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Thymus Dependent Lymphocytes in Leprosy. I. T Lymphocyte Subpopulations Defined by Monoclonal Antibodies[1]

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Immunological disturbances have often been described in leprosy patients—more so in the lepromatous (LL) than in the tuberculoid (TT) end of the clinicopathological spectrum of the disease ([9]). Negative skin test responses to *Mycobacterium leprae* or its antigens, purified protein derivative of *M. tuberculosis* (PPD), and dinitrochlorobenzene (DNCB), as well as low-to-negligible *in vitro* lymphocyte responses to the same antigens or mitogens characterize untreated LL patients. Lack of circulating *M. leprae*-sensitive lymphocytes ([8]), macrophage defects in antigen presentation ([11]), and suppressor cells ([2, 13, 14, 16]) have all been cited as playing a role in the pathogenesis of leprosy. The nature of the suppressor cell(s) is not known, and both macrophages and T lymphocytes have been reported to be involved. Data from experimental murine mycobacteriosis have also suggested defects in both the macrophages and lymphocytes ([5, 7, 12, 20]). The elucidation of the mechanisms of the immunological unresponsiveness in lepromatous leprosy patients will have profound clinical and experimental implications.

Erythema nodosum leprosum (ENL) occurs as a complication in patients with the low resistant type of leprosy. Histological features of the lesions as well as demonstration of immune complexes around blood vessels with vasculitis have been the basis of the concept that ENL is a clinical manifestation of an Arthus phenomenon ([23]). During the complication, specific *in vivo* and *in vitro* responses to *M. leprae* remain low; while responses to dinitrochlorobenzene (DNCB) and mitogens have been reported to be significantly higher than in patients without ENL ([1, 17]). These later findings and clinical observations indicate that ENL may be associated with an imbalance of T lymphocyte immunoregulation ([15]). Indeed, an imbalance of T cell subsets has been reported in LL patients with a history of recent ENL attack ([1]).

This paper describes the results of using monoclonal antibodies to characterize peripheral blood T lymphocytes in leprosy patients. Peripheral blood lymphocytes carrying the cytotoxic/suppressor cell phenotype, OKT 8, were found to be increased only in patients with lepromatous leprosy. Patients with symptoms and signs of ENL, on the other hand, had an inversion of the OKT 4^+/OKT 8^+ ratio, indicating a decrease of suppressor cells. This disturbance was acute and tended to revert to LL figures with clinical improvement.

MATERIALS AND METHODS

Untreated leprosy patients attending the All Africa Leprosy Rehabilitation and Training Centre (ALERT, Addis Ababa, Ethiopia) were referred to us after thorough clinical examination. Patients were clinically classified according to the Ridley-Jopling scale ([19]) and, whenever possible, a skin biopsy was examined to confirm the classification. Normal healthy individuals who had had several years of exposure to leprosy patients served as the control group. These individuals had no clinical signs of leprosy. Patients with ENL were taken into the study while they still had fresh nodules and/or other clinical features of the syndrome.

Peripheral blood was drawn by a venipuncture and lymphocytes isolated on a Fi-

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coll-Isopaque gradient ([4]). Cells were resuspended at a concentration of 0.5 × 10^6/ml in RPMI 1640 culture medium containing 20% normal human serum, penicillin, streptomycin, and glutamine. Lymphocytes were cultured in triplicate with whole washed *M. leprae* at a concentration of 10^6 bacilli/ml; with PPD at a concentration of 10 μg/ml, and with phytohemagglutinin (PHA) at a concentration of 2 μg/ml. *M. leprae* were isolated from a subcutaneous nodule of an untreated lepromatous leprosy patient ([3]). One μCi tritiated thymidine (2 Ci/ml, Amersham, England) was added 18 hr before termination of cultures and the degree of radioactive thymidine incorporation measured in a liquid scintillation counter (Intertechnique, Plaisir, France). PHA cultures were terminated after 72 hr and the PPD and *M. leprae*-stimulated cells were harvested after seven days.

A portion of the lymphocytes was resuspended in ice-cold RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (Flow Laboratories, England) at a concentration of 5 × 10^6 cells/ml. Monoclonal antibodies Orthoclone OKT 3 PAN, Orthoclone OKT 4 IND, and Orthoclone OKT 8 SUP were produced and kindly donated to us by the Immunobiology Division, Ortho Pharmaceutical Corporation, Raritan, New Jersey, U.S.A. These antibodies have been extensively studied, and their specificity characterized ([18]). The lyophilized antibodies were reconstituted with 1 ml sterile 0.9% w/v NaCl and stored in 200 μl aliquots at −70°C until used. Single aliquots were thawed only once and used within one month. Two hundred μl of cells (5 × 10^6 cells/ml) were mixed with 5 μl of reconstituted monoclonal antibodies and incubated in an ice bath for 30 min.

The cells were then washed by centrifugation and reincubated with 100 μl of fluorescent-labelled, rabbit anti-mouse immunoglobulins (Lot 011A code F232, DAKO Immunoglobulins A/S, Copenhagen, Denmark). After 30 min of incubation, the cells were again washed and a drop of phosphate-buffered saline, pH 7.2, containing 30% v/v glycerol was added to the cell pellet. The pellet was then disrupted by mild agitation and positively staining cells were counted using a fluorescent microscope. Cells were considered positive when a granular surface staining was seen. At least 100 cells were counted before a percentage of positively staining cells was calculated. Absolute numbers of the T cell subpopulations were not determined. For controls, normal mouse serum was used as the primary antibody instead of the monoclonal antibodies.

The Student's *t* test was used for statistical analysis; p values of less than 0.01 were taken as being significant.

RESULTS

Eighty-two individuals were taken into the study, ranging in age from 13 years to 45 years. Thirteen individuals served as controls. The 69 leprosy patients were classified as: lepromatous (LL) or borderline lepromatous (BL), 25; borderline tuberculoid (BT), 18; and clinical evidence of erythema nodosum leprosum (ENL), 26.

Results of the *in vitro* lymphocyte transformation tests using *M. leprae,* PHA, and PPD are shown in Table 1. It can be seen that of the leprosy patients only those with BL-LL disease show reduced responses to PHA and PPD. In contrast, patients with ENL (who were either BL or LL) had statistically higher responses to both PHA and PPD. The response to *M. leprae* was not significantly different between BT patients and healthy contacts. Both BL and LL patients with or without ENL had significantly lower lymphoproliferative responses to *M. leprae.*

Orthoclone OKT 3 PAN stained 64% of all peripheral blood mononuclear cells of healthy leprosy contacts. There were no significant differences among the three patient groups so far as these cells were concerned (Table 2). Since this surface antigen is found in all T cells, it is concluded that there is no major difference in the total lymphocyte population when comparing leprosy patients with the healthy contact group. In all groups, the sum of the percentage of OKT 4^+ and OKT 8^+ cells was slightly less or equal to the OKT 3 PAN positive cells, indicating that cells expressing more than one surface antigen were not seen. The percentage of OKT 8 SUP positive cells was significantly increased in BL-LL patients in comparison with normal controls. The increase in this lymphocyte subset was accompanied by a decrease in the percentage

TABLE 1. In vitro *lymphoproliferative responses in leprosy patients.*

Group	No.	Antigens		
		M. leprae	PPD	PHA
Controls	13	30,962 ± 700[a]	56,427 ± 870	66,486 ± 830
BT	18	29,840 ± 1,624	55,697 ± 1,569	66,873 ± 1,302
BL-LL	25	3,585 ± 345[b]	46,531 ± 1,129[b]	52,945 ± 1,250[b]
ENL	26	3,361 ± 242[b]	69,588 ± 829[b]	92,771 ± 1,129[b]

[a] Results expressed as the mean ± standard error of the mean of the differences in counts per minute between stimulated and unstimulated cultures.
[b] Significantly different from controls, $p < 0.01$, Student's *t* test.

of OKT 4 IND positive lymphocytes in these patients. Patients with ENL, on the other hand, had a marked decrease in the percentage of OKT 8 SUP positive cells. The ratio of inducer to suppressor/cytotoxic lymphocytes has been shown to reflect the peripheral T lymphocyte balance while eliminating the possibility of interference by non-T lymphocyte contaminants. The Figure shows the distribution of this ratio. It can be seen that lepromatous leprosy (BL-LL) patients had a reduced ratio of inducer to suppressor/cytotoxic lymphocytes; while patients with ENL had a significantly higher than normal value. This ratio was not significantly different when comparing BT patients with normal controls.

DISCUSSION

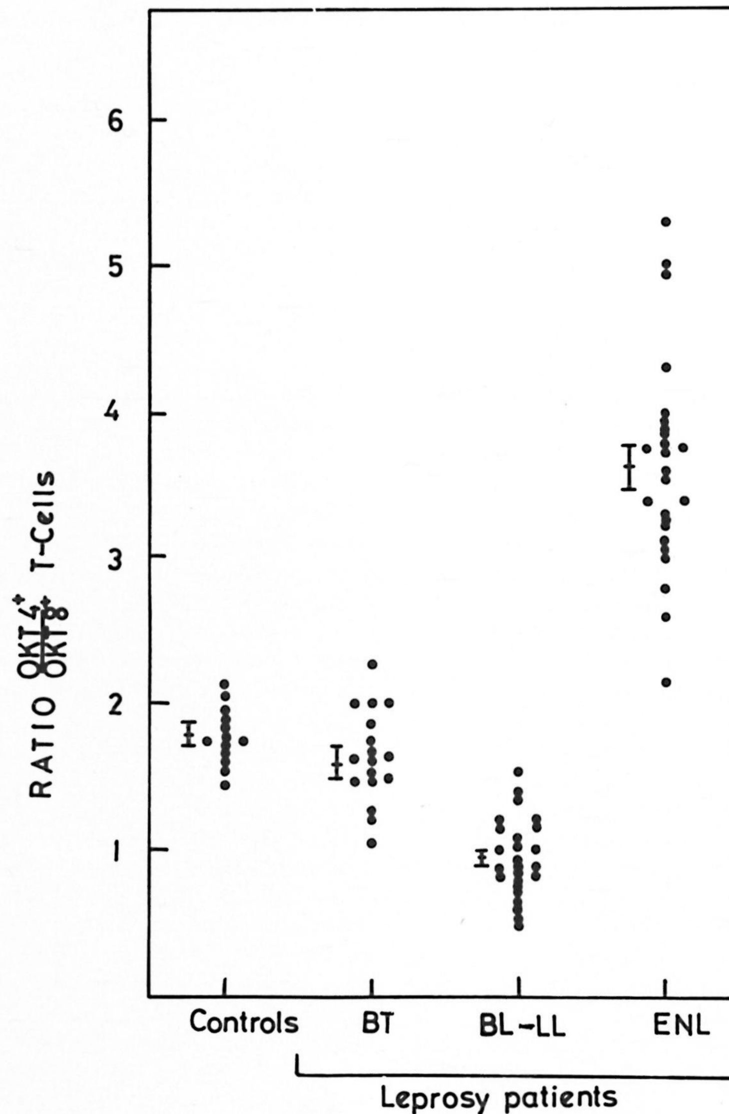
A cellular immune deficiency specific for *M. leprae* is characteristic of low resistant leprosy ([9]). This deficit has not been explained clearly, but recent evidence in support of suppressor mechanisms has been presented ([13, 14]). Both clinical and experimental observations strongly indicate the role of suppressor mechanisms in the pathogenesis of leprosy. The nature of these mechanisms, however, remains unknown. Reports of suppressor macrophages have been published ([11, 16]) but this has not been confirmed by others ([21]). That macrophages from LL patients are capable of destroying *M. leprae* upon stimulation ([9]) argues in favor of a primarily T lymphocyte defect. Our data show that the percentages of the total numbers of circulating T lymphocytes remain within normal values in leprosy patients. This, therefore, implies that the T cell defect is probably functional rather than absolute.

Several investigations have shown that certain surface markers can be used to identify lymphocyte subpopulations with defined functional properties. Monoclonal antibodies raised against human lymphocytes have been used to characterize such cells, and thus OKT 3 PAN stains all human peripheral blood T lymphocytes ([18]). This antibody stained 64% of peripheral blood mononuclear cells; while OKT 4 IND, which stains human inducer/helper T cell subsets, stained 41% of peripheral blood mononuclear cells of the control group. The OKT 4 IND positive cells were significantly reduced in BL and LL patients but not in BT patients. OKT 8 SUP which preferentially stains human suppressor/cytotoxic T

TABLE 2. *T lymphocyte subsets in leprosy patients defined by mononuclear antibodies.*

Group	No.	T cell subsets		
		OKT 3+	OKT 4+	OKT 8+
Controls	13	64.18 ± 0.38[a]	41.28 ± 0.85	22.39 ± 0.72
BT	18	65.14 ± 1.02	39.14 ± 1.02	24.95 ± 0.83
BL-LL	15	65.43 ± 0.61	31.17 ± 0.71[b]	33.31 ± 0.99[b]
ENL	26	65.19 ± 0.51	49.75 ± 0.65[b]	13.86 ± 0.69[b]

[a] Results expressed as a mean ± standard error of the mean percentage of peripheral blood mononuclear cells staining positively.
[b] Significantly different from controls, $p < 0.01$, Student's *t* test.



THE FIGURE. Proportion of inducer and suppressor cells in peripheral blood of leprosy patients (Mean ± S.E.M.). Results in patients with BL-LL or ENL differ significantly from the control group ($p < 0.01$). BT versus controls are not significant.

lymphocytes, on the other hand, showed a significant increase in this T cell subset in lepromatous patients. This is in contrast to the data of Bach, *et al.* ([1]) who reported no deviation in the lepromatous patients. It must be noted, however, that their patients had all been treated; whereas our patients were untreated. Prolonged chemotherapy has been shown to decrease or even eliminate the nonspecific immunologic unresponsiveness in lepromatous patients ([9]).

The induction of suppressor cells in leprosy is not well understood. It is known, however, that mycobacterial components, for example, PPD, are capable of inducing antigen-specific suppressor cells ([20]). Furthermore, *M. leprae* have recently been shown to be able to suppress *in vitro* lymphoproliferative responses to mitogens ([22]). Antigenic analysis of *M. leprae* shows that this bacillus shares most antigens with other mycobacteria ([10]). Since the anergy seen in at least treated LL patients is remarkably specific to *M. leprae,* it is possible that certain antigenic determinants of *M. leprae* are capable of inducing a suppressor system

specific for *M. leprae*. The suppressor T cell we identified in this study, however, is antigen nonspecific and our findings should be taken with caution with regard to the role of these cells in the pathogenesis of lepromatous leprosy. Moreover, a clear functional correlation of the T cell markers used in this study is still being debated.

ENL lesions have many histological features similar to the Arthus phenomenon ([23]). This, plus the demonstration of immune complexes around the blood vessels, forms the basis of the concept that ENL is a clinical manifestation of the Arthus reaction. However, many questions cannot be answered by this concept, and recently it has been proposed that the initiation of ENL may at least be due to an acute imbalance of T lymphocyte immunoregulation ([15]). Our findings and those of Bach, *et al.* ([1]) show that during ENL an inversion of the normal inducer/suppressor cell balance occurs. This finding, however, does not exclude the involvement of immune complexes in ENL, especially during the perpetuation phase. In three patients where analysis of T cell subsets was done during and after recovery from ENL a normalization of the T cell subsets was seen with clinical recovery from ENL. Whether this disturbance is primary or secondary to ENL remains to be answered by long-term studies. Although there was an increase in nonspecific hypersensitivity during ENL, specific *M. leprae* responses remained low. The mechanisms for this specific anergy are not known at present, but the anergy could be acquired. Similar findings have been reported in animals heavily infected with mycobacteria ([6]). Bach, *et al.* ([1]) have proposed failure to elicit specific memory cells to *M. leprae* during primary sensitization as being the main mechanism for this anergy.

SUMMARY

Monoclonal antibodies recognizing different human T lymphocyte subpopulations were used to characterize peripheral blood T lymphocytes in patients with leprosy. An increase in the suppressor T lymphocyte subpopulation was seen only in lepromatous leprosy (BL-LL) patients. In contrast, patients who had erythema nodosum leprosum (ENL) showed a disturbance in immunoregulation seen as a decrease of the suppressor cell percentage and manifested by an increase in *in vitro* lymphoproliferative responses to both PPD and PHA. This imbalance was seen to normalize as patients improved clinically. There was no deviation from the normal values of the total T lymphocyte population.

It is suggested, therefore, that ENL may be associated with an acute imbalance of T lymphocyte subpopulations. Since the suppressor T lymphocyte indentified by the mononuclear antibody used is antigen nonspecific, the significance of these suppressor cells in the pathogenesis of leprosy remains unclear.

RESUMEN

Se usaron anticuerpos monoclonales que reconocen diferentes subpoblaciones de linfocitos T humanos, para characterizar a los linfocitos T de la sangre periférica de pacientes con lepra. Sólo se observó un incremento en la población de linfocitos T supresores en los pacientes con lepra lepromatosa (LL). En contraste, los pacientes con eritema nodoso leproso (ENL) mostraron disturbios en su inmunoregulación manifestados como una disminución en el porcentaje de células supresoras y como un incremento *in vitro* de las respuestas linfoproliferativas inducidas con PPD y con fitohemaglutinina (PHA). Estas alteraciones tendieron a desaparecer cuando los pacientes mejoraron clínicamente. No se encontraron anormalidades en los valores de linfocitos T totales. Se sugiere que el ENL puede estar asociado a un desbalance crítico en las subpoblaciones de linfocitos T. Puesto que los linfocitos T supresores identificados con los anticuerpos monoclonales empleados no son específicos para un antígeno en particular, no esta claro el significado de estas células supresoras en la patogénesis de la lepra.

RÉSUMÉ

En vue de caractériser les lymphocytes T du sang périphérique chez des malades atteints de lèpre, on a utilisé les anticorps monoclonaux dirigés contre des sous-populations différentes de lymphocytes T humains. On a observé une augmentation dans la sous-population de lymphocytes T "suppressors" uniquement chez les malades atteints de lèpre lépromateuse LL. Par contre, les malades qui présentaient un érythème noueux lépreux (ENL) présentaient des troubles de la régulation immunitaire sous forme d'une diminution du pourcentage de cellules "suppressors"; ces troubles entrainaient une accentuation des réponses lymphoprolifératives *in vitro*, à la fois avec le P.P.D. et avec la phytochemaglutinine (PHA). On a observé que ce déséquilibre revenait à la normale lorsque les malades présentaient une amélioration clinique. On n'a noté aucune déviation des valeurs normales de la population totale des lymphocytes T.

On suggère dès lors que l'érythème noueux lépreux (ENL) peur être associé avec un déséquilibre aigu des sous-populations de lymphocyte T. Du fait que le lymphocyte T "suppressor", identifié par l'anticorps monoclonal utilisé dans cette étude était un antigène non spécifique, la signification des cellules "suppressor" dans la pathogénèse de la lèpre reste non élucidée.

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