Mechanism of Immunosuppression in Leprosy: Presence of Suppressor Factor(s) from Macrophages of Lepromatous Patients

PADMINI R. SALGAME, P. R. MAHADEVAN,* AND NOSHIR H. ANTIA

The Foundation for Medical Research, Worli, Bombay-400018 India

Human peripheral blood mononuclear cell proliferation induced by *Mycobacterium leprae* could be inhibited by the suppressor factor in the lysate of the macrophages of lepromatous leprosy patients. Macrophages from normal subjects and tuberculoid patients did not show production of a suppressor factor. Inhibition occurred only when the factor was present in the initial stages of lymphocyte culture. The factor is heat stable and nondialyzable. Proliferation induced by some mycobacteria and concanavalin A could also be blocked by the factor. Interestingly, blastogenic response by a few other antigens and phytohemagglutinin could not be inhibited by the suppressor factor. Mononuclear cells pretreated with such lysate from lepromatous macrophages for 24 h could induce suppressive activity in the cells in vitro in an autologous system. Treatment of these cells with carbonyl iron after the induction phase, to remove phagocytic cells, did not abolish their suppressive activity. The lepromatous macrophage lysate also generated suppressive activity in a T-lymphocyte-enriched population of normal subjects. These studies are interpreted to indicate that immunosuppression in lepromatous patients is produced by both macrophages and T lymphocytes. The exact phase in which either of these cells acts as a suppressor may be different. Specific suppression by macrophages to *M. leprae* can be an early event, and nonspecific suppression by T lymphocytes may be a later event in the course of lepromatous leprosy.

Human leprosy exhibits a wide spectrum, ranging from the tuberculoid type with high immunity to the lepromatous type showing depressed cell-mediated immunity. Attempts to delineate the mechanisms of anergy in lepromatous leprosy have resulted in conflicting data. Early workers indicated a defective lymphocyte population as being responsible for the low immune response to *Mycobacterium leprae* in lepromatous leprosy (8, 17). Hirschberg (11) identified the lepromatous macrophage as being the defective cell in leprosy. This was confirmed in another study with HLA-matched individuals (20). Our earlier studies (24) have also indicated that an impairment in macrophage metabolism could be responsible for lowered cell-mediated immunity in lepromatous leprosy.

There is now growing evidence for the importance of suppressor cell-mediated immunoregulation in mycobacterial infections (6, 14, 28). Lepromin-induced reduction in response to stimulation of leukocytes by concanavalin A (ConA) has been reported in most lepromatous patients (18). The authors have also indicated that adherent cells and T cells are involved in mediating this suppressor activity. Two types of

immunosuppressive populations have also been identified in experimental murine leprosy infection (4). A direct requirement for both adherent cells and T lymphocytes for inducing suppression in mycobacterial infections was suggested by Wadee et al. (26).

In the present study we have investigated the possibility of a macrophage-derived inhibitory product negatively modulating lymphocyte proliferation. The study of this product could make it possible to examine the mechanism of immunosuppression in an in vitro system.

MATERIALS AND METHODS

Subjects. Leprosy patients were classified according to the Ridley and Jopling classification (22). Normal controls were healthy volunteers, living in the same environment, who may have had various degrees of exposure to leprosy patients.

Antigens. *M. leprae* was harvested from nodules of untreated lepromatous leprosy patients by a technique developed earlier in our laboratory (1). Purified protein derivative (PPD) was obtained from the Staten Serum Institute, Copenhagen, Denmark; *Mycobacterium bovis* BCG was obtained from Glaxo Pharmaceuticals Ltd., Greenford, England. *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium*

scrofulaceum, Mycobacterium kansasii, and *Mycobacterium vaccae* were cultivated in Middlebrook 7H10 medium. ICRC, a cultivable acid-fast isolate obtained from a lepromatous nodule (2), was cultivated in Dubos medium. The bacilli were harvested in their logarithmic phase.

Preparation of macrophage lysate. Macrophages were cultivated in vitro from the blood of bacteriologically positive lepromatous leprosy patients by a method described in detail earlier (24). After 5 days in culture, the macrophages were scraped off glass with a rubber policeman. Cells were suspended in a known volume of saline, and the cell count was determined. The cells were exposed to six cycles of freezing and thawing. Intracellular material thus released is referred to as lysate. Lysate thus obtained was passed through a sterile Millipore filter (pore size, 0.22 μm; Millipore Corp., New Bedford, Mass.) to remove cell debris. Lysate was stored frozen until use. Lepromatous macrophage lysate is referred to, for convenience, as LL lysate. Lysates from the macrophages of tuberculoid patients (TT lysate) and normal volunteers (N lysate) were prepared as described above. However, *M. leprae* was added in vitro to these cultures before preparing the lysate so as to simulate as closely as possible the conditions prevailing in the lepromatous leprosy patients. Lysates were also prepared from macrophages of long-term-treated, bacillus-negative lepromatous patients, with or without adding *M. leprae* to the macrophages. The lysate prepared without *M. leprae* is referred to as C-LL(-ve) lysate, and lysate with added *M. leprae* is referred to as ML-LL(-ve) lysate. Lysate was also made from macrophage cultures after adding *M. leprae* and cycloheximide. Cycloheximide was present during the entire period of 48 h before lysate was prepared. LL lysate was checked for heat stability at 56°C for 15 min (H-LL lysate). LL lysate was dialyzed for 24 h in the cold against distilled water. The dialysate is referred to as D-LL lysate.

Nonspecific esterase. The method of Koski et al. (15) was used for the identification of macrophages.

Lymphocyte proliferation assay. Mononuclear cells from healthy individuals were separated over a Ficoll-Triosil gradient. A cell count was taken and checked for viability with trypan blue. The cell suspension was adjusted to 10^6 cells per ml in culture medium (minimal essential medium + 20% AB serum). A sample (100 μl) was distributed into each well of a microtiter plate, and 50 μl of antigen or mitogen was distributed into each of the appropriate wells. Mitogen-treated cultures were harvested on the third day, and antigen-exposed cultures were harvested on the sixth day. Each culture combination was prepared in triplicate. At 18 h before the cultures were harvested, 0.5 μCi of [^{3}H]thymidine (specific activity, 9.8 Ci/mM) was added to each well. Cells were harvested on fiber-glass filter disks, and total thymidine incorporation was determined by the liquid scintillation counting system. Results were expressed as counts per minute, calculated as experimental counts per minute minus base-line counts per minute. The concentrations of ConA and PPD used were 8 and 4 μg/ml, respectively. The concentration of phytohemagglutinin (PHA) used was 5 μg/ml. *M. leprae* and the other mycobacteria were used at a dose of 3×10^6 bacilli per ml.

Induction of suppressor cells. Mononuclear cells (3×10^6 cells per ml) in complete culture medium (minimal essential medium + 20% AB serum) were incubated with LL lysate (prepared from 3×10^5 cells) for 24 h at 37°C in a 5% CO_2 atmosphere. Control cells were incubated without LL lysate. After the induction, cells were washed twice in minimal essential medium, suspended in fresh medium, and incubated with 50 μg of mitomycin-C (Sigma Chemical Co., St. Louis, Mo.) per ml for 45 min at 37°C in a 5% CO_2 atmosphere. Cells were washed three times in minimal essential medium and were suspended in complete medium at a concentration of 10^6 cells per ml.

Responder cells. For autologous cultures, samples of mononuclear cells from each subject were stored in complete medium at 4°C during the induction period. Cells were washed before use.

Suppressor assay. Assays were performed with flat-bottomed microtiter plates. Experiments were performed in triplicate, and each well contained 100 μl of responder cells (10^5 cells) and 100 μl of suppressor or control cells treated with mitomycin-C. Antigen or mitogen was added to each well in 50-μl doses. Concentrations used were the same as those given above. Plates were incubated at 37°C in 5% CO_2 in air. At 18 h before harvesting, 0.5 μCi [^{3}H]thymidine was added to each well. The cells were processed for scintillation counting to determine total incorporation of thymidine. Results were expressed as counts per minute, calculated as the counts per minute of responder cells in the presence of mitomycin-C-treated cells and the stimulant minus the counts per minute of responder cells in the presence of mitomycin-C-treated cells alone.

Separation of phagocytic cells from the stimulator cell population. Sterilized carbonyl iron (5 mg/ml) was added to the LL lysate-induced suppressor cell population. Cells were subsequently incubated at 37°C for 45 min, during which time they were agitated at 15-min intervals. After incubation, the cells were again layered on Ficoll-Triosil to remove carbonyl iron-ingested cells. The iron-ingested cells settled to the bottom. Cells at the interphase were collected and tested for suppressor activity. Morphological examination by light microscopy of the cells at the interphase showed about 1% monocyte contamination. Before separation, 8 to 10% monocytes were present.

Separation of T lymphocytes and suppressor cell induction. A T-lymphocyte-enriched population was obtained from normal subjects by the nylon wool column technique (9). The T-lymphocyte-enriched population was exposed for 24 h to LL lysate and was checked for inhibitory activity to ConA stimulation as described above.

RESULTS

Effect of macrophage lysate on normal lymphocyte proliferation. When LL lysate was added to normal mononuclear cell cultures activated with *M. leprae*, substantial suppression of blastogenesis was observed. When TT lysate or N lysate was added to the normal culture, no inhibition was observed (Fig. 1). Macrophage lysate from lepromatous patients negative for acid-fast bacilli when prepared without addition of *M. leprae*
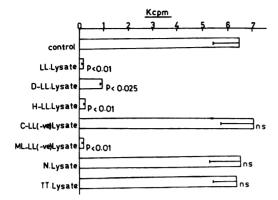
FIG. 1. Proliferative responses to *M. leprae* as measured by [³H]thymidine incorporation of normal control mononuclear cells, normal cells exposed to lepromatous, tuberculoid, and normal lysate, dialyzed LL lysate, and LL lysate treated at 56°C for 15 min. Normal mononuclear cells were also exposed to lysate from lepromatous patients who were negative for acid-fast bacilli with [ML-LL(-ve) lysate] and without [C-LL(-ve) lysate] the addition of *M. leprae* in vitro. *P* was calculated between experimental samples and controls with the Student *t*-test.

in vitro [C-LL(-ve) lysate] did not show inhibitory activity. However, when *M. leprae* was added to these macrophage cultures [ML-LL(-ve) lysate], there was production of suppressor factor (Fig. 1). The addition of cycloheximide to macrophage cultures exposed to *M. leprae* prevented the production of the suppressor factor (Table 1). Further macrophage lysate prepared in the presence of autoclaved *M. leprae* had no suppressor activity (Table 1). The inhibitory

TABLE 1. Effect of cycloheximide and autoclaved *M. leprae* on the production of the inhibitory factor in lepromatous macrophages

LL-lysate[a]	Δ cpm				P[b]
	1	2	3	4	
C	3,608	8,242	3,670	5,098	
ML	125	3,067	112	1,566	<0.0025
CY + ML	2,800	6,929	3,681	6,784	NS[c]
AML	3,512	8,126	3,427	5,001	NS

[a] Cells from normal healthy controls were stimulated with *M. leprae* in the presence of lysate of macrophages from lepromatous bacillus-negative patients (C); lysate of macrophages from the same patient, exposed to *M. leprae* in vitro (5 × 10⁶ bacilli per ml) for 48 h before the lysate was prepared (ML); lysate prepared from macrophage cultures 48 h after cycloheximide (1 μg/ml) and *M. leprae* were added (CY + ML); and lysate prepared from macrophages treated with autoclaved *M. leprae* for 48 hours (AML).
[b] Compared with values for C.
[c] NS, Not significant.

factor was nondialyzable, and moreover, the factor retained its activity on being heated at 56°C for 15 min (Fig. 1).

Effect of addition of LL lysate to normal mononuclear cell culture at different time intervals. The lymphocyte proliferation assay permitted us to explore the temporal events in the exposure of lysate to normal mononuclear cell cultures for mediating the suppression. LL lysate was added to mononuclear cell cultures at the initiation of culture and at 24, 48, 72, and 96 h after culture initiation. In all cases, [³H]thymidine was added 96 h after the initiation of culture. It was observed that inhibitory activity decreased as the time interval of addition of the lysate to the culture was delayed, inhibition being observed up to 48 h. The percentages of inhibition observed at 0, 24, and 48 h were 60, 59, and 50%, respectively. At 72 h there was partial inhibition (25.5%), after which no inhibition was observed (Fig. 2). Apparently, suppressor factor activity is manifested in early events associated with lymphocyte proliferation.

Specificity of action of LL lysate. We also studied the specificity of the inhibition caused by LL lysate. LL lysate significantly suppressed ConA-mediated lymphocyte proliferation (Table 2). Proliferation to mycobacterial antigens *M. scrofulaceum, M. avium, M. intracellulare*, and *M. kansasii* was also blocked (Fig. 3). However, blastogenesis induced by *M. vaccae, M. bovis* BCG, and PPD was not inhibited by the LL
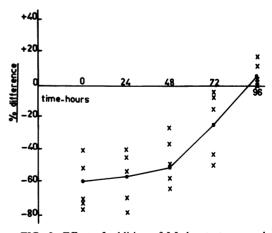

FIG. 2. Effect of addition of LL lysate to normal mononuclear cell cultures at various time intervals ranging from 0 to 96 h after activation with *M. leprae*. The percent difference was calculated as [(counts per minute − control counts per minute)/(control counts per minute)] × 100, where counts per minute represents the antigen-stimulated culture and experimental counts per minute represents the culture stimulated with antigens and LL lysate added at various time intervals. ●, Mean value.

lysate (Fig. 3). Normal lymphocyte blastogenesis to PHA was also not affected by LL lysate (Table 2). In the five experiments with ICRC, stimulation was inhibited by the LL lysate in only one case (Fig. 3).

Ability of LL lysate to activate suppressor cells in vitro. LL lysate was added to a normal mononuclear cell culture to induce suppressor activity. It was capable of inducing a suppressor cell population when tested in coculture experiments (Fig. 4 and 5). The level of suppression was 67 ± 11% for the ConA response and 86 ± 7% for the M. leprae response (Table 3). The suppressor cells were nonspecific in their action as they inhibited both ConA- and M. leprae-mediated stimulation (Fig. 4 and 5). Mononuclear cells of tuberculoid patients also showed significant suppression in the presence of autologous cells treated with LL lysate (Fig. 4 and 5). The level of suppression observed was 82 ± 11% for the ConA response and 86 ± 11% for the M. leprae response (Table 3). Results for long-term-treated, bacillus-negative lepromatous patients were similar to those for normal volunteers and tuberculoid patients; when stimulated with ConA, their mononuclear cells were also suppressed in the presence of autologous cells pretreated for 24 h with LL lysate (Fig. 4 and Table 3). Suppressor activity in lepromatous patients was tested with ConA stimulation only as their response to M. leprae is never regained even after long-term treatment. In lepromatous bacillus-positive patients, response to M. leprae and to ConA is very low; hence, when we tested for suppressor activity, there were no increased levels of suppression above those already present (Table 4).

Effect of removal of phagocytic macrophages from suppressor cell population on inhibitory activity. Cells treated with LL lysate retained their suppressive activity even after depletion of the phagocytic cells. Suppression in the presence of LL lysate alone was 57% in controls; in carbonyl iron-treated cells it was 50%. In the
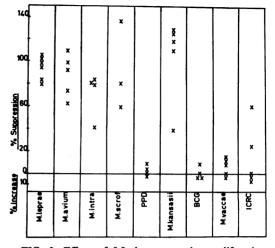

FIG. 3. Effect of LL lysate on the proliferative responses of normal mononuclear cells to various mycobacteria. Data have been presented as increase or suppression, calculated as [(counts per minute of antigen + LL lysate culture − counts per minute of antigen culture)/(counts per minute of antigen culture)] × 100. M. intra, M. intracellulare; M. scrof, M. scrofulaceum.

tuberculoid and lepromatous group, the corresponding values were 77 and 73% suppression for LL lysate and 79 and 75% suppression for phagocyte-depleted cells (Table 5).

Effect of LL lysate on T-lymphocyte-enriched cells. LL lysate was capable of inducing suppressive activity in the T-lymphocyte-enriched cell population (Table 6).

DISCUSSION

These experiments demonstrate that lepromatous macrophages produce immunosuppressive factors which could be responsible for the lowered cell-mediated immune response observed in lepromatous patients. We have preliminary data to show that a sonicate of M. leprae antigen

TABLE 2. Effect of LL lysate on ConA and PHA stimulation of normal mononuclear cells

Stimulation of cells	cpm[a]				
	Expt				Mean ± SEM
	1	2	3	4	
Control	1,821	1,790	266		1,292 ± 54
ConA	34,842	40,432	35,235		36,836 ± 1,835
ConA + LL lysate	2,693	1,986	1,552		2,077 ± 333
Control	1,821	2,569	2,433	266	1,772 ± 528
PHA	83,610	56,653	69,828	30,538	61,282 ± 10,335
PHA + LL lysate	111,804	55,747	63,054	28,086	64,672 ± 17,422

[a] Mean of triplicate cultures for columns 1 through 4. For experiments with ConA, $P < 0.0025$ (comparing results with and without LL lysate); results of experiments with PHA were not statistically significant.

TABLE 3. Effect of LL lysate on induction of suppressor cells from mononuclear cells[a]

Individuals supplying cells	ConA				M. leprae			
	Δ cpm in cells:		% Suppression[c]		Δ cpm in cells:		% Suppression[c]	
	Control	Treated[b]			Control	Treated[b]		
Normal	38,236 ± 7,032	14,323 ± 5,012	67 ± 11		4,749 ± 1,125	863 ± 555	86 ± 7	
Tuberculoid	31,459 ± 10,003	3,263 ± 1,853	82 ± 11		4,586 ± 1,073	769 ± 541	86 ± 11	
Lepromatous	20,129 ± 5,452	3,776 ± 1,414	82 ± 7					

[a] Suppression of ConA and M. leprae responses of mononuclear cells from normal, tuberculoid, and lepromatous people. All values show means ± standard errors of the mean.
[b] Preincubated in LL lysate.
[c] Calculated as [1 − (counts per minute of treated cells/counts per minute of control)] × 100.

had stimulatory capacity similar to that of whole M. leprae. The ability of M. leprae lysates to inhibit whole M. leprae-induced proliferation may give information to determine whether the factor is bacterial or derived from the host cell. However, the results presented in Table 1 and Fig. 1 for normal and tuberculoid macrophage lysates and cycloheximide-treated lepromatous macrophage lysates also give certain indications whether the lysate is a host cell or a bacterial product. Lysates prepared from normal and tuberculoid macrophages exposed to M. leprae in vitro did not show any inhibitory activity. These results support the conclusion that the suppressor factor is not a direct product of M. leprae. Lysate made from cycloheximide-treated macrophages and macrophages exposed to heat-killed M. leprae also do not show any inhibitory activity. This also favors the possibility that the observed effects are not due to bacillary products but most likely are macrophage products. C-LL(-ve) lysate showed no inhibitory action, but the addition of M. leprae to the macrophage cultures induced the production of the suppressor factor. These findings suggest that an active interaction between the lepromatous macrophages and the bacilli is required for the induction of the suppressor factor. The inhibitor is heat stable and nondialyzable. In vitro production of this factor was not affected by concomitant incubation with indomethacin, a prostaglandin synthetase inhibitor, suggesting that the factor is not a prostaglandin (data not shown).

Among the monocyte factors that have suppressor activity are the dialyzable and the heat-stable factors (5, 27). Furthermore, arginase and unlabeled thymidine (21) are also immunosuppressive in various in vitro systems. Our experiments, in which we have shown that the require-
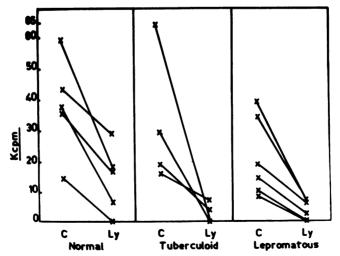

FIG. 4. Suppression of ConA mitogenic responses of mononuclear cells from normal, tuberculoid, and lepromatous groups. Cells pretreated for 24 h with LL lysate were added to autologous cells and exposed to mitogen. Each value is the mean counts per minute of triplicate cultures. Values are shown for cells pretreated with LL lysate (Ly) and for untreated control cells (C).
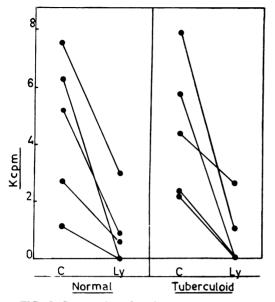
FIG. 5. Suppression of *M. leprae* responses of peripheral blood mononuclear cells from normal and tuberculoid groups. Cells pretreated for 24 h with LL lysate were added to autologous cells and exposed to *M. leprae*. Each value is the mean counts per minute of triplicate cultures from each individual. Values are shown for cells pretreated with LL lysate (Ly) and for untreated control cells (C).

ment of lysate in the initial hours of culture generates suppressor activity, may rule out the possibility that thymidine or arginase is involved in the suppression.

Another interesting observation in our study was the specificity of action of the lysate. A lepromatous patient who is in the early and very active stages of the disease may be anergic to mitogens and a host of antigens, including *M. leprae* (3, 19). This correlates with our observation that mitogenic and antigenic stimulation of

TABLE 4. Suppression of ConA response of mononuclear cells from lepromatous patients[a]

Preincuba-tion	2nd culture	cpm			
		Expt			Mean ± SEM
		1	2	3	
Control	None	265	2,158	1,406	1,276 ± 540
	ConA	3,532	6,157	2,915	4,201 ± 1,013
LL lysate	None	461	2,154	2,723	1,779 ± 692
	ConA	400	7,340	4,435	4,058 ± 2,050

[a] Suppressive activity of mononuclear cells from lepromatous patients, positive for acid-fast bacilli, when exposed to LL lysate, as tested in an autologous system.

TABLE 5. Effect on inhibitory activity of removing phagocytic cells from suppressor cell population[a]

Preincubation	Δ cpm in cell populations:		
	Normal (2)[b]	Tuber-culoid (3)	Lep-roma-tous (2)
Control	31,195	21,880	27,496
LL lysate	13,526 (57)[c]	4,070 (77)	7,485 (73)
LL lysate + carbonyl iron	16,151 (50)	6,576 (79)	6,977 (75)

[a] Mononuclear cultures exposed to LL lysate were treated with carbonyl iron to remove phagocytic cells or were left untreated and were checked for suppressive activity to ConA stimulation in an autologous system. All cells were subjected to a second culture in ConA.

[b] Numbers in parentheses show numbers of experiments.

[c] Numbers in parentheses show percentages of suppression, calculated as [1 − (counts per minute of treated sample/counts per minute of control)] × 100.

normal mononuclear cells was inhibited by LL lysate. However, normal lymphocyte proliferation mediated by *M. vaccae*, *M. bovis* BCG, PPD, and (to a certain extent) ICRC was not inhibited by LL lysate. These antigens may somehow overcome the suppressive action of the lysate. At this stage we do not know the exact mechanisms involved.

Several studies have emphasized a crucial role for macrophages in regulating T-cell-dependent reactivities (10, 23). First, macrophages may be required to present antigens or mitogens to T cell recognition units in a manner required for T cell activation. Second, macrophages may modulate T cell reactivity through the liberation of soluble immunoregulatory molecules. These sol-

TABLE 6. Effect of LL lysate on T-lymphocyte-enriched normal mononuclear cells[a]

Preincubation	Δ cpm in expt:	
	1	2
Control	38,225	42,019
LL Lysate		
Mononuclear cells	6,433 (83)[b]	9,316 (78)
T-lymphocyte-enriched cells	6,384 (83)	8,122 (81)

[a] Whole mononuclear cells or T-lymphocyte-enriched cells were pretreated with LL lysate for 24 h and tested for suppressive activity to ConA stimulation in an autologous system.

[b] Numbers in parentheses indicate percentage of suppression, calculated as explained in Table 5, footnote c.

uble products may exert a direct effect on responding T cells. Alternatively, as the data presented here suggest, some immunoregulatory material liberated by macrophages may indirectly affect responding T cell subsets. The studies presented here indicate that the macrophage or its products and T cell interaction is required to generate suppression. Although this circular interaction between macrophages and T cells may seem unduly complicated, it is consistent with the known complexities of cell interaction originally proposed by Jerne (13).

Cells treated with carbonyl iron after the induction phase still showed suppressive activity to ConA stimulation. These results suggest that the suppressor cell induced in vitro is a lymphocyte. T lymphocyte enrichment methods show that the suppressor lymphocyte is a T cell. However, further enrichment methods for T lymphocytes and T suppressor lymphocytes are in progress. The action of the suppressor cells generated in vitro was nonspecific in its action as it inhibited both ConA and *M. leprae* stimulation.

In other experiments, we have shown that the lysate also inhibits certain macrophage functions (manuscript in preparation). Taking all the above data into consideration, we hypothesize that in vitro, macrophages of the susceptible individual infected with *M. leprae* produce immunosuppressors which act nonspecifically and systemically by altering macrophages and T lymphocytes. Huchet and Florentine (12) have suggested that a low dose of *M. bovis* BCG would mainly favor the induction of suppressor macrophages, whereas larger doses could lead to the induction of T cells in addition. Mehra et al. (18) have also reported that *M. leprae*-induced suppression in vitro was produced by an adherent cell and an E rosetting cell. Over and above the T-suppressor cells induced by lepromin, the presence of these cells in lepromatous patients in the above study could be due to in vivo activation of T cells by certain products released from the monocyte. The authors do show the presence of adherent suppressor cells in vitro also.

These experiments are important in our understanding of the abnormal immune responses in mycobacterial diseases. They could indicate a central role for both macrophages and T cells. Adherent cells of patients with disseminated fungal infection released a soluble factor that was capable of inhibiting the blastogenic response of normal T cells, probably by generating T-suppressor cells (25). Further, in experimental malaria and *Trypanosoma brucei* infection, both macrophages and T cells have been shown to be affected by the parasites, resulting in immunodepression (7, 16).

In leprosy, which presents a complex disease spectrum, there may well be more than one immunological mechanism operative in regulation of the immune response to leprosy bacilli.

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