Immune Response to *Mycobacterium leprae*: Plaque-Forming Cells in Mice

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Intravenous immunization with a cell extract of *Mycobacterium leprae* produced a primary immune response of considerable magnitude, followed by an equally large response after secondary stimulation, as measured by assay of plaque-forming cells (PFC). Infection with *M. leprae* or immunization with cell extract by the footpad route produced a lower level of response than that seen in the intravenous group. Identical patterns of response, although not of the same magnitude, were observed after both primary and secondary challenges in the two footpad groups, one infected with viable *M. leprae* and the other immunized with *M. leprae* cell extract. The secondary response after a booster dose to all these groups appeared to be an enhanced immunoglobulin M response. Control studies confirmed that the immune response was a direct result of the host-parasite interaction and that the PFC observed resulted from stimulation of antibody-forming cells by antigens of *M. leprae*. The similarity in time of appearance of peak PFC levels in the two footpad groups may be attributed to the live challenge passing through a latent phase. Alternatively, the challenge is known to contain a large proportion on nonviable cells, and it may also contain soluble *M. leprae* antigens. Studies of the cross-reactivity of the antigens have extended previous observations on antigens shared between *M. leprae* and other mycobacterial species. Use of the two antigen-containing fractions of the *M. leprae* cell extract has suggested that one of the fractions contains some shared antigens, whereas the other has an antigen specific to *M. leprae*.

Several studies of plaque-forming cells (PFC) in mycobacterial infections have been reported. Parlett and Chu (11) described the immunological response in mice and rabbits to mycobacterial antigens; Larson and his associates (7) assayed PFC in rabbits immunized with viable BCG and oil-cell wall vaccines; and Okuyama and Morikawa (10) studied the PFC response in rabbits immunized with BCG. We studied the PFC response in mice infected with *Mycobacterium leprae* and also in mice injected with cell extracts prepared from *M. leprae* derived from human lepromata. Attention was directed chiefly to the early immunoglobulin M (IgM) response in *M. leprae* infection of mice.

**MATERIALS AND METHODS**

**Mice.** Locally bred BALB/c mice were used throughout.

**Immunization.** Mice of one group were inoculated in the right hind footpad, each with $5 \times 10^4$ *M. leprae* harvested during logarithmic multiplication in mice by the method of Shepard (13). Two groups of mice were inoculated with cell extract of *M. leprae* derived from human lepromata, prepared by the method described previously (9). One group of mice was given 0.1 ml of a 1:1 mixture of the cell extract with Freund incomplete adjuvant in the right hind footpad. To mice of the second group, 0.1 ml of cell extract (without adjuvant) was administered intravenously in a tail vein. Two groups of mice were used as controls. One group was given a suspension obtained from the footpads of uninfected mice each into the right hind footpad. The second group was not inoculated. Fifteen days after primary immunization, some of the mice from each group were injected a second time with the same material administered into the left hind footpad or intravenously.

**Collection of splenic cells.** Four mice of each group were sacrificed at intervals, beginning on day 3 after inoculation and continuing to day 40. The spleens were removed, and the splenic cells were teased into sterile Hanks balanced salt solution with sterile blunt forceps. The resulting suspension was passed through sterile, 20- to 27-gauge needles until a homogenous, single cell suspension was obtained. The suspensions were centrifuged in the cold at $600 \times g$ for 5 min; and the sediments were resuspended in fresh, sterile Hanks balanced salt solution to obtain a cell count of $10^6$/ml. Splenic cell suspensions were stored at 4°C until used.

**Antigen-coated sheep erythrocytes.** Antigens
were conjugated to sheep erythrocytes with carbodiimide reagent (6), by the modification of the method of Johnson et al. (5) described by Golub et al. (3). Five ml of 50% sheep erythrocytes (Serveel-Texas Biological Laboratories, Fort Worth, Tex.) was washed three times in conjugation buffer and resuspended in the same volume of buffer. To 1 ml of this suspension, 0.5 ml of a solution of 200 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide per ml (Ott Chemical, Muskegon, Mich.) and 1 ml of the respective antigens were added, and the mixture was incubated at 4 °C for 1 h. The antigen-coated sheep erythrocytes were washed three times with the buffer, resuspended in a concentration of 50%, and stored at 4 °C until used. The antigens used in the assay included cell extracts from M. leprae derived from human lepromata, M. leprae obtained from infected armadillo tissue (generously supplied by E. E. Storrs, Gulf South Research Institute, New Iberia, La.), M. avium, M. intracellulare, M. paratuberculosis, M. smegmatis, M. kansasii, and M. marinum. Other antigens were fractions obtained by the chromatographic separation of the cell extract of the M. leprae obtained from human lepromata, and of an extract of normal human tissue.

Hemolytic plaque assay. Hemolytic plaque assays to detect cells producing antibodies to M. leprae were performed in 10-cm-diameter, sterile, plastic dishes. The dishes were partially filled with a base agar layer prepared by suspending 2.8% Noble agar in distilled water and autoclaving. Equal volumes of the melted agar and 2 × Hanks tissue culture medium (Difco) were mixed, and 5 ml of this mixture was poured into each plate; plates were stored at 4°C prior to use. Agar for the uppermost layer was prepared in a concentration of 1.4% in distilled water and mixed in equal proportions with 2 × Hanks tissue culture medium, distributed in sterile test tubes with plastic caps, and stored at 4°C until used.

Direct assay. One-tenth milliliter of the splenic cell suspension was poured onto the base agar layer. Diethylamine ethylene dextran in a concentration of 0.85 mg/ml was added to the melted top layer to block any anticomplementary action of the agar. Sensitized (antigen-coated) sheep erythrocytes were added to the melted agar in a concentration of 0.1 ml/ml of agar; this mixture was then overlayed on the cell suspension and mixed to form a monolayer. The mixture was allowed to harden, and the plates were incubated at 37°C. After 1 h, the plates were removed from the incubator, and 2 ml of a 1:10 dilution of freshly reconstituted guinea pig complement (Texas Biological Laboratories, Inc., Fort Worth, Tex.) was added. The plates were then reincubated for 1 to 2 h at 37°C, and the resulting clear plaques were counted under a stereomicroscope.

Indirect assay. It is generally accepted that the plaques appearing after the addition of complement indicate cells producing 19S antibody. Sterzl and Riha (14) and Dresser and Wortis (1) have shown that plaques can be obtained with cells producing 7S antibody if the system is amplified by the addition of antibody to 7S gamma globulin. In the present study, indirect plaques were detected by the addition of commercially obtained antisera to mouse immunoglobulins. The assay was carried out employing the procedures already described, except that 1-ml amounts of a 1:40 dilution of the antoglobulin serum were added after the first incubation, and the plates were reincubated for 1 h prior to the addition of the complement.

RESULTS

The experiments were designed to determine the patterns of the primary and secondary immune responses at the spleen cell level of three groups of mice, one infected with M. leprae in the footpad, one immunized with M. leprae cell extract by the intravenous route, and the third given the cell extract mixed with Freund incomplete adjuvant in the footpad. Appropriate control groups were also studied.

Four mice of each group were sacrificed at each interval beginning on day 3 after the primary immunization. The last assay was carried out on day 40 after the primary challenge. The results (Fig. 1) represent the change with time of the number of PFC in animals challenged with M. leprae in the footpad. Each point in the graph represents the average number of PFC from four animals and for three different experiments. In the primary stimulation group, PFC were detected as early as day 3, with a subsequent increase that peaked on day

![Graph](http://iai.asm.org/.../infection.png)

Fig. 1. Primary and secondary immune responses in mice inoculated with 5 × 10⁵ "viable" M. leprae into a hind footpad, as measured by the PFC response of spleen cell suspensions. Symbols: •, primary response; O, secondary response.
10 and declined thereafter to the base level on about day 25. After the secondary challenge had been administered, the number of PFC increased beginning on day 2 and peaked on day 5. This response was shorter lived than the primary response, although the maximal number of PFC was larger.

Figure 2 represents the data from the mice immunized with *M. leprae* cell extract in Freund incomplete adjuvant by the footpad route. Each point represents the average number of PFC for four animals and for two different experiments. In this group also, the peak for the primary response appeared on day 10 after immunization, and the secondary peak appeared on day 5 after the secondary challenge. Tapering of the immune response occurred at about the same rate as in the infected mice, but the magnitude of the responses appeared to be smaller than in the infected group.

Figure 3 shows the response of mice immu-

**Fig. 2.** Primary and secondary immune responses in mice administered *M. leprae* cell extract in Freund incomplete adjuvant into a hind footpad, as measured by the PFC response of spleen cell suspensions. Symbols: ●, primary response; ○, secondary response.

**Fig. 3.** Primary and secondary immune responses in mice administered *M. leprae* cell extract intravenously, as measured by the PFC response of spleen cell suspensions. Symbols: ●, primary response; ○, secondary response.

nized with the cell extract by the intravenous route; each point is an average of the number of PFC for four animals and for two different experiments. Here, the peak response to primary immunization appeared on day 3 and was larger than in the other two systems. The secondary response also appeared to peak on day 3 after secondary challenge; the maximal number of plaques was about the same as that after primary immunization. The decline in the number of PFC began on about day 5 for both the primary and secondary responses. There was no apparent difference between the two responses in the time of the peak response and the rate of decline.

Assay of indirect plaques, with specific antimouse globulin antisera, was restricted to a few intervals, mainly on the days of peak response, and was carried out only in the mice infected with *M. leprae*. This procedure revealed about the same number of plaques as was found in the direct assay, and the rate of appearance of the plaques appeared to follow closely the rate at which the direct plaques appeared.

In Table 1, the results are summarized of
studies in which the ability of the spleen cells harvested from mice infected with \textit{M. leprae} in the footpad to form plaques upon exposure to a variety of mycobacterial antigens was examined. These studies were performed with spleens obtained from mice 10 days after inoculation. Because the number of PFC varied with the time from inoculation to assay, the results are expressed in terms of the number of PFC found after exposure to a given antigen as a percentage of the largest number of PFC found in response to any antigen by each spleen cell suspension. These results show that each spleen cell suspension responded with the same number of plaques to the cell extract prepared from \textit{M. leprae} derived from armadillo tissue as to the cell extract prepared from the organisms obtained from human lepromata. The spleen cell suspensions also gave a large response to fraction C obtained by chromatographic separation of the cell extract prepared from \textit{M. leprae} obtained from human tissue. Current studies in this laboratory have shown that fraction C possesses a single antigen that has been found to be specific for \textit{M. leprae} in hypersensitivity studies conducted on guinea pigs sensitized with \textit{M. leprae} and other mycobacteria (R. G. Navalkar, P. J. Patel, and R. R. Dalvi, J. Med. Microbiol., in press). Very few plaques are formed when cells obtained from previously uninfected animals or from animals inoculated into the footpads with mouse footpad material containing no \textit{M. leprae} are exposed to \textit{M. leprae} cell extract, and when cells from mice infected with \textit{M. leprae} are exposed to an extract of human tissue containing no organisms. The formation of plaques when spleen cells obtained from mice infected with \textit{M. leprae} are exposed to cell extracts prepared from other mycobacteria indicates the presence of cross-reacting antigens, confirming the results of earlier studies of the antigenic mosaic of \textit{M. leprae} (9).

\section*{DISCUSSION}

The present study reports the numbers of IgM antibody-forming cells in mice infected with \textit{M. leprae} or immunized with the cell extract of \textit{M. leprae} by two different routes. In the infected mice, immunocyte proliferation was detected as early as day 3 after challenge, and reached a peak about day 10. A similar pattern appeared on secondary stimulation, although the peak was reached earlier than in the primary response and was considerably greater in magnitude. The decline of both the primary and secondary responses began soon after peak levels had been achieved.

Similar responses were observed in the mice given \textit{M. leprae} cell extract either intravenously or in Freund adjuvant into the footpads. That the responses to small numbers of whole bacterial cells were so large was unexpected. It has been shown that the great majority of \textit{M. leprae} harvested during logarithmic multiplication in mouse footpads are not viable (8); in addition, the infected footpad tissues from which the suspensions of \textit{M. leprae} were prepared may contain bacterial antigens released by autolysis during the course of the infection. Thus, the inoculum of \textit{M. leprae} may have contained a considerable quantity of readily available antigen. In this regard, the specificity of the responses to the several challenges must be considered.

The small numbers of plaques in the control cultures demonstrate that the PFC did not represent a response to normal human tissue antigens present in the cell extract as minor contaminants or to mouse tissue antigens, nor do they reflect the presence of heterophile antibodies naturally occurring in mouse serum. The studies performed with the other mycobacterial antigens appear to confirm the specificity of the responses observed. Of the two antigen-positive fractions of \textit{M. leprae} cell extract used in this study, fraction A was able to induce as many plaques as were produced by the cell extracts of the cultivable mycobacteria,

\begin{table}
\centering
\begin{tabular}{|l|l|}
\hline
Mycobacterial antigens used for sheep erythrocyte sensitization & Plaques detected (\%)
\hline
\textit{M. leprae} (human) cell extract & 100
\hline
\textit{M. leprae} (armadillo) cell extract & 100
\hline
\textit{M. avium} cell extract & 40
\hline
\textit{M. paratuberculosis} cell extract & 38
\hline
\textit{M. intracellularum} cell extract & 42
\hline
\textit{M. leprae} (human) cell extract (control 1)* & 6
\hline
\textit{M. leprae} (human) cell extract (control 2)* & 11
\hline
Normal human tissue extract (control 3)* & 6
\hline
\end{tabular}
\caption{Assay of PFC in mice infected with \textit{M. leprae}}
\end{table}
whereas fraction C produced a much larger number of plaques. Fraction A contains more than one antigen, whereas only one antigen may be demonstrated in fraction C by immunodiffusion, and studies of delayed hypersensitivity and passive cutaneous anaphylaxis conducted on these fractions suggest that the single antigen of fraction C is specific to *M. leprae* (Navalkar, Patel, and Dalvi, J. Med. Microbiol., in press).

The more rapid development of the secondary response and the rapid decline of both the primary and the secondary responses were striking. In the secondary response, the number of PFC increased rapidly after stimulation, and reached a peak in about half the time required for the peak of the primary response. It is possible that the large number of PFC developing within so short a time represented the sum of newly produced, antibody-producing cells and the restimulation of previously primed cells. Parlett and Chu (11) did not find such an anamnestic response, but Larson et al. (7) and Okuyama and Morikawa (10) reported such a phenomenon in their studies. Pearlman (12) and Finkenstein and Uhr (2) have suggested that a rapid decrease in PFC within a very short time after the peak may result from inhibition by early production of 7S antibody. This formulation is consistent with the decline of PFC not only in the groups of mice immunized by the footpad route, but also in those immunized intravenously. The number of indirect PFC appeared equal to the number of direct PFC in the few assays performed. It is very likely that a mixture of both 19S and 7S antibodies was produced. Wortis and his associates (15) have reported that nearly 57% of direct PFC found in the mouse spleen can also be detected by the indirect assay. Whether these antibody-producing cells are 7S producers remains to be established.

The studies reported herein suggest that an IgM response to an antigenic stimulus, whether with whole *M. leprae* or with cell extracts of the organisms, is triggered very early in the course of infection or immunization. This response declines very rapidly after reaching a peak and remains below detectable levels thereafter. The secondary response appears more likely to be an enhanced IgM response than a true IgG response. Hemagglutination studies in which 2-mercaptoethanol-treated sera have also been assayed indicate that this suggestion may be a valid one (unpublished data). It is possible that an IgG response would result from a later secondary challenge, after suppression of the IgM response was complete. Studies of this possibility are currently in progress.

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**LITERATURE CITED**