Enhanced Cell-mediated Immune Responses in Erythema Nodosum Leprosum Reactions of Leprosy[1]

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"Reactions" in leprosy patients are occasional episodes of significant inflammation. While erythema nodosum leprosum (ENL) occurs in lepromatous leprosy patients, "lepra reactions" may occur anywhere in the leprosy spectrum except in the two polar types ([22]). It is widely assumed that there is an immunological basis for these reactions since the position of the patient on the leprosy spectrum is related to the degree of cell-mediated immunity against *Mycobacterium leprae* ([19]). Tuberculoid patients show a high degree of cell-mediated immune (CMI) responses in contrast to the relative absence of these responses in lepromatous leprosy patients. This anergy in lepromatous patients has been found to be specific for *M. leprae* which does not revert even after treatment ([2, 20]).

ENL is characterized by crops of tender, erythematous, subcutaneous nodules and, histologically, the lesions show perivascular infiltration of polymorphonuclear cells along with histiocytes containing fragmented bacteria ([16]). Since the ENL reaction occurs in patients with depressed CMI and increased mycobacterial antigen load, it has been suggested that ENL is a clinical manifestation of the Arthus phenomenon ([22, 23]). This is supported by the presence of immunoglobulins ([23]) and complement ([10]) in the lesions and circulating immune complexes and complement degradative products ([1]).

Evidence of enhanced immune responses to dinitrochlorobenzene (DNCB) during ENL reaction ([14]) and lack of immune complex deposits and complement products in the lesions of some patients ([10, 22, 23]), however, suggests that it may be an imbalance in T-cell functioning which results in the ENL reaction. To pursue this assumption, we have studied the leukocyte migration inhibition (LMI) responses to whole and sonicate *M. leprae,* mitogen phytohemagglutinin-P (PHA), and purified protein derivative (PPD) of tuberculin in lepromatous patients, both during and after an ENL reaction. Enumeration of early T cells ([24]) was done simultaneously. There is clear evidence that *M. leprae*-specific CMI responses were enhanced during ENL reactions and slowly returned to lower levels afterward. However, there was no indication of skin reactivity to an intradermal injection of soluble *M. leprae* antigens during the ENL episode.

MATERIALS AND METHODS

Patients. A total of 77 leprosy patients in the age group 17–60 years were studied. The patients were selected from the outpatient departments of the Sivananda Rehabilitation Home, Kukatpally, and the Dhoolpet Leprosy Research Centre, Karwan, both in the city of Hyderabad, India. The patients were classified according to the Ridley-Jopling scale ([17]). Thirty-nine patients who did not have reactions at the time of examination were classified as lepromatous or borderline lepromatous leprosy. Of this group, 20 patients were fresh cases with no previous history of antileprosy treatment, and 19 patients had been treated with dapsone for less than 1 year. Forty-four lepromatous patients with ENL reactions were studied. However, only 22 out of the 44 ENL patients were followed after subsidence of the reaction. This included six patients who were followed from the LL state to the post-ENL stage. Their results are included along with other patients since their responses showed similar patterns.

An ENL patient blood sample was taken before starting treatment with anti-inflammatory drugs or steroids; whereas a post-ENL patient blood sample was taken after

[1] Received for publication on 5 February 1986; accepted for publication in revised form on 20 August 1986.

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ensuring that the patient had not been taking anti-inflammatory drugs or steroids for the last 3 or 7 days, respectively.

Leukocyte migration inhibition test (LMIT). A slightly modified technique of Soborg and Bendixen ([18]) was followed to measure the inhibition of leukocyte migration to mitogen PHA, *M. leprae* antigens, and PPD. Briefly, to 12 ml of acid citrate dextrose (ACD) anticoagulated blood, 6 ml of 3% gelatin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in saline was added in a culture tube. After thorough mixing, it was incubated at 37°C for 45 min in a slanting position to sediment erythrocytes. The clear leukocyte-rich plasma was aspirated into another tube and centrifuged to pellet the leukocytes. The leukocytes were then washed three times with minimum essential medium (MEM; Bios, Bombay, India) and finally resuspended in 1 ml of MEM to give a concentration of 3×10^7 cells/ml. The cell suspension was loaded into uniform capillaries (Arthur H. Thomas Co., Philadelphia, Pennsylvania, U.S.A.), plugged with modeling clay at one end, and centrifuged at $150 \times g \times 5$ min. The capillaries were then cut at the cells-medium interphase and were kept in polystyrene chambers (Laxbro, Pune, India). The chambers were immediately filled with MEM containing 20% fetal calf serum (FCS) with or without mitogen or antigens. They were then sealed with coverslips and incubated at 37°C for 18 hr. Each test was run in triplicate. The area of migration was measured with a planimeter. The migratory index was calculated as follows:

$$\text{migratory index} = \frac{\text{average area of migration with antigen or mitogen}}{\text{average area of migration with medium alone}}$$

Of the reagents used, phytohemagglutinin-P (PHA) was obtained from Difco Laboratories, Detroit, Michigan, U.S.A., and the purified protein derivative (PPD) of tuberculin (mammalian) was obtained from the Staten Serum Institut, Copenhagen, Denmark. Armadillo-derived whole and sonicate *M. leprae,* Batch no. AB51, was supplied by Dr. R. J. W. Rees, National Institute for Medical Research, London.

PHA, 10 μg/ml; PPD, 25 μg/ml; and 2.5×10^7 bacilli/ml of whole and sonicate *M. leprae* were found optimal in the LMIT among tuberculoid patients (data not shown), and were used as the optimal dose in the present study.

Enumeration of early T and total T lymphocytes. Early T and total T lymphocytes were enumerated by the methods of Wybran, *et al.* ([24]) and Jondal, *et al.* ([6]), respectively. Briefly, to 6 ml of ACD anticoagulated blood, 3 ml of 3% gelatin in saline was added. After thorough mixing, it was incubated at 37°C for 45 min in a slanting position to sediment the erythrocytes. Leukocyte-rich plasma was layered on Histopague (Sigma) and was centrifuged at $1000 \times g \times 30$ min. The band of lymphocytes was aspirated into another tube, and the cells were washed three times with MEM. The lymphocytes were resuspended in MEM to give a concentration of 10×10^6 lymphocytes/ml. Viability was checked with 0.2% trypan blue (ICN K&K Laboratories, Plainview, New York, U.S.A.), and 0.1 ml of the lymphocyte suspension (1×10^6 lymphocytes) were aliquoted into six tubes. To this suspension, 25 μl of FCS absorbed with sheep red blood cells (SRBC) was added. An equal volume of 1% SRBC in MEM was then added to the above suspension and incubated at 37°C in a water bath for 10 min. All of the tubes were spun at $60 \times g \times 5$ min. Three tubes were incubated at 4°C for 1 hr (early T cells), and the remaining tubes were incubated overnight at 4°C (total T cells). After incubation, the cell pellet was carefully and gently resuspended. The lymphoctyes were stained with 0.1% aqueous toluidine blue, and the rosettes were counted in a hemocytometer (Neubauer) at 450× magnification. A rosette-forming cell was defined as a lymphocyte surrounded by at least five SRBC. In each test, 200 lymphocytes were counted, and the results expressed as percentages.

Skin test. One-tenth ml of leprolin (soluble protein fraction of armadillo-derived *M. leprae* antigen, Batch no. AB23R, kindly supplied by Dr. R. J. W. Rees, National Institute for Medical Research, London) was injected intradermally into the volar aspect of the forearm of 10 LL, 12 ENL, and 2 post-ENL patients. Any early reaction after 48 hr was noted.

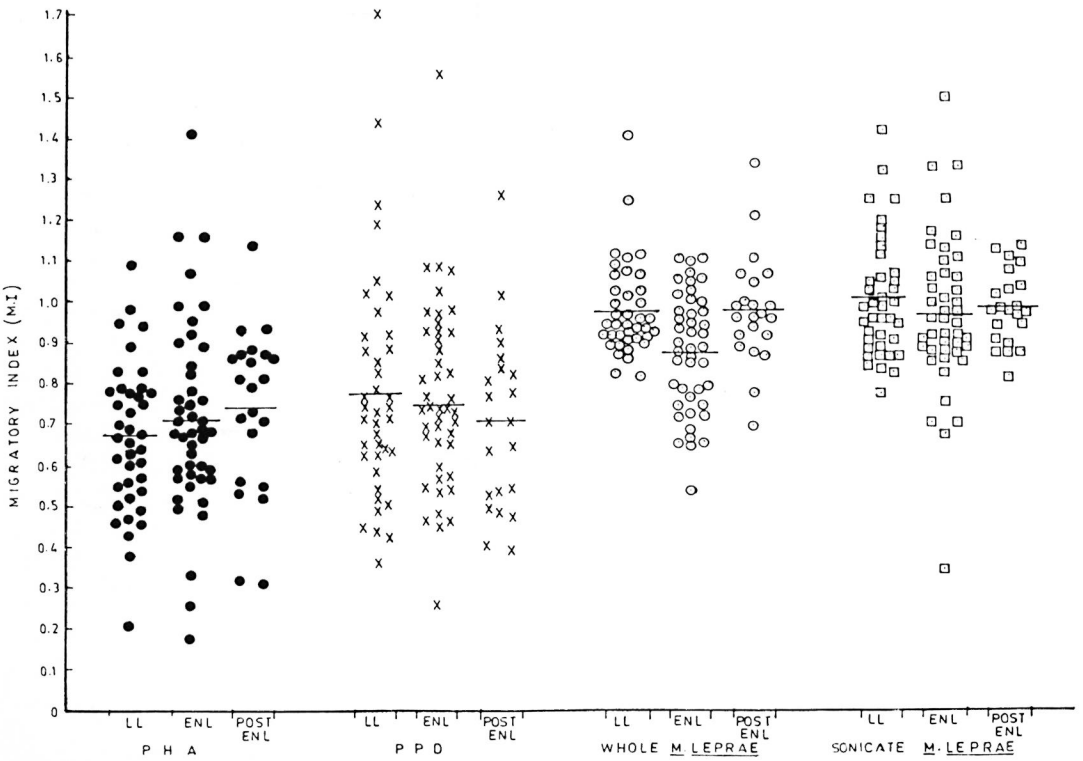

FIG. 1. Individual migratory indices of lepromatous (LL), ENL, and post-ENL patients to PHA, PPD, whole *M. leprae,* and sonicate *M. leprae.* Horizontal lines indicate migratory index.

Statistical analysis. One-way analysis of variance by a nonparametric method (Kruskal-Wallis test) was done to test the significance of variation among the groups. The two-sampled Student's *t* test was used to determine the significance of differences in the results between groups. A correlation coefficient (r) was calculated, and the significance was expressed ([3]).

RESULTS

Figure 1 shows the LMI responses to PHA, PPD, whole and sonicate *M. leprae* of the LL, ENL, and post-ENL leprosy patients. Since the distribution of the LMI responses of the patients are skewed, the nonparametric method (Kruskal-Wallis analysis of variance) was used and gave an F value of 7.8579 to whole *M. leprae,* indicating the difference in the mean migratory index at $p < 0.001$ level of significance. However, no significant difference was observed in the mean migratory index to PHA (F value of 0.8784), PPD (F value of 0.6674), or sonicate *M. leprae* (F value of 0.5878). Whole *M. leprae* elicited an increased response from the ENL group (0.87 ± 0.022), and this was highly significant ($p < 0.001$) compared to LL patients (0.97 ± 0.018). Furthermore, this enhanced response returned to a lower level than that of LL patients in post-ENL patients (0.97 ± 0.029), indicating the transitory nature of the enhancement. Sonicate *M. leprae* responses were poor in all of the groups and, also, there were no significant differences among the groups. The LMI responses obtained with PHA and PPD were uniform in all of the groups, but were higher compared to the *M. leprae* antigens. There was no crossreactivity between PPD and whole *M. leprae* ($r = 0.2176$) in these patients. A difference of 0.1 migratory index (10% inhibition) between the groups was significant.

One-way analysis of variance among the groups of leprosy patients gave an F value of 6.6869 for early T lymphocytes, indicating the difference in their mean percentages at $p < 0.01$ level of significance; whereas an F value of 1.2240 was obtained for total

T lymphocytes, indicating no significant differences in the mean percentages. The percentage of early T lymphocytes (Fig. 2) also increased in ENL patients (47.68 ± 1.78) which was highly significant ($p < 0.001$) when compared to the LL group (38.96 ± 1.67). However, their number did not come down significantly in the post-ENL group (45.66 ± 2.30) and remained high compared to the LL group. The number of total T lymphocytes was the same in all three groups.

In contrast, dermal reactions to leprolin were negative in ENL patients and, for that matter, were uniformly negative in all groups.

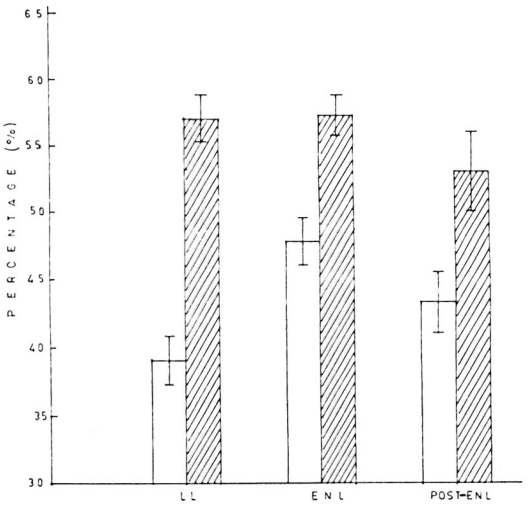

FIG. 2. Early (□) and total (▨) T lymphocytes in the peripheral blood of LL, ENL, and post-ENL patient groups. Bars represent mean; lines on bars represent standard error of the mean.

DISCUSSION

The present study indicates that CMI responses are enhanced during the inflammatory episode of ENL in lepromatous leprosy patients and return to LL levels once the episode is over. Although responses were high with PHA and PPD, they were identical in all of the groups, implying that during reaction *M. leprae*-specific responses are enhanced. The LMIT measures lymphokine production *in vitro* and its effect on leukocyte migration. Using this test, we have observed significantly enhanced responses (lower migratory indices) with whole *M. leprae* during ENL. However, there may be a differential effect of the leukocyte inhibitory factor on different species of neutrophils, and since neutrophils are increased in ENL, this could affect the pattern of migration. In an earlier study, we have shown that there is good correlation between Mitsuda reaction and migratory indices in different types of leprosy ([8]), indicating that the LMIT is an *in vitro* correlate of delayed-type hypersensitivity. Similar observations were also made by others ([12, 18]).

Varied results have been reported using lymphocyte transformation (LT) responses to PHA and PPD. Rea, *et al.* ([15]) showed no difference between ENL and LL patients; whereas others ([11, 21]) have reported significantly increased PHA and PPD responses in ENL patients than in LL patients. The emergence of antigen-reactive T cells in lepromatous leprosy patients during ENL has been reported recently using both the LMIT and leukocyte transformation test (LTT) assay systems ([7]). Studies on T-cell subsets ([11, 21]) have also shown increased ratios of T4/T8 in ENL patients, and apparent deinhibition of interleukin-2 (IL-2) synthesis *in situ* in ENL lesions ([13]).

Lim, *et al.* ([9]) have shown that early T cells increase significantly in ENL compared to LL patients. We have confirmed this observation in this study, supporting our data with LMITs of the same patients. That early T cells are an *in vitro* correlate of human delayed-type hypersensitivity has been shown by Felsburg and Edelman ([4]). Also, studies with cell-mediated immunodeficiency diseases reveal that their cell-mediated immunocompetence is reflected by the percentage of early T cells ([5]).

Interestingly, there were no *in vivo* dermal responses to leprolin in ENL patients. This might mean that there are two different types of hypersensitivity reactions or that different types of antigenic determinants are involved in differentiating *in vivo* and *in vitro* responses. An alternate explanation for the lack of dermal response could be that T-dependent antibody pathways are involved. These studies, although emphasizing the role of antigen-specific responses in ENL episodes, do not provide any evidence to indicate that these altered responses are the cause of ENL reactions.

SUMMARY

Cell-mediated immune (CMI) responses were measured in 39 lepromatous, 44 erythema nodosum leprosum (ENL), and 22 post-ENL patients. The leukocyte migration inhibition test was used to measure CMI responses to mitogen phytohemagglutinin-P (PHA), crossreacting antigen purified protein derivative (PPD) of tuberculin, and armadillo-derived whole and sonicate *Mycobacterium leprae*. "Early T" lymphocytes of the peripheral blood were also enumerated using the rosetting technique. Significantly enhanced immune responses (lower migratory indices) were found to whole *M. leprae* during ENL. Although responses were high with PHA and PPD, they were identical in all of the groups, indicating that during ENL reaction *M. leprae*-specific responses are enhanced. "Early T" lymphocytes also showed a significant increase in ENL reactions compared to lepromatous patients. However, there was no response to the leprolin skin test in ENL patients in contrast to the enhanced *in vitro* CMI responses.

RESUMEN

Se midieron las respuestas immunes celulares (RIC) en 39 pacientes lepromatosos, en 44 pacientes con eritema nodoso leproso (ENL), y en 22 pacientes post-ENL. La prueba de inhibición de la migración de leucocitos se usó para medir las RIC contra el mitógeno fitohemaglutinina P (PHA), contra el antígeno de reacción cruzada PPD, y contra *Mycobacterium leprae* derivado de armadillos íntegro y sonicado. Usando la técnica de las rosetas también se enumeraron los linfocitos T "tempranos" de la sangre periférica. Durante el ENL se encontraron respuestas inmunes significativamente aumentadas (bajos índices de migración) contra el *M. leprae* íntegro y aunque las respuestas fueron altas con PHA y PPD, ellas fueron idénticas en todos los grupos, indicando que las respuestas específicas contra el *M. leprae* se incrementan durante la reacción ENL. Los linfocitos T "tempranos" también mostraron un incremento significativo durante las reacciones ENL. Sin embargo y en contraste con las RIC incrementadas *in vitro*, los pacientes con ENL no mostraron reactividad a la prueba dérmica de la leprolina.

RÉSUMÉ

Les résponses d'immunité à médiation cellulaire (CMI) ont été mesurées chez 39 malades lépromateux, 44 sujets atteints d'érythème noueux lépreux (ENL) et chez 22 malades après un tel épisode d'ENL. L'épreuve d'inhibition de la migration leucocytaire a été utilisée pour mesurer les réponses de l'immunité à médiation cellulaire à la phytohémagglutinine-P (PHA), un mitogène, ainsi qu'à un antigène de réactions croisées, le dérivé protéïnique purifié (PPD) de la tuberculine et au bacille de la lèpre, soit intact dérivé du tatou, soit traité aux ultrasons. On a également procédé à la numération des "lymphocytes précoces T" du sang périphérique, au moyen de la technique de la rosette. Un renforcement significatif des réponses immunitaires (indices de migration plus faibles) a été observé pour le bacille entier au cours de l'ENL. Quoique les réponses notées avec la PHA et le PPD soient plus fortes, elles étaient cependant identiques dans tous les groupes, ce qui indique qu'au cours de la réaction d'ENL, les réponses spécifiques à *Mycobacterium leprae* sont stimulées. Les "lymphocytes précoces T" ont également montré une augmentation significative dans les réactions d'ENL, par comparaison avec les malades lépromateux. Néanmoins, aucune réponse n'a été observée à l'épreuve cutanée à la lépromine, contrairement à ce qui était noté pour les réponses d'immunité à médiation cellulaire *in vitro*.

Acknowledgments. We gratefully acknowledge the help of Dr. G. V. Siva Somnath and Dr. A. Beine, Sivananda Rehabilitation Home, and Dr. J. N. A. Stanley and Dr. J. M. H. Pearson, Dhoolpet Leprosy Research Centre, Hyderabad, for providing us with blood samples and also classifying the patients. Our profound thanks are due to Dr. R. J. W. Rees, formerly Head, Laboratory for Leprosy and Mycobacterial Research, National Institute for Medical Research, London, for supplying us with armadillo-derived *M. leprae* antigens. Finally, Dr. T. Dharma Rao acknowledges the financial support of the Council of Scientific and Industrial Research, New Delhi, in the form of a Senior Research Fellowship.

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