

Immunity to Leprosy. 1. The Proliferative Response of Murine T Lymphocytes to *Mycobacterium leprae*¹

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The development of a vaccine against leprosy requires the specific induction of protective immunity to antigens of *Mycobacterium leprae*. The strategy followed successfully in vaccine research in a number of diseases has been to establish resistance to disease using animal models and then to extrapolate to trials in man. There are certain aspects of the disease process in leprosy that are unique from the viewpoint of developing a vaccine. These arise from a series of major problems: the inability to grow *M. leprae* in culture, the slow growth of *M. leprae* in host organisms, the lack of readily available experimental hosts that reflect the spectrum of the disease in humans, and the long duration of the disease process.

It has been known since the pioneering studies of Shepard^(15, 16) that viable *M. leprae* injected into the foot pads of mice will multiply to yield approximately 10⁶ organisms but that, after a period of months, this bacterial growth ceases. It is generally considered that the immune system in normal mice controls the growth of *M. leprae* through the expression of cell-mediated rather than humoral immunity^(6, 13). Mice in which cell-mediated immunity is compromised (thymectomy and irradiation, or athymic nude mutation) show enhanced susceptibility to infection with *M. leprae*^(2, 4, 14). Although much is known of the serological crossreactivities between *M. leprae* and other mycobacteria, as well as a unique phenolic glycolipid antigen^(1, 7, 9, 11), the T cell populations involved in the control of growth of *M. leprae* in mice and the antigenic determinants these T cells recognize have not been defined.

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The work described in this paper is aimed at the establishment of an assay to examine the response of different subclasses of murine T cells to *M. leprae* antigens. Mice were immunized with *M. leprae* at the base of the tail, and lymphocytes from draining lymph nodes subsequently removed in order to determine the response to *M. leprae* antigens in a microculture assay. Lymphocytes harvested from draining lymph nodes can be induced to proliferate by antigen in culture, providing a quantitative measurement of the response⁽³⁾. We describe here the basic parameters of this assay system. These techniques will allow the identification of *M. leprae*-derived antigens seen by T cells. These studies pave the way for the establishment of a library of T cell lines which can be used to identify the major T cell-specific antigens of *M. leprae*, as well as the effector classes of T cells which respond to these antigens.

MATERIALS AND METHODS

Animals. C57BL/10J, the congenic strains B10.M, B10.S, B10.A, B10.A(2R), B10.A(3R), B10.A(4R), B10.A(5R), BALB/cJ, C3H/HeN and BALB/c × C57BL/6J(CDF₁) mice were obtained from the breeding facility maintained by the Department of Immunobiology at the Auckland Medical School, Auckland, New Zealand. Mice 6–10 weeks old were used for all experiments.

Antigen. Lyophilized, irradiated armadillo-derived *M. leprae* was provided by Dr. P. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A.^(1, 9). This was used as antigen by suspending in sterile saline and stored at 4°C until used, either for immunization of mice or as antigen in culture assays.

Immunization procedure. Antigen suspended in saline was emulsified in Freund's incomplete adjuvant (FIA) and injected subcutaneously at the base of the tail. The antigen concentration was adjusted so that

the total dose of antigen delivered in a standard 50 μ l injection was varied between 0.01 μ g and 200 μ g. Control animals were injected with 50 μ l of sterile saline emulsified in FIA.

Lymph node preparation. Mice were sacrificed from 2 to 30 days after injection. Inguinal and para-aortic lymph nodes were removed aseptically under a dissecting microscope and placed in culture medium.

Lymphocyte cultures. Cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 50 units/ml penicillin, and 50 μ g/ml streptomycin sulfate. Cell suspensions were prepared under the dissecting microscope (by teasing with forceps) after adherent fat had been removed. Lymph node cells (LNC) were washed once and resuspended in standard medium, counted, and diluted to different concentrations between 10^6 /ml and 4×10^6 cells/ml. Cells were dispersed into flat-bottomed microtiter wells, and 10 μ l of antigen in saline was dispensed into each well to give a range of final antigen concentrations in the microwells from 6 μ g/ml to 100 μ g/ml. The total culture volume was 200 μ l. Control microwells contained cells but no antigen. Cultures were incubated for varying times at 37°C in an atmosphere of 6% CO₂ in humidified air.

After incubation, the cell cultures were pulsed with 0.5 μ Ci of ³H-thymidine (2 mCi/mMole; New England Nuclear Corp., Boston, Massachusetts, U.S.A.) for 6 hr and then harvested on glass-fiber filter paper. The results are expressed as mean counts per minute of triplicate cultures. Standard deviations were less than 10% of the mean.

Mitogenic responses. Cells were also cultured with lipopolysaccharide (LPS) or concanavalin A (ConA) ranging from 0.6 μ g/ml to 10 μ g/ml, and radiolabeled and harvested as detailed elsewhere (¹⁰).

T cell depletion. Lymph node cell suspensions were prepared in culture medium and affinity-purified, anti-Thy-1 monoclonal antibody prepared from T.24-31.7 ascites was added to give a final cytotoxic titer of 10^5 (¹⁰). After incubation at 37°C for 10 min, the cells were pelleted by centrifugation, resuspended in culture medium, and spleen-absorbed guinea pig complement added. After incubation at 37°C for a further 30 min, the cells were washed twice and

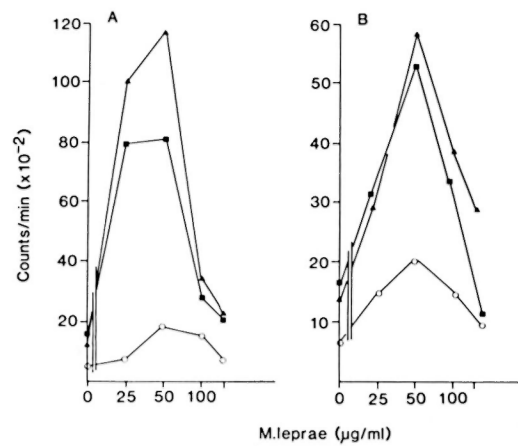


FIG. 1. Effect of cell density. CDF₁ mice were immunized with A) 20 μ g and B) 100 μ g *M. leprae* antigen in FIA, and draining lymph nodes harvested after 18 days and challenged with 25–150 μ g/ml *M. leprae* antigen in culture. After 4 days in culture, the proliferative response was measured by ³H-TdR incorporation after a 6 hr pulse. ○ = cultures at 2×10^6 cells/ml, ● = cultures at 3×10^6 cells/ml, ▲ = cultures at 4×10^6 cells/ml.

then cultured with *M. leprae* antigen or mitogen. To determine the efficiency of T cell depletion, a small aliquot of these cells was also stained with fluorescein-labeled anti-Thy-1 antibody (¹⁰). All treated lymph node cells used in the experiments described here contained less than 1% Thy-1+ cells.

RESULTS

Effect of cell density. CDF₁ mice were injected subcutaneously at the base of the tail with either 20 μ g or 100 μ g of *M. leprae* in FIA. After 18 days, draining lymph node cells were harvested and cultured in microwells using different cell concentrations: 2×10^6 , 3×10^6 , and 4×10^6 cells/ml. *M. leprae* antigen was titrated into cultures in the range of 25–150 μ g/ml. After 4 days in culture, cells were labeled with ³H-thymidine and the radioactive incorporation measured (Fig. 1). Lymph node cells prepared from mice primed with both 20 μ g (Fig. 1A) and 100 μ g (Fig. 1B) *M. leprae* in FIA showed maximal stimulation at a density of 4×10^6 cells/ml in culture using challenging doses of *M. leprae* antigen in the range of 25–50 μ g/ml.

Effect of antigen concentration used for *in vivo* immunization. The effect of the con-

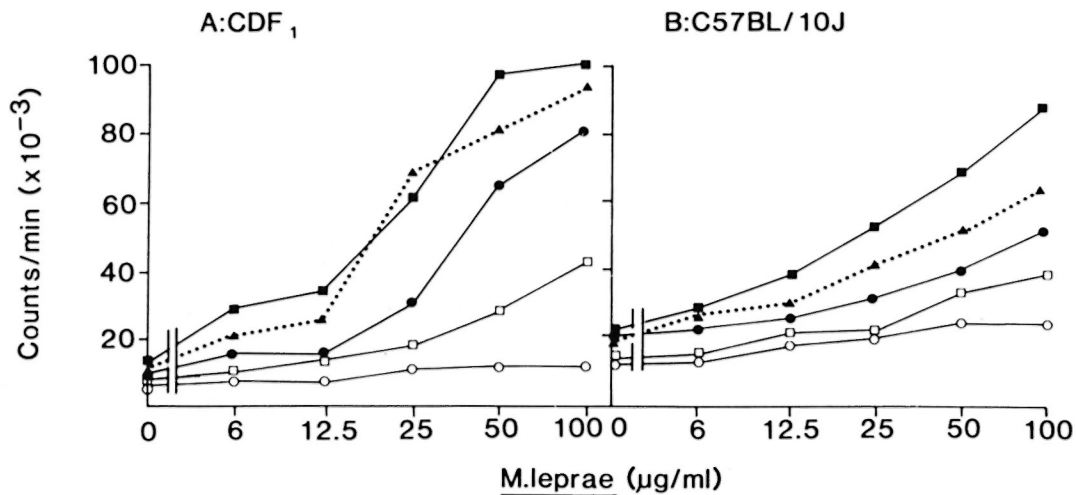


FIG. 2. Effect of antigen dose. A) CDF₁ and B) C57BL/10J mice were injected with doses of *M. leprae* antigen from 0.1 µg to 20 µg per animal. After 12 days, draining lymph node cells were harvested and cultured for 3 days at a cell density of 4×10^6 with *M. leprae* antigen ranging from 6–100 µg/ml in culture. The proliferative response was then measured by ³H-TdR incorporation after a 6 hr pulse. Mice were immunized with: ○ = FIA/saline or □ = 0.1 µg, ● = 1.0 µg, ▲ = 10 µg, ■ = 20 µg *M. leprae* antigen in FIA. (Points are means of triplicate cultures with standard deviations less than 10% of the mean count.)

centration of *M. leprae* used to immunize mice was determined. CDF₁ and C57BL/10J mice were injected with *M. leprae* antigen from 0.1 to 20 µg per mouse in FIA. After 12 days lymph nodes were harvested and cell cultures prepared using a final density of 4×10^6 cells/ml. *M. leprae* antigen was titrated into each microculture in a range of 6–100 µg/ml. After 3 days, cells were labeled with ³H-thymidine and the radioactive uptake measured. The results expressed show the response of CDF₁ (Fig. 2A) and C57BL/10J (Fig. 2B) lymph node cells, and are similar. Lymph nodes from mice immunized with saline in FIA did not respond to *M. leprae* antigen in this culture assay. At all immunizing doses of antigen used (0.1 to 20 µg per mouse), subsequent challenge with *M. leprae* antigen in culture yielded a proliferative response. The magnitude of the *in vitro* response increased as the dose of antigen used to immunize the mice was raised. Our experience from many experiments is that immunization of mice with 1.0 µg to 20 µg of *M. leprae* in FIA, followed by culturing of lymph node cells with 25 µg/ml to 100 µg/ml of *M. leprae* antigen *in vitro*, gives a reproducible proliferative response assay.

Effect of time of immunization. A group of mice was immunized with 100 µg of *M. leprae* antigen. At various times for the following 28 days, lymph nodes were harvested and cultured at a density of 4×10^6 cells/ml with 100 µg/ml of *M. leprae* antigen. Radioactive incorporation of ³H-thymidine was determined after 4 days in culture. The data presented in Figure 3 are a summary of the results obtained from immunizing and assaying a group B10.M mice. Each point represents the mean of triplicate cultures and for each time two sets of data are shown. The open circles represent the response of lymph node cultures in the absence of added *M. leprae* antigen. This gives an indication of the background that may result from the carryover of *M. leprae* antigen from the lymph nodes to the cell culture. The closed circles represent the same cell population cultures with 100 µg/ml of *M. leprae* (Fig. 3).

Following *in vivo* immunization of mice, it was 4–5 days before lymph node cells were rendered responsive to challenge *in vitro* with *M. leprae* antigen in culture. Maximum responsiveness varied but was generally in the range of 6–15 days after immunization. Thereafter, lymph node cells

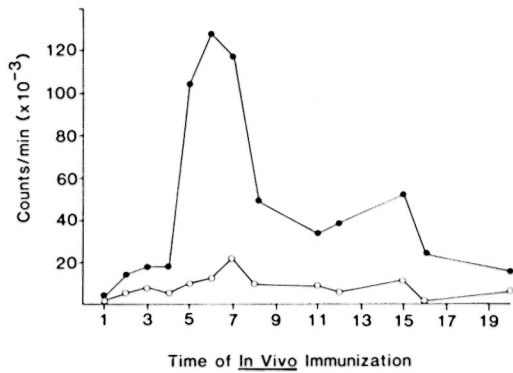


FIG. 3. Effect of time of immunization. A group of B10.M mice were immunized with 100 µg of *M. leprae* antigen and draining lymph node cells harvested from 1–20 days after priming and cultured at a density of 4×10^6 cells/ml with *M. leprae* antigen at 100 µg. After 3 days in culture, proliferative responses were measured by ³H-TdR uptake following a 6 hr pulse. The maximum response of lymph node cultures to *M. leprae* antigen is plotted against day of harvest of lymph nodes after *in vivo* immunization (● = lymph node cells cultured with *M. leprae* antigen, ○ = without *M. leprae* antigen). (Cultures were performed in triplicate and standard deviations were less than 10% of the mean.)

immunized *in vivo* began to lose their ability to respond to antigen in subsequent culture. The addition of *M. leprae* antigen to primed lymph node culture always resulted in a significant increase in the proliferative response (Fig. 3).

Kinetics of *in vitro* proliferative response. The kinetics of the proliferative response of immunized lymph node cells to *M. leprae* antigen in cell culture was followed using a number of mouse strains. Groups of B10.M, B10.S, B10.P, and C57BL/10J mice were immunized with 100 µg of *M. leprae* antigen emulsified in FIA and 12 days later, draining lymph nodes were harvested and cultured in the presence of 100 µg/ml of *M. leprae* antigen (Fig. 4). After various incubation periods over a period of 6 days, as indicated, cultures were labeled with ³H-thymidine and the radioactive uptake measured (Fig. 4).

The results showed several points: First, there was a clear difference in the responses of the strains; B10.M mice gave a high response, while B10.S, B10.P and C57BL/10J showed lower responses in that order. Second, these strain differences were observed

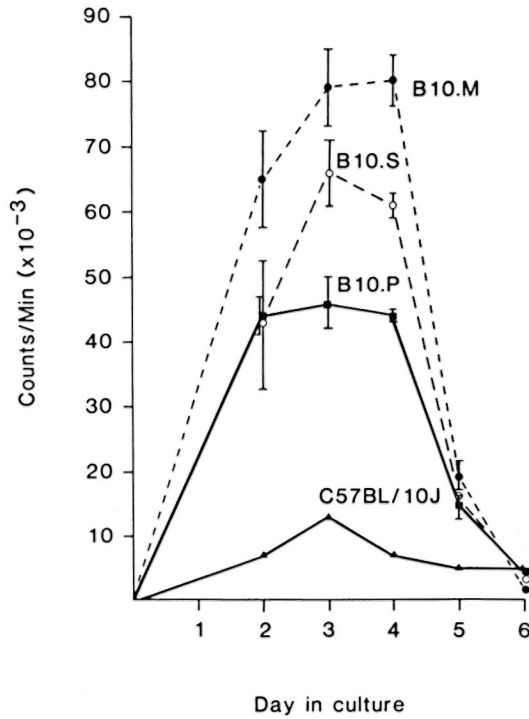


FIG. 4. Kinetics of *in vitro* response. B10.M, B10.S, B10.P, and C57BL/10J mice were immunized with 100 µg of *M. leprae* antigen. After 12 days, draining lymph node cells were cultured at a density of 4×10^6 cells/ml with *M. leprae* antigen at 100 µg/ml. After incubation periods ranging from 2–6 days, different cell cultures were labeled with ³H-TdR and incorporation measured after a 6 hr pulse. The maximum proliferative response is plotted against time of lymph node cells in culture in days. (Cultures were performed in triplicate and standard deviations were less than 10% of the mean.)

throughout the 6-day period. Third, our observations from many experiments are consistent with this T cell proliferative response reaching a maximum between days 3 and 6 of culture. After that time, the response decreased. We have observed very little variation in kinetics using different strains of mice in similar experiments.

Strain survey. A survey was carried out to compare the proliferative response of a number of inbred strains of mice to *M. leprae* antigen. All groups of mice were age matched, and the data for each strain described here have been reproduced at least three times.

All mice were immunized with 100 µg of *M. leprae* antigen in FIA, and lymph node

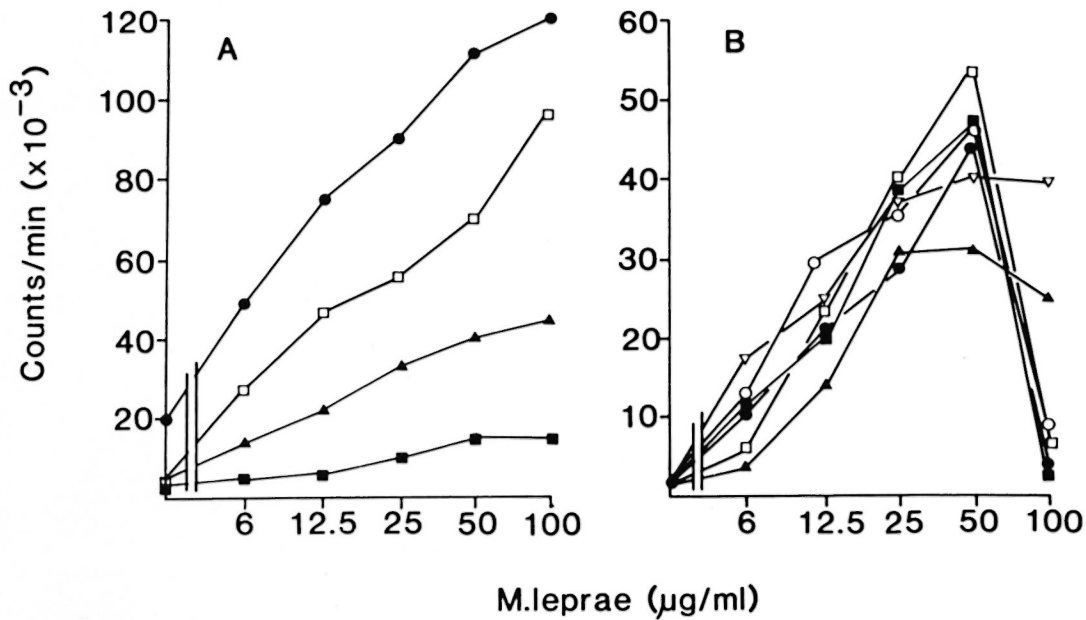


FIG. 5. Responses of different inbred strains of mice to *M. leprae*. Groups of mice of different inbred strains were immunized with 100 µg of *M. leprae*. After 12 days, draining lymph node cells were cultured at a cell density of 4×10^6 /ml with *M. leprae* antigen titrated from 6–100 µg/ml. After 3 days' incubation, cell proliferation was measured by ^3H -TdR incorporation using a 6 hr pulse time.

Group A

- = B10.M
- = B10.S
- ▲ = C57BL/10J
- = BALB/cJ

Group B

- ▽ = C57BL/10J
- ▲ = B10.A
- = B10.A(2R)
- = B10.A(3R)
- = B10.A(4R)
- = B10.A(5R)

cells prepared for culture 12 days later. For each strain of mouse, lymph node cells were titrated in culture with 6–100 µg/ml of *M. leprae* antigen. The cell density was always 4×10^6 cells/ml. In Figure 5A, the proliferative responses of C57BL/10J, BALB/cJ, B10.S, and B10.M are compared. There are differences in responses that are statistically significant. While BALB/c and C57BL/10J are low-responder strains, the congenic strains B10.S and B10.M consistently appeared as high-responder strains. The data for Figure 5B compare the responses of C57BL/10J mice to those of the congenic strains B10.A, B10.A(2R), B10.A(3R), B10.A(4R), and B10.A(5R), and there are statistically significant differences among the strains in this group of mice.

Specificity of response. To test the antigen specificity of the proliferative response, groups of mice were separately immunized

with keyhole limpet hemacyanin (KLH) or *M. leprae* in FIA. The primary dose of KLH was 100 µg and of *M. leprae* 10 µg per mouse. After 10 days, lymph nodes were harvested and cells cultured with 25, 50, or 100 µg/ml of *M. leprae* antigen or KLH at a density of 4×10^6 cells/ml. On day 3, the cultures were labeled with ^3H -thymidine and the radioactive uptake determined (Fig. 6). Mice immunized with *M. leprae* and challenged with *M. leprae* antigen but not KLH *in vitro* showed a proliferative response. Mice immunized with FIA alone did not respond to *M. leprae* or KLH. Mice immunized with KLH in FIA responded to KLH but not to *M. leprae* (Fig. 6).

Identification of the responding cells. To analyze the involvement of T cells in the proliferative response, mice were immunized with 100 µg of *M. leprae*, and 12 days later lymph node cells prepared. At that

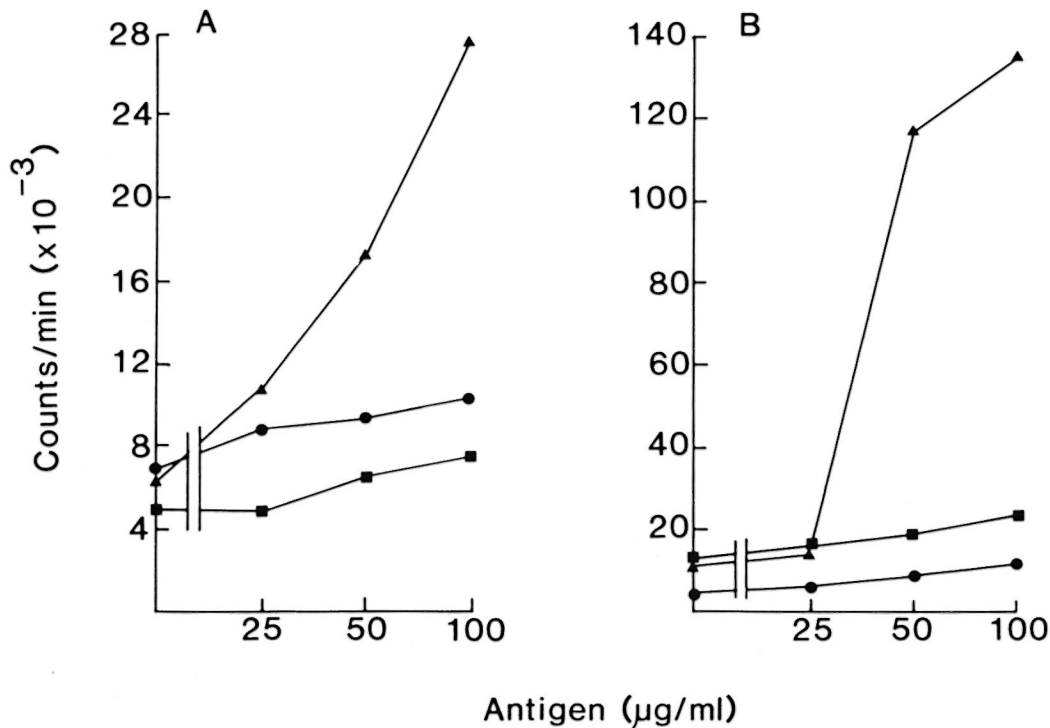


FIG. 6. Specificity of proliferative response. CDF₁ mice were immunized with 10 µg of *M. leprae* antigen, or 100 µg of keyhole limpet hemocyanin (KLH) antigen, or FIA in saline. After 10 days, draining lymph node cells were cultured with either 25, 50, or 100 µg/ml of *M. leprae* antigen, or KLH antigen for 3 days. Cultures were then labeled with ³H-TdR for 6 hr and radioactive uptake measured. (Cultures were performed in triplicate and standard deviations were less than 10% of the mean.)

Group A	Group B
▲ = <i>M. leprae</i> -primed cells cultured with <i>M. leprae</i> antigen.	▲ = KLH-primed cells cultured with KLH antigen.
● = <i>M. leprae</i> -primed cells cultured with KLH antigen.	■ = KLH-primed cells cultured with <i>M. leprae</i> antigen.
■ = FIA-primed cells cultured with <i>M. leprae</i> antigen.	● = FIA-primed cells cultured with KLH antigen.

time, some of the cells were treated with anti-Thy-1 antibody and complement as detailed earlier. Both treated and control cell cultures were then prepared separately with *M. leprae* antigen, or the lymphocyte mitogens ConA or LPS. All cultures were radiolabeled on day 3, and the results are presented in Figure 7. Untreated lymph node cells responded to *M. leprae* (Fig. 7A), ConA (Fig. 7B), and LPS (Fig. 7C). However, treatment of lymph node cells with anti-Thy-1 antibody resulted in the loss of responsiveness of the cells to *M. leprae* and ConA, but not to LPS.

Spleen cells. Spleen cells from CDF₁ mice primed with 25, 100, or 250 µg of *M. leprae*

antigen with FIA at the base of tail showed no increased proliferation in response to *M. leprae* antigen *in vitro* compared with spleen cells from the control unprimed mice (data not shown).

DISCUSSION

Lymphocytes prepared from inguinal and para-aortic lymph nodes of mice immunized in the base of the tail with *M. leprae* respond specifically to the same antigen when subsequently challenged in culture. The proliferative response assay described here varies with the dose of antigen used for immunization and the concentration of antigen used in culture. Maximum re-

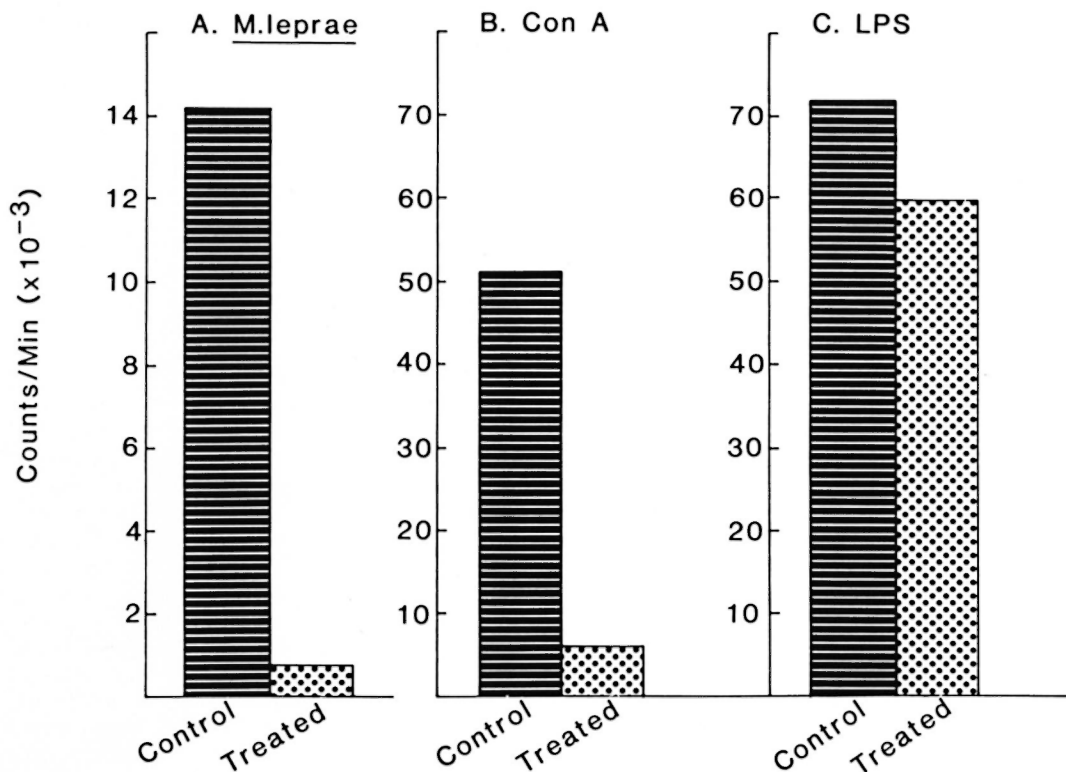


FIG. 7. Identification of responding cells in the proliferative response. CDF₁ mice were immunized with 100 μg of *M. leprae* antigen. After 12 days draining lymph node cells were prepared for culture. *M. leprae*-immunized cells were then treated with monoclonal anti-Thy-1 antibody and complement (see Materials and Methods) and challenged in culture with *M. leprae* antigen, ConA, or LPS.

responses are reproducibly obtained using 20–100 μg of *M. leprae* to immunize the mice, and 25–100 $\mu\text{g}/\text{ml}$ in subsequent culture challenge. The length of the immunization period is important. Lymph node cells respond well to *M. leprae* 5–10 days after *in vivo* immunization, following which the response declines. The *in vitro* proliferative response reaches a maximum generally 3–5 days after culture. The observations made in this study confirm and extend those previously published by Haregowoin and Louis⁽⁸⁾. They used bacillary numbers rather than dry weight of lyophilized armadillo-derived antigen in their experiments, so direct comparison is difficult. They found that higher doses (10^6 organisms) used for priming and higher doses (10^6 organisms) *in vitro* culture were inhibitory. It has been our experience that higher than 50 $\mu\text{g}/\text{ml}$ of *M. leprae* antigen *in vitro* may be inhibitory to proliferation. The onset of the inhibition appears

to be quite abrupt, and some variation has been observed from experiment to experiment (Figs. 1A and 1B, Fig. 2A, Fig. 6A). This variation may be related to minor variations in antigen concentration or culture conditions. Their kinetic experiment showed a peak response on days 6 and 7, slightly later than the finding reported here. They showed specificity of the cells to proliferate to the priming antigen, and that the proliferative response was primarily of T cells. No comment is made in their study of the differences in magnitude of the proliferative response in different strains of mice.

The development of these murine proliferative assays is necessary to begin to evaluate T lymphocyte responses to *M. leprae*. In this assay, there is a preferential response of T lymphocytes⁽³⁾ which allows a simple quantitative measure of T cell responses to *M. leprae* antigens. A preliminary survey of the responsiveness of selected mouse strains

to *M. leprae* antigens using this assay reveals differences. For example, C57BL/10J lymph node cells are lower responders than cells from the congenic B10.M strain which is a higher responder. These data imply that the expression of genes in the mouse major histocompatibility locus is important in T cell responses to *M. leprae*. By analogy, this approach may be important for considering how genes located in the HLA region of humans influence immunity to *M. leprae* and, also, in determining sensitivity or resistance to the disease process.

There are three central questions to the involvement of T lymphocytes in disease protection: a) What is the chemical nature of the *M. leprae* antigen? b) What subclasses of T lymphocytes respond to antigenic determinants? c) Which of these T cell responses play a role in protective immunity to *M. leprae*?

The development of a T cell proliferative assay is a first step in answering these questions. By a combination of biochemical and new recombinant DNA techniques, antigenic determinants from *M. leprae* that elicit T cell responses can be identified. This assay system is a relatively simple way of measuring responses to these determinants. By using interleukin-2 to establish long-term cultures of antigen-specific T cells (5, 8, 12, 17), it should be possible to determine the phenotypes of cells that respond to *M. leprae* antigens and to determine their effector functions. This may reveal whether select antigens elicit responses in different subclasses of T cells. Finally, knowledge of T cell responses in murine lymphocytes can be applied to human T lymphocytes. In particular, a scientific rationale for the development of vaccines to provide selective T cell immunity can be established.

SUMMARY

A microculture assay is described for measuring the response of murine T lymphocytes to *Mycobacterium leprae* antigens. Mice were immunized with *M. leprae* in Freund's incomplete adjuvant at the base of the tail, and after five days lymphocytes from draining lymph nodes were harvested and cultured with *M. leprae* antigens. Radioactive thymidine uptake was used to quantitate the antigen-induced proliferation of the lymphocytes. The effects of cell

density and antigen concentration on the kinetics of the proliferative response were determined. We present evidence that it is the T lymphocytes that proliferate in response to antigen in microcultures, and that the lymphocyte response varies with the mouse strain used. This assay system can be used to identify the antigens of *M. leprae* that elicit T cell responses and the effector function of the *M. leprae*-specific T cell involved.

RESUMEN

Se describe un sistema de microcultivo para medir la respuesta de los linfocitos T murinos a los antígenos del *Mycobacterium leprae*. Los ratones fueron inmunizados inyectando *M. leprae* en adyuvante incompleto de Freund, en la base de la cola. Cinco días después, se cosecharon linfocitos de los ganglios regionales y se cultivaron con *M. leprae*. Para cuantificar la linfoproliferación inducida por antígeno se midió la incorporación de H³T. Después de establecer las condiciones óptimas para el ensayo se encontró que son los linfocitos T los que proliferan en respuesta al antígeno y que la respuesta de los linfocitos varía con la cepa de ratón que se utilice. Este sistema puede usarse para identificar los antígenos del *M. leprae* que inducen las respuestas de las células T, y la función efectora de las células T reactivas al *M. leprae*.

RÉSUMÉ

On décrit ici une méthode d'évaluation par microculture visant à mesurer la réponse des lymphocytes T murins aux antigènes de *Mycobacterium leprae*. Des souris ont été immunisées par *M. leprae* dans l'adjuvant incomplet de Freund, par inoculation à la naissance de la queue. Après 5 jours, on a procédé à la récolte des lymphocytes dans les ganglions lymphatiques afférents; ceux-ci ont été alors cultivés en présence d'antigènes de *M. leprae*. La capture de thymidine radioactive a été utilisée pour quantifier la prolifération des lymphocytes causée par les antigènes. On a déterminé l'action de la densité cellulaire et de la concentration en antigènes sur la cinétique de la réponse proliférative. On fournit des données qui démontrent que ce sont les lymphocytes T qui prolifèrent suite à la présence d'antigènes dans les microcultures, et que cette réponse lymphocytaire varie selon la souche de souris utilisée. Ce système d'évaluation peut être utilisé pour identifier les antigènes chez *M. leprae* qui induisent une réponse des cellules T et pour mettre en évidence le rôle des cellules T spécifiques de *M. leprae* qui sont impliquées dans cette réaction.

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