Leprosy XII. Quantitative Analysis of Thymus-Derived Lymphocyte Response to Phytohemagglutinin in Leprosy [1, 2]

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There are several indications that patients with lepromatous leprosy have decreased capacity for cell-mediated immunity responses. These include: a) depressed delayed skin reactivity to mycobacterial antigens and other microbial antigens ([2]), b) depressed capacity to develop contact sensitivity to chemical sensitizers ([20]), c) a depressed capacity to reject allogeneic skin grafts ([7]), and d) depressed mitogen-induced transformation of peripheral blood lymphocytes ([3, 4, 8, 13, 19, 21]). It is generally believed that the lymphocyte from patients with lepromatous type of leprosy do not undergo transformation as freely as do those from normal persons when phytohemagglutinin (PHA) is added in tissue culture ([4]). However, there are some different views on this point ([1, 12]). The conventional method for studying PHA responses requires the isolation of lymphocytes from a large volume of blood and prolonged (three to seven days) incubation at 37°C. Under these circumstances it is often difficult to repeat this test frequently for precise and prompt evaluation. Further, the initial isolation of lymphocytes in the peripheral blood in the conventional method may result in random selection of lymphocyte subpopulations and provide uncontrollable variables in each experiment. Moreover, the absolute number of lymphocytes in the peripheral blood varies in different persons, and on different days, and even at different times of day in the same person. Therefore, the conventional methods of stimulation of isolated lymphocytes by PHA that do not take into account the total number of responding units per milliliter of blood may be fraught with great error. We wished to re-examine the lymphocyte transformation to PHA in leprosy patients using a method that could reflect T cell presence and functions in quantitative terms and that was simple to perform and that would require small amounts of blood and only a short period for evaluation. The test described by Park and Good ([5]) which uses a very small amount of whole blood directly incubated with PHA was chosen for this evaluation. This report presents the results of the application of this PHA stimulation test for induction of responses of patients with various types of leprosy including *erythema nodosum leprosum* (ENL) and drug (DDS) resistant lepromatous patients.

MATERIALS AND METHODS

Method. The method used for this study is a new micromethod for evaluating lymphocyte response to PHA. Briefly 0.5 ml of venous blood was collected into a sterile, capped polystyrene tube and five units of heparin were added. Fifty μl of this blood was added into the sterile tube and mixed with 50 μl of tissue culture medium (RPMI 1640) containing 50 μg of PHA-M. To the control tube, 50 μl of blood and 50 μl of tissue culture medium without PHA-M were added. Both PHA and control tests were performed in triplicate. After 24 hours incubation, 0.5 μCi of [methyl-^{3}H] dT in 50 μl of tissue culture medium was added to each tube and mixed gently by tapping the tube. The tubes were incubated for another 14 hours under the same conditions. At the end of the second incubation, 3 ml of distilled water was added to the culture tube. To induce rapid lysis of the erythrocytes the distilled H_2O and the tissue culture was vigorously mixed for 30 seconds. The entire mix-

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ture was then quickly filtered through a millipore filter by use of the apparatus described by Robbins *et al* ([18]). The filter, with entrapped white blood cells and erythrocyte ghosts, was washed successively with cold physiologic saline and 4 ml of 5% trichloracetic acid. The filter paper was then carefully transferred with forceps to the bottom of the glass specimen vial which contained 0.1 ml of 0.2N KOH to dissolve the collected precipitate from the filter paper. The tube was placed in a standard scintillation vial and then counted in a liquid scintillation counter.

Materials. *Patients.* A total of 35 patients with various types of leprosy were studied: 20 had active lepromatous type, 9 inactive lepromatous type, 4 borderline type and 2 indeterminate type leprosy. Their disease's activity or inactivity was determined by clinical manifestations, histologic findings and bacteriologic study. The classification of each patient was based on the Ridley and Jopling scales ([17]). All the patients had been treated with antileprosy chemotherapeutic agents such as diaminodiphenylsulfone, B663 (phenazine dye) and rifampicin for various periods before the blood was collected. The ratio of male to female was two to one and the racial background varied: 7 were Caucasians, 6 Mexican-Americans, 2 Cubans, 2 Filipinos, 1 Samoan, 1 Hawaiian, 1 Negro (from Trinidad), 1 Chinese, and 15 were of mixed races, mostly of South American origin.

Twelve patients with lepromatous type of leprosy have proved to be drug (DDS) resistant. Seven of the patients with active lepromatous type were complicated by ENL. Five active lepromatous patients were receiving steroid therapy (20 mg to 40 mg prednisolone/per day) for varying periods.

Blood samples. The samples of venous blood were drawn with sterile plastic syringes containing heparin and were transported by air from the leprosarium (Carville, La.) to the University of Minnesota. The samples were kept at room temperature during transportation. As a control, the blood was simultaneously collected from two or three healthy persons. Venous blood samples were shipped in small groups of four or five samples and two or three control bloods handled in exactly the same way were included with each shipment.

RESULTS

The results of the PHA test for each group are summarized in Table 1. The mean PHA response of patients with active lepromatous leprosy showed low values but it is difficult to compare the response of one group with that of another because we found different background counts in the unstimulated samples. Therefore, a stimulation index (number of counts after stimulation with PHA/ the unstimulated control sample) for each patient was calculated. To correct for a skewed distribution, logarithmic transformation of the individual indices was used since the radioactive counts of the responding cells are distributed as an exponential function. The mean of the indices of each group was recorded and the results are compared in Table 2. The response of the active lepromatous group showed the lowest value in this study and the other groups with inactive lepromatous, borderline and indeterminate leprosy fell within normal range for PHA responses when these were compared with the mean of the control group (0.91 ± 0.2).

It is interesting, however, that the mean of the inactive lepromatous group falls between the mean value for the active lepro-

TABLE 1. *Mean of PHA-test obtained by a micromethod for various types of leprosy.*

Group	No. subjects	Responses	
		Control Mean ± S.D.	With PHA Mean ± S.D.
Control (healthy)	7	109 ± 31	1246 ± 1291
Active LL	20	306 ± 515	1114 ± 960
Inactive LL	9	736 ± 642	3683 ± 2283
BB Group	4	221 ± 207	2965 ± 2009
I Group	2	324 ± 40	3353 ± 88

TABLE 2. *Stimulation index*[a] *and log data for each group of PHA test.*

Group	No. subject	S.I.[a] Mean ± S.D.	Log[b] (S.I.) Mean ± S.D.
Control	7	9.9 ± 6.9	0.91 ± 0.20
Active LL	20	6.7 ± 4.8	0.61 ± 0.30[c]
Inactive LL	9	10.3 ± 9.9	0.80 ± 0.40
BB Group	4	18.3 ± 17.6	1.01 ± 0.49
I Group	2	10.3 ± 0.6	1.01 ± 0.03

[a] Stimulation Index = PHA/cont.

[b] In order to correct for the skewed distribution of the radioactive counts, a logarithmic transform of the individual indices was used.

[c] Statistical analysis by Student's test is significant difference between control group and active LL group ($p < 0.05$).

TABLE 3. *Drug resistance patient group and no drug resistant group of active LL.*

Group	No.	Control Mean ± S.D.	PHA Mean ± S.D.	S.I. Mean ± S.D.	Log (S.I.) Mean ± S.D.
Drug (DDS) resistant	12	324 ± 654	904 ± 713	5.7 ± 4.4	0.57 ± 0.38
Not drug resistant	8	278 ± 216	1430 ± 1172	6.7 ± 0.4	0.64 ± 0.45

TABLE 4. *ENL complicated patient group and patient group without ENL.*

Group	No.	Control Mean ± S.D.	PHA Mean ± S.D.	S.I. Mean ± S.D.	Log (S.I.) Mean ± S.D.
With ENL	7	143 ± 89	1359 ± 608	10.4 ± 4.3	0.95 ± 0.25
Without ENL	13	394 ± 626	983 ± 1081	3.8 ± 3.1	0.42 ± 0.37

Statistical analysis by Student's test shows significant differences between these groups ($p < 0.05$).

TABLE 5. *Steroid treated group and no steroid-treated group.*

Group	No.	Control Mean ± S.D.	PHA Mean ± S.D.	S.I. Mean ± S.D.	Log (S.I.) Mean ± S.D.
Steroid Tx	5	222 ± 101	1496 ± 632	8.7 ± 5.3	0.78 ± 0.50
No steroid Tx	15	334 ± 595	987 ± 1015	5.2 ± 4.2	0.58 ± 0.47

matous group and that of the borderline leprosy group.

The results of further studies of active lepromatous leprosy patients who were drug (DDS) resistant, ENL-complicated, and steroid treated are summarized in Tables 3, 4, and 5, respectively. The mean of the drug (DDS) resistant group showed a lower value than that of the drug (DDS) sensitive group (Table 3). The mean of ENL-complicated patient group was higher than the mean of the patient group not complicated by ENL (Table 4). This difference is significant ($p < 0.05$). No difference was noted between the group of patients with active lepromatous leprosy who had been given steroids and the group that was untreated by steroid.

DISCUSSION

Oppenheim has argued that the transformation of lymphocyte culture *in vitro* with PHA may be an index of the capacity of an individual to mount a cell-mediated immune response ([14]). Lymphocyte stimulation by

PHA has also been recommended as a means of testing the overall adequacy of the thymus-dependent lymphocytes ([5]). However, at least five broad functionally different groups of lymphocytes exist in peripheral blood: a) thymus-derived cells (T lymphocytes), b) bone marrow derived cells (B lymphocytes), c) lymphocytes that can ultimately function as monocytes, d) primitive stem cells, and e) "partially" differentiated or committed stem cells ([6, 11]). Until recently, it has been impossible to separate the different functional components of the lymphocyte population. For example, Ficoll-Hypaque density gradient separation contains all functional fractions in apparently random distribution. Thus, for the study of the subpopulations very special fractionation technics which take advantage of the functional characteristics of each population must be used. Thus to isolate the lymphocytes by Ficoll-Hypaque gradient fractionation technics, although yielding what looks morphologically to be quite a uniform lymphocyte population, regularly contains lymphocytes of all functional classes. Also, variables in the conventional methodology for recording responses to PHA stimulation on the whole, leave much to be desired. On a practical basis, in the method used in this study, an effort is made to evaluate the proliferative response obtained to PHA per unit of whole blood and thus to evaluate the functional T lymphocyte population and its proliferative response as a reflection both of the number of T cells and their capacity to respond to this stimulation. Influences of erythrocytes and heparin on the T lymphocyte responses obtained have been discussed elsewhere ([15]). It is of concern that monocytes or monocyte precursors, as well as lymphocytes, can be stimulated to respond by proliferation with PHA ([16]). Nonetheless, our results with this test for PHA responsiveness of T cells for various types of leprosy patients seem to be consonant with those obtained by others using more conventional technics; the mean of the active lepromatous patient groups showed the lowest value in this study, which is significantly lower than responses obtained by control group's cells. In other types of leprosy (inactive lepromatous type, borderline, and indeterminate types), the mean responses to PHA stimulation fell within the normal range. It is interesting that the results with inactive lepromatous leprosy revealed improved capacity to respond to PHA in view of the possibility entertained by many that lepromatous leprosy might reflect an inherent immunologic deficiency.

Twelve of the active lepromatous cases which had proved refractive to chemotherapy with DDS showed as a group a lower response to PHA than did other patients with active disease who were responding to the drug. At present we do not know whether there is any relation between the low response to PHA and development of drug resistance. However, the small PHA response and the low level of T lymphocytes obtained from the quantitative T lymphocyte study by two entirely different technics ([9, 10]) for this same patient group taken with the findings recorded here indicates that patients with lepromatous leprosy who are drug resistant have a markedly depressed immunological capacity *vis-a-vis* the T lymphocytes. Thus, for some reason, the low T cell immunologic potential and resistance of lepromatous patients to treatment with DDS seem to be related.

The study of seven cases of leprosy complicated with ENL showed a higher value for PHA response than was observed in those not complicated by ENL. This difference of PHA responsiveness for these two groups of patients with active lepromatous leprosy is also similar to that obtained from enumeration of T lymphocytes by the two other methods.

Because the results for PHA responses in this study are consistent with the findings obtained by quantitation of T cells using immunological markers and the demonstrated capacity of T cells to form rosettes with sheep red blood cells after incubation at 37°C ([9]), the view that the results of PHA testing by the method used reflects the number of T lymphocytes in the circulating blood gains support. As a consequence of these findings, it would appear that one can, in future studies with lepromatous patients, use the proliferative responses of whole blood to PHA as an indication of the adequacy or inadequacy of the T cell population both with respect to its numbers and with respect to its capacity to respond to stimulation.

Although the results of PHA testing have revealed the inadequacy of the T cell populations of patients, for example, in groups of patients with lepromatous leprosy, the use

of the test to define adequacies or inadequacies of T cell population in individual patients is not entirely satisfactory. This is in all likelihood a function of as yet uncontrolled variable.

SUMMARY

The immune status of various leprosy patients was evaluated by using a micromethod to evaluate lymphocyte responses to phytohemagglutinin (PHA). In our study, whole blood was used and the degree of response to PHA stimulation was expressed in terms of unit volume of blood. A markedly decreased response to PHA stimulation was noted in patients with active lepromatous leprosy. Patients with active lepromatous leprosy who have been proved drug (DDS) resistant showed less response than did those of drug sensitive patients with active lepromatous disease, while the patients with active lepromatous leprosy complicated by *erythema nodosum leprosum* (ENL) showed higher response than did those of patients with no complicated ENL.

Comparing the results obtained to those obtained using other methods for T cell analysis indicates that these results reflect the number of T lymphocytes in the leprosy patient. Thus, this simple method is of value in assaying the presence and responses of T lymphocytes in the leprosy patient.

RESUMEN

Se evaluó la situación immunológica de varios enfermos con lepra, utilizando un micrométodo para medir las respuestas linfocitarias a la fitohemaglutinina (PHA). Para este estudio se utilizó sangre completa, y el grado de respuesta a la estimulación con PHA se expresó en terminos de unidad de volumen de sangre. En los pacientes con lepra lepromatosa activa se encontró una respuesta francamente disminuída a la estimulación con PHA. Los pacientes con lepra lepromatosa activa que habían demostrado ser resistentes a drogas (DDS) presentaron una respuesta menor que los pacientes con lepra lepromatosa activa que fueron sensibles a drogas, mientras que los pacientes con lepra lepromatosa activa complicada con eritema nodoso leproso (ENL) mostraron una respuesta más alta que los pacientes que no presentaban complicaciones de ENL.

Al comparar los resultados obtenidos por nosotros con los resultados que se obtienen utilizando otros métodos para el análisis de las células T, se observa que estos resultados reflejan el número de linfocitos T en los pacientes con lepra. Por lo tanto, este método simple es de valor para determinar la presencia y respuestas de los linfocitos T en el paciente con lepra.

RÉSUMÉ

L'état immunitaire de divers malades de la lèpre a été étudié en utilisant une microméthode permettant d'évaluer la réponse lymphocytaire à la phytohémagglutinine (PHA). Au cours de cette étude, on a utilisé du sang entier; l'importance de la réponse à la stimulation par la phytohémagglutinine a été exprimée en unité de volume de sang. Une diminution notable de cette réponse à la stimulation à la phytohémagglutinine a été observée chez des malades souffrant de lèpre lépromateuse active. Les patients présentant une lèpre lépromateuse active qui s'était révélée résistante au médicament (DDS) ont montré une réponse moins nette que les malades lépromateux actifs sensibles au médicament; les individus atteints d'une lèpre lépromateuse active compliquée par un érythème noueux lépreux (ENL) ont présenté une réponse plus forte que ceux sans complication de ce genre.

La comparaison de ces résultats obtenus par cette méthode avec ceux d'autres méthodes permettant d'analyser les cellules-T, indique que ces résultats correspondent au nombre de lymphocytes-T chez les malades de la lèpre. Dès lors, on considère que cette méthode simple est utile pour déceler la présence et suivre la réponse des lymphocytes-T chez le malade de la lèpre.

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