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Cell-Mediated Immune Response in *Mycobacterium leprae* Infected Mice[1,2]

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Studies on cell-mediated immune response in leprosy patients have been carried out by various workers ([2,3,7,8,10,13,17,18,28,29]) wherein it has been shown that patients in advanced stages of leprosy, i.e., lepromatous leprosy, were deficient in their ability to express delayed-type hypersensitivity to both homologous as well as heterologous antigens. This immune deficiency was further confirmed through studies on the ability of the patients' lymphocytes to undergo blastogenesis in the presence of PHA as well as lepromin-like preparations derived from the leprosy bacillus ([14]). However, the recent work of Rea *et al* ([23]) appears not to be in agreement with these observations.

All of the studies mentioned above have been conducted on leprosy patients. Although Rees *et al* ([24]) had established the mouse model introduced by Shepard ([26]) to be in the BB or BT spectrum of human leprosy, studies on immune response either on cellular or humoral level were not undertaken till recently ([21]). However, this model has been extensively used for screening various antileprosy drugs ([11,16,22,24]). Present studies were undertaken to evaluate the mechanisms of cell-mediated immune response in mice infected with *M. leprae*. This work complements earlier studies on the humoral response of similarly infected mice ([21]).

MATERIALS AND METHODS

Mice. Six to eight week old female inbred strains of BALB/c mice were used throughout the experiments. These mice were bred and raised in the laboratory of Dr. L. Levy, USPHS Hospital, San Francisco, California.

Infection and immunization. The infection and immunization were carried out in Dr. Levy's laboratory, prior to receipt of the animals by our laboratory. Three groups of mice were studied. Sixty mice in each group were assigned randomly. Groups A and B were inoculated in the right hind foot pad each with 5×10^3 viable and nonviable *M. leprae* respectively, harvested during logarithmic multiplication in mice by the method of Shepard ([26]) and as used by Levy *et al* ([15]). Group C consisted of normal uninoculated mice. For the nonviable challenge, the *M. leprae* were killed by five repeated cycles of freeze-thawing.

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Skin tests. Since both the infection and immunization were done in the foot pad of the animals, it was decided to do the skin tests in the ears of the animals. Seventy-two hours prior to a scheduled sacrifice, skin testing materials were injected intradermally into the ears, using a tuberculin syringe with a 27 gauge needle. Extent of swelling and ear thickness were measured with a Schnelltaster (Schnelltaster system, Kroplin, W. Germany) at 0, 6, 18, 24, and 48 hours after intradermal injections. Prior to skin testing, the thickness of the normal ears was measured. This ranged between 0.3 and 0.5 mm. A difference in excess of 1.0 mm in measurement between the normal ear and the test ear was considered as a significant reaction.

The amount of 0.03 ml each of *M. leprae* cell extract (20 μg/ml protein), preparation of which has been described previously ([20]), PPD (25 μg/ml, kindly supplied by the U.S.-Japan Cooperative Medical Science Program, NIAID, through the Center for Disease Control, Atlanta, Georgia) and Dharmendra lepromin were used as skin testing antigens at the concentrations mentioned, with sterile saline used as a control. The skin test antigen was placed in one ear of a given animal and saline was injected in the contralateral ear. At the time of sacrifice, both ears were excised and maintained in a tissue fixative for future histopathologic studies. The tests were read "blind" by two individuals not aware of the placement of particular materials in a given ear.

Lymphocyte transformation (LT). Four mice in each group were sacrificed by cervical dislocation at biweekly intervals beginning one week post-infection. Prior to sacrifice, blood was collected from both eyes by intraorbital puncture and the pooled plasma thus collected was maintained at -20°C for use in immunoglobulin and serum protein assays. Inguinal and mesenteric lymph nodes were collected and pooled in each group. The nodes were teased out gently on a 200 mesh stainless steel screen into sterile Hanks' tissue culture medium using sterile blunt forceps. The single cell suspensions thus obtained were centrifuged in cold at 600×g and washed three times. The final cell pellet was suspended at a concentration of 10^6 viable cells/ml of RPMI-1640, supplemented with penicillin-streptomycin, L-glutamine and 10% heat inactivated normal human serum (Difco Laboratories, Detroit, Michigan). The cell suspension was cultured in one milliliter volumes of RPMI-1640 in 16 × 125 mm upright glass tubes fitted with plastic closures. Cell cultures, in triplicate, were stimulated by the addition of phytohemagglutinin (PHA-Difco) to a final concentration of 10 μl/ml, lipopolysaccharide (LPS-Difco) at 100 μg/ml and *M. leprae* cell extract at 20 μg/ml. To avoid repeated freeze-thawing, all the stimulating agents were aseptically diluted in Hanks' balanced salts solution (HBSS) to obtain the required concentration of each material in 0.1 ml of HBSS. The diluted fluids were stored in sterile provials (Cooke Laboratories, Alexandria, Virginia) at -70°C. As a standard procedure the contents of each single vial were used during an experiment immediately after thawing. All cultures were incubated for a total period of 96 hours at 37°C in 5% CO_2 atmosphere.

Isotope incorporation. All lymphocyte cultures were pulse-labeled by adding 1 μCi/ml of ^{3}H-Tdr (specific activity: 1.9 Ci/mmol, Schwarmann) in 0.1 ml HBSS approximately 24 hours before culture termination. Determination of label uptake was done as described by Colley and DeWitt ([5]). Briefly, cells were washed twice in ice-cold 0.15 M NaCl and precipitated twice in cold 5% (w/v) trichloroacetic acid (TCA) for ten minutes. The final TCA-insoluble precipitates were dried overnight at room temperature and dissolved in 0.4 ml of hydroxide of hyamine 10x (ICN Isotope and Nuclear Division, Cleveland, Ohio). The dissolved material was transferred with 2 ml of absolute ethanol to 20 ml capacity scintillation vials containing 10 ml of scintillation fluid (5 gm PPO and 1 gm POPOP per liter toluene). All samples were counted in a Beckman LS-150 liquid scintillation counter with a counting error of 5% or less. Since the degree of quenching was constant for all samples, the results are expressed as counts per minute (cpm) per culture.

Each of the tests was done identically in two separate experiments and the data was calculated as the average of reading obtained for the two experiments.

RESULTS

Course of infection. No significant differences were observed in spleen weights of

animals in all three groups throughout the course of experiments, neither were any gross pathologic changes noted.

Skin reactivity. Table 1 shows the data on skin tests carried out as a measure of delayed-type hypersensitivity (DTH). A positive skin reactivity to the homologous cell extract antigen was observed in the mice seven weeks after immunization with the killed *M. leprae*. However, this response was transient in nature and diminished to the base level within a short time. Compared to this, positive skin reactions were observed at the 11th week among the animals in the infected group. Once developed, this positivity to the homologous antigen persisted up to the 15th week, i.e., the time of termination of the experiments. PPD gave either weakly positive or negative skin reactions in all the three groups. In the infected and immunized groups, the Dharmendra antigen gave reactions that were of a

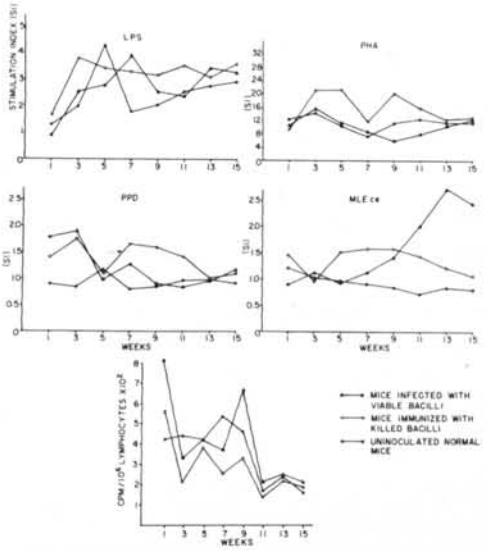

FIG. 1. Transformation of lymphocytes from normal and *M. leprae* infected and immunized mice, with PHA, LPS, PPD and *M. leprae* cell extract.

TABLE 1. *Skin reactions in* M. leprae *infected mice.*

Group[a]	Skin test antigens[b]	Reading in mm/week of sacrifice							
		1	3	5	7	9	11	13	15
A	Mle ce	—	0.50 E	0.50 E	0.52 E	1.04 E.S	1.53 E.S	1.55 E.S	1.56 E.S
	PPD	—	—	0.50 E	—	0.51 E	1.04 E.S	1.00 E	1.05 E.S
	Lepromin	ND	0.50 E	0.50 E	0.50 E	0.50 E	—	1.04 E.S	1.00 E.S
	Saline	—	—	—	—	—	—	—	—
B	Mle ce	—	0.50 E	0.52 E	1.15 E.S	1.00 E.S	0.65 E	0.52 E	0.55 E
	PPD	—	—	—	0.50 E	1.00 E.S	0.52 E	0.54 E	—
	Lepromin	—	—	0.50 E	0.50 E	0.52 E	0.52 E	0.45 E	0.48 E
	Saline	—	—	—	—	—	—	—	—
C	Mle ce	—	0.50 E	—	—	—	0.52 E	—	—
	PPD	—	—	—	—	—	—	—	—
	Lepromin	—	0.52 E	0.50 E	0.50 E	0.48 E	0.53 E	0.55 E	0.52 E
	Saline	—	—	—	—	—	—	—	—

[a] A = foot pad infection with 5×10^3 live *M. leprae*.
B = immunization with 5×10^3 killed *M. leprae*.
C = uninfected normal mice.
[b] Mle ce = *M. leprae* cell extract; lepromin = Dharmendra antigen.
(—) = no measurable swelling (S) or erythema (E).
ND = not done.
Note: A difference of 1 mm or more in ear thickness (S) accompanied by erythema (E) is considered to be a positive reaction.

low order, and in the control group this antigen showed only erythema but no visible or measurable swelling. Skin reactions to saline were negative in all animals throughout the course of these investigations.

Lymphocyte transformation. Figure 1 gives the data on lymphocyte transformation studies. The results are an average of triplicate cultures for two different experiments. The lymphocyte stimulation index (SI) was calculated as counts per minute (cpm) obtained in a stimulated culture of lymphocytes of mice in a given group/cpm obtained in a nonstimulated culture of lymphocytes of mice in the same group.

The responses to LPS, a nonspecific B cell mitogen ([12]), as well as to PHA, a nonspecific T cell mitogen ([6, 12, 30]), were quite uniform and persistent in lymphocyte cultures from animals of all the three groups all through the entire period of these studies. This served as a measure of positive control. Lymphocytes from the infected animals which had positive skin reactions transformed in the presence of homologous cell extract antigen. The development, however, of positive skin reactivity and lymphocyte transformation in the infected group, were not evident until the 11th week post-infection. Lymphocytes from the immunized animals, which showed positive skin reactions, exhibited blast formation when stimulated with the homologous antigen. This transformation was seen as early as the fifth week, remained constant up to the ninth week, and declined thereafter.

DISCUSSION

The experiments were designed to determine the kinetics of cell-mediated immunity in mice infected with *M. leprae*. In an attempt to follow the course of infection, spleen weights were recorded for each animal sacrificed at a specific time interval. No significant differences were observed in the spleen weights of mice in all the three groups at any given interval. The foot pad infection of mice with *M. leprae* produces a self-limiting disease which remains confined to the foot pad only and does not become systemic till about one or two years after infection ([24]). It is likely that the spleen weights of the animals remained unchanged because of the localization of infection within the foot pad, especially since the experiments were terminated at about the 15th week post-infection.

Positive skin reactivity to homologous antigen observed at the 11th week among the animals in the infected group indicates the development of skin reactivity at this stage of bacillary multiplication. Once developed, the reactivity persisted up to the 15th week, i.e., till the termination of the experiments. In the group of animals immunized with the killed *M. leprae*, the onset of delayed hypersensitivity was early as compared to the infected group. The decline in this sensitivity at a later juncture can be attributed to diminution in the antigenic stimulus as time progressed. Animals in Group C (uninfected, normal) did not elicit positive skin reactions with either the *M. leprae* cell extract or PPD. These animals did, however, exhibit erythema only at the site of injection when the Dharmendra antigen was used. A possible explanation of this could be the presence of particulate matter, both bacillary as well as tissue, in this antigenic preparation. The weakly positive skin reactions to this antigen seen in the infected and the immunized groups of animals, could be due to the fact that the Dharmendra antigen used has been in storage for several years before it was used and hence its potency may have diminished considerably. Abe *et al* ([1]) state that the lepromin preparation made in their laboratory and maintained in the cold for five years had a diminished potency as judged by the ability to detect the late reaction in patients in comparison with a portion of the same preparation lyophilized and kept for the same period. It is very likely that this may be true of Dharmendra lepromin.

It was felt that the LT test might be able to detect sensitivity despite the fact that the early skin tests were negative. Such observations have been reported by other investigators in patients as well as in infected animals ([3, 4, 27]). Failure of the lymphocytes from the infected animals to respond to an appreciable level to specific antigenic stimuli in the early phase cannot be explained satisfactorily at this time. It might be possible that although the lymphocytes were sensitized, the sensitization was of such a low order as not to be at an appreciable level. Alternatively, it is likely that at the level of infection induced, the lymphocytes were not sensitized during the early phase, due to insufficient accessibility of the antigen. How-

ever, transformation was seen to occur as the multiplication of bacilli progressed and reached a plateau level. It has been observed by other workers that lymphocyte stimulation ratio to *M. leprae* antigens was elevated only in the later stages of infection ([31]).

Evans and Levy ([9]) studied the histopathology of *M. leprae* infected mouse foot pads and observed the changes occurring at the plateau time, which are associated with onset of cellular immunity. Histologically they observed a) the persistence of mononuclear cells infiltrate, and b) the indication that mononuclear cells containing organisms were activated during killing of organisms. Observations made during present studies appear to fit well with these studies. Resistance to intracellular pathogens is vested in a cellular mechanism that involves the interplay of macrophages and specifically sensitized lymphocytes ([19]). The lymphocyte SI to the homologous cell extract antigen seemed to increase by about the 11th week after infection. The positive skin reactivity together with the elevated SI indicate the production and proliferation of antigen-reactive lymphocytes. These lymphocytes in turn govern the activities of mononuclear phagocytic cells through production of lymphokines. Occurrence of all these events coincides with the time of plateau of *M. leprae* in the foot pad of the infected mouse.

The observations made in the present study indicate that induction of skin reactivity in the mouse model correlates well with the ability of the lymphocytes to undergo blast formation in the presence of either a nonspecific mitogen or a specific antigenic stimulus. It is apparent that the response of the mouse is basically a T cell rather than a B cell one. These observations parallel our earlier studies on the humoral response in respect of the production and enhancement of IgM and the low level of IgG throughout the experimental course, despite secondary challenges being given at various intervals ([20]).

SUMMARY

Positive skin reactions to homologous antigens in mice infected with *Mycobacterium leprae* were observed at the time that the bacillary multiplication was nearing the plateau level. Once developed, the skin reactivity persisted for a considerable length of time. Lymphocyte transformation studies indicated that the lymphocytes from infected animals were unable to respond to specific antigenic stimuli in the early phase of infection. Possible explanation for this failure could be that the lymphocytes although sensitized, were not capable of responding to the stimuli due to a rather low level of sensitization. The elevated lymphocyte stimulation index in the later stages of infection, coupled with the skin reactivity indicates the production and proliferation of antigen-reactive lymphocytes which in turn govern the activity of mononuclear phagocytic cells through the production of lymphokines. Occurrence of all the events appears to coincide with the time closer to the plateauing of *M. leprae* in the foot pad of the mouse. It is suggested that the cell-mediated immune response in the *M. leprae* infected mouse is basically a T cell rather than a B cell response.

RESUMEN

En ratones infectados con *Mycobacterium leprae* se observaron intradermoreacciones positivas contra antígenos homólogos hacia el tiempo en el que la multiplicación bacilar estuvo cercana a su meseta. Una vez desarrollada, la reactividad en piel persistió durante un periodo considerable de tiempo. Los estudios de transformación de linfocitos indicaron que los linfocitos de los animales infectados fueron incapaces de responder hacia estímulos antigénicos específicos durante la fase temprana de la infección. Una posible explicación para esta falta de respuesta podría ser que los linfocitos, aunque sensibilizados, no respondieron a los estímulos debido a un bajo nivel de sensibilización. El elevado índice de estimulación de los linfocitos en los estados tardíos de la infección, asociado con la reactividad en piel, señalan la producción y proliferación de linfocitos reactivos al antígeno los cuales, a su vez, gobiernan la actividad de los fagocitos mononucleares a través de la producción de linfocinas. La aparición de estos eventos parece coincidir con el tiempo en el cual el crecimiento del *M. leprae* en los cojinetes plantares del ratón alcanza su meseta. Se sugiere que la respuesta inmune mediada por células en el ratón infectado con *M. leprae* es básicamente una respuesta de células T más que de células B.

RÉSUMÉ

Chez des souris infectées par *Mycobacterium leprae*, on a observé des réactions cutanées

positives à des antigènes homologues au moment où la multiplication bacillaire atteignait son plateau. Une fois apparue, la réactivité cutanée persiste pour une considérable période de temps. Des études de transformation lymphocytaire ont montré que les lymphocytes provenant d'animaux infectés ne répondaient pas à des stimulations antigéniques spécifiques pendant la phase précoce de l'infection. Une explication possible pour cette absence de réponse pourrait être le fait que les lymphocytes, malgré qu'ils soient sensibilisés, ne sont pas capables de répondre aux simulations par suite d'un niveau de sensibilisation relativement bas. L'observation d'un index de stimulation lymphocytaire élevé au cours des stades tardifs de l'infection, étant associée avec la réactivité cutanée, témoignent d'une production et d'une prolifération de lymphocytes réagissant aux antigènes, ces phénomènes pourraient à leur tour déterminer l'activité des cellules phagocytaires mononucléaires, par le truchement de la production de lymphokines. L'apparition de ces phénomènes semble coïncider avec le moment où la croissance de *M. leprae* atteint un plateau dans le coussinet plantaire de la souris. On suggère que la réponse immunitaire cellulaire chez des souris infectées par *M. leprae* est essentiellement une réponse par les cellules T plutôt qu'une réponse par les cellules B.

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