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## Transformation of Leprous Lymphocytes by Leprolin, Tuberculin and Phytohemagglutinin<sup>1-2</sup>

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In states of delayed sensitivity, it is well known that circulating immune lymphocytes respond to specific antigens with blast cell transformation.<sup>4</sup> This phenomenon is thought to be broadly related to cellmediated immunity but does not necessarily correlate with tests for delayed sensitivity (<sup>10</sup>).

There appears to generalized impairment of delayed sensitivity and cellmediated immunity in leprosy patients, especially those with lepromatous leprosy (<sup>1</sup>,-<sup>13</sup>). In lepromatous leprosy there is a depression in the lymphocyte transformation response to PHA (<sup>2, 4, 11</sup>), PPD (<sup>2</sup>) and streptolysin 0 (<sup>2, 12</sup>). In the present investigation transformation of leprous lymphocytes induced by PHA, PPD and the specific antigen "leprolin," a soluable extract of *Mycobacterium leprae*, was studied.

## MATERIALS AND METHODS

Unless specified otherwise the following materials and methods were used throughout this investigation.

Human subjects. The volunteers studied included seven healthy "normal" subjects, nine tuberculoid patients and thirteen lepromatous patients. Although tuberculin skin tests were not done, essentially all subjects were assumed to be tuberculin sensitive since previous surveys had shown that 98% or more of all patients and healthy attendants in the leprosarium were tuberculin positive. All were well-nourished males ranging from 30 to 50 years of age. The patients were under treatment in the Leprosarium, Shin-Tsong, Lo Sheng Taiwan and Leprosy Unit, 813 Army Hospital, Tau-Yan, Taiwan. The type and status of their disease was determined on

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<sup>4</sup> The term "delayed sensitivity" is used in preference to the more commonly used term "delayed hypersensitivity."

the basis of physical findings, tests for acidfast organisms in skin lesions and the lepromin test. Individuals who were consistently negative for one year or longer to scraping tests for acid-fast organisms in skin lesions were classified as "inactive." All patients were, or had been, under treatment with DDS or B.663.

Tissue culture medium. The tissue culture medium used was medium 199 (Difco) containing 50% heat-inactivated pooled normal group AB human plasma, 100 units of penicillin/ml and 100  $\mu$ g of streptomycin/ml.

Lymphocytes. Thirty milliliters of fasting blood were drawn from each subject and transferred to a 50 ml test tube containing 2 ml of heparin solution (100 units/ml) and 4 ml of 6% polyvinylpyrrolidone. The tube was allowed to stand in a vertical position at 37° C for one hour to allow the red cells to settle. The supernatant was collected with a Pasteur pipette, centrifuged at 55 X g for five minutes and the sedimented macrophages (monocytes) and granulocytes were discarded; the supernatant was centrifuged at 550 X g for six minutes and the red cells contaminating the lymphocyte-rich sediment were lysed by osmotic shock (8). The lymphocytes were washed twice with Hank's balanced salt solution (HBSS) and resuspended in the tissue

culture medium described above to achieve a concentration of  $10^6$  cells/ml. Over 90% of the cells were lymphocytes, the majority of which were viable by the dye exclusion method.

Antigens and phytohemagglutinin. A soluble extract of M. leprae, "leprolin," was prepared by a modification of the method of Castro and Arcuri (3). Lepromatous nodules from several patients with active disease were autoclaved at 15 1b for 15 min. They were trimmed, cut into pieces, dried overnight in an oven at 37° C and ground in a mortar to yield fine granules. The bacilli were recovered in chloroform by several cycles of extraction during an hour of grinding, defatted by washing with ether, suspended in physiological saline and subjected to sonification (DC 10 amp) for ten minutes. The material was centrifuged at 2,400 X g for 30 min and the supernatant (leprolin) was collected; its protein content was determined by the method of Kalckar (9). The leprolin was stored at -20° C and aliquots of the same batch were used for all of the present studies. The preparation elicited Fernandez reactions in the several tuberculoid patients tested. Purified protein derivative of tuberculin (PPD) was generously donated by Dr. Chow of the Taiwan Serum and Vaccine Institute. Phytohemagglutinin

	Transforming agent added			
Lymphocyte Donor	PHA-M 10 µ1/ml	PPD 10 µg/ml	Leprolin 0.4 mg/ml	Medium (Control)
N-1ª	51.0 <sup>b</sup>	0.0	3.5	2.3
N-2	22.0	8.0	2.1	0.0
N-3	25.0	4.4	0.0	0.0
N-4	35.0	1.1	2.0	0.5
N-5	36.0	0.0	1.0	3.6
N-6	40.0	0.2	0.1	0.2
N-7	44.6	0.3	0.5	4.0
Mean	36.2	2.0	1.3	1.5

TABLE 1. Transformation of normal lymphocytes induced with PHA, PPD and leprolin.

\* N designates normal healthy subject.

<sup>b</sup> Each number designates the average percent of transformed cells in duplicate specimens.

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Lymphocyte Donor	Transforming agent added			
	PHA-M 10 μ1/ml	$\begin{array}{c} \mathrm{PPD} \\ \mathrm{10} \ \mu \mathrm{g/ml} \end{array}$	Leprolin 0.4 mg/ml	Medium (Control)
T-1ª	12.7 <sup>b</sup>	2.0	. 18.0	0.4
T-2	18.0	16.0	22.0	8.0
T-3	16.0	16.0	17.8	9.0
T-4	17.7	10.0	12.0	8.0
T-5	26.0	28.2	20.0	4.9
T-6	8.0	3.0	4.0	5.4
T-7	20.8	27.0	8.0	3.4
T-8	21.0	14.0	9.0	6.0
T-9	15.0	2.0	4.4	1.0
Mean	17.2	13.1	12.8	5.1

TABLE 2. Transformation of tuberculoid lymphocytes induced with PHA, PPD and leprolin.

<sup>a</sup> T designates tuberculoid patient.
<sup>b</sup> Each number designates the average percent of transformed cells in duplicate specimens.

M (PHA-M) was obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

Transformation test. Three milliliters of the lymphocyte suspension were added to each of a series of screw-capped test tubes (125 x 16 mm). The desired quantities of PHA-M, PPD or leprolin were then added to respective tubes and incubation was carried out at 37° C in a CO<sub>2</sub> incubator under an atmosphere containing 5% CO2 and 95% air. The tests were run in duplicate or triplicate. After four days the tubes were centrifuged lightly to sediment the cells, and smears were prepared from each speci-

Lymphocyte Donor	Transforming agent added			
	PHA-M 10 µl/ml	PPD 10 µg/ml	Leprolin 0.4 mg/ml	Medium (Control)
L-1 <sup>a</sup>	$0.2^{b}$	0.7	1.3	0.4
L-2	0.2	0.0	0.1	0.2
L-3	1.6	0.6	1.8	0.4
L-4	6.0	1.7	2.4	5.0
L-5	3.0	1.2	0.7	1.5
L-6	8.0	1.0	4.0	1.6
L-7	1.7	.0.9	0.7	0.7
L-8	8.6	4.0	18.0	3.8
L-9	6.0	3.0	4.0	3.0
Mean	3.9	1.5	3.7	1.8

TABLE 3. Transformation of lepromatous lymphocytes induced with PHA, PPD and leprolin.

L designates lepromatous patient.

<sup>b</sup> Each number designates the average percent of transformed cells in duplicate specimens.

TABLE 4. Transformation of normal, tuberculoid and lepromatous lymphocytes induced with

Lymphocyte Donors	Transforming agent added			
	PHA-M 10 µl/ml	PPD 10 µg/ml	Leprolin 0.4 mg/ml	Medium (Control)
Healthy Subjects	36.2ª	2.0	1.3	1.5
Tuberculoid Patients	17.2	13.1	12.8	5.1
Lepromatous Patients	3.9	1.5	3.7	1.8

PHA, PPD and leprolin. (Summary of Tables 1, 2, and 3).

\* Each value designates the average percent of transformed cells among lymphocyte specimens from groups of subjects as follows: seven healthy subjects, nine tuberculoid patients, nine lepromatous patients.

men and stained with Giemsa stain. A total of 1000 nucleated cells were counted and the average per cent of blast cells was recorded.

### RESULTS

The results of the first set of experiments designed to measure the transformation response of normal, tuberculoid and lepromatous lymphocytes to PHA, PPD and leprolin are presented in Tables 1 to 3 and are summarized in Table 4. The average percentages of transformed lymphocytes among the various groups (Table 4) show that, as compared with normal lymphocytes, PHA transformation of lymphocytes from both groups of leprosy patients was markedly reduced; the reduction being far the greater in the case of lepromatous lymphocytes, which showed essentially no transformation. With respect to leprolin and PPD, normal lymphocytes and lepromatous lymphocytes also showed little or no transformation; in contrast tuberculoid lymphocytes commonly showed a significant transformation response.

Evidently the failure of normal lymphocytes to respond to PPD in our tests was

Lymphocyte Donor	Transforming agent added			
	PHA-M 10 µl/ml	$\begin{array}{c} \mathrm{PPD} \\ 10 \ \mu \mathrm{g/ml} \end{array}$	Leprolin 0.4 mg/ml	Medium (Control)
L1-10 <sup>n</sup>	$6.5^{\mathrm{b}}$	7.0	2.0	3.6
L1-11	25.8	10.0	15.4	1.2
L1-12	14.5	10.0	5.6	7.0
L1-13	21.4	16.0	16.0	5.0
Mean	17.1	10.8	12.0	4.2

TABLE 5. Transformation of lymphocytes from patients with inactive lepromatous leprosy induced with PHA, PPD and leprolin.

\* L1 designates lepromatous patient with inactive disease.

<sup>b</sup> Each number designates the average per cent of transformed cells in duplicate specimens.

not due to a lack of specific skin sensitivity for experience has shown that in Taiwan most healthy Chinese and patients in the Lo Sheng Leprosarium and the Army Hospital are tuberculin positive. Others have also noted that delayed hypersensitivity reactions of the skin do not necessarily correlate with *in vitro* lymphocyte transformation reactions (see review by Oppenheim [<sup>10</sup>]).

Another experiment was conducted using four patients with drug-arrested (inactive) lepromatous leprosy. Although the number of patients was small the results presented in Table 5 (compare with results in Table 3) are significant and suggest that arrest of lepromatous leprosy by DDS treatment is accompanied by the appearance of lymphocytes which have regained in significant degree their capacity to transform in response to either the nonspecific agent PHA or the specific agents leprolin and tuberculin. This has been confirmed by later work in which transformation was assessed by determining H<sup>3</sup>-thymidine incorporation as a measure of DNA synthesis (7).

As controls for our experiments, we have observed that neither heparin nor extracts of leprous or normal lymphocytes prepared by freezing and thawing influence PHAtransformation of leprous or normal lymphocytes ( $^{7}$ ). Also the possibility that lepromatous lymphocytes might be unable to respond to PHA because of absorbed antigen-antibody complexes appears to have been ruled out by our observation that treatment of the cells with trypsin did not enhance their response to PHA ( $^{7}$ ).

## DISCUSSION

The results of the present experiments confirmed the observation of Dierks and Shepard (<sup>4</sup>) that the capacity of lymphocytes of leprosy patients to transform in the presence of PHA is markedly depressed, especially in the case of lepromatous patients with active disease.

The results also demonstrated that, tuberculoid lymphocytes commonly show substantial transformation responses to leprolin and PPD, while lepromatous lymphocytes commonly show little or no transformation response to these agents. The observed transformation response of tuberculoid lymphocytes to leprolin parallels the well-established fact that tuberculoid patients commonly mount positive Fernandez and Mitsuda reactions, whereas lepromatous patients do not.

The failure of other investigators (4) to demonstrate substantial transformation of tuberculoid lymphocytes with preparations of *M. leprae*, presumed to contain soluble derivatives of the organisms, may have been due to the problem of securing potent leprolin. It has been our experience that some batches of leprolin lack activity suitable for in vitro studies on lymphocytes such as lymphotoxin production (6) and inhibition of macrophage migration (5). For unknown reasons other preparations have been satisfactory. It seems unlikely that tissue antigens contaminating the leprolin preparation used in the present study contributed to transformation since transformation responses were essentially nil in the case of normal lymphocytes treated with leprolin.

general, among the lymphocyte In preparations from each of the various tuberculoid patients there was parallelism between transformation induced with leprolin and PPD (exceptions were patients numbered T-1 and T-7). It is possible that this parallelism and the marked capacity of tuberculoid lymphocytes to respond to PPD as compared to normal lymphocytes resulted from cross reactivity between antigens of M. leprae and M. tuberculosis. Since almost all healthy Chinese adults and patients with tuberculoid leprosy show a positive skin test to tuberculin, it may be presumed that, as compared to the healthy subject, the tuberculoid patient has the stimulus of cross-reacting M. leprae antigens added to the baseline stimulus afforded by antigens of M. tuberculosis. This could explain our results showing that the lymphocytes of tuberculoid patients responded to PPD whereas the lymphocytes of healthy subjects did not respond. However, an alternative possibility is that the populations of circulating lymphocytes capable of responding to specific antigens or to nonspecific stimuli of various sorts are

more abundant or active in tuberculoid patients than healthy subjects. This is suggested by the greater transformation shown in control medium by tuberculoid lymphocytes than by normal lymphocytes. The possibility that the concentration of PPD used was toxic appears to be ruled out by the observation that tuberculoid lymphocytes showed good transformation responses to PPD. The suppressing effect of autologous serum reported by Bullock and Fasal (<sup>2</sup>) was also avoided by the use of homologous group AB human plasma.

The possibility that the mechanism responsible for the low capacity of lepromatous lymphocytes to respond to PHA, PPD or leprolin rests with a reduction in the number of thymus-dependent lymphocytes in the circulation is suggested by the finding of Turk and Waters (<sup>14</sup>) who observed that thymus-dependent lymphocytes in the paracortical areas of lymph nodes become depleted in lepromatous leprosy. An alternative possibility is that lepromatous leprosy in some unknown way suppresses the qualitative immunocompetence of circulating lymphocytes.

### SUMMARY

The observation of other investigators that lymphocytes from leprosy patients, especially lepromatous patients have a lowered capacity to transform in the presence of phytohemagglutinin (PHA) and PPD has been confirmed. Whereas, lymphocytes derived from most tuberculoid patients showed a substantial transformation response to either leprolin, PHA-M, or PPD, lymphocytes derived from lepromatous patients showed little or no transformation response to these agents. In contrast, lymphocytes from normal subjects showed good transformation responses to PHA-M but negligible responses to leprolin and PPD. Lepromatous lymphocytes partially regained their capacity to transform in the presence of either PHA-M, leprolin or PPD when the disease became inactive as the result of chemotherapy.

#### RESUMEN

Se ha confirmado la observación de otros investigadores de que los linfocitos de pacientes con lepra, especialmente pacientes lepromatosos, tienen disminuída la capacidad de transformación en presencia de fitohemaglutinina (PHA) y PPD. Mientras que los linfocitos derivados de la mayor parte de los pacientes tuberculoides mostraron una notable transformación en respuesta ya sea a la leprolina, PHA-M, o PPD, los linfocitos derivados de pacientes lepromatosos mostraron una respuesta de transformación muy disminuída o no mostraron respuesta ante estos agentes. En contraste, los linfocitos de sujetos normales mostraron buenas respuestas de transformación a la PHA-M pero respuestas casi negativas ante la leprolina y el PPD. Los linfocitos lepromatosos recuperaron parcialmente su capacidad para transformarse en presencia ya sea de PHA-M, leprolina o PPD, cuando la enfermedad se hizo inactiva a consecuencia de quimioterapia.

#### RÉSUMÉ

On a confirme dans cette étude l'observation faite par d'autres chercheurs à savoir que les lymphocytes provenant de malades atteints de lépre et particulièrement ceux provenant de malades lépromateux, présentent une capacité diminuée à la transformation en présence de phytohémagglutinine (PHA) et de PPD. Alors que les lymphocytes dérivés de la plupart des malades tuberculoides témoignaient d'une transformation marquée en présence de léproline, de PHA-M, ou de PPD, les lymphocytes obtenus chez les patients lépromateux ne présentaient pas de transformation, ou seulement peu de transformation, en présence de ces produits. Au contraire, les lymphocytes obtenus chez des individus sains témoignaient d'une transformation satisfaisante en présence de PHA-M mais d'une réponse négligable en présence de léproline ou de PPD. Les lymphocytes lépromateux pouvaient partiellement regagner leur capacité à la transformation, en présence de PHA-M, de lépropline, ou de PPD, lorsque la maladie devenait inactive par suite de la chimiothérapie.

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