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An indomethacin sensitive suppressor factor released by macrophages of leprosy patients

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Abstract. Reduction in F_c receptor expression as assayed by 'erythrocyte' rosetting of macrophage cultures from long term treated lepromatous leprosy patients (bactereologically negative) was seen in the presence of viable *Mycobacterium leprae.* Macrophages with and without intracellular bacilli demonstrated this reduction. On the basis of this observation the conditioned medium of *Mycobacterium leprae* infected macrophage cultures of lepromatous patients, were tested on macrophages from normal individuals for [^{3}H]-leucine incorporation and antigen specific physical interaction with lymphocytes. Both these parameters showed decreased values as compared to the controls which were not exposed to this conditioned medium. Lymphocyte transformation to *Mycobacterium leprae* in leucocyte cultures of normal individuals was also reduced in the presence of the conditioned medium from lepromatous patients' macrophages. The indication that this factor may be a prostaglandin was suggested by the observation that its synthesis was inhibited by indomethacin. Its importance in the non-specific depression in cell-mediated immunity seen in lepromatous patients is discussed.

Keywords. Macrophage; lepromatous leprosy; prostaglandins.

Introduction

An aberrant macrophage response to *Mycobacterium leprae* in lepromatous leprosy patients was reported earlier by us. Amongst these were a reduced ability to express F_c receptors, a significant reduction in protein synthesis and a negative macrophage-lymphocyte interaction in the presence of *M. leprae.* It was observed specifically in the first two systems that macrophages containing intracellular *M. leprae* seemed to exhibit a suppressive effect on macrophages within the same culture not harbouring intracellular bacilli (Birdi *et al.,* 1979, 1983).

The experiments presented here provide indications for the presence of a suppressor factor released by infected lepromatous macrophages, whose activity is recoverable from the spent culture medium. Various workers have demonstrated the indomethacin sensitive nature of prostaglandin synthesis (Goodwin, 1981). On the basis of the inhibition of the factor by indomethacin it could be suggested that this factor was a prostaglandin. This factor is distinct from the intracellular factor not affected by indomethacin reported previously (Salgame *et al.,* 1983).

Abbreviations used: BI+ve, Bacteriologically positive; BI–ve bacteriologically negative; EA rosetting, erythrocyte rosetting; SRBC, sensitized sheep erythrocytes; MEM, minimal essential medium; SI, stimulation index.

Materials and methods

Choice of patients

Leprosy patients were classified according to the Ridley and Jopling classification (Ridley and Jopling, 1966). Lepromatous patients have been further subdivided as bacteriologically positive (BI+ve) *i.e.* those that harbour acid-fast bacilli in skin smears, and bacteriologically negative (BI – ve) *i. e.* those patients who do not show any acid-fast bacilli in skin smears.

Source of M. leprae

Biopsies of nodules from lepromatous patients were homogenized and then trypsinized. The *M. leprae* thus obtained after differential centrifugation was washed with saline, stored at 4°C and used within a week (Ambrose *et al.,* 1978).

Macrophages

Mononuclear cells were isolated from heparinized peripheral blood by sedimentation in 6% Dextran. Macrophages were freed from most of the other cells by adherence to glass. The macrophages thus obtained were maintained for 7 days in minimal essential medium (MEM) containing 40% human AB serum. The culture medium was changed every 48 h.

Preparation of conditioned medium

Macrophage cultures maintained for 7 days from lepromatous patients were infected *in vitro* with 5×10^6 *M. leprae*/tube as enumerated by the method of Hanks *et al.* (1964). After 24 h the excess *M. leprae* was washed off and the culture was maintained for an additional 48 h after which the medium was collected free from cells. The conditioned medium obtained from $0{\cdot}3\times10^6$ lepromatous macrophages was added to each normal culture.

F_c mediated erythrocyte rosetting (*'EA' rosetting*)

M. leprae (5×10^6) were added to each Leighton tube culture. The cultures were incubated for 24 h before the excess *M. leprae* was washed Goff. Macrophages were further maintained for 72 h after *M. leprae* infection before 'EA' rosetting was carried out, using sensitized sheep erythrocytes (SRBC) (Birdi *et al.,* 1983). In brief, SRBC in a 2% suspension in MEM were sensitized with an equal volume of goat anti-SRBC antibody. A suspension of 1% sensitized SRBC was overlaid onto the macrophage monolayer and allowed to rosette for 30 min at 37°C under 5% CO_2. Nonrosetted SRBC were removed by washing, and the monolayers were fixed in 2·5% glutaraldehyde and stained with Ziehl-Neelsen acid-fast stain to identify *M. leprae.* The

percentage of cells with two or more SRBCs attached was determined. A total of 200 cells were counted. The conditioned medium was added 24 h prior to 'EA' resetting where required. The percentage of cells rosetted with three or more SRBCs was determined.

Antigen specific macrophage-lymphocyte physical interaction

Mononuclear cells from peripheral blood were isolated on a Ficoll-Triosil gradient. The cells so obtained consisted of 80% to 90% lymphocytes and 10–20% macrophages. The cells were then resuspended in MEM containing 20% human AB serum in a concentration of 4×10^6 cells/ml and distributed into Leighton tubes containing coverslips. The conditioned medium was added along with 3×10^6 *M. leprae*/tube and the cells were incubated at 37°C for 18 h. The non-rosetted lymphocytes were washed off and the cells were fixed in 2·5% gluteraldehyde and stained with Ziehl-Neelsen acid-fast stain. The percentage of macrophages with 2 or more lymphocytes adhering to it was determined.

Effect of macrophage conditioned medium on [^3H]-leucine incorporation by macrophages

Macrophage monolayer cultures maintained for 7 days were exposed to the conditioned medium from various sources. Control cultures were maintained to which no conditioned medium was added *in vitro*. The cells were then labelled for 3 h with 1-μCi/ml [^{3}H]-leucine (sp. act. 7·7 Ci/mmol) and further processed for scintillation counting. Radioactivity was measured in the trichloroacetic acid insoluble portion. Incorporation of the precursor added in control cultures was taken as the index of baseline incorporation in the macrophages. Per cent difference between experimental and control cultures was calculated.

Lymphocyte transformation

Mononuclear cells were separated over a Ficoll Triosil gradient. A cell count was taken by diluting with Turks fluid and the suspension was also checked for viability using 0·3% trypan blue. The cell suspension was adjusted to 1×10^6 cells/ml in culture medium (MEM+20% AB serum). Aliquots of 0·1 ml were distributed in each well of a microtitre plate. The *M. leprae* (3×10^6/ml) was distributed in 0·1 ml amounts into each well. The cultures were harvested on the sixth day. Each culture combination was set up in triplicate. Eighteen h prior to harvesting the cultures, 1 μCi/ml [^{3}H]-thymidine (sp. act. 9·8 Ci/mmol) was added to each well. Cells were processed for scintillation counting to determine the incorporation of [^{3}H]-thymidine and the stimulation index (SI) was defined as the ratio of radioactivity incorporated in the experimental over that of control.

Treatment with indomethacin

Indomethacin (1 μg/ml) was added to the cultures for a period of 24 h prior to the assay.

Treatment with cycloheximide

The effect of cycloheximide (2μg/ml Sigma no. C6255) was tested using 'EA' resetting assay on macrophages of lepromatous (BI–ve) patients. Two protocols were used. In one set of experiments, *M. leprae* and cycloheximide were added simultaneously and incubated overnight. These cultures were maintained in medium containing cycloheximide after the excess antigen was washed off for 48 h after which 'EA' rosetting was done.

In a second set of experiments 24 h after *M. leprae* infection of the macrophage cultures, cycloheximide was added 48 h before 'EA' rosetting was carried out.

Results

Effect on 'EA'-rosetting

Macrophages from *M. leprae* infected cultures of lepromatous patients were divided into two populations—macrophages containing intracellular bacilli and those without. Since reduced rosetting was seen in both populations, it was probable that *M. leprae* interacted with the macrophages producing a factor that reduced the F_c activity of the macrophage in which it resided. Simultaneously these macrophages also secreted the inhibitory factor into the surrounding medium which reduced the rosetting capacity of other macrophages (figure 1).

This was confirmed by assessing the inhibitory function of the secreted factor on all the three macrophage parameters.

Effect on [^3H]-leucine incorporation into normal macrophages by the conditioned medium obtained from M. leprae infected macrophage cultures

The conditioned medium obtained from *M. leprae* infected macrophage cultures from

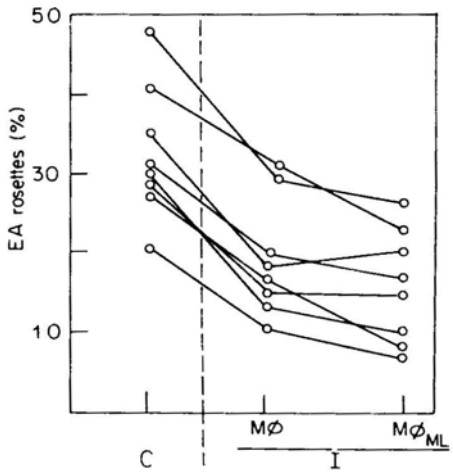

Figure 1. 'EA' rosetting of macrophages from lepromatous (BI—ve) patients in the presence of viable *M. leprae.* Each line represents 3 sets of values from a single patient. The variation in each vertical column represents the range of values.
C, [a]Control, uninfected macrophage culture. I, *M. leprae* infected macrophage culture. Mϕ, [b]Macrophages with no intracellular *M. leprae.* $M\phi_{ML}$, [c]Macrophage with intracellular *M. leprae, a: c,* $P < 0{\cdot}005$; *b: c,* not significant.

normal individuals and tuberculoid patients did not decrease the [^{3}H]-leucine incorporation in normal macrophages (figure 2). However in the presence of the conditioned medium from *M. leprae* infected macrophage cultures of lepromatous patients, the [^{3}H] -leucine incorporation of normal macrophages was reduced. If the macrophages from lepromatous (BI–ve) patients were infected with heat-killed *M. leprae,* no reduction in [^{3}H]-leucine incorporation was noted.

Effect on macrophage-lymphocyte normal interaction by the conditioned medium obtained from M. leprae infected macrophage cultures of lepromatous patients

Normal interaction in the presence of antigen (*M. leprae,* PPD) was reduced if the lepromatous conditioned medium was introduced into the system but the levels were not lower than the baseline value of 10%. Therefore it appears that the factor in the conditioned medium is not specific in function (figure 3).

Effect on lymphocyte proliferation

M. leprae induced lymphocyte proliferation of normal individuals was inhibited in the presence of the conditioned medium prepared from lepromatous (BI—ve) macrophage infected *in vitro* with *M. leprae* (table 1). However the supernatant collected from lepromatous (BI—ve) macrophages alone had no suppressor activity.

Effect of cycloheximide on the production of the inhibitory factor/s

The requirement for protein synthesis by the macrophages from lepromatous (BI—ve) patients was assessed using a protein synthesis inhibitor-cycloheximide. It is evident from the data presented in figure 4A that the levels of 'EA' rosetting are restored in both

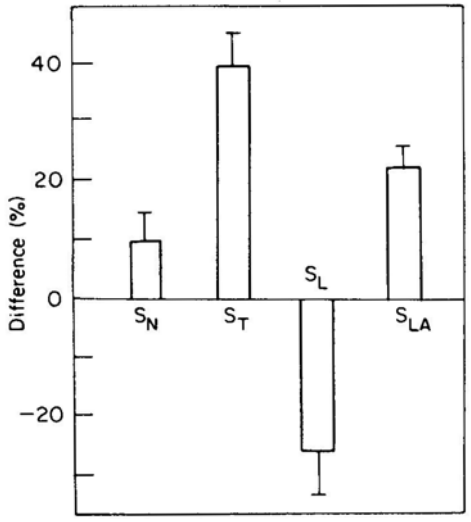

Figure 2. Effect of *M. leprae* infected macrophage supernatant on [^{3}H]-leucine incorporation by normal macrophages. S_N, Spent medium from macrophage cultures of normal subjects infected with viable *M. leprae.* S_T, Spent medium from macrophage cultures of tuberculoid patients infected with *M. leprae.* S_L, Spent medium from macrophage cultures of lepromatous patients infected with *M. leprae.* S_{LA}, Spent medium macrophage cultures of lepromatous patients infected with autoclaved *M. leprae.* The results are expressed as mean ± SE (n = 5).

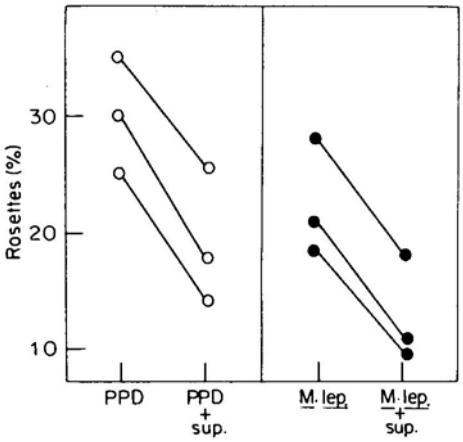

Figure 3. Efect of lepromatous macrophage supernatant on normal macrophage-lyphocyte interaction to *M. leprae* or purified tuberculin (PPD). Each line represents values from a single patient Sup, Conditioned medium from macrophage cultures of lepromatous patients infected with *M. leprae* PPD.

Table 1. The effect of the culture supernatant on *M. leprae* induced lymphocyte proliferation in normal individuals.

Type of supernatant added[a]	[^{3}H]-Thymidine incorporation[b] (mean cpm ± S.E.) $n = 4$	*P* value
Control	6432 ± 1277	
M. leprae	2503 ± 794	$P < 0{\cdot}05$
Indomethacin	7404 ± 1583	NS
Indomethacin + *M. leprae*	7708 ± 1664	NS

[a]Supernatant was collected from bacillary negative lepromatous macrophages. Supernatant was assayed for suppressive activity in a normal lymphocyte proliferation assay to *M. leprae* antigen.

Control, Supernatant from macrophage cultures not exposed to *M. leprae.*

M. leprae, Supernatant from macrophage cultures exposed to *M. leprae.*

Indometacin, Supernatant from macrophage cultures exposed to indomethacin only.

M. leprae+indomethacin, Supernatant from macrophage cultures exposed to *M. leprae* + indomethacin.

[b]The difference in response between normal peripheral blood mononuclear cells stimulated with *M. leprae* and the unstimulated background count.

macrophage populations *i.e.* those containing intracellular *M. leprae* and those without, if the drug is added along with *M. leprae.*

However if bacilli were added 48 h prior to the addition of cyclohemixide, only the macrophages with no intracellular bacilli showed improved levels of rosetting (figure 4B). These kinetic results therefore implicate two factors that mediate suppression. The first is a stable early interaction product contained interacellularly. The second is continously produced and secreted by the macrophages with intracellular *M. Leprae* and mediates amplification.

Effect of indomethacin on the factor secreted by the macrophages

'*EA*' *rosetting*: The possibility that this factor was a prostaglandin was investigated by checking if its synthesis was blocked by indomethacin.

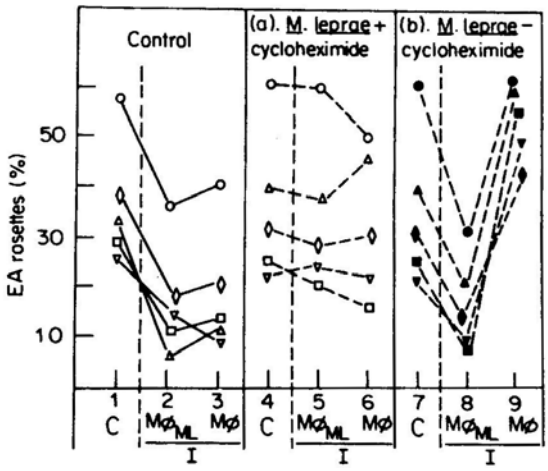

Figure 4. Effect of cycloheximide on the production of the inhibitory factor. M. lep + cycloheximide: Both added simultaneously M. lep — cycloheximide: *M. leprae* added 24 h prior C, Uninfected macrophage cultures. I, *M. leprae* infected macrophage cultures. Mϕ, Macrophages without intracellular *M. leprae.* Mϕ $_{ML}$, Macrophages with intracellular *M. leprae.* (O),Untreatedcultures; (●), culture treated with cycloheximide. Each symbol in all the 3 panels denotes a single patient. 1:4, Not significant; 5:6, not significant; 8:9, $P < 0{\cdot}005$; 4:5, not significant; 7:8, $P < 0{\cdot}005$.

"EA' rosetting of macrophages from lepromatous (BI—ve) patients infected with *M. leprae in vitro* and treated with indomethacin was determined. With this reagent present in the culture, macrophages devoid of intracellular acid fast bacilli did not show any reduction in their capacity to form rosettes with sensitized SRBC. However, cells harbouring *M. leprae* continued to show the same low level of rosetting values similar to those seen in indomethacin free cultures (figure 5).

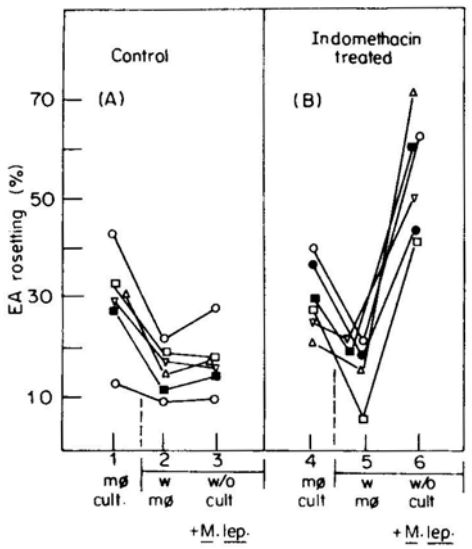

Figure 5. 'EA' rosetting of macrophages from lepromatous (BI—ve) patients. **A.** Control cultures. **B.** Indomethacin treated cultures. Each symbol represents one lepromatous patient, w, Macrophage with intracellular *M. leprae,* w/o, Macrophages without intracellular *M. leprae,* mϕ cult, Macrophage cultures from lepromatous (BI —ve) patients, mϕ cult. + M. lep, Macrophage cultures from lepromatous (BI—ve) patients infected with *M. leprae.* 1:2, $P < 0{\cdot}01$; 2:3, not significant; 5:6, $P < 0{\cdot}001$; 2:5, not significant; 3:6, $P<0{\cdot}001$.

Lymphocyte proliferation assay: The supernatants of indomethacin treated lepromatous cultures failed to show any inhibitory action on a normal *M. leprae* induced lymphocyte proliferation assay (table 1).

Discussion

It is clear from the data presented that macrophages of lepromatous leprosy patients on incubation with *M. leprae* release a factor that alters some basic functions of normal macrophages.

The factor released in macrophage culture medium is specific for an interaction between viable *M. leprae* only and macrophages from a lepromatous patient. The depression was not seen with the conditioned medium from tuberculoid or normal individuals macrophages infected with 5×10^6 *M. leprae*/culture or heat killed *M. leprae* and in earlier studies on F_c receptor expression (Birdi *et al.,* 1983).

Studies carried out in our laboratory have also demonstrated the presence of a specific intracellular suppressor factor in the lysed preparations of lepromatous macrophages infected with viable *M. leprae* (lysate) which was capable of altering normal macrophage function. The extracellular factor reported in this paper differs from the product retained within the infected macrophage (Salgame *et al.,* 1980).

Studies by Preston (1979) demonstrated 2 factors in *M. lepraemurium* infected mice, one able to activate and the other able to suppress. In resistant mice the activating factor played a major role while in susceptible mice the suppressor factor was dominant. It is possible that the increase in [^{3}H]-leucine seen with the conditioned medium from tuberculoid patients is due to a similar factor.

Addition of indomethacin in our system did not increase 'EA' rosette values of cells containing bacilli, though it did augment 'EA' rosette formation in uninfected cells. In the former therefore there are two possibilities. The first is that the decrease in rosette forming cells in bacillary positive cells is not due to prostaglandin or that an intracellular pool of prostaglandin exists within the macrophage which reduces the rosetting capacity of the cell. If the latter were true, then the continuous release of prostaglandin formed prior to indomethacin addition would not be inhibited by an antagonist of prostaglandin synthesis and a depression would be apparent in the rosetting activity of the uninfected cells. This is not so (figure 5). Therefore this line of argument supports the first possibility that non-prostaglandin materials may be responsible only for reduced rosetting in the cells containing bacilli.

The kinetic results with cycloheximide show that the intracellular indomethacin resistant factor may be produced in the early stages of interaction since a lapse of 48 h between antigen addition and cycloheximide addition does not result in an increase of rosetting activity of cells harbouring acid fast bacilli. However cells not harbouring intracellular bacilli do not show any depression suggesting that the second factor is indomethacin sensitive and is continuously produced and secreted.

This subserves the notion that the two factors *viz.* lysate and prostaglandin act as independent entities and that suppression brought on by one is independent of the action of the other. Turcotte and Lemieux (1982) are supportive in their observations that in BCG infected mice the suppression is mediated by two different mechanisms.

The presence of a suppressor factor has also been reported by Satish *et al.* (1983).

Recent results obtained by Bahr *et al.* (1981) suggested that a normal prostaglandin dependent indomethacin sensitive regulatory mechanism was absent from the peripheral blood nonnuclear cells of lepromatous patients. Our data show that the production of a specific suppressor factor is not affected by indomethacin. It is therefore probable that the lymphocyte transformation test utilized by them detected in lepromatous (BI+ve) patients the indomethacin resistant factor as identified by us. It is conceivable that in these patients immunodepression would be modulated mainly through the specific suppressor factors that are not prostaglandins (lysate).

It is not clear whether the difference between the normal and lepromatous supernatant is qualitative or quantitative. It is probable that the amount of regulatory factor released by lepromatous macrophages far exceeds that produced by normal macrophages or that it makes an appearance at earlier stages of infection. Either way it culminates in a premature suppression of the immune response by macrophages uneffected by *M. leprae,* adding to the several abnormal sequence of events occurring inside the cells infected with *M. leprae.*

The possibility exists that the non-specific anergy seen in extremely bacilliferous lepromatous patients is due to the non-specific depression of the immune response by prostaglandins released by infected macrophages. On treatment, these lepromatous patients demonstrate a reduction in their bacillary load and regain their cell-mediated immunity to unrelated antigens but continue to show a specific anergy to *M. leprae.*

The importance of the regulation of the immuno response by prostaglandins has been emphasized by the finding that the altered immune response in Hodgkin's disease may be caused by the increased production of prostaglandins (Goodwin *et al.* al., 1977). Studies carried out by us have demonstrated an altered macrophage topography in lepromatous patients. These observations conform with those of Oropeza-Rendon *et al.* (1980) who have shown that prostaglandins alter the configuration of the macrophage membrane. Concanavalin A activated cells have shown that monocyte suppressive activity is predominantly mediated through the release of prostaglandin E_2 which induces suppressor *T* cells. The induction of *T*-suppressor cells in lepromatous leprosy could be the result of a similar mechanism.

Studies carried out by Shand *et al.* (1981) demonstrate that *T* lymphocytes do not require to undergo proliferation or differentiation to function as suppressor cells since colchicine could prevent suppressor activity. This result indicates the probability that it is a membrene-linked phenomenon. Thus prostaglandin E_2 maybe acting via this mechanism in the induction of suppressor *T* cells.

While prostaglandins appear to suppress cell mediated immunity directly, in humoral immunity they are necessary but not sufficiency for inhibition since direct addition of prostaglandin to plaque forming cells *in vitro* is generally not suppressive but prostaglandin synthetase inhibitors enhance humoral responses (Robertson, 1981). It is accepted that in lepromatous leprosy the humoral response is not reduced and there is suppression only of CMI responses.

It is likely therefore that *M. leprae* not only evades the host immune defence system but also activates a suppressor mechanism *via* the host's own surveillance network. Prostaglandin synthesis may be one such mechanism that is affected.

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