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In vitro suppression of interleukin 2 production by *Mycobacterium leprae* antigen

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SUMMARY

The suppressive activity of three different lots and sources of *Mycobacterium leprae* (*M. leprae*) was studied by measuring the inhibitory effect on interleukin 2 (IL-2) production in normal subjects. All three *M. leprae* preparations had suppressive activity on IL-2 production when peripheral blood mononuclear leucocytes (PBML) were stimulated with the mitogens PHA-P or Con A in a dose response. *M. leprae* also had suppressive activity on IL-2 production when PBML were stimulated with the specific antigen, PPD. The inhibitory activity of *M. leprae* on IL-2 was not due to the direct interaction of *M. leprae* and IL-2 because direct mixing of IL-2 with different concentrations of *M. leprae* did not alter the activity of IL-2. Incorporation of *M. leprae* for 0, 6 and 12 h in PHA-P and PBML cultures had no inhibitory effect on IL-2 production; however, after 14, 16 and 18 h of *M. leprae* incorporation, significant inhibitory effects were noted on IL-2 production. The suppressive mechanism of *M. leprae* was studied by incorporating *M. leprae* into PBML or adherent cells. The suppressive activity could be detected in both *M. leprae*-stimulated PBML and *M. leprae*-stimulated monocyte supernatant fluids. The suppressive mechanism of *M. leprae* was further evaluated by incorporating 1 and 2 μg/ml of indomethacin in PBML containing PHA-P and *M. leprae*. The suppressive activity of *M. leprae* was significantly diminished by indomethacin, suggesting that the inhibitory effect of *M. leprae* may result from the induction of PBML and adherent cells to produce the immunosuppressive activity of prostaglandin(s).

Keywords interleukin 2 *Mycobacterium leprae* antigen indomethacin

INTRODUCTION

Many immunologic studies of lepromatous leprosy patients have concluded that humoral-mediated immunity (HMI) is generally unimpaired, or elevated, whereas cell-mediated immunity (CMI) is impaired. The reasons for the defective mechanism of CMI in lepromatous leprosy patients' immune response to *M. leprae* is still unclear. Early studies suggested a generalized anergy or unresponsiveness of CMI to various antigen and chemical sensitization (Waldorf *et al.*, 1966; Bullock, 1968). Later studies showed that the patients were only unresponsive to *M. leprae* antigens, while manifesting quite normal responses to other antigens (Turk & Bryceson, 1971; Myrvang *et al.*, 1973; Mendes *et al.*, 1974). Lack of circulating T lymphocytes in lepromatous leprosy patients capable of responding to *M. leprae* by proliferation was reported by Godal *et al.* (1971). The unresponsiveness of CMI in lepromatous leprosy patients due to suppressor T cells or monocytes, has been suggested by some investigators (Mehra *et al.*, 1979; 1980; 1984; Nelson *et al.*, 1987). However, other studies have conflicting results to the suppressor T cell hypothesis (Bjune, 1979; Nath & Singh, 1980; Stoner *et al.*, 1982). Our findings showed that CD8 cells from lepromatous leprosy patients failed to suppress immunoregulation of B cell response to pokeweed mitogen (PWM) (Bullock *et al.*, 1982). Recently, Kaplan *et al.* (1987) have shown that *M. leprae* antigens suppressed T cell proliferation in response to mitogens and antigens in both lepromatous and tuberculoid leprosy patients, as well as controls never exposed to *M. leprae* or *M. leprae*-endemic areas. Therefore, the suppressive mechansim of *M. leprae* antigen to CMI of human subjects is still poorly understood by researchers.

In this study we have shown that *M. leprae* induces suppression of IL-2 production or secretion from Con A- or PHA- or PPD-stimulated PBML from normal donors. The inhibitory effects of *M. leprae* may result from the induction of prostaglandin(s) production from monocytes of the normal donors since indomethacin is able to diminish the inhibitory effect of *M. leprae*.

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MATERIALS AND METHODS

Patients and controls

Leprosy patients were classified by standard clinical and histological criteria (Ridley & Jopling, 1966). All patients were free of other serious disease and erythema nodosum leprosum, and none had received corticosteroid drugs. Controls were male and female healthy volunteers in our department or blood bank donors.

Isolation of PBML

Peripheral blood from laboratory personnel or leprosy patients was drawn into a heparinized tube (10 U/ml of blood). PBML were isolated by Ficoll-Hypaque density gradient centrifugation (Boyum, 1968), washed twice with RPMI 1640 medium and resuspended with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), 10 mM HEPES, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 5×10^{-5} M 2-mercaptoethanol. In all, 10% FCS was used as a supplement in preference to autologous or pooled human serum. The concentration of PBML was adjusted to 1×10^6 cells/ml with supplemented RPMI 1640 medium.

Separation of monocytes

PBML (4×10^6 cells) in 2·0 ml supplemented RPMI media were added onto a 35×10 mm plastic tissue culture plate, incubated at 37°C 5% CO_2 for 2–4 h. Non-adherent cells were washed off three times with supplemented RPMI media. PBML, non-adherent and adherent cells were stained with non-specific esterase stain (NSE). Adherent cells were NSE-positive for 89·86 ± 2·61% (Mean ± s.d. for seven subjects). The exact number of monocytes (5×10^5/ml) adhering on the plate was calculated and the total reached by adding a certain volume of supplemented RPMI media.

Mitogens and antigens

Concanavalin A (ConA) was obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.). Phytohaemagglutinin-P (PHA-P) was purchased from Wellcome (Wellcome Reagents Limited, Wellcome Research Laboratories, Beckenham, UK). Armadillo-derived, freeze-dried *M. leprae* lots were obtained as follows: Lot 1 from Dr R. J. W. Rees (IMMLEP Bank); Lot 2 from Dr Thomas Gillis, Gillis W. Long, Hansen's Disease Center, Carville, LA.; and lot 3 through Contract No. 1 AI-52582 from Dr Patrick J. Brennan, Department of Microbiology, Colorado State University, Fort Collins, CO. Purified proteins derivative of tuberculin (PPD) (2 mg/ml) was obtained from Connaught International Division (Ontario, Canada) and was diluted in RPMI 1640 medium for use as indicated. Indomethacin was purchased from Sigma.

Culture conditions

We achieved optimal conditions for IL-2 production when 1×10^6/ml PBML were stimulated with 40 μg/ml ConA or with 2 μg/ml PHA-P, for an incubation period of 18 h. Using the same cell concentration, we achieved optimal conditions for IL-2 production with 20 μg/ml PPD and an incubation period of 48 h. The inhibitory effect of *M. leprae* for IL-2 production was assayed by incorporating various amounts of *M. leprae* into culture systems containing 1×10^6/ml PBML and 20 μg/ml ConA, 1×10^6/ml PBML and 1 μg/ml PHA-P or 1×10^6/ml PBML and 10 μg/ml PPD. The doses of ConA or PHA-P or PPD were suboptimal doses. *M. leprae* 100 μg/ml was added after 0, 2, 4, 6, 12 and 18 h incubation of PBML and PHA-P with a final incubation period of 18 h. Therefore, *M. leprae* was incorporated and in contact with PBML and PHA-P for 18, 16, 14, 12, 6 and 0 h, respectively, with the same total incubation period of 18 h for all cultures. The suppressive effect of cultured supernate on IL-2 production was measured by incorporating such supernate in PBML stimulated with PHA. The effect of indomethacin was determined by adding 0, 1 and 2 μg/ml indomethacin into culture containing 1×10^6/ml PBML, 1 μg/ml PHA-P and 100 μg/ml *M. leprae*. The percentage suppression of IL-2 production was calculated as follows:

$$\text{Suppressive activity of } M.\ leprae \text{ in cell culture} = 100 - \left(\frac{\text{IL-2 from culture contain stimulator and suppressor}}{\text{IL-2 from culture contain only stimulator}}\right) \times 100$$

$$\text{Suppressive activity of supernate from PBML or monocytes} = 100 - \frac{\text{IL-2 from culture incubated with supernate of culture with } M.\ leprae}{\text{IL-2 from culture incubated with supernate of culture without } M.\ leprae} \times 100$$

Assay of IL-2

Levels of IL-2 in culture supernatant fluid were determined by IL-2-dependent cells, 3-day ConA blasts (Granelli-Piperno, Vassalli & Reich, 1981). After two-fold serial dilutions of each cultured supernatant fluid were performed in 100 μl of supplemented RPMI 1640 medium in a microtitre tissue culture plate (Linbro Co., New Harven, CN), 100 μl containing 2×10^4 cells of 3-day ConA blasts were added into each cultured well. After 24 h of incubation at 37°C, 5% CO_2, each microtitre well was pulsed with 0·2 μCi of ^{3}H-TdR (Amersham International, UK). Cell cultures were harvested 18 h later onto glass fibre strips. The ^{3}H-TdR incorporation was determined by liquid scintillation counting (LS 3801, Beckman Liquid Scintillation Counter). Levels of IL-2 were expressed in units/ml by comparison with commercial standard human IL-2 (Human interleukin 2, ultrapure, Genzyme, MA).

Preparation of 3-day ConA blasts

Three-day ConA blasts were prepared from inbred strains of BALB /c mice, 8–12 weeks old, with some modifications of the methods of Granelli-Piperno *et al.* (1981) and Makonkawkeyoon *et al.* (1987). Briefly, mouse spleens were removed by aseptic techniques, crushed and passed through a stainless steel mesh into RPMI 1640 medium. Erythrocytes were lysed by 0·83% ammonium chloride solution, spleen cells were washed twice and resuspended in supplemented RPMI medium to a concentration of 1×10^6 cells/ml. One millilitre of spleen cell suspension was stimulated with 5 μg/ml ConA (Sigma) at 37°C, 5% CO_2 for 3 days. The cells were washed twice, resuspended and adjusted to 2×10^5 cells/ml with supplemented RPMI 1640 medium.

RESULTS

Stimulating or suppressive effects of various concentrations of *M. leprae* or PPD on IL-2 production or secretion in normal controls or leprosy patients are shown in Table 1. Concentrations of *M. leprae* ranging from 1–100 μg/ml in PBML were not able to stimulate IL-2 production in PPD-positive normal

Table 1. Interleukin 2 production responses to *M. leprae* or PPD in normal or leprosy patients

Concentration of antigens (μg/mls)	IL-2 production from PBML (ct/min)			
	Normal* (8)	TT (4)	BT (3)	BL (2)
M. leprae				
0	261±77	171±34	159±39	181±75
1	257±155	235±32	228±22	262±52
5	287±141	328±89	260±64	330±9
10	304±131	402±132	282±45	232±17
20	348±153	498±171	386±153	312±42
40	337±170	531±74	364±107	331±21
80	380±226	586±101	439±194	305±25
100	268±108	597±48	427±196	325±30
PPD				
10	13241±8054	ND	ND	ND

Results are expressed as mean±s.d.

Number of subjects is given in parentheses. *All normal controls were PPD$^+$ (skin test).

TT tuberculoid leprosy; BT borderline leprosy; BL borderline lepromatous.

ND not done.

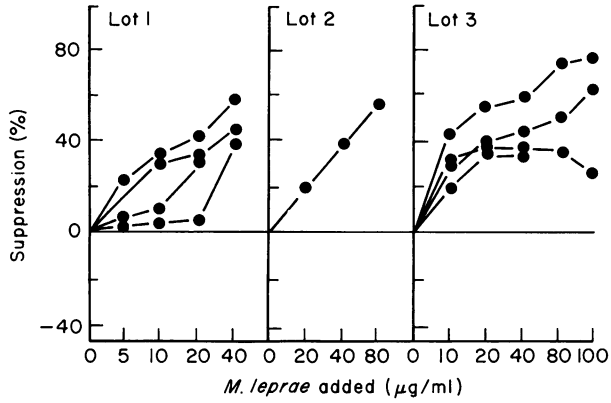

Fig. 1. Suppression of IL-2 production from PBML stimulated with PHA-P by three different lots of *M. leprae*. Each line represents one normal subject.

controls, TT, BT and BL, while 10 μg/ml PPD in PBML of the same normal control group strongly stimulated IL-2 production. Figure 1 shows *M. leprae*-induced suppression of IL-2 production when normal peripheral blood mononuclear leucocytes are stimulated with 1 μg/ml PHA-P. Three preparations of *M. leprae* from three different sources as mentioned in Materials and Methods, were used to evaluate the suppressive activity of *M. leprae*. All three *M. leprae* preparations had suppressive activity for IL-2 production. Each line represents one normal subject. Figure 2 shows very similar suppressive activity of *M. leprae* for IL-2 production when Con A is used as the stimulating agent. There is also a strong suppressive activity of *M. leprae* for IL-2 production when stimulated with 10 μg/ml of PPD as shown in Fig. 3. Figure 4 shows that the suppressive activity of all three preparations of *M. leprae* is not due to the

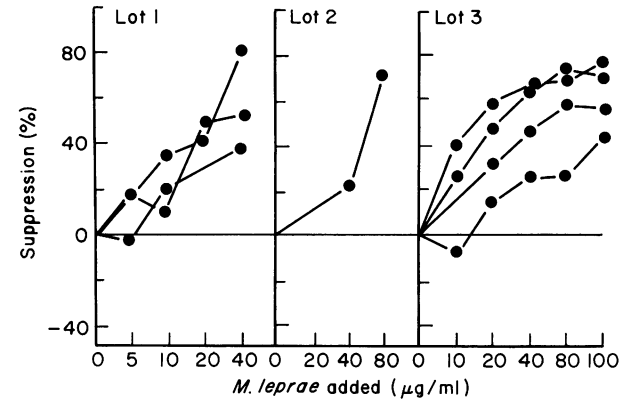

Fig. 2. Suppression of IL-2 production from PBML stimulated with ConA by three different lots of *M. leprae*. Each line represents one normal subject.

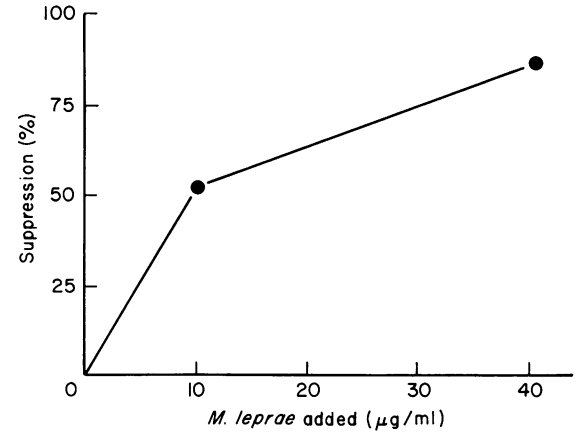

Fig. 3. Suppressive activity of *M. leprae* lot 3 on IL-2 production of normal PBML stimulated with PPD.

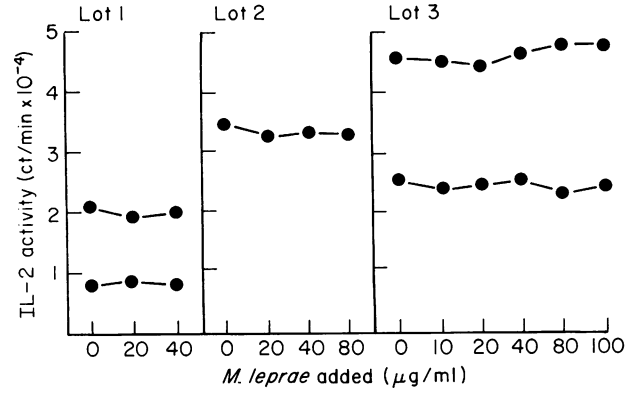

Fig. 4. Various concentrations of all three lots of *M. leprae* were not able to directly inhibit or diminish IL-2 activity.

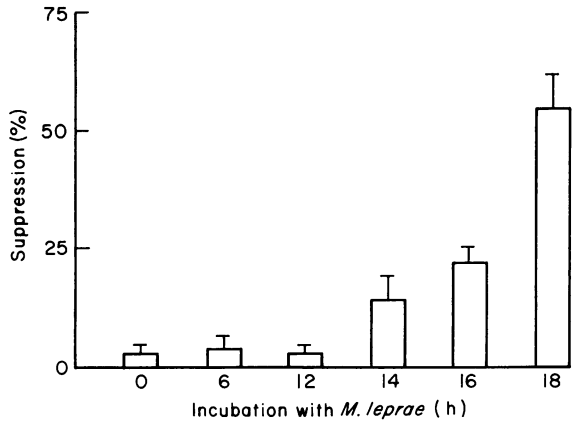

Fig. 5. Suppressive activity of IL-2 production by *M. leprae* in different incubation periods in cell culture. The mean and standard deviation represents the average of four normal subjects.

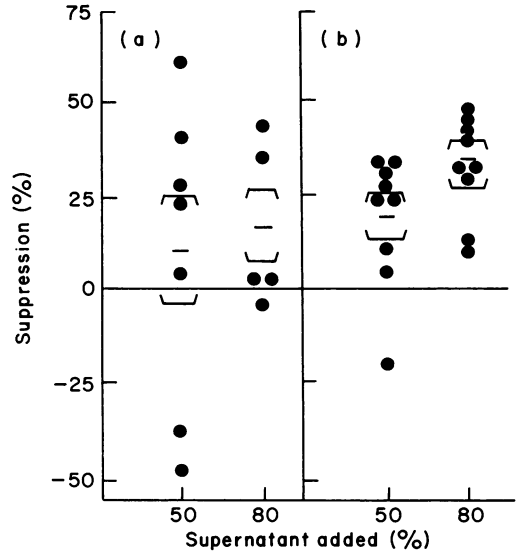

Fig. 6. Suppressive activity of IL-2 production by supernatant from PBML stimulated with 40 μg/ml *M. leprae* (a) and supernatant from *M. leprae*-stimulated monocytes or adherent cells (b).

binding of IL-2 to *M. leprae*. One can see that when our laboratory produced human IL-2 in the absence or in the presence of different concentrations of *M. leprae*, the IL-2 activity is not altered by *M. leprae*. Figure 5 illustrates the suppressive effect of *M. leprae* in different time periods of incubation in cell culture. When *M. leprae* was incorporated in cell culture for 0, 6 and 12 h of incubation there was very little suppression of IL-2 production. However, after 14 h of incubation with *M. leprae*, the suppressive activity was more than 10%, after 16 h more than 20%, and after 18 h it rose to more than 50%. The supressive mechanism of *M. leprae* was further studied by incorporating 0 and 40 μg/ml *M. leprae* with 1×10^6/ml PBML or 5×10^5 monocytes/ml. The suppressive activity can be found in the supernatant fluid of both PBML and monocyte culture as shown in Fig. 6 (a and b respectively). The

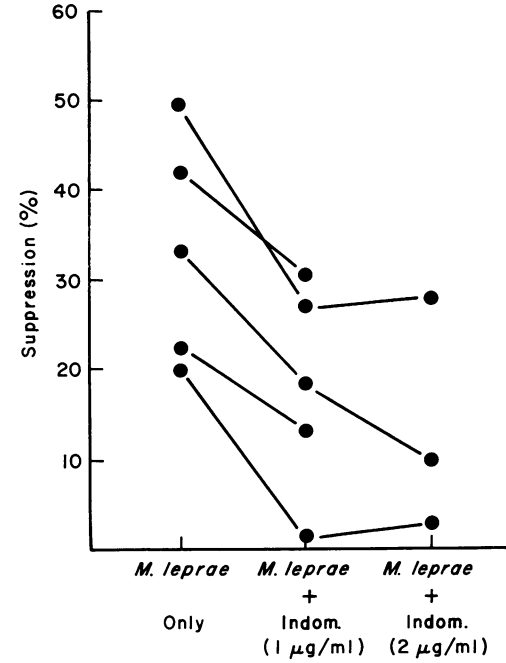

Fig. 7. Suppressive activity of IL-2 production by *M. leprae* can be inhibited or diminished by indomethacin. Each line represents one normal subject.

mechanism of the suppressive activity of *M. leprae* on IL-2 production was further evaluated by incorporating 0, 1, and 2 μg/ml indomethacin in PBMLs containing PHA and *M. leprae*. Figure 7 shows that indomethacin is able to inhibit or diminish the suppressive activity of *M. leprae* on IL-2 production in every normal subject tested.

DISCUSSION

It is generally known that the different clinical forms of leprosy are clearly associated with varying host defense and cellular immunity to *M. leprae*. The highest bacterial load is seen in lepromatous patients but reduces as one approaches the tuberculoid pole. Cellular immunity is defective both *in vivo* and *in vitro* in lepromatous leprosy. This immunologic defect leading to the reduced or absent cellular immunity to *M. leprae* in lepromatous patients is not fully understood. Previous studies of lepromatous leprosy suggest that there is a generalized anergy or unresponsiveness for CMI to various antigens. A lack of sensitization of lepromatous patients to chemical allergens, such as dinitrochlorobenzene (DNCB) and picryl chloride, has been reported (Waldorf *et al.*, 1966; Bullock, 1968). Subsequent studies have shown that, in the early stages of disease, lepromatous patients, although unresponsive to *M. leprae* antigens, appear quite capable of manifesting normal responses to other skin test antigens such as coccidioidin, histoplasmin, and PPD (Myrvang *et al.*, 1973; Mendes *et al.*, 1974). Interestingly, such patients have been shown to convert to tuberculin-positive after vaccination with BCG (Lowe & McNulty, 1953). The latter evidence suggests that the immunologic defect in lepromatous leprosy patients is a specific unresponsiveness to *M. leprae* antigens. Godal *et al.* (1971) have shown that lepromatous leprosy patients lack circulating T lymphocytes capable of responding to *M. leprae* by proliferation.

Evidence for suppression of the immune response by suppressor T cells or monocytes in lepromatous leprosy patients has been presented (Mehra *et al.*, 1979; 1980). Exposure of T cells and monocytes from patients with lepromatous, but not tuberculoid leprosy, to lepromin preparations suppressed the *in vitro* mitogenic response of their lymphocytes. It was also shown that the unique *M. leprae* phenolic glycolipid I is capable of inducing suppression of mitogenic responses of lepromatous patients' mononuclear leucocytes *in vitro* (Mehra *et al.*, 1984). However, other attempts to find disease-related suppression in lepromatous leprosy have provided conflicting results (Bjune, 1979; Nath & Singh, 1980). When lymphocytes from lepromatous patients were mixed with HLA-matched cells from lepromin-positive donors, there was no suppression observed (Stoner *et al.*, 1982). From our previous study we have shown that CD8 cells from lepromatous leprosy patients, when co-cultured with normal B cells plus normal CD4 cells, failed to suppress B cell response to PWM stimulation, while normal B cells plus normal CD4 cells, were suppressed by normal CD8 cells (Bullock *et al.*, 1982).

A number of reports have suggested that the mononuclear leucocytes of lepromatous leprosy patients cannot function normally due to the monocyte-derived suppressor factors (Salgame, Mahadevan & Antia, 1983; Sathish *et al.*, 1983; Birdi *et al.*, 1984a; b). Our study resulted from the initial finding that when we tried to stimulate PBML from normal controls (PPD^+), tuberculoid (TT), borderline tuberculoid (BT), and borderline lepromatous (BL) leprosy with various concentrations of *M. leprae* to determine lymphocyte transformation and IL-2 production. Response of lymphocyte transformation to 1 μg/ml *M. leprae* stimulation was quite good in most cases tested (data not shown). However, all normal, TT, BT and BL tested cases did not produce any, or produced very little IL-2 when stimulated with *M. leprae*. Therefore, the inhibitory effect of *M. leprae* to IL-2 production in normal subjects was studied in more detail. In this study we found that all three preparations of *M. leprae* from three different sources have suppressive activity in suppressing IL-2 production of normal PBML when stimulated with PHA or Con A or PPD. The suppressive activity varies proportionally to doses of *M. leprae*. All *M. leprae* preparations do not adsorb or bind to IL-2 or directly inactivate IL-2 activity. The suppressive activity of *M. leprae* will only occur when it is incorporated in the culture for at least 14 h. However, when *M. leprae* is incorporated for only 0, 6 and 12 h in culture, there is no significant inhibition of IL-2 production from PBML. This suggests that early incorporation of *M. leprae* in culture can inhibit IL-2 production. However, when IL-2 is produced, the *M. leprae* cannot inhibit or stop the IL-2 production and secretion.

The mechanism of suppressive activities of *M. leprae* was further evaluated by incorporating indomethacin into culture containing normal PBML with PHA-P and *M. leprae*. Culture supernatant fluid was assayed for IL-2 activity. All tested normal subjects showed that indomethacin was able to reduce significantly the suppressive activity of *M. leprae*. This result suggests that the suppressive effects of *M. leprae* may come from the induction of prostaglandins (PGs) production by normal PBML stimulated with *M. leprae*, since PGs in turn inhibit lymphokine formation and T cell mitogenesis (Parker, 1986). We tried to see which cell type is responsible for producing the suspected prostaglandin(s). We then incorporated *M. leprae* with normal PBML and adherent monocytes. Each culture supernatant fluid was mixed with normal PBML and PHA-P to determine any suppressive activity of each supernate. There was suppressive activity in supernatant fluid of PBML stimulated with *M. leprae*, but the suppressive activity of supernatant fluid from *M. leprae*-stimulated adherent monocytes was greater.

Recently, Kaplan *et al.* (1987) showed that *M. leprae* antigens, either particulate or soluble fractions, are able to suppress T cell proliferation in response to mitogens or antigens in both lepromatous and tuberculoid leprosy patients, as well as normal controls never exposed to *M. leprae* or *M. leprae*-endemic areas. Prasad, Mishra & Nath (1987) showed that PGL-I induces a general suppressive effect on mitogenic responses across the leprosy spectrum and is not unique to the lepromatous leprosy type. They also showed that PGL-I, in the same concentrations, has both a stimulatory and suppressive role in some healthy and lepromatous subjects.

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