

## Peripheral Blood T Lymphocyte Subsets in Leprosy<sup>1</sup>

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The introduction of monoclonal antibodies directed against T lymphocyte subsets has provided a tool for quantifying T lymphocytes in peripheral blood (<sup>12</sup>). Thus in blood studies total (or pan) T cells (Leu 1, Leu 4, OKT3, OKT11-positive +), a helper/inducer subset (Leu 3, OKT4-positive +) and a suppressor/cytotoxic subset (Leu 2, OKT8-positive +) can be judged to be increased or decreased by percentages or absolute numbers. Also, abnormalities in the ratio of phenotypic helper/inducer to phenotypic suppressor/cytotoxic cells, a helper: suppressor ratio, can be demonstrated.

Because of the putative importance of immunological mechanisms in the pathogenesis of leprosy, and because of the varieties of granulomatous responses and reactional states which constitute the broad clinical canvas of leprosy, the study of peripheral blood T cell subsets in patients with leprosy is logical. To date, at least six investigations have been reported. Bach (<sup>1</sup>) and Wallach (<sup>18</sup>) and their associates and Mshana, *et al.* (<sup>8</sup>), reporting their data in percentages, found that active lepromatous patients without erythema nodosum leprosum (ENL) had significant decreases in the OKT4+ subset, significant increases in the OKT8+ subset, and significant decreases in their helper:

suppressor ratios. In contrast, lepromatous patients with ENL had increased OKT4+ percentages, decreased OKT8+ percentages, and increased helper:suppressor ratios. Van Voorhis, *et al.* (<sup>17</sup>) and Gonzales-Amaro, *et al.* (<sup>5</sup>) in studies of eight and ten patients, respectively, found no abnormality. Bullock, *et al.* (<sup>4</sup>) in nonreactional lepromatous patients found a significant, and proportionate, reduction in the absolute numbers in the three phenotypes, without a disturbance in the helper:suppressor ratio.

In the present paper we report our study of blood T cell subsets in 122 leprosy patients, representing three granulomatous postures and three reactional states, as well as some patients with long-term dapsone therapy.

### MATERIALS AND METHODS

Patients were classified according to the histopathological and clinical criteria of Ridley and his associates (<sup>13-15</sup>) as TT (polar tuberculoid), BT (borderline with tuberculoid features), BB (borderline), BL (borderline with lepromatous features), and LL (lepromatous, either polar or subpolar). Three reactional states were recognized: erythema nodosum leprosum (ENL), Lucio's reaction, and reversal reaction, and were diagnosed according to criteria previously published (<sup>9</sup>).

Each patient with a reactional state was in reaction when studied. Furthermore, those with ENL had not yet received thalidomide; those with reversal reactions had not yet received steroids, and those with Lucio's reactions had received neither chemotherapeutic nor anti-inflammatory agents. Lepromatous patients under continuous chemotherapy for eight or more years who had no clinical evidence of a granulomatous response or a reactional state were considered to be inactive. BT patients with

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three years or more of continuous dapsone therapy who had no clinical evidence of a granulomatous response were considered to be inactive. Normal controls were judged to be normal on the basis of history and physical examination. The immunologically abnormal controls were patients with systemic lupus erythematosus, diagnosed by criteria published elsewhere (2).

**Flow cytometry analysis of lymphocyte subpopulations (6).** Peripheral blood was collected in EDTA Vacutainer® tubes (Becton, Dickinson & Co., Rutherford, New Jersey, U.S.A.) and maintained at room temperature until processed (less than 24 hr). In standardizing the procedure, it was found that cells stored in EDTA-anticoagulated whole blood show little or no change in the immunologic phenotype for 24 hr or longer. In actual practice, all specimens from patients or controls were obtained in the morning and were processed in the afternoon of the same day.

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (OKT3, 4, 8, and 11; Ortho Diagnostic Systems, Inc., Raritan, New Jersey, U.S.A.) were used to stain the cells directly. One hundred microliter aliquots of anticoagulated whole blood containing  $1-2 \times 10^6$  cells were incubated with 10  $\mu$ l of the FITC-labeled antibody for 30 min at 4°C, exposed to an ammonium chloride lysing reagent (Ortho) for erythrocyte removal, and washed with 2 ml of phosphate buffered saline (Gibco, Santa Clara, California, U.S.A.) containing 5% bovine calf serum and 0.1% sodium azide. The remaining nucleated cells were analyzed directly.

Peripheral blood samples from healthy donors were analyzed concurrently with patient specimens. FITC-labeled goat anti-mouse IgG (1:80 dilution) was used as a background control for T cell antibodies. A cell line, KM-3 (R. J. Billing, University of California at Los Angeles, California, U.S.A.), which expressed cALLA antigen, was used as a positive control.

Flow cytofluorometric analysis of labeled fluorescent cells was accomplished using a Spectrum III Cytofluorograph (Ortho) operated at 488 nm wavelength and 90 mw for fluorescein excitation. Cells were selected for analysis with each antibody on the basis of forward and right angle scatter which

made it possible to distinguish lymphocytes from monocytes, granulocytes, and cellular debris (6). The fluorescence intensity of the cells in the lymphocyte clusters, selected on the cathode ray terminal display screen, was measured and displayed as a histogram, plotting fluorescence versus cell number. Background fluorescence was determined using the above controls. The percentage of positive cells refers to the percentage of lymphocytes with more intense fluorescence than 99% of the background controls. Fluorotrol (Ortho), artificial fluorescent particles, was used for instrument calibration. Thus the percentage of OKT3+, OKT4+, and OKT8+ cells was determined by immunofluorescent flow cytometry. On the same phlebotomy specimen, the absolute lymphocyte count was determined by a conventional whole blood leukocyte count and differential. The numbers of each T cell phenotype per cubic millimeter were calculated.

To validate the analysis of lymphocytes identified by flow cytometry of unseparated cells, studies were conducted by staining with OKM-1 (Ortho) monoclonal antibody, which identifies monocytes and polymorphonuclear leukocytes. These studies demonstrated that the lymphocyte clusters contained greater than 95% of the total lymphocytes of the normal controls and 90% of the lymphocytes of the patients. Also, differences in the shape and position of the lymphocyte clusters in patients and controls were sought, but were not found.

For statistical analysis groups were compared by the Student *t* test.

## RESULTS

The results are summarized in The Table. Values for numbers of cells per cubic millimeter, percentage of each phenotype, and the helper: suppressor ratio are reported.

Normal controls, tested concurrently with the patients, consisted of five clinic personnel and 18 household contacts of the patients. The abnormal controls, 27 patients with systemic lupus erythematosus (SLE), when compared to the control group showed a selective, significant deficiency in the OKT4+ subset, both in absolute numbers and in the percentage of total lymphocytes. Associated with this selective deficiency was a secondary but significant

THE TABLE. Mean  $\pm$  S.D. of numbers (or percentages) of lymphocytes and T lymphocyte subsets and the mean  $\pm$  S.D. helper: suppressor ratio in patients and controls.

Group	Lymphocytes (mm <sup>-3</sup> )	Pan T OKT3+	Helper/ inducer OKT4+	Suppressor/ cytotoxic OKT8+	Helper/ suppressor ratio
Control N = 23	2550 $\pm$ 932	1662 $\pm$ 636 (68 $\pm$ 12)	1015 $\pm$ 395 (40 $\pm$ 10)	682 $\pm$ 330 (27 $\pm$ 8)	1.7 $\pm$ 0.7
SLE N = 27	1475 $\pm$ 790 <sup>a</sup>	1040 $\pm$ 545 <sup>b</sup> (65 $\pm$ 13)	470 $\pm$ 295 <sup>a</sup> (32 $\pm$ 10) <sup>b</sup>	510 $\pm$ 365 (32 $\pm$ 8) <sup>c</sup>	1.2 $\pm$ 0.5 <sup>b</sup>
LL no reaction N = 24	1619 $\pm$ 637 <sup>a</sup>	1040 $\pm$ 491 <sup>a</sup> (64 $\pm$ 12)	619 $\pm$ 345 <sup>a</sup> (36 $\pm$ 11)	425 $\pm$ 226 <sup>b</sup> (26 $\pm$ 8)	1.5 $\pm$ 0.5
LL no reaction, no treatment N = 12	1636 $\pm$ 681 <sup>b</sup>	1047 $\pm$ 493 <sup>b</sup> (64 $\pm$ 13)	692 $\pm$ 402 <sup>c</sup> (37 $\pm$ 13)	444 $\pm$ 226 <sup>c</sup> (25 $\pm$ 8)	1.6 $\pm$ 0.6
LL with ENL N = 19	2015 $\pm$ 819	1301 $\pm$ 547 (65 $\pm$ 8)	791 $\pm$ 375 (40 $\pm$ 10)	518 $\pm$ 258 (26 $\pm$ 7)	1.7 $\pm$ 0.8
LL with long- term treatment N = 18	2001 $\pm$ 618	1334 $\pm$ 558 (65 $\pm$ 11)	881 $\pm$ 321 (43 $\pm$ 7)	518 $\pm$ 234 (27 $\pm$ 11)	1.8 $\pm$ 0.7
LL with Lucio's N = 4	1851 $\pm$ 608	1242 $\pm$ 325 (71 $\pm$ 8)	804 $\pm$ 219 (47 $\pm$ 16)	436 $\pm$ 200 (24 $\pm$ 10)	2.8 $\pm$ 1.7
Borderline lepomatous N = 13	1813 $\pm$ 447 <sup>c</sup>	1210 $\pm$ 375 <sup>c</sup> (69 $\pm$ 10)	666 $\pm$ 230 <sup>b</sup> (37 $\pm$ 7)	560 $\pm$ 222 (31 $\pm$ 10)	1.4 $\pm$ 0.6
Reversal reaction N = 13	2308 $\pm$ 766	1330 $\pm$ 556 (56 $\pm$ 13) <sup>b</sup>	650 $\pm$ 239 <sup>b</sup> (29 $\pm$ 8) <sup>b</sup>	637 $\pm$ 288 (30 $\pm$ 11)	1.2 $\pm$ 0.7
Tuberculoid N = 18	2269 $\pm$ 769	1534 $\pm$ 544 (69 $\pm$ 9)	896 $\pm$ 414 (39 $\pm$ 10)	655 $\pm$ 226 (30 $\pm$ 6)	1.4 $\pm$ 0.7
Tuberculoid with long-term treatment N = 13	2123 $\pm$ 644	1377 $\pm$ 460 (65 $\pm$ 8)	821 $\pm$ 369 (38 $\pm$ 8)	592 $\pm$ 225 (28 $\pm$ 8)	1.5 $\pm$ 0.8

<sup>a</sup> p < 0.001.

<sup>b</sup> p < 0.01.

<sup>c</sup> p < 0.05.

lymphopenia, OKT3+ cytopenia, increased percentage of the OKT8+ subset, and a greatly reduced helper: suppressor ratio. However, the absolute numbers of the OKT8+ subset did not vary significantly from the normal controls because of its increased percentage.

Twenty-four active lepomatous patients (LL), polar or subpolar, were studied. Twelve were untreated, four were relapsing off chemotherapy, and eight had been receiving chemotherapy from 1–4 years with a median of two years. As judged by mean values, these patients, when compared to normal controls, were proportionately and significantly lymphopenic, OKT3+ cytopenic, OKT4+ cytopenic and OKT8+ cytopenic. In contrast to SLE patients, they had normal helper: suppressor ratios be-

cause of the nonselective, proportionate decrease in all phenotypes. Expressed as a percentage, the distribution of the T cell subsets did not vary significantly from that of the normal controls. Significant differences were observed between the untreated, relapsing, and treated subgroups of patients. Because of the particular interest in untreated lepomatous patients, the data from these 12 are given separately in The Table. The mean values of the 12 untreated patients were similar to those of the aggregated 24 patients with active lepomatous disease. As judged by pretreatment biopsy specimens, the relapsing-treated patients were comparable to the untreated patients. For example, of the 12 untreated patients, 3 were polar and 8 subpolar lepomatous; in the relapsing-treated group, 3 were polar, 7 subpolar, and

in 2 the pretreatment biopsy specimens were not available for review. Also, the range of the bacillary load as judged by the biopsy index, 4.2 to 6+, was similar pretreatment. In the eight individuals who were on treatment, tissues were not evaluated at the time of phlebotomy. The four relapsing patients had a biopsy index ranging from 4.5 to 6+.

Of great interest was the fact that two groups of lepromatous patients did not show these decreases. In 19 patients with active ENL, 4 untreated and 15 dapsone-treated for a median of two years and a maximum of four years, and in 18 inactive lepromatous patients who had been receiving dapsone therapy continuously for eight years or longer, the mean values did not differ significantly from those of the normal controls.

The four lepromatous patients with Lucio's reaction demonstrated a tendency toward increased helper: suppressor ratios. However, this was too small a group to demonstrate statistically significant differences.

Two groups of leprosy patients, borderline lepromatous and reversal reaction, mimicked SLE patients in having an apparently selective deficiency in the OKT4+ subset. The 13 borderline lepromatous (BL) patients (12 untreated, 1 treated for six weeks) had a significant selective deficiency of their helper/inducer subsets with a secondary significant lymphopenia and pan T cytopenia. In addition, they had normal OKT8+ mean values, as found in SLE. Although the helper: suppressor ratio was less than normal, larger numbers of patients will be needed to learn if this is significant. The 13 patients with reversal reactions (2 BT, 2 BB, 9 BL at the time of study) had a significant deficiency in their OKT4+ subsets both in absolute numbers and in percentages, and secondary, significant decreases in the percentages of their pan T phenotypes. Again, more patients are needed to learn if the apparently low helper: suppressor ratio could be significant.

Tuberculoid (BT) patients, whether active (17 untreated, one treated for one month) or inactive (on continuous dapsone therapy for three years or more) did not differ significantly from normal controls.

The kinds of distribution found in this study are exemplified by those of the OKT4+ subset in controls and active lep-

romatous patients. In the 23 normal controls, absolute values ranged from a low of 439 per mm<sup>3</sup> to a high of 1927, and the median value, 950, was close to that of the mean, 1015; three individuals had values less than one standard deviation below the mean, or 620; four individuals had values greater than one standard deviation above the mean, or 1410. In contrast, in the 24 active lepromatous patients, the absolute values ranged from a low of 246 per mm<sup>3</sup> to a high of 1507, and the median value, 435, was lower than that of the mean, 619; two individuals had values smaller than one standard deviation below the mean, or 274; three had values greater than one above the mean, or 964. Twelve (50%) of the active lepromatous patients had values that were less than that of the smallest control value.

#### DISCUSSION

Three abnormal patterns of T cell subsets have been identified in this study. Active lepromatous patients have a lymphopenia, a proportionate reduction in the numbers of each of the three T cell subsets, but insignificant changes in T cell subsets expressed as percentages and in the helper: suppressor ratio. This pattern is in accord with the findings of Bullock, *et al.* (4). Borderline lepromatous patients have a selective deficiency of the OKT4+ subset with an apparent secondary lymphopenia and OKT3+ cytopenia, but no OKT8+ cytopenia, a pattern similar to that seen in SLE. Finally, patients with reversal reactions have a selective OKT4+ deficiency, both in absolute numbers and percentages, with an apparent secondary reduction in the percentage of OKT3+ cells, but no lymphopenia or OKT8+ cytopenia. Because all three patterns have in common a deficiency in the OKT4+ subset, and because this OKT4+ subset deficiency could be the central abnormality in at least two of the three, we do not know if the three patterns represent variants of one fundamental abnormality or if each is distinctive in and of itself.

The absence of significant abnormalities in lepromatous patients with ENL or with prolonged chemotherapy or in borderline tuberculoid patients indicates that the observed abnormalities are a consequence of leprosy and not predisposing causes.

The mechanism(s) responsible for the



three abnormal patterns is not known. Conceivably they could arise as a consequence of extensive tissue infiltration which characterizes lepromatous and borderline lepromatous leprosy, as a result of altered lymphocyte percolation secondary to lymphoid tissue involvement with leprosy (<sup>3</sup>), as some combination thereof, or by totally unrelated mechanisms, such as antilymphocyte antibodies (<sup>2</sup>). Findings of a predominance of the suppressor/cytotoxic phenotype in lepromatous tissue (<sup>7</sup>) would indicate that tissue infiltration in and of itself is not a good explanation for the observed proportionate reduction. However, the heavy lymphocytic infiltration of borderline lepromatous tissues (<sup>13</sup>) and the 2:1 predominance of the helper/inducer phenotype in these tissues (<sup>7</sup>) are not inconsistent with the pattern of selective helper/inducer deficiency with a secondary lymphopenia and pan T cytopenia. Also, because reversal reactions are probably delayed-type hypersensitivity reactions, the selective deficiency of the helper/inducer subset might be a consequence of a high turnover of this subset. Until kinetic data are available, these considerations will remain speculative. The complexities of the immunological network allow for many hypothetical explanations of our observations (<sup>2</sup>).

Functional consequences of these abnormalities are also speculative. However, it is conceivable that some of the immunological differences between lepromatous patients with and without ENL, which include DNCB responsiveness (<sup>9</sup>), serum migration inhibition activity (<sup>11</sup>), and cell-mediated lympholysis (<sup>16</sup>), could reflect or be associated with the differences in peripheral blood T cell subset distribution.

In details our results failed to confirm the findings of Bach, *et al.* (<sup>1</sup>) Wallach, *et al.* (<sup>18</sup>), and Mshana, *et al.* (<sup>8</sup>). Because those investigators did not provide data in absolute numbers, the magnitude of our differences is unknown. Bullock, *et al.* (<sup>4</sup>) used methods similar to those of Bach, Wallach, Mshana and their associates (<sup>1, 8, 18</sup>) but had results similar to ours. Therefore, methods used would appear to be an unlikely explanation for the differences. Geographic/ethnic factors have been invoked to explain the puzzling differences in immunological findings between clinically evidently similar

groups of leprosy patients (<sup>10</sup>), and this may be appropriate in the present instance. We did observe two ENL patients with high helper:suppressor ratios, 3.3 and 3.5, but we also observed two with low ratios, both 0.6. Furthermore, no relationship between the severity of ENL and helper:suppressor ratio values could be discerned.

In a broader perspective, our findings are similar to the others in that active lepromatous patients without ENL differ greatly from lepromatous patients with ENL, and in that with prolonged chemotherapy abnormalities disappear.

### SUMMARY

Peripheral blood T lymphocyte subsets were measured by flow cytometry in 122 patients with leprosy, in 23 normal controls, and in 27 patients with systemic lupus erythematosus (SLE). Active lepromatous patients not in reaction showed a significant lymphopenia and a significant proportionate reduction in the number of OKT3-positive (pan T), OKT4-positive (helper/inducer), and OKT8-positive (suppressor/cytotoxic) cells, but no alteration in distribution as judged by percentage and no abnormality in the helper:suppressor ratio. Borderline lepromatous subjects not in reaction had a significant selective deficiency in the number of cells of the OKT4-positive subset, with a significant but secondary lymphopenia and OKT3-positive cytopenia, a pattern similar to that found in SLE patients. Patients undergoing reversal reactions had a selective deficiency in the OKT4-positive subset in both absolute numbers and as a percentage of total lymphocytes, and a secondary deficiency in the percentage of OKT3-positive cells. No abnormalities were demonstrated in patients with active erythema nodosum leprosum, lepromatous patients with long-term treatment, and untreated or treated borderline tuberculoid patients.

### RESUMEN

Por citometría de flujo se cuantificaron las subpoblaciones de linfocitos T periféricos en 122 pacientes con lepra, en 23 controles sanos y en 27 pacientes con lupus eritematoso sistémico (LES). Los pacientes lepromatosos activos no reaccionales mostraron una importante linfopenia y una reducción proporcional en el número de células OKT3-positivas (pan T), OKT4-

positivas (cooperadoras/inductoras) y OKT8-positivas (supresoras/citotóxicas), sin alteración en su distribución según se deduce del porcentaje y de la normalidad en la relación cooperadoras:supresoras. Los pacientes con lepra intermedia sin reacción tuvieron una significativa deficiencia selectiva en el número de células OKT4-positivas, con una linfopenia significativa pero secundaria y con citopenia de células OKT3-positivas, un patrón similar al encontrado en los pacientes con LES. Los pacientes con reacciones reversas tuvieron una deficiencia selectiva en las subpoblaciones OKT4-positivas, tanto en números absolutos como en el porcentaje total de linfocitos, y una deficiencia secundaria en el porcentaje de células OKT3-positivas. No se demostraron anomalías en los pacientes leptomatosos con tratamiento prolongado ni en los pacientes intermedios-tuberculoideos tratados o no tratados.

### RÉSUMÉ

Chez 122 malades atteints de lèpre, 23 témoins normaux, et 27 malades souffrant de lupus erythémateux systémique (SLE), on a mesuré les sous-ensembles de lymphocytes-T du sang périphérique, par des mesures de cytométrie dynamique. Les malades lépromateux actifs mais non réactionnels présentaient une lymphopénie significative, de même qu'une réduction proportionnelle significative du nombre de cellules OKT3 positives (pan T), OKT4 positives (adjuvantes, inductrices) et OKT8 positives (suppressives, cytotoxiques); par contre, on n'a observé aucune altération dans la distribution de ces cellules, ainsi qu'on peut en juger par leur pourcentage; aucune anomalie n'a été de plus notée dans le ratio adjuvant-supprimeur. Les malades atteints de lèpre lépromateuse borderline, sans être en réaction, présentaient une carence sélective significative dans le nombre de cellules du sous-ensemble OKT4 positives, accompagnées d'une lymphopénie significative mais secondaire, et d'une cytopénie pour les cellules OKT3 positives; ce profil est semblable à celui que l'on trouve chez les malades atteints de lupus érythémateux systémique. Les malades qui passaient par des réactions reverses témoignaient d'une carence sélective dans le sous-ensemble de cellules OKT4 positives, tant en nombre absolu qu'en pourcentage du nombre total de lymphocytes, de même d'une carence secondaire dans le pourcentage des cellules OKT3 positives. Aucune anomalie n'a été démontrée chez les malades souffrant d'érythème noueux lépreux actif, pas plus que chez les malades lépromateux ayant été traités durant longtemps, chez ceux qui n'avaient pas été traités, ou chez les patients tuberculoïdes borderline traités.

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