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Evaluation of *Mycobacterium leprae* Immunogenicity Via Adoptive Transfer Studies

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The immune response of mice to live, heat-killed, or autoclaved *Mycobacterium leprae* was investigated. After sensitization with 10^7 organisms in each group, recipient mice were transfused with the sensitized splenocytes 28 days later. A selected number of these mice were infected with 5×10^3 *M. leprae*, and the remaining animals were sacrificed at scheduled intervals for evidence of cell-mediated immunity to the *M. leprae* cell extract. Data from these and the bacteriological assays showed that all three materials induce cell-mediated immunity and also extend protection against the *M. leprae* challenge but not against a *Listeria monocytogenes* challenge. Adoptive immunity against *M. leprae* was expressed equally effectively in both non-irradiated animals and those sublethally (500 R) irradiated. This study reveals that, after adoptive transfer of immunity, a bacillary restriction occurs with concomitant onset of delayed hypersensitivity and that the protection observed could be specifically directed against an *M. leprae* challenge.

In the search for a leprosy vaccine, a general approach taken by Shepard is the use of *Mycobacterium leprae* itself (13, 15). This approach has resulted in establishing the fact that *M. leprae* has immunoprotective qualities whether it is administered as a viable or as a nonviable vaccine. Immunity is manifested as the restriction or prevention of multiplication of the organisms in an inoculum of 5,000 *M. leprae* in the footpads of mice.

The purpose of this study was to assess acquired immunity to *M. leprae* via adoptive transfer studies. Lymphocyte transformation and delayed hypersensitivity, indices of cell-mediated immunity, were used to measure transfer of sensitivity of lymphocytes sensitized to various *M. leprae* preparations, either viable, killed by heat, or autoclaved. Transfer of protective immunity, specific as well as nonspecific, was also assessed.

MATERIALS AND METHODS

Experimental animals. Six- to eight-week-old female BALB/c mice purchased from Harlan Sprague-Dawley, Inc., Indianapolis, Ind., were used throughout the studies.

Immunization of donors. In the cell-mediated immunity kinetics studies, four groups of mice were studied. One group consisted of animals inoculated in the right hind footpad with 10^7 viable *M. leprae*. A second group was inoculated with 10^7 *M. leprae* killed by exposure to heat (60°C) for 30 min. A third group consisted of animals inoculated with 10^7 *M. leprae* killed by autoclaving. The fourth group consisted of normal, uninoculated mice.

Cell transfer. Twenty-eight days after immunization, donor mice were killed by cervical fracture, and their spleens were removed aseptically. The splenic cells were teased into sterile Hanks tissue culture medium with sterile, blunt forceps. The resulting suspension was passed through sterile 20- to 27-gauge needles to obtain a homogeneous single cell suspension. The suspension was centrifuged in the cold at $600 \times g$ for 5 min, and the sediment was resuspended in fresh, sterile Hanks tissue culture medium to obtain the desired concentration. Before transfer, one group of recipients was sublethally irradiated by total body exposure to 500 rads from a ^{137}Cs source. All lymphoid cell recipients were injected intravenously with 10^8 cells per animal.

***M. leprae* infection.** The spleen cell recipients reserved for infection were injected via the left hind footpad with 5×10^3 *M. leprae* in 0.02 ml of phosphate-buffered saline. Bacterial assays were performed at 120 days and are presented according to the method described by Shepard (14). When these studies were repeated later, duplicate footpads were sent to A. M. Dhople, Florida Institute of Technology, Melbourne, for bacterial assay to ensure the validity of our observations.

Lymphocyte transformation. Five mice in each group were sacrificed at predetermined intervals, and their spleens were processed to obtain a final cell pellet, which was resuspended at a concentration of 10^6 cells per ml of RPMI 1640 tissue culture medium containing 100 U of penicillin and 100 μg of streptomycin per ml of medium supplemented with 0.3% glutamine and 10% heat-inactivated fetal calf serum (Difco Laboratories, Detroit, Mich.). All cultures were incubated in triplicate at 37°C in a 5% CO_2 in air mixture after the addition of sonicated *M. leprae* cell extract (25 μg of protein per ml) and phytohemagglutinin (10 μl/ml). All tubes were cultured for 96 h after pulse-labeling with 0.1 μCi of tritiated thymidine (specific activity, 1.9 Ci/mmol; Schwarzmann) in 0.1 ml of Hanks balanced salt solution approximately 20 h before culture termination. Cells were then washed twice in ice-cold 0.15 M NaCl and precipitated twice in cold 5% (wt/vol) trichloroacetic acid for 10 min. The final trichloroacetic acid-insoluble precipitates were dried overnight at room temperature and dissolved in 0.4 ml of Hyamine hydroxide. The dissolved material was transferred with 2 ml of absolute ethanol to 5.0 ml of scintillation fluid and counted in a Beckman liquid scintillation counter with a counting error of 5% or less.

Preparation of *M. leprae* sonicates. Suspensions of armadillo-derived *M. leprae* were pooled and washed three times with sterile distilled water. The final pellet was weighed and resuspended in a known amount of sterile distilled water to obtain a concentration of 1.2 mg/ml (moist weight) and sonicated in a cup-horn sonicator (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.) with continuous pulsing for 5 min at 4°C. Sonication was repeated three times within 3 min after

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each treatment. At the end of this period, the disrupted material was smear-stained with acid-fast strain to determine the extent of breakage. If found unsatisfactory, sonication was repeated until maximum breakage was observed. In no instance was there total breakage. The material was then centrifuged in the cold at low speed to sediment heavy particles. The supernatant was collected under sterile conditions in known portions, distributed in sterile vials, and maintained at 0°C until used.

Skin tests. Skin tests were done in the footpads of mice 72 h before each scheduled sacrifice. The skin testing materials were injected intradermally with a tuberculin syringe through a 27-gauge needle. The extent of erythema and induration was measured with dial gauge calipers (Schenell-taster system; Kropelin, West Germany) at 0, 6, 18, 24, and 48 h postinjection. *M. leprae* cell extract was used as the skin testing antigen, and sterile, normal saline was injected in the contralateral footpad as a control. A difference of at least 1.0 mm in measurement between the footpad injected with cell extract and the footpad injected with saline was considered a positive reaction.

Nonspecific immunity. A mouse-virulent culture of *Listeria monocytogenes* was stored at −70°C. Recipients of *M. leprae*-sensitized spleen cells were inoculated with 10^4 *L. monocytogenes* via the tail vein. Seventy-two hours later, spleens and livers were removed and homogenized, and appropriate dilutions of homogenates were made for plating. The number of viable *L. monocytogenes* in each organ was determined by quantitative culture on tryptic soy agar.

Statistical evaluation. Groups were compared by the Mann-Whitney U test (2); the data are presented as arithmetic means.

RESULTS

Skin test studies. The results obtained in these studies are a measure of delayed-type hypersensitivity. Recipients of normal spleen cells in the control groups did not show positive reactions to either normal saline or *M. leprae* cell extract. Animals adoptively sensitized to live, heat-killed, and autoclaved *M. leprae* developed swelling at the site of injection of the cell extract as early as the first week post-adoptive transfer. Swelling was evident at 6 h after injection and usually persisted beyond 48 h. The data in Fig. 1 show the swelling at 48 h since there was no significant increase observed thereafter. The reaction was stronger at week 3 after adoptive transfer in the test groups (Fig. 2).

Lymphocyte transformation. Figure 3 gives the data on lymphocyte transformation studies. The results are an average of triplicate cultures for three different experiments. The lymphocyte stimulation index was calculated as counts per minute obtained in a stimulated culture of lymphocytes of mice in a given test group divided by counts per minute obtained in a nonstimulated culture of lymphocytes of mice in the same group. Lymphocytes from the recipients that received sensitized splenocytes from each of the three experimental groups and had positive skin reactions transformed in the presence of *M. leprae* cell extract.

The results of in vitro transformation in the presence of *M. leprae* cell antigen appear to correlate well with results obtained in the skin test studies. The responses to phytohemmagglutin, a nonspecific T-cell mitogen, however, appeared to fluctuate with time. In all three groups, reactivity to *M. leprae* antigens lasted approximately 5 weeks. A loss of skin reactivity to *M. leprae* antigens, together with the loss of in vitro activity of lymphocytes in the animals studied, seems to indicate a loss of specific reactivity in the absence of antigen.

***M. Leprae* protection studies.** Figure 4 shows the results of *M. leprae* harvests after a 4-month incubation period after a standard footpad inoculation of mice with 5×10^3 acid-fast bacilli. The harvests from the various groups are compared with those of the recipients of normal spleen cells. Bacillary counts that ranged from zero to less than 10^4 were considered negative. The recipients of spleen cells from syngeneic mice that were immunized with viable or nonviable *M. leprae* via the hind footpad demonstrated a significant resistance to bacillary multiplication ($P < 0.01$) as compared to controls. An additional group that was exposed to 500 rads before cell transfer demonstrated higher resistance to bacillary multiplication. Very little variation was seen within the different experimental groups.

Nonspecific resistance. Nonspecific resistance experiments were conducted to determine the nature of immunity demonstrated by animals that were recipients of spleen cells from animals immunized to one of the various *M. leprae* preparations. Two weeks after adoptive transfer of *M. leprae*-sensitized splenocytes, the recipients were challenged intravenously with 10^4 *L. monocytogenes*. The listerial counts in the spleens and livers on day 3 after infection indicated no differences between the test and control groups (Fig. 5). It therefore appeared that the *M. leprae*-sensitized spleen cells were not able to mediate resistance to *L. monocytogenes* infection.

DISCUSSION

Data obtained in these studies indicate several aspects. First, although sensitization of the recipient host occurs

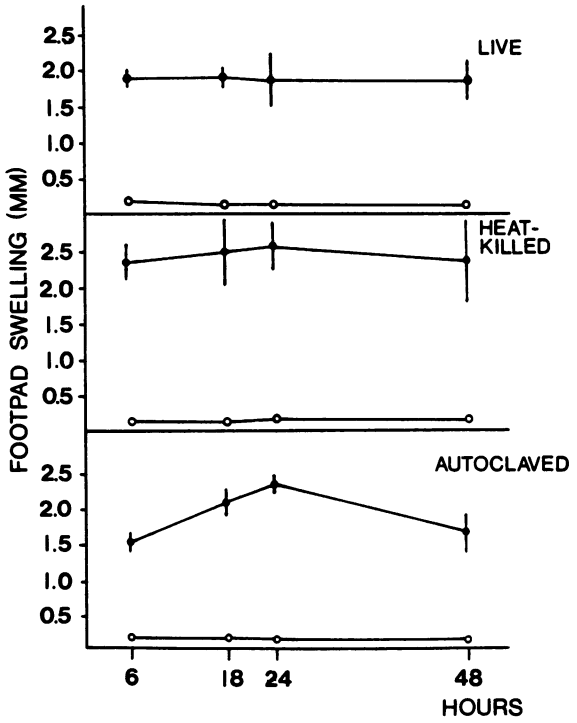

FIG. 1. Kinetics of footpad swelling in mice administered *M. leprae* cell extract (●) or saline (○) 3 weeks after the intravenous infusion of 10^8 splenocytes from mice immunized with 10^7 live *M. leprae* or *M. leprae* killed by exposure to 60°C or autoclaving. The points and bars represent the means and standard deviations of 12 mice.

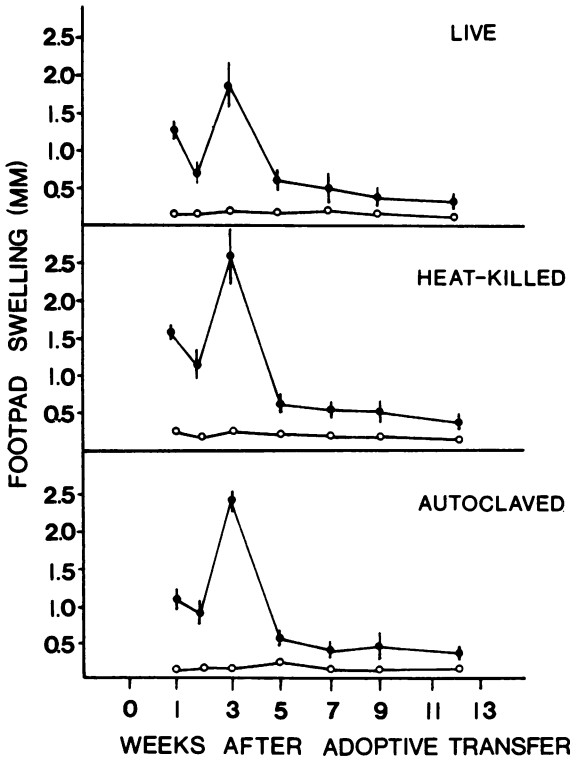

FIG. 2. Kinetics of footpad swelling in mice administered *M. leprae* cell extract (●) or saline (○) at various intervals after the intravenous infusion of 10^8 splenocytes derived from mice immunized with 10^7 live *M. leprae* or *M. leprae* killed by exposure to 60°C or autoclaving. The points and bars represent the means and standard deviations of 12 mice.

through adoptive transfer of antigen-stimulated lymphocytes, this sensitization appears to decline with time. Such a decline can be explained on the basis of diminishing immunological memory by the sensitized splenocytes. Evidence in this direction has been presented by several workers (3, 4; M. W. Chase, Fed. Proc. 22:617, 1963). Second, if induction of sensitivity is a parameter of immunity, it will be noted that all three preparations of *M. leprae*, namely, heat-killed, autoclaved, and viable, appear to be equally immmunogenic, which is basically in agreement with Shepard's observations (15). Lymphocyte transformation studies which correlated well with the onset, development, and decline of skin reactivity also tend to confirm these findings.

Experiments designed to assess the ability of the sensitized splenocytes to confer immunity, either of the homologous or heterologous type, have indicated that the splenic cells from *M. leprae*-sensitized mice, offer protection against an *M. leprae* infection but not against *L. monocytogenes* challenge. This immunity is manifested as restriction of multiplication of a challenge infection in the recipients. That *M. leprae* retains its immunogenicity despite the heat treatment is further confirmed through the footpad bacillary counts. The group that was irradiated before receiving immune spleen cells from mice previously immunized with heat-killed *M. leprae* was used to determine whether irradiation is an essential facet in protection against *M. leprae*. Other investigators have demonstrated that such treatment of recipients before the transfer of lymphoid cells facilitates the expression of adoptive immunity effectively (7). Several other investigators, however, have shown that protection can be imparted to non-irradiated normal recipients as well (10–12). The data in this study are essentially in agreement with the latter observations and reveal that effective immunity can be established either with or without prior irradiation of the recipients, although the former may facilitate a better expression of protection than the latter, as can be seen from the number of bacillary-negative animals in the irradiated group. Negative status as expressed here denotes bacillary counts from zero to 10^4. Our results on the immunogenicity of *M. leprae* are in agreement with those of Shepard et al. (15), who reported findings in mice that were immunized 28 days before the challenge infection. However, there are basic differences between the two approaches. In the Shepard system, the additive effects of adjuvant activity in *M. leprae* and the ability of these organisms to persist in the tissues, even when dead, appear to serve as a constant immunological stimulus in the vaccinated animals (15). In our system, however, such continuous antigenic stimulus is either reduced or perhaps even absent in the adoptively challenged recipients, thus demonstrating the immune capabilities of the sensitized lymphocytes. The possibility that some soluble antigen could have been transfused with the

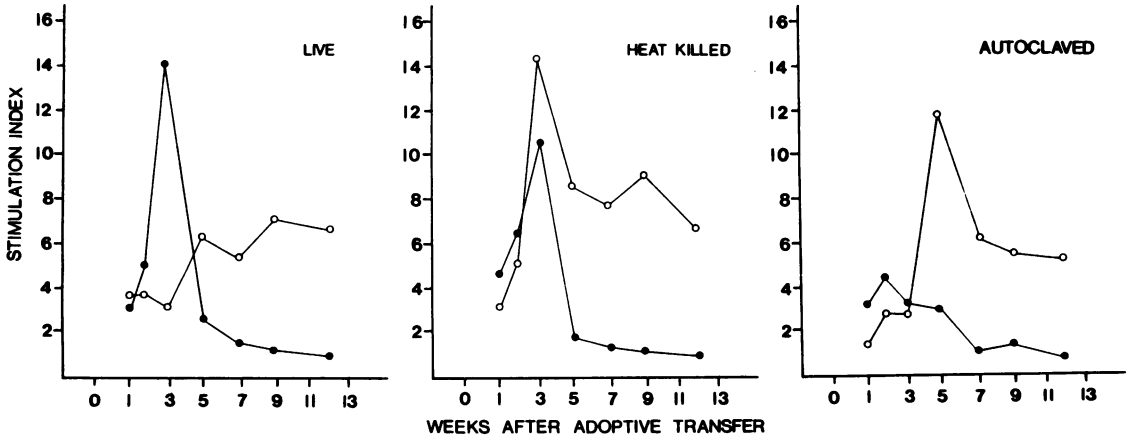

FIG. 3. Blastogenic responses of lymphocyte cultures derived at various intervals from recipients of 10^8 splenocytes adoptively transferred from mice immunized with 10^7 live *M. leprae* or *M. leprae* killed by exposure to 60°C or autoclaving and cultured in the presence of *M. leprae* cell extract (○) or phytohemagglutinin (●).

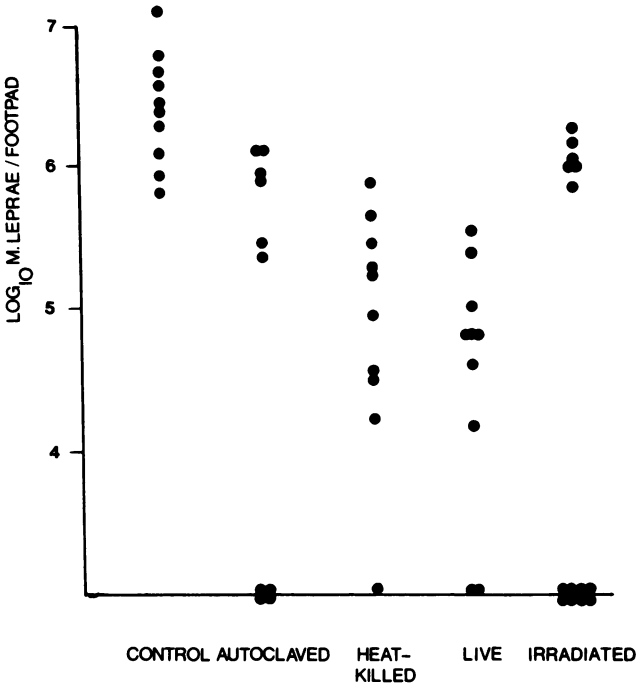

FIG. 4. Multiplication of *M. leprae* at 120 days after a 5×10^3 challenge in the footpads of mice that were the recipients of 10^8 splenocytes from mice immunized with 10^7 live, heat-killed, or autoclaved *M. leprae*. An additional group consisted of mice sublethally irradiated before adoptive transfer of splenocytes from mice immunized with heat-killed *M. leprae*.

sensitized splenocytes does exist. However, the antigen that is released by both viable and nonviable organisms into the host system could be limited considering the short period of time between immunization and transfusion. Whether such a limited amount of antigen could have assisted in the expression of adoptive immunity is not clear at this time. What is most intriguing is the fact that the sensitized state of the transferred cells persisted despite a low-level challenge of *M. leprae* used for infection. Studies by Lefford (7) on adoptive protection in tuberculosis have indicated a similar phenomenon. A significant difference between the two studies however, is the route and dose level of infection. Whereas Lefford used a challenge of 10^4 to 10^5 viable R_1R_v organisms administered intravenously, in our studies the route was the footpad and the challenge dose was 5×10^3 organisms.

The fluctuation observed in the response to phytohemagglutinin, a nonspecific T-cell mitogen, is difficult to explain adequately at this time. One likelihood is that this variation could be due to stimulation of a transient population of suppressor cells that appeared at certain intervals. However, we do not have experimental data to either confirm or refute this. A similar situation has been observed in tumor immunity wherein suppressor cells appear at certain intervals, thus promoting tumor enhancement (5). It is not clear whether a similar situation could have occurred in our experiments.

Although the spleen cells from *M. leprae*-immunized mice were capable of imparting immunity against *M. leprae* infection, they were unable to confer such resistance against *L. monocytogenes*. The following factors in the experimental design should be considered. (i) The challenge infections were administered 2 to 3 weeks after adoptive transfer. The adoptively acquired immune response, as evidenced by delayed hypersensitivity and lymphocyte transformation experiments, was at its highest level during that period. However, this was in response to the *M. leprae* antigen. The listerial challenge may have come at a time when the level of adoptive immunity needed to resist such an infection could have waned. (ii) *L. monocytogenes* is very different antigenically from *M. leprae* and therefore may not have stimulated specifically sensitized lymphocytes to *M. leprae* to extend protection.

The studies reported here indicate that *M. leprae*, despite its physiological state, that is, either killed or viable, is capable of inducing delayed-type hypersensitivity as well as conferring protection against a homologous challenge, thus confirming Shepard's initial observations. Second, this im-

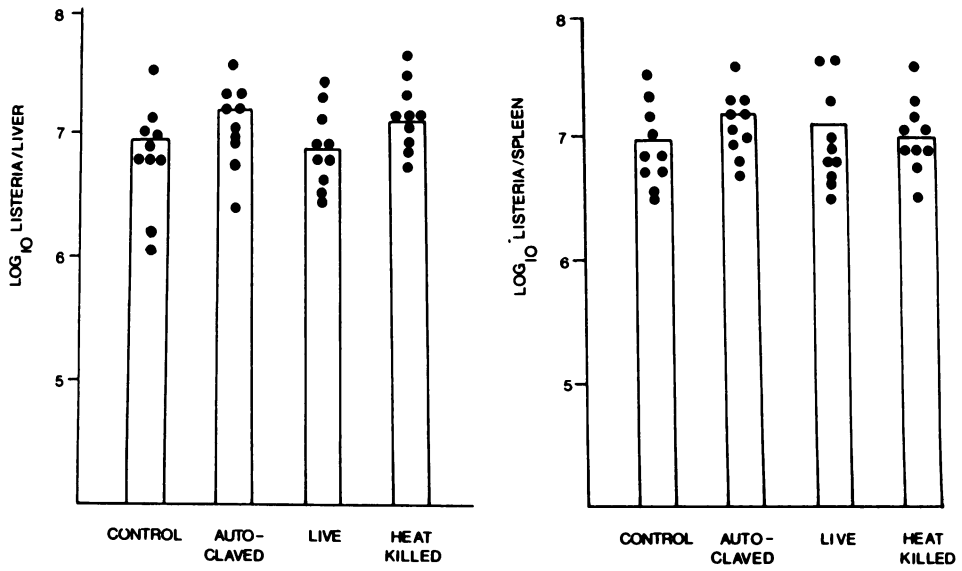

FIG. 5. Multiplication of *L. monocytogenes* at 72 h after a 10^4 intravenous challenge in the livers and spleens of mice that were the recipients of 10^8 splenocytes from mice immunized with 10^7 autoclaved, heat-killed, or live *M. leprae*.

munity, which can be adoptively transferred to normal recipients, persists for a prolonged period. The studies also indicate that this immunity can be expressed effectively in normal recipients as well as in those irradiated before challenge.

In addition, in these studies the protection extended to the host animals appears to be directly related to *M. leprae*, since no protection was seen against *L. monocytogenes* infection. It is not clear whether cross-protection could have occurred if other mycobacteria had been used as infecting pathogens. It is also important to recognize that, although the skin reactivity is transient in nature, the sensitized lymphocytes have the capability to express antibacterial immunity that prevails for the 120-day duration of the experiments. It is difficult at this time to offer a logical explanation for this situation, except that perhaps delayed-type hypersensitivity and antibacterial immunity are two separate phenomena, not entirely interdependent, at least as far as *M. leprae* infection is concerned. It is also likely that two distinctly different moieties of antigens are concerned, both heat stable and immunoactive, with one inducing skin sensitivity and the other imparting immunity. It should be noted that the immune response observed in our experiments was at a predetermined interval, that is, after the expression of skin reactivity. It would be of interest to determine whether this could be true at other intervals such as before the onset or after a decline of skin reactivity.

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