INTERNATIONAL JOURNAL OF LEPROSY

Volume 54, Number 1
Printed in the U.S.A.

Immunity to Leprosy. III. The *in vitro* Induction of B Lymphocyte Proliferation by Mycobacteria[1]

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Lymphocytes can be induced to proliferate and differentiate to effector cells by polyclonal mitogens which act on large numbers of cells, or by antigens which are specific for lymphocytes expressing appropriate antigen-binding receptors. It has been known for many years that the cell walls of bacteria contain many substances which are mitogenic or antigenic for lymphocytes. Classical examples are the lipopolysaccharide (LPS) of the Gram-negative bacterial membrane ([1, 10, 17, 22]), and the purified protein derivative (PPD) of *Mycobacteria tuberculosis* ([5, 20]). Tuberculin antigen, derived from mycobacteria, has been widely used in diagnostic studies ([11, 12]). The chemistry of antigenic determinants from mycobacteria, whether shared by species or species-specific, is poorly understood ([5]). Our research is directed towards determining the antigens found on *Mycobacterium leprae* which are responsible for eliciting T-cell responses in mice. We have recently described the T-cell proliferative responses of murine T lymphocytes to *M. leprae* ([6]) and shown that the H-2D gene region of the major histocompatibility complex exerts a strong effect on the magnitude of *in vitro* T-cell proliferative responses ([7]). In extending this work to compare the antigenic reactivities of other mycobacterial species to *M. leprae,* we have observed that some, but not all, mycobacteria act directly as potent lymphocyte mitogens in cultures. If *in vitro* techniques are to be used to analyze T-cell responses to antigenic determinants, mitogenic properties of mycobacteria must be recognized if present in order to understand the mechanisms of lymphocyte stimulation. Since it is known that the purified protein derivative from *M. tuberculosis* is a B-cell mitogen ([15, 20]), we were also concerned that other mycobacteria may also contain similar B-cell-stimulating products.

In this paper, we compare the mitogenic properties of 14 different species of mycobacteria, showing that several of these mycobacteria act as classical polyclonal B-cell mitogens. We discuss the mitogenic substances of mycobacteria and the methods that can be used to analyze T-cell responses to mycobacterial antigens.

MATERIALS AND METHODS

Animals. F_1 hybrid BALB/c × DBA/2J (CDF_1) mice, C3H/HeN, and C3H/HeJ mice were obtained from the breeding facility maintained by the Department of Immunobiology in the Auckland School of Medicine. Mice from 6–10 weeks of age were used for all experiments.

Mycobacteria. *M. leprae* purified from armadillo tissue was kindly provided after irradiation and lyophilization by Dr. P. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A. *M. gordonae, M. scrofulaceum, M. vaccae, M. avium-intracellulare, M. bovis, M. phlei, M. chelonei, M. smegmatis, M. fortuitum, M. avium, M. marinum, M. kansasii, M. tuberculosis* and *M. bovis* strain Calmette Guérin were obtained from the typed collection of the National Tuberculosis Reference Laboratory, Greenlane Hospital, Auckland, New Zealand. These species of mycobacteria were all prepared by culturing on Löwenstein-Jensen medium. Each mycobacterial preparation was separately harvested from the medium, suspended in sterile saline, washed twice in RPMI 1640 tissue culture medium,

[1] Received for publication on 12 July 1985; accepted for publication in revised form on 16 December 1985.

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and washed once each in sterile saline, distilled water, and in 0.1 M ammonium acetate. The mycobacteria were lyophilized and then sterilized by irradiation (60,000 Rad) at the ICI Tasman Vaccine Laboratory, Wellington, New Zealand. All mycobacteria were then stored at 4°C under sterile conditions until use.

Lymphocyte cultures. For use as antigen, lyophilized irradiated mycobacteria were weighed out and resuspended in sterile saline at a concentration of 2 mg/ml. Single cell suspensions derived from spleen, thymus, and lymph node tissues were cultured in RPMI 1640 culture medium supplemented with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate. Cell suspensions at 4×10^6 cells/ml were dispersed in 200 μl cultures with 10 μl of mycobacterial antigen at concentrations ranging from 6 μg/ml to 100 μg/ml. After incubation for 3 days at 37°C in 8% CO_2 with humidified air, triplicate cultures were pulsed with tritiated thymidine, harvested on glass-fiber filter paper, and the radioactivity measured in a liquid scintillation counter.

T-cell and adherent-cell depletion. Lymph node cell suspensions were prepared as described above in 5 ml of culture medium. 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 ([13]). After incubation at 37°C for 10 min, the cells were pelleted by centrifugation, resuspended in 5 ml of culture medium, and 0.25 ml of spleen-absorbed guinea pig complement added. After incubation at 37°C for a further 30 min, the cells were washed twice and then cultured with *M. leprae* antigen or mitogen as detailed above. To determine the efficiency of T-cell depletion, a small aliquot of these cells was also stained with fluorescein-labeled anti-Thy-1 antibody ([13]). All of the lymph node cells treated with anti-Thy-1 antibody and used in the experiments described here contained less than 1% Thy-1+ cells. Adherent cells were removed by passing the cells through columns of Sephadex G-10 ([14]).

Detection of mouse immunoglobulin. The levels of mouse antibody in the culture supernatants were determined by enzyme-linked immunosorbent assays (ELISA) as described elsewhere ([4,24]). Goat anti-mouse immunogobulin in phosphate buffered saline (PBS) (pH 7.2) was dried on microtiter plates (1 μg/well), fixed with methanol, and the plates then incubated with a buffer containing 50% horse serum in PBS for 15 min. The culture supernatants (100 μl/well) were incubated for 45 min, the supernatants were then removed, and the plates washed extensively with PBS containing 0.5% Tween 20. Mouse immunoglobulin was detected by the addition of a urease-conjugated goat anti-mouse immunoglobulin for 20 min, then extensive washing with PBS 0.5% Tween 20, and a final rinse with deionized water. The plates were developed by adding urea substrate (0.2 mM EDTA, 8 mg bromocresol purple, 100 mg urea, in 100 ml deionized water, pH 4.8), and incubating for 1–2 hr at 37°C, then measuring absorbance at 540 and 690 nm.

RESULTS

Mitogenic effects of mycobacteria. The suspensions of irradiated mycobacteria in saline were cultured with normal lymph node cells prepared from CDF_1 mice at concentrations ranging from 6–100 μg/ml. After 3 days in culture, the cells were incubated with radioactive thymidine. The data presented in Figure 1 show the results obtained using *M. leprae, M. kansasii, M. bovis,* and *M. gordonae. M. gordonae* was clearly mitogenic in lymph node cell cultures; whereas *M. kansasii* and *M. leprae* showed no significant proliferative effects. *M. bovis* stimulated an intermediate proliferative response.

Similar experiments were performed a number of times to compare the effects of 14 different mycobacterial species in lymph node cultures. To present the results in a compact manner, the mitogenic index has been calculated in lymph node cultures incubated with 100 μg/ml of each mycobacteria and presented in Figure 2. The mitogenic index (radioactive uptake at any one mycobacterial concentration divided by background uptake of lymph node cells lacking mycobacteria) rose in proportion to the mycobacterial concentration over a range from 6–100 μg/ml, but the relative mitogenicity of the bacteria to each other remained the same as that shown in the results

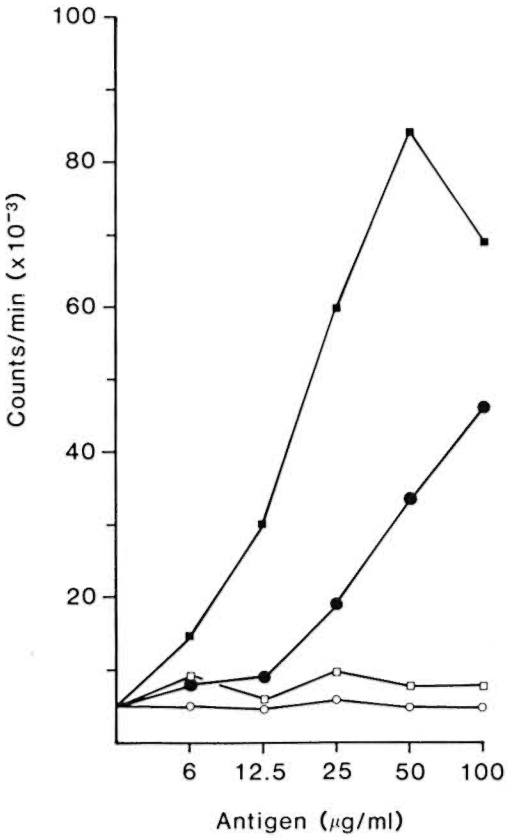

FIG. 1. Mitogenic effects of mycobacteria in CDF_1 lymph node cultures. Suspensions of *M. gordonae* (■), *M. bovis* (●), *M. leprae* (□) and *M. kansasii* (○) were cultured in normal lymph node cultures at concentrations of 6–100 μg/ml. After 3 days, the cultures were radiolabeled with ^{3}H-thymidine for 6 hr.

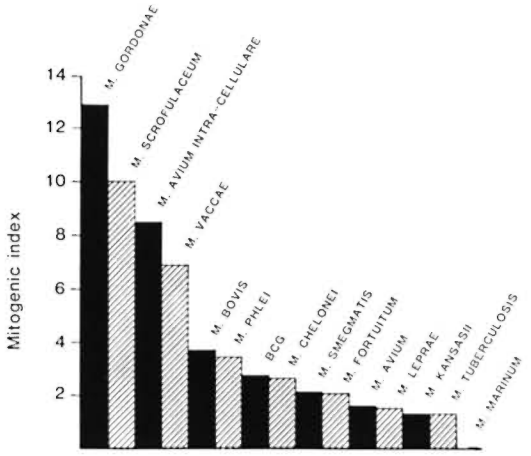

FIG. 2. Comparative mitogenic effects of 14 mycobacterial species in lymph node cultures. Each mycobacterium was cultured at a concentration of 100 μg/ml in CDF_1 lymph node cultures. After 3 days, the cultures were radiolabeled with ^{3}H-thymidine for 6 hr. The mitogenic index (uptake of radioactivity in cultures containing mycobacteria divided by the background incorporation in cultures lacking mycobacteria) is presented.

in Figure 2. These mitogenic effects have been consistent using spleen or lymph node cultures in many experiments. As a result, the mycobacteria could have been arbitrarily divided into three mitogenic groups: high, intermediate, and low.

Mycobacteria which showed little or no mitogenicity included *M. leprae, M. marinum, M. kansasii, M. tuberculosis, M. fortuitum, M. smegmatis, M. avium* and *M. chelonei.* Mycobacteria which elicited strong mitogenic responses included *M. avium-intracellulare, M. gordonae,* and *M. scrofulaceum.* Mycobacteria which stimulated mitogenic responses intermediate between the other two groups included *M. bovis, M. vaccae,* and *M. phlei* (Fig. 2). We have compared the kinetics of mitogenic responses elicited by these mycobacteria in lymph node cultures and found that these groupings did not change with time in culture. Further, sonicates prepared from *M. gordonae, M. scrofulaceum,* and *M. vaccae* are mitogenic, and the mitogenicity is resistant to the action of trypsin and pronase (data not shown).

Effect of mycobacteria in spleen cell cultures. The data presented in Figure 3 are the mitogenic indices of mycobacteria determined using CDF_1 spleen cells cultured with 100 μg/ml of mycobacteria under exactly the same conditions as those described for lymph node cells (Fig. 2). The pattern of mitogenicity was essentially the same in the spleen cell cultures as that observed for the lymph node cultures. The major difference was that *M. vaccae* appeared as a strong mitogen in spleen cultures (Fig. 3) but as an intermediate mitogen in lymph node cultures (Fig. 2).

Characterization of mitogen response. One problem in the preparation of mycobacteria is the potential for the contamination of the cultures with Gram-negative bacteria. To test whether the mitogenic responses were due to the presence of contaminating lipopolysaccharide from Gram-negative bacteria, the responses to various mycobacteria were compared using C3H/

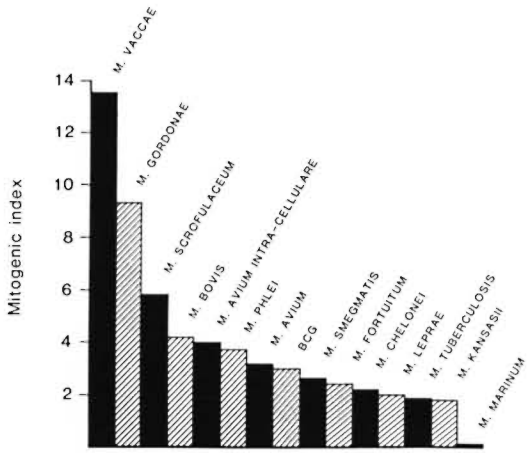

FIG. 3. Comparative mitogenic effects of 14 mycobacterial species in spleen cultures. Each mycobacterium was cultured at a concentration of 100 μg/ml in CDF_1 spleen cultures. After 3 days, the cultures were radiolabeled with ^{3}H-thymidine for 6 hr. Mitogenic indices were calculated as described in legend for Figure 2.

HeN and C3H/HeJ spleen cultures. C3H/HeJ mice have been shown to contain a gene, *Lps*^{r}, which renders lymphocytes unresponsive to LPS ([21]). Spleen cultures prepared from C3H/HeJ and responder C3H/HeN mice were cultured with *M. scrofulaceum*. The data presented in Figure 4 show that *M. scrofulaceum* induced mitogenic responses in both C3H/HeN and C3H/HeJ spleen cultures, indicating that the stimulating substance was not LPS. Identical response patterns were observed using *M. gordonae* and *M. vaccae* (data not shown). To further determine whether T cells were required for the mitogenic response elicited by *M. scrofulaceum*, a sample of C3H/HeN spleen cells was treated with anti-Thy-1 antibody and complement before culture. The responses of normal and T-cell-depleted C3H/HeN spleen cells to *M. scrofulaceum* were identical, indicating that the mitogenic response was not T-cell dependent (Fig. 4A).

The data presented in Figures 4B and 4C are the controls for the experiment shown in Figure 4A. First, to test the effectiveness of the T-cell depletion, normal C3H/HeJ, normal C3H/HeN, and anti-Thy-1-treated C3H/HeN spleen cells were incubated with various concentrations of concanavalin A (ConA) for 48 hr, and the radioactive thymidine uptake measured (Fig. 4B). The data shown in Figure 4B revealed that both C3H/HeN and C3H/HeJ spleen cells responded mitogenically to ConA, but that the anti-Thy-1 treatment of C3H/HeN spleen cells prior to culture resulted in a complete loss of responsiveness to ConA. To show that C3H/HeJ spleen cells were unresponsive to LPS, the data presented in Figure 4C show that both normal and anti-Thy-1-treated C3H/HeN spleen cells responded to *Escherichia coli* K235 LPS, but that the C3H/HeJ spleen cells failed to respond to LPS. These experiments have been repeated using *M. gordonae* and *M. vaccae* and have given identical results (data not shown). In other experiments, splenocytes from C3H/HeJ mice, T-cell depleted by prior injection with anti-Thy-1 antibody *in vivo* ([14]), were harvested and the adherent cells were removed by passage through columns of Sephadex G-10 ([14]). These T-cell- and adherent-cell-depleted spleen cells gave proliferative responses in the presence of *M. gordonae, M. scrofulaceum,* and *M. vaccae* comparable with those of untreated C3H/HeN spleen cells (data not shown). These studies establish that neither adherent cells nor Thy-1+ cells are required for the mitogenic response to *M. scrofulaceum, M. gordonae,* and *M. vaccae,* and that the active entity is not lipopolysaccharide. Further, we have repeatedly tested thymocyte cultures for mitogenic responsiveness to mycobacteria. Thymocyte cultures do not respond mitogenically to any of the mycobacteria species used here.

B-cell responses to mycobacteria. To determine the effects of mitogenic mycobacteria on B cells, CDF_1 spleen cells were depleted of T cells by treatment with anti-Thy-1 antibody and complement. Cells were then cultured with concentrations of *M. scrofulaceum* and *M. gordonae* ranging from 5–100 μg/ml. After 3 days, triplicate cultures were assayed for radioactive thymidine uptake to measure mitogenic responses (Fig. 5A). A further set of triplicate cultures assayed immunoglobulin synthesis to determine whether the mitogenic response was accompanied by polyclonal antibody production (Fig. 5B). The data presented in Figure 5 show that in T-cell-depleted spleen cultures *M. scrofulaceum* acts as a polyclonal B-cell mitogen.

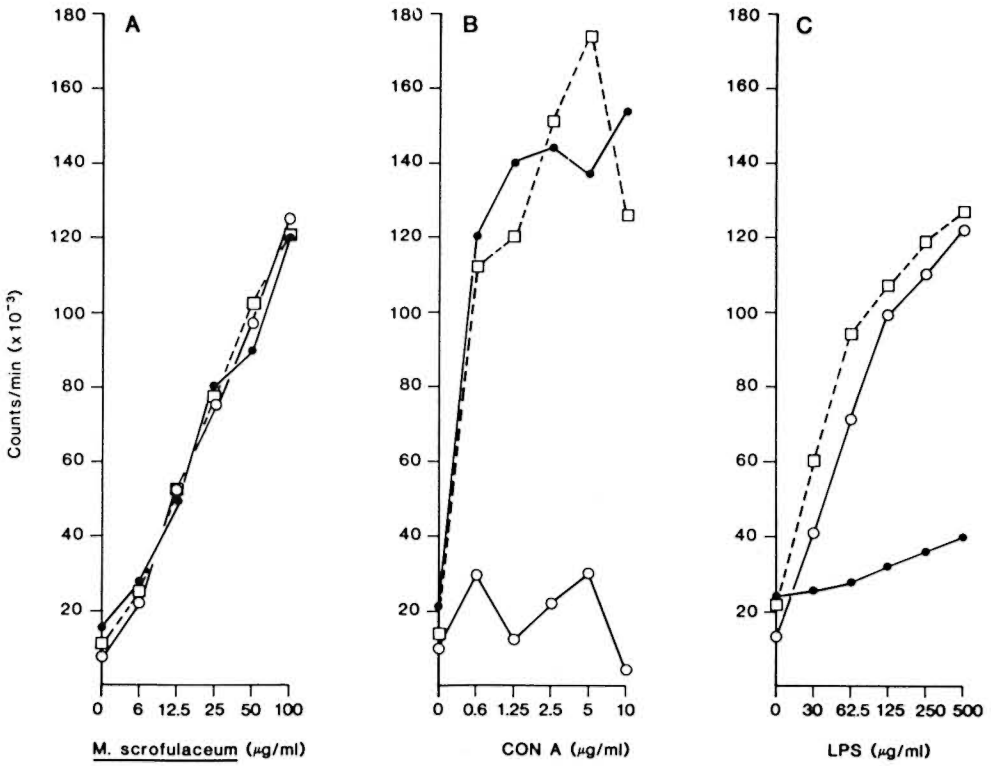

FIG. 4. Nature of the mitogenic activity of *M. scrofulaceum.* Spleen cultures were prepared using C3H/HeJ spleen cells (●), C3H/HeN spleen (□), or anti-Thy-1-treated C3H/HeN spleen cells (○). **A)** Cultures were incubated with 6–100 μg/ml of *M. scrofulaceum.* **B)** Cultures were incubated with 0.6–10 μg/ml of ConA. **C)** Cultures were incubated with 30–500 μg/ml LPS.

DISCUSSION

The characteristics of the proliferative response elicited by several species of irradiated mycobacteria in murine lymph node and spleen cultures described in this paper show some similarity to those elicited by PPD ([6, 11]). The question that arises is whether the mitogenic substances responsible for the effects described are due to PPD, or a PPD-like substance. The strong mitogenic properties of *M. gordonae, M. scrofulaceum* and *M. vaccae* can be seen using viable mycobacteria, irradiated and lyophilized mycobacteria, sonicated mycobacteria, or pronase-treated sonicates (data not shown). These polyclonal stimulations are much greater (>10-fold) than those reported for PPD (<3-fold) elsewhere ([6, 11]). We have consistently observed that the mitogenicity of PPD is much less than those of whole mycobacteria described here.

All species of mycobacteria contain multiple antigens. Cell wall polysaccharides, proteins, and peptides have all been shown to be antigenic. Mycobacteria are widely used because of their multiple effects on the immune response due to the adjuvant properties of substances such as the muramyl dipeptide component of the cell wall ([23]). We think it likely that the mitogenic effects elicited by *M. scrofulaceum, M. gordonae, M. vaccae,* and *M. bovis* are due to a substance not related to PPD. One of the distinctive properties of mycobacteria is that a range of glycolipids have been identified immunologically on the outer cell wall ([2, 3, 9]). Thus far, three immunogenic glycolipid groups have been described. A specific phenolic glycolipid has been chemically defined and found to be a unique antigen for *M. leprae* ([9]). A glycopeptidolipid group has been associated with the *M. avium/M. scrofulaceum* complex ([2, 3]). A third group, the lipooligosaccharides, have been character-

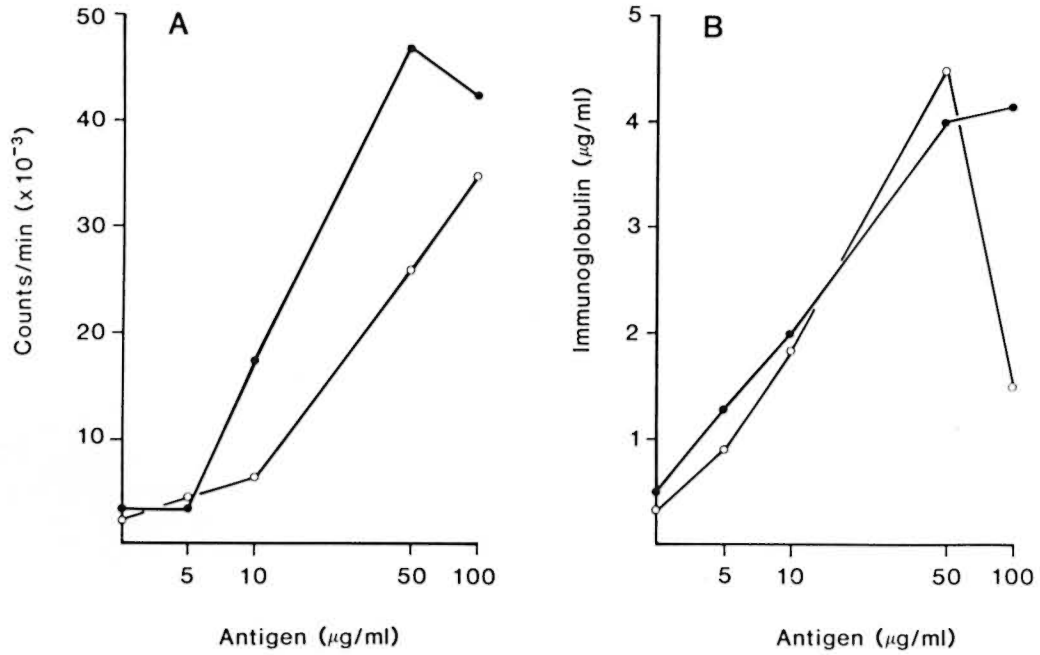

FIG. 5. Polyclonal mitogenic effects of *M. scrofulaceum* and *M. gordonae*. CDF_1 spleen cells were depleted of T cells by treatment with anti-Thy-1 antibody and complement and cultured with 5, 10, 50, and 100 μg/ml of *M. scrofulaceum* (●) and *M. gordonae* (○). **A)** ^{3}H-thymidine uptake in cultures after 3 days. **B)** Mouse immunoglobulin concentration in culture supernatants after 3 days.

ized from *M. kansasii* [8]. In view of the known B-cell mitogenic properties of lipid A from Gram-negative bacteria [1, 10, 17, 22], there may exist glycolipid structures common to some species of mycobacteria which are also polyclonal B-cell mitogens.

The mycobacteria that we have tested do not readily fall into a group with common features, are ubiquitous, and have been found all over the world. They are found in all groups according to the classification scheme of Runyon, *et al.* [16].

The particular issue underlying this study that is important to our own research concerns the nature of the antigens that elicit T-cell-derived responses. Mycobacterial proteins readily induce cell-mediated hypersensitivity as observed in delayed skin-test reactions and a variety of *in vitro* assays of cellular hypersensitivity [18, 19]. Immunization also leads to antibody responses to a variety of mycobacterial polysaccharides and proteins, but humoral responses do not appear to play a significant role in providing protection against the disease bacillus. The problem in leprosy is to determine how cell-mediated immune responses can lead to protective immunity, and which specific antigens are responsible for eliciting protective immune responses.

The importance of these results to our work is the following. We have described the use of an *in vitro* T-cell proliferative assay to *M. leprae* in murine lymph node cultures [6] and are attempting to use this assay to define the antigenic determinants of *M. leprae* that induce T-cell responses [7]. We have not observed that whole mycobacteria or sonicates act as T-cell mitogens [7] and because of that the immune responses to antigenic determinants can be readily observed. In preparing to screen different species of mycobacteria for T-cell-specific antigens that show crossreactivity with antigens from *M. leprae*, it was apparent that several mycobacteria, namely, *M. scrofulaceum, M. vaccae* and *M. gordonae*, are potent polyclonal B-cell mitogens. We are unaware of other such reports of these effects in the literature. *In vitro* assays such as the T-cell proliferation response are valuable techniques to determine the antigens

with which T lymphocytes react. It is not yet clear whether those antigens on mycobacterial cell surfaces which are recognized by T cells are the same as or different from those against which antibodies are generated. However, it is a reasonable hypothesis that the antigens recognized by lymphocytes *in vitro* are the same as the antigens seen *in vivo*. Since most lymphocyte culture assays measuring proliferative responses contain mixtures of B and T lymphocytes, the presence of a polyclonal mitogen for one of these cell types could make the interpretation of experiments difficult. By recognizing that polyclonal B-cell mitogens exist in some species of mycobacteria, it is possible to adapt the lymph node culture assay to selectively screen for either antigenic or mitogenic responses.

SUMMARY

The development of murine proliferative response assays has been initiated to begin to evaluate T-lymphocyte responses to the antigens of *Mycobacterium leprae*. In this study, *M. leprae* and 13 related strains of mycobacteria have been tested for stimulatory effects in proliferation assays using murine spleen, thymus or lymph node cultures. A number of mycobacteria were found to directly stimulate the proliferation of spleen and lymph node cells of all mouse strains tested including C3H/HeJ mice. Thymocyte cultures showed no response. The mitogenic effects of mycobacteria in spleen cultures were not dependent upon the presence of T cells or adherent cells, and resulted in the production of antibody-forming cells. Thus, these bacteria acted as polyclonal B-cell mitogens and could be readily distinguished from the lipopolysaccharide of Gram-negative bacteria by their mitogenic activity on C3H/HeJ spleen cells. The species of mycobacteria which exhibit direct mitogenic effects in spleen and lymph node cultures are a particular problem when specific immune responses to the antigens of these bacteria are compared. Such comparisons are necessary if *in vitro* assays are to be used to determine the nature of cross-reactive antigens between *M. leprae* and other mycobacteria.

RESUMEN

Iniciamos el desarrollo de ensayos para evaluar la respuesta proliferativa de linfocitos T murinos contra los antígenos del *Mycobacterium leprae*. En este estudio se probó la capacidad del *M. leprae* y de otras 13 micobacterias relacionadas para estimular la respuesta proliferativa de las células de bazo, de timo y de ganglios linfáticos murinos. Se encontró que varias micobacterias estimularon la proliferación de las células del bazo y de los ganglios linfáticos de todas las cepas de ratón probadas, incluyendo a la C3H/HeJ. Los timocitos no respondieron. Los efectos mitogénicos de las micobacterias en los cultivos esplénicos no fueron dependientes de la presencia de células T o de células adherentes y dieron como resultado la producción de células formadoras de anticuerpos. Estas bacterias actuaron como mitógenos policlonales de los linfocitos B y pudieron ser distinguidos fácilmente del lipopolisacárido de bacterias Gram-negativas por su actividad mitogénica sobre células de bazo C3H/HeJ. Las especies de micobacterias que tienen un efecto mitogénico directo sobre las células del bazo y de los ganglios linfáticos constituyen un problema sobre todo cuando se trata de comparar las respuestas inmunes específicas a los antígenos de esas bacterias. Tales comparaciones son necesarias en los ensayos *in vitro* diseñados para determinar la naturaleza de los antígenos compartidos por *M. leprae* y otras micobacterias.

RÉSUMÉ

On a entamé le développement d'épreuves portant sur la réponse proliférative murine en vue de commencer à évaluer les réponses des lymphocytes-T aux antigènes de *Mycobacterium leprae*. Dans cette étude, on a étudié *M. leprae* et 13 souches apparentées de mycobacteries pour mettre en évidence les effets stimulants, au cours d'épreuves de prolifération utilisant des rates de rat, le thymus, ou des cultures de ganglions lymphatiques. On a observé qu'un certain nombre de mycobactéries pouvait directement stimuler la prolifération des cellules spléniques et des cellules provenant des ganglions lymphatiques dans toutes les souches de souris qui ont été étudiées, y compris la souche C3H/HeJ. Les cultures de thymocyte n'ont montré aucune réponse. Les effets mitogènes des mycobactéries en culture de rate ne dépendaient pas de la présence de cellules-T ou de cellules adhésives; ils étaient la conséquence de la production de cellules produisant des anticorps. Dès lors, il apparaît que ces bactéries jouent le rôle de mitogènes pour les cellules-B polyclonales; elles pourraient être facilement distinguées du lipopolysaccharide des bactéries Gram-négatives, par leur activité mitogénique sur les cellules spléniques de la souche C3H/HeJ. Les espèces de mycobactéries qui témoignent d'effets mitogènes directs dans les cultures de rate et de ganglions lymphatiques présentent un problème particulier, si l'on veut comparer les réponses immunologiques et spécifiques à leurs antigènes. De

telles comparaisons sont cependant nécessaires pour les épreuves *in vitro,* dont le but est de déterminer la nature des antigènes présentant des réactions croisées chez *M. leprae* et chez d'autres mycobactéries.

Acknowledgments. This work was supported by the New Zealand Leprosy Trust Board and the South Pacific Trust Fund of the New Zealand Medical Research Council. We are grateful to the Leprosy Trust Board for their enthusiastic support and encouragement. We thank Dr. P. Brennan, Fort Collins, Colorado, U.S.A., for providing us with *Mycobacterium leprae.*

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