Cellular Immunity in Patients With Leprosy. Circulating T Lymphocytes and Their Response to PHA in Leprosy[1]

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There is considerable evidence that patients with the lepromatous form of leprosy (LL) have impaired specific as well as nonspecific cellular immunity ([1, 10, 23, 28]); however, depression of nonspecific cellular immunity is not a constant finding even in untreated lepromatous leprosy. Thus, in LL patients from Venezuela and Mexico, the skin reactivity to chemical sensitizers, as well as the response of lymphocytes to PHA, were found to be normal ([3, 16]).

The mechanism responsible for the defect in cellular immunity is not understood. Some authors believe that it is genetically determined ([25]), a hypothesis supported by the different response of lymphocytes to PHA of Chinese LL patients as compared to Malaysians or Indians ([15]). Others consider that the defect indicates a state of immunologic tolerance ([8]) or immunologic deviation ([27]). Secondary factors such as the state of nutrition and frequency of intercurrent infection have also been considered to play a role in the cellular defect ([4]).

From various studies it has been concluded that the site of the defect is either on T lymphocytes ([10, 24]) or at the *M. leprae*-induced lymphocyte-macrophage interaction ([9]). The existence of a serum factor responsible for the impairment is also claimed by some investigators ([2, 15]). Reduction of circulating T lymphocytes has been considered to be responsible for the defect but results of studies of lymphocyte populations are not in complete agreement. Thus, reduction of the T lymphocyte population, as determined by the "E-rosette" technic after long incubation is reported by some investigators ([7]); whereas Nath *et al* ([14]) found that the reduction involved only a subpopulation or T lymphocytes detectable after incubation for one hour. The present study was undertaken in order to determine whether lymphocytes from lepromatous patients show a reduced response to phytohemagglutinin (PHA), and if so, whether this is due to the presence of a serum factor or to reduction in the number of circulating T lymphocytes.

MATERIALS AND METHODS

Patient and control population. The study was carried out on 42 inpatients at the Public Hospital for Infectious Diseases, Athens. According to the Ridley-Jopling scale ([18]), 9 were classified as LL, 23 as BL, and 8 as BT cases (Table 1). All LL cases were bacteriologically positive and displayed reactions at the time of study ([17]) as shown in Table 2. Fifteen of the BL patients were bacteriologically positive, of whom 11 showed reactions (Table 3). Only two BT cases were bacteriologically positive and one showed reactions (Table 4). All patients were treated with 4,4 diaminodiphenylsulfone (DDS) for variable time periods. In addition, four LL cases and five BL cases were given thalidomide concurrently. Cortisone had not been given to any of the patients for at least six months preceding the investigation. Thirteen healthy subjects served as controls.

Lymphocyte preparation. Twenty milliliters of heparinized (500 IU, LEO) venous blood were mixed with an equal volume of 0.9% NaCl, layered on 30 ml of Lymphoprep (Nyegaard, Oslo) (9.6 W/V sodium metrizoate + 5.6% Ficoll) and centrifuged at 400 g for 30 minutes. Cells at the interface were removed and washed three times with Eagle's Minimum Essential Medium (MEM). Spinner Modified (Difco 5839, Difco supplemen-

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TABLE 1. *Summary of data on patients and normal subjects.*

Clinical state	No. subjects	Sex M	Sex F	Age (mean)	Duration of disease/years	Treatment DDS	Treatment Thalidomide
LL	9	6	3	49	13—44	all	4
BL	23	9	14	50	4—43	all	5
BT	8	5	3	52	2—31	all	—
Normal	13	8	5	54	—	—	—

TABLE 2. *Study of peripheral blood lymphocytes in patients with LL. ENL,* erythema nodosum leprosum; *EN, exacerbation nodule; RR, reversal reaction; PHA-CPM, PHA transformation of lymphocytes,* ^{14}*C-thymidine incorporation; ER, nonimmune rosettes.*

No.	Reactions	PHA-CPM counts/min.	ER %	Absolute number ER/mm^3	Absolute number lymphocytes/mm^3
1	ENL	5374[a]	58	1234	2128
2	EN	5056	56	705	1260
3	ENL	17051[a]	55	660	1200
4	ENL	4932	56	627	1120
5	EN	1769	50	767	1534
6	ENL	7113	49	960	1960
7	ENL	9533[a]	55	1032	1876
8	RR	7277[a]	55	737	1340
9	ENL	2380	48	835	1740

[a] Patients on thalidomide.

tary literature, 1962, p 278), pH 7.2 enriched with 0.3 glutamine. The cells were suspended in the same medium and lymphocytes were counted.

Lymphocyte transformation by phytohemagglutinin. Lymphocytes at a concentration of 2×10^6 in 4 ml MEM supplemented with 5% glutamine, 25% homologous serum (from a healthy donor, blood group AB),[3] and antibiotics (penicilline 100 u/ml, streptomycine 100 γ/ml) were cultured in triplicate in 15 ml glass culture tubes. One tenth milliliter PHA-M (Difco) was added to each tube and incubation was carried out in an atmosphere of 5% CO_2 in the air at 37°C. Twenty-four hours before harvesting, 0.1 μCi ^{14}C-thymidine (specific activity 188 μCi per mM) was added. At the end of this period the cells were recovered by centrifugation and washed in 0.9% NaCl. The cells were dried, transferred into scintillation fluid as described by Simberkoff *et al* ([21]) and the radioactivity measured in a liquid scintillator counter.

Spontaneous rosettes. The technic suggested by Hudson ([11]) was followed with minor modifications. One tenth milliliter of a suspension containing 1×10^7 washed lymphocytes per ml of MEM was mixed with 0.2 ml of a 2% suspension of washed sheep red blood cells (SRBC) and 0.1 ml of fetal calf serum (absorbed with SRBC). The mixture was spun at 150 g for ten minutes and incubated at room temperature for one hour followed by 18 hours at 4°C. The cells were resuspended gently, mixed with one drop of 0.4% brilliant cresyl blue and counted. Rosettes developed under these conditions were found to be stable. Lymphocytes covered with at least four SRBC were considered as E-rosettes.

RESULTS

Transformation of lymphocytes by PHA. The response of lymphocytes to PHA as indicated by the incorporation of ^{14}C-thymidine was expressed as counts per minute (cpm). Lymphocytes from all the groups of leprosy patients showed a diminished response when compared with lymphocytes from normal subjects (Table 5, Fig. 1). The difference in counts between LL and BL patients on the

[3] The same serum was used throughout the study.

TABLE 3. *Study of peripheral blood lymphocytes in patients with BL. DR, downgrading reaction; ENL,* erythema nodosum leprosum; *RR, reversal reaction; PHA-CPM, PHA transformation of lymphocytes, ^{14}C-thymidine incorporation; ER, nonimmune rosettes.*

No.	*M. leprae*	Reactions	PHA-CPM counts/min.	ER %	Absolute number ER/mm^3	Absolute number lymphocytes/mm^3
1	+	RR	3996[a]	48	1183	2464
2	+	DR	14520[a]	60	828	1380
3	+	RR	4066[a]	51	949	1860
4	+	RR	16817	64	1142	1785
5	+	DR	11644	50	650	1300
6	+	DR	9121[a]	53	700	1320
7	+	RR	5164	49	882	1800
8	+	RR	17014[a]	65	1176	1809
9	+	RR	6773	60	1138	1896
10	+	ENL	6751	60	1152	1920
11	+	ENL	7969	65	1420	2184
12	+	0	8102	60	1188	1980
13	+	0	8304	60	738	1230
14	+	0	4608	50	1125	2250
15	+	0	5565	58	696	1200
16	—	0	8968	64	1478	2310
17	—	0	7026	60	1188	1980
18	—	0	8526	60	1458	2430
19	—	0	9708	62	2325	3750
20	—	0	3471	49	1338	2730
21	—	0	8934	60	1286	2144
22	—	0	9203	63	1071	1700
23	—	0	9342	61	1347	2208

[a] Patients on thalidomide.

TABLE 4. *Study of peripheral blood lymphocytes in patients with BT. DR, downgrading reaction; ENL,* erythema nodosum leprosum; *RR, reversal reaction; PHA-CPM, PHA transformation of lymphocytes, ^{14}C-thymidine incorporation; ER, nonimmune rosettes.*

No.	*M. leprae*	Reactions	PHA-CPM counts/min.	ER %	Absolute number ER/mm^3	Absolute number lymphocytes/mm^3
1	+	DR	16336	55	1139	2070
2	+	0	7907	56	1310	2340
3	—	0	7076	60	1872	3120
4	—	0	11664	59	857	1452
5	—	0	21575	58	835	1440
6	—	0	8120	59	1381	2340
7	—	0	9780	60	1896	3160
8	—	0	8990	62	2232	3600

one hand and normal controls on the other as evaluated by the Milcoxon-White test ([20]) was significant ($p < 0.01$) and between BT and normal controls was also significant ($p < 0.05$), but to a lesser degree. No difference was observed between bacteriologically positive and negative BL cases.

As is indicated in Tables 2, 3, and 4, no relation was evident between lepra reactions and lymphocyte response to PHA. Lymphocytes from patients treated with thalidomide showed about the same degree of unresponsiveness to PHA stimulation as those from patients of the same group who were taking only DDS.

The spontaneous incorporation of ^{14}C-thy-

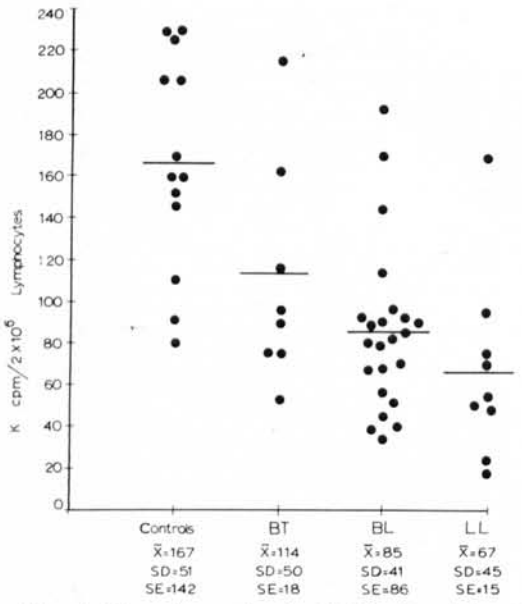

FIG. 1. Transformation by PHA of peripheral blood lymphocytes in the groups of patients and controls. K cpm = counts per minute divided by 100, x = mean value, SD = standard deviation, SE = standard error of mean.

midine without PHA or other mitogen was estimated in eight BL and two BT cases as well as in three normal controls. No significant difference was noted between patients and normal controls.

Spontaneous rosette-forming T cells. The percentage and absolute number of rosette-forming lymphocytes, assumed to be T cells in the different groups, are shown in Tables 2-5. The difference in mean values of the percentage of T lymphocytes is significant only between LL patients and controls ($p < 0.01$). However, the mean absolute number of T lymphocytes, estimated from the absolute number of circulating lymphocytes, compared with the absolute number of T lymphocytes of normal controls was found to be significantly decreased in both LL ($p < 0.01$) and BL ($p < 0.05$) patients. A significant difference in mean absolute value was also observed between LL and BL ($p < 0.01$) cases and between LL and BT cases ($p < 0.01$). Reduction in the total population of circulating lymphocytes was found in both LL and BL cases. The difference in means between LL and normal controls gave a p value of less than 0.01, and, that between BL and normal controls a p value of less than 0.05 (Table 5).

In order to determine whether the number of circulating T lymphocytes is influenced by the presence of *M. leprae*, the results of the cell counts obtained on BL cases were analyzed according to the bacteriologic findings in these patients. In the bacteriologically positive and negative cases the mean values were 992 (SEM 115) and 1436 (SEM 135) per mm^3, respectively. The difference between them was statistically significant ($p < 0.01$). When compared with normal controls, only bacteriologically positive cases showed a significant decrease ($p < 0.01$).

DISCUSSION

In leprosy the immunologic system plays an important role in the cure of the disease and in the development of its various clinical features.

Most investigators accept that in LL there is a defect at the cellular level manifest as relative unresponsiveness to PHA stimulation ([5, 6, 10, 22, 28]). However, Ulrich *et al* ([26])

TABLE 5. *Study of peripheral blood lymphocytes in leprosy patients and normal subjects.*

Clinical state	No. subjects	PHA/trans. cpm[a] (mean ± SEM[b])	E-rosettes % absolute	E-rosettes no/mm^3	Blood lymphocytes/mm^3
LL	9	6721 (± 1520)	54 (± 1.20)	840 (± 66.5)	1573 (± 122)
BL	23	8504 (± 863)	58 (± 1.2)	1146 (± 74.5)	1984 (± 118)
BT	8	11431 (± 1788)	59 (± 0.8)	1440 (± 182)	2440 (± 283)
Normal	13	16731 (± 1424)	60 (± 1.8)	1507 (± 128)	2492 (± 171)

[a] cpm = counts per minute.
[b] SEM = standard error of mean.

found no difference between a group of 24 patients with bacteriologically positive LL of long duration and normal controls in their responses to PHA and PWM. Normal transformation in leprosy was also previously reported by Seagren *et al* [19].

Specificity of the immunologic defect in leprosy has also been noted. Thus, Myrvang *et al* [13] in an extensive study found that the response to *M. leprae* measured by skin sensitivity and *in vitro* methods showed a continuous decrease from strong responses in the polar tuberculoid (TT) group to virtually negative responses in the polar LL group. On the other hand, the response to BCG and PPD decreased only slightly towards the lepromatous pole of the spectrum.

In all groups of patients included in our study, a significant decrease in the response to PHA was observed, most markedly in those with LL. However, the unresponsiveness among BL cases was not related to the presence or absence of *M. leprae*.

The defective T lymphocyte response to the nonspecific mitogen PHA could not be attributed to DDS since all patients were on DDS treatment. Also age cannot be held responsible because the mean age was nearly the same in all groups of patients and in controls (Table 1).

Our results thus confirm that in leprosy there is a defect of the T lymphocytes in response to the nonspecific mitogen PHA. This finding is not likely to be attributed to suppressive effect of autologous serum as reported by Bullock and Fasal [2], since we avoid this by washing the lymphocytes prior to culture and adding homologous serum from a healthy AB donor. However, plasma factors may still be important in the suppression; they may have been firmly bound to the lymphocytes initially and not be removed by washing.

The possibility that the depressed response to PHA of lymphocytes from cases of lepromatous leprosy is associated with a reduction in the circulating T lymphocyte population is supported by the studies of Dwyer *et al* [7], Nath *et al* [14] and Lim *et al* [12]. Reduced response to PHA in our cases could be attributed to a reduced number of circulating T lymphocytes only in LL cases in which both the percentage and absolute number of E-rosettes were reduced. In BL cases the percentage of these cells was within the normal range and there was a reduction only in absolute number of E-rosettes formed. The possibility that depressed responsiveness is due to reduction of a subpopulation of T lymphocytes is not, however, excluded by the technics employed in this study.

Turk and Waters [25] observed that in BL cases who had received intensive therapy, *M. leprae* was not demonstrable in peripheral lymph nodes, and the paracortical areas were repopulated by small lymphocytes. In our bacteriologically negative BL cases, the mean values for circulating T lymphocytes were not significantly different from those of normal controls. Thus, T lymphocyte deficiency does not appear to be a genetic defect because the number of E-rosettes returned to normal in the bacteriologically negative BL patients.

SUMMARY

The ability of peripheral blood lymphocytes from polar lepromatous (LL), borderline lepromatous (BL) and borderline tuberculoid (BT) patients to transform *in vitro* in the presence of phytohemagglutinin was found to be significantly reduced.

A significant reduction in the percentage and absolute number of T lymphocytes was observed in LL cases. In BL cases the number of T lymphocytes was decreased, but the reduction was proportional to the reduction in the total lymphocyte population and was observed only in bacteriologically positive cases.

RESUMEN

Se encontró que la capacidad de los linfocitos de sangre periférica de los pacientes con lepra lepromatosa (LL), lepra cercana a la lepromatosa o "borderline" lepromatosa (BL) y lepra cercana a la tuberculoide o "borderline" tuberculoide (BT), para transformarse *in vitro* en presencia de fitohemaglutinina, estuvo reducida en forma importante.

En los casos de LL se observó una reducción importante en el porcentaje y en el número absoluto de linfocitos T. En los casos BL el número de linfocitos T estuvo disminuído pero la reducción fue proporcional a la reducción en la población linfocítica total y ésto sólo se observó en los casos bacteriológicamente positivos.

RÉSUMÉ

On a observé une réduction significative dans la capacité de transformation *in vitro* en présence de

phytohemagglutinine chez les lymphocytes du sang périphérique provenant de malades atteints de lèpre lépromateuse polaire (LL), de lèpre lépromateuse borderline (BL) et de lèpre tuberculoïde borderline (BT).

Dans les cas LL, on a observé une réduction significative dans le pourcentage et dans le nombre absolu de lymphocytes T. Dans les cas BL, le nombre de lymphocytes T était diminué, mais la diminution était proportionnelle à la réduction de la population totale de lymphocytes; de plus, cette diminution n'a été observée que chez les cas bactériologiquement positifs.

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