Lymphotoxin Production by Lymphocytes from Leprosy Patients[1, 2]

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Although it is the consensus that lymphocytes play a major role in both antitissue and antimicrobial cellular immunity, the manner by which they effect defense against microbes and the rejection of foreign grafts is not known.

When lymphocytes are stimulated in appropriate ways, for example immune lymphocytes by specific antigen or nonimmune lymphocytes by phytohemagglutinin (PHA), the suspending medium acquires various biological activities presumed to result from the secretion and/or passive liberation of one or more substances (4). One common attribute of supernatants derived from stimulated lymphocytes is their capacity to kill cultured mammalian cells (5). The substance presumed to cause this cytotoxicity has been called lymphotoxin (10). The various biologically active components present in the supernatants of stimulated lymphocytes have not been isolated and characterized to the point where specific activities can be assigned to them. Thus lymphotoxin may be more than one component or it could be one component with more than one activity. Precisely what population(s) of lymphocytes may be capable of synthesizing and secreting lymphotoxin is not known. However, it is probable that lymphocytes which are abnormal with respect to various activities, such as the capacity to transform under the influence of PHA and to produce the macrophage migration-inhibition factor (MIF) in the presence of specific antigen, are also deficient in their capacity to form lymphotoxin.

Since the limited capacity of lymphocytes from leprosy patients to transform when stimulated by PHA and bacterial antigens has been well documented (1, 3, 7), we deemed it worthwhile to determine the capacity of lymphocytes from leprosy patients to produce lymphotoxin in the presence of PHA and leprolin.

MATERIALS AND METHODS

Human volunteers. The volunteers studied included 16 lepromatous patients, 19 tuberculoid patients and 6 healthy subjects. All were well-nourished adult males in good general physical condition and all patients were under DDS treatment at the Lo Sheng Leprosarium, Shin-Tsong, Taiwan. Their disease status was judged on the basis of physical findings, tests for acid-fast organisms in skin lesions and the lepromin (Mitsuda type) test.

Lymphocytes. Forty milliliters of venous blood were drawn from each subject and transferred to a tube containing 2 ml of 6% polyvinylpyrrolidone and 200 units of heparin. After standing for 1 hour at 37° C the supernatant plasma was withdrawn, chilled in an ice bath and centrifuged at 55 g for 3 min to sediment macrophages and granulocytes. The red cells contaminating the supernatant plasma were destroyed by osmotic shock (8) and the remaining lymphocytes were sedimented at 550 g for 5 min, washed three times in medium 199 (Difco, containing 100 units of penicillin and 100 μg streptomycin/ml) and suspended in medium 199 supplemented with 30% heat-inactivated group AB human serum to give a concentration of 2 x 10^6 cells per ml. The final preparation contained approximately 95% lymphocytes; more than 95% of

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these cells were viable.

Phytohemagglutinin. PHA-M obtained from the Difco Laboratories was used in this investigation. It was added to lymphocyte cultures to give concentrations of 10, 20 and 40 μl per ml.

Leprolin. A soluble extract of *M. leprae* "leprolin" was prepared by a modification of the method of Castro and Arcuri (2). Lepromatous nodules from several patients with active disease were autoclaved at 15 lb for 15 min. They were trimmed, cut into pieces, dried in an oven at 37° C overnight, and ground in a mortar to yield fine granules. The bacilli were harvested by repeated extraction with chloroform during an hour of grinding, defatted by washing with ether, suspended in physiological saline and sonicated for 10 minutes. The sonicated material was centrifuged at 2,400 g for 30 min and the supernatant (leprolin) was collected; its protein content was determined by the method of Kalckar (9). The preparation gave early Fernandez reactions in several tuberculoid patients tested. It was stored at —20° C until use and the same batch of leprolin was used throughout the present study. It was added to lymphocyte cultures to give concentrations of 18, 36, and 72 μg/ml.

Production of lymphotoxin. Two milliliter aliquots of each lymphocyte suspension containing different amounts of added phytohemagglutinin or leprolin were distributed in culture tubes in duplicate. Lymphocyte cultures without any added stimulating agent were used as negative controls. After incubating the preparations for five days on a roller drum at 37° C, the cells were sedimented by centrifugation at 550 g for 5 min and the supernatants were collected.

Assay for lymphotoxin. The supernatants were diluted with Eagle's minimal essential medium (Difco) containing 100 units penicillin and 100 μg streptomycin/ml, 5% calf serum (Difco), and tested for cytotoxic activity on 3-day-old HeLa cell cultures established with 1 x 10^5 cells in Eagle's minimal essential medium containing 10% calf serum. After two days of incubation at 37° C, the adhering cells were flushed from the glass into the overlying culture medium and mixed to break up clumps with the aid of a Pasteur pipette. Viable cell counts were conducted by observing 500 to 1000 cells treated with trypan blue. The per cent of dead cells proved to be the best indicator of lymphotoxin activity and was taken as a rough measure of lymphotoxin in the supernatant.

RESULTS

Lymphotoxin production in response to PHA-M. Suspensions of lymphocytes were prepared from individual blood samples taken from seven lepromatous patients, seven tuberculoid patients and three healthy subjects respectively. The supernatants derived from these lymphocyte preparations were diluted with three volumes of Eagle's medium and tested for lymphotoxin as outlined above.

The supernatants produced by the lymphocytes of healthy subjects in the presence of phytohemagglutinin killed most of the HeLa cells within 48 hours. Cytopathic changes were first noted at 24 hours, at which time most of the cells had rounded up and some had detached from the glass. Within the cells that remained adherent many small vacuoles formed and coalesced to produce one or more large vacuoles which compressed the nucleus to one side of the cell. The cells enlarged and finally disintegrated and disappeared from the glass.

The lymphotoxin activity of supernatants produced by leprous lymphocytes was impaired substantially, impairment being most marked in the case of lymphocytes from lepromatous patients. The results are summarized in Tables 1-3. Because the supernatants produced in the absence of stimulating agent also killed a few HeLa cells, the figures presented designate the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of stimulating agent.

Lymphotoxin production in response to leprolin. The suspensions of lymphocytes were prepared from nine lepromatous patients, twelve tuberculoid patients and three normal healthy subjects. In this set of experiments, the supernatants were diluted with an equal volume of Eagle's medium.

TABLE 1. *Cytotoxic activity of supernatants of PHA-treated lymphocytes from lepromatous leprosy patients.*

Lymphocyte donor	Concentration of PHA-M used to produce supernatant		
	10 μl/ml	20 μl/ml	40 μl/ml
1	39.5[a]	40.1	32.3
2	36.9	45.5	15.5
3	8.5	—	—
4	3.8	7.5	6.1
5	4.6	8.2	5.5
6	3.3	− 0.6	6.9
7	− 1.2	7.5	4.3
Mean ± S.D.	13.6 ± 17.0	18.0 ± 19.5	11.8 ± 10.8

[a] Each value given for supernatants produced in the presence of PHA-M designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of PHA-M.

The data on lymphotoxin production by lymphocytes stimulated with leprolin presented in Tables 4-6 show that no detectable lymphotoxin was produced by lepromatous lymphocytes and barely detectable amounts were produced by tuberculoid lymphocytes.

As shown in Tables 1-6, the capacity of lymphocytes to produce lymphotoxin appears to be impaired in both types of leprosy patients. However, application of Student's t-test showed that only the impairment of lepromatous lymphocytes is statistically different from the control group. In the experiments dealing with PHA-M, lepromatous lymphocytes differ from tuberculoid lymphocytes and from normal lymphocytes but the difference between tuberculoid lymphocytes and normal lymphocytes is not significant. In experiments dealing with leprolin, tuberculoid lymphocytes differ from lepromatous lymphocytes and normal lymphocytes but the difference between lepromatous lymphocytes and

TABLE 2. *Cytotoxic activity of supernatants of PHA-treated lymphocytes from tuberculoid leprosy patients.*

Lymphocyte donor	Concentration of PHA-M used to produce supernatant		
	10 μl/ml	20 μl/ml	40 μl/ml
1	83.7[b]	85.1	90.4
2	79.9	73.9	75.5
3	78.3	77.9	—
4	21.1	—	18.9
5	92.1	78.5	78.1
6	92.1	85.5	76.7
7	12.7	12.3	13.3
Mean ± S.D.	65.7 ± 33.9	68.9 ± 28.1	58.8 ± 33.6

[b] Each value given for supernatants produced in the presence of PHA-M designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of PHA-M.

TABLE 3. *Cytotoxic activity of supernatants of PHA-treated lymphocytes from normal subjects.*

Lymphocyte donor	Concentration of PHA-M used to produce supernatant		
	10 μl/ml	20 μl/ml	40 μl/ml
1	90.3[c]	91.1	91.6
2	57.8	90.9	88.6
3	88.7	90.3	91.0
Mean ± S.D.	78.9 ± 18.3	90.8 ± 0.42	90.4 ± 1.59

[c] Each value given for supernatants produced in the presence of PHA-M designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of PHA-M.

normal lymphocytes is not significant. The results of the statistical analysis are summarized in Tables 7 and 8.

DISCUSSION

The low capacity of lymphocytes from lepromatous patients to form lymphotoxin in the presence of leprolin was not unexpected and is in accord with the concept that general impairment of delayed sensitivity and cellular immunity, a characteristic of lepromatous leprosy, is often associated with abnormalities in lymphocyte function [5].

The observation that tuberculoid lymphocytes in the presence of leprolin produced, at most, only negligible amounts of lymphotoxin was unexpected in view of the fact that most tuberculoid patients can mount a Fernández reaction. However, the findings correlate with the report of Dierks and Shepard ([3]) that in the presence of disrupted *M. leprae* the transformation rates of both lepromatous and tuberculoid lymphocytes are low and with similar observations on transformation made in our laboratories with the same batch of leprolin used in the present tests ([7]). Our previous observation that even lepromatous lymphocytes produce small amounts of MIF in the presence of leprolin ([6]) is not necessarily in conflict with the present finding, since MIF may not be identical with lymphotoxin and moreover, the macrophage migration-inhibition test may have greater sensitivity than the test for lymphotoxin using HeLa cells. With respect to the local delayed sensitivity reaction it should be emphasized that this reaction is probably very complex and that the contributions of various leukocytes and lymphocyte effector molecules to the reaction are not known. Consequently correlation between the production of various effector molecules by lymphocytes stimulated with antigen and delayed cutaneous sensitivity responses may vary greatly.

[5] The term "delayed sensitivity" is used in preference to the more commonly used term "delayed hypersensitivity."

SUMMARY

The limited capacity of leprous lymphocytes to produce lymphotoxin in response to both the specific antigen, leprolin, and the nonspecific agent, PHA-M, follows the same general pattern of depressed responses to these agents previously reported for lymphocyte transformation and the production of the macrophage migration-inhibitory factor.

RESUMEN

La capacidad limitada de los linfocitos leprosos para producir linfotoxina en respuesta tanto al antígeno específico, leprolina, como al agente no específico, PHA-M, sigue el mismo patrón general de respuestas deprimidas ante aquellos agentes que se había presentado previamente para la transformación linfocitaria y la producción de factor de inhibición de migración de macrófagos.

TABLE 4. *Cytotoxic activity of supernatants of leprolin-treated lymphocytes from lepromatous leprosy patients.*

Lymphocyte donor	Concentration of leprolin used to produce supernatant		
	18 μg/ml	36 μg/ml	72 μg/ml
1	0.9[d]	1.4	1.3
2	0.1	1.1	1.1
3	1.3	1.1	1.2
4	0.2	0.1	1.2
5	0.1	0.1	0.4
6	0.3	− 0.1	− 0.2
7	0	− 0.2	− 1.1
8	0.2	1.3	1.5
9	—	2.4	1.7
Mean ± S.D.	0.4 ± 0.5	0.8 ± 0.88	0.8 ± 0.9

[d] Each value given for supernatants produced in the presence of leprolin designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of leprolin.

RÉSUMÉ

Les lymphocytes de malades atteints de lèpre témoignent d'une capacité restreinte à produire une lymphotoxine lorsqu'on les stimule soit par l'antigène spécifique, à savoir la léproline, soit par le PHA-M, qui est un agent non-spécifique. Ceci s'inscrit dans le même schéma général d'une déficience dans la capacité de réagir à l'égard de ces agents, telle qu'elle a été rapportée auparavant pour la transformation lymphocytaire et pour la production d'un facteur inhibant la migration des macrophages.

TABLE 5. *Cytotoxic activity of supernatants of leprolin-treated lymphocytes from tuberculoid leprosy patients.*

Lymphocyte donor	Concentration of leprolin used to produce supernatant		
	18 μg/ml	36 μg/ml	72 μg/ml
1	8.3[e]	6.4	10.2
2	5.9	6.6	6.8
3	2.5	5.5	5.5
4	2.3	1.9	1.5
5	1.9	0.5	2.6
6	3.6	2.3	0.6
7	1.0	1.9	3.8
8	1.9	4.1	7.1
9	3.5	4.4	2.1
10	3.7	4.8	5.2
11	3.7	3.3	2.3
12	3.2	3.1	5.1
Mean ± S.D.	3.5 ± 1.97	3.7 ± 1.91	4.4 ± 2.78

[e] Each value given for supernatants produced in the presence of leprolin designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding control cultures containing supernatant prepared in the absence of leprolin.

TABLE 6. *Cytotoxic activity of supernatants of leprolin-treated lymphocytes from normal subjects.*

Lymphocyte donor	Concentration of leprolin used to produce supernatant		
	18 μg/ml	36 μg/ml	72 μg/ml
1	0.7[f]	1.3	0.6
2	− 0.2	0.1	− 0.3
3	0.2	0.3	0.6
Mean ± S.D.	0.2 ± 0.45	0.6 ± 0.64	0.3 ± 0.5

[f] Each value given for supernatants produced in the presence of leprolin designates the average per cent of dead cells in the cultures in excess of any value for dead cells noted in the corresponding cultures containing supernatant prepared in the absence of leprolin.

TABLE 7. *Statistical analysis of data presented in Tables 1–3.*

Experimental groups	Concentration of PHA-M used to produce supernatant		
	10 μl/ml	20 μl/ml	40 μl/ml
Lepromatous vs Tuberculoid	$p < 0.01$	$p < 0.01$	$p < 0.01$
Lepromatous vs Normal	$p < 0.001$	$p < 0.001$	$p < 0.001$
Tuberculoid vs Normal	$p > 0.5$	$p > 0.2$	$p > 0.1$

TABLE 8. *Statistical analysis of data presented in Tables 4–6.*

Experimental groups	Concentration of leprolin used to produce supernatant		
	18 μg/ml	36 μg/ml	72 μg/ml
Lepromatous vs Tuberculoid	$p < 0.001$	$p < 0.001$	$p < 0.01$
Lepromatous vs Normal	$p > 0.5$	$p > 0.7$	$p > 0.3$
Tuberculoid vs Normal	$p < 0.02$	$p < 0.02$	$p < 0.05$

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