **118**:195–204.
11. **Shepard, C. C., and D. H. McRae.** 1968. A method for counting acid-fast bacteria. Int. J. Lepr. **36**:78–82.
12. **Shepard, C. C., R. Van Landingham, and L. L. Walker.** 1976. Immunity to *Mycobacterium leprae* infections in mice stimulated by *M. leprae*, BCG, and graft-versus-host reactions. Infect. Immun. **14**:919–928.
13. **Shepard, C. C., A. Y. Youmans, and G. P. Youmans.** 1977. Lack of protection afforded by ribonucleic acid preparations from *Mycobacterium tuberculosis* against *Mycobacterium leprae* infections in mice. Infect. Immun. **15**:733–736.