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Alterations in the Membrane of Macrophages from Leprosy Patients

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Macrophage cultures pulsed with viable *Mycobacterium leprae* were assessed for erythrocyte rosetting in three groups of individuals, i.e., normal subjects, and tuberculoid and lepromatous patients. Of these, only the lepromatous group showed a reduction in rosetting ability after infection with *M. leprae*. The specificity of such a reduction pattern was confirmed by using various mycobacteria to infect the macrophages. A threshold effect was noted in all three groups. Although a reduction was obtained in the amount of rosetting of macrophages from lepromatous patients with 10^4 acid-fast bacilli per culture, tuberculoid and normal macrophages resisted such an effect with as large a dose as 20×10^6 to 30×10^6 and 30×10^6 bacilli per culture, respectively. The *M. leprae*-caused alterations in macrophages from lepromatous patients were reversible by treatment with trypsin and colchicine. Cytochalasin B and Tween 80 were unable to alter the pattern. Treatment of cells with neuraminidase was inconclusive since it enhanced rosetting values of both control and infected cultures. These manipulations were significant in elucidating the target point of the host (macrophage) and parasite (*M. leprae*) interaction and in delineation of the external and internal effects upon the macrophages. Both *M. leprae* and macrophages were participants in Fc reduction, as treatment of the former with rifampicin and of the latter with cyclocheximide significantly augmented the rosetting ability. In conclusion, it appears that *M. leprae*, upon entering a lepromatous macrophage, initiates the production of a protein which acts via the microtubules to alter membrane topography. It is possible that the altered membrane prevents effective macrophage-lymphocyte interaction. This could be one of the mechanisms by which cell-mediated immunity is suppressed in lepromatous leprosy.

In lepromatous leprosy, the patient is incapable of developing a cell-mediated immune response against *Mycobacterium leprae*, and bacilli are found to reside intracellularly in large numbers in macrophages. This parasitism results from an interaction between the macrophages and *M. leprae*, and it has been suggested (7) that the outcome of this interaction may culminate in the symptoms and pathology associated with this spectrum of the disease.

We have studied several parameters to determine the altered metabolism of the infected macrophage, such as protein synthesis (3) and the production of a suppressor factor (12). In this paper, an attempt has been made to ascertain how the parasitized macrophage communicates with its surroundings. Since receptors are the prime mode of cellular communication, in the present study attention has been focused on the Fc receptors as markers for the integrity of the macrophage membrane.

The Fc receptors are of considerable significance in the phagocytosis of opsonized particles, antibody-dependent cellular cytotoxicity (14), and have been implicated in the etiology of the inflammatory signals in granuloma formation (2). The importance of this receptor in the participation of the macrophage in the immune response makes it an ideal marker for this study.

MATERIALS AND METHODS

Choice of patients. Leprosy patients were classified according to the Ridley and Jopling classification (10). The lepromatous patients were further subdivided into long-term-treated lepromatous patients who were bacteriologically negative (BI-ve), i.e., did not demonstrate acid-fast bacilli (AFB) in skin smears, and bacteriologically positive patients who demonstrated AFB in their skin smears.

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

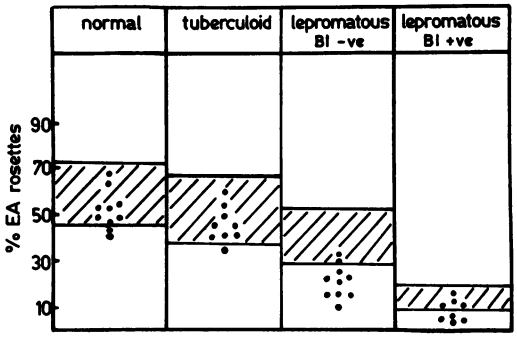

FIG. 1. Infection of macrophages from normal subjects and from leprosy patients in vitro with viable *M. leprae*. The hatched area represents the range of percentage of EA rosetting of uninfected cultures, whereas each dot (●) represents the rosetting levels of macrophages (from individual patients) infected with *M. leprae*. BI + ve, Bacteriologically positive patients.

enriched macrophage culture checked morphologically by nonspecific esterase staining.

Source of *M. leprae*. Biopsies of nodules from lepromatous patients were homogenized and then treated with trypsin. The *M. leprae* cells thus obtained after differential centrifugation were washed with saline, stored at 4°C, and used within a week (1).

Infection of macrophage monolayers. A total of 5×10^6 bacilli were added to each Leighton tube culture. The cultures were incubated for 24 h before the uningested *M. leprae* were washed off.

Fc-mediated EA rosetting. Infected cultures were maintained for 72 h before erythrocyte rosetting (EA rosetting) was carried out. Sheep erythrocytes (SRBC) in a 2% suspension in minimal essential medium 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.

Treatment with trypsin. The macrophage monolayer was treated with trypsin (no. 0152; Difco Laboratories) for 10 min at 37°C under 5% CO_2. The concentrations of trypsin used were 0.125 and 0.05%.

Treatment with neuraminidase. The macrophages were treated with 5 U of neuraminidase (no. N2876; Sigma Chemical Co.) per ml for 12 min at 37°C under 5% CO_2.

Treatment with colchicine or cytochalasin B. Macrophages were exposed to 10^{-5} M colchicine (no. C9754; Sigma) or 10 μg of cytochalasin B (no. C6762; Sigma) per ml for 1 h at 37°C under 5% CO_2. EA rosetting of the macrophages after the above treatments was carried out to determine the effect of each substance.

Treatment with Tween 80. Tween 80 (0.1%; no. P1754; Sigma) was added to the macrophages for 1 h at 37°C under 5% CO_2.

Treatment with cycloheximide. Macrophages were incubated with 2 μg of cycloheximide (no. C6255; Sigma) per ml. In one set of experiments, *M. leprae* and cycloheximide were added simultaneously and incubated overnight. These macrophage cultures were maintained in medium containing cycloheximide after excess *M. leprae* was washed off. EA rosetting was carried out the next day.

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

Treatment with rifampicin. Rifampicin (10 μg/ml; no. R3501; Sigma) was added to the macrophage culture 24 h before *M. leprae* infection. After the excess *M. leprae* was washed off, the cultures were maintained for an additional 48 h in medium containing rifampicin.

RESULTS

Infection of macrophages from normal subjects and from leprosy patients in vitro with viable *M. leprae*. There were no significant differences in the numbers of macrophages from normal subjects and tuberculoid and lepromatous patients that phagocytized *M. leprae*. The values were 52, 58, and 72%, respectively.

Macrophages from normal subjects and from long-term-treated tuberculoid patients did not show any significant difference in EA rosetting of macrophages after *M. leprae* infection when compared with control cultures. However, a significant decrease ($P < 0.005$) was seen in treated lepromatous (BI-ve) patient macrophages infected with *M. leprae* when compared with a control culture of the same patient (Fig.

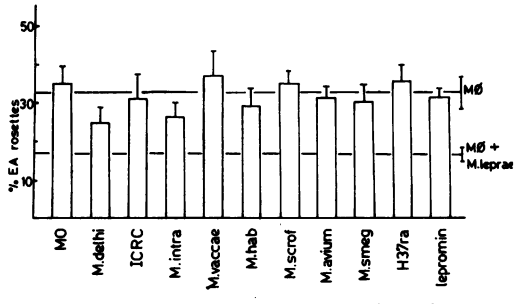

FIG. 2. Infection of macrophages from lepromatous patients with other mycobacteria. The results are expressed as the mean of three experiments. MO, Cultivable mycobacterium from a nodule of a lepromatous patient. Supplied by L. Kato, Institute of Microbiology, University of Montreal, Quebec, Canada. M. delhi, Isolated from soil. Supplied by G. P. Talwar, All India Institute of Medical Sciences, New Delhi, India. ICRC, Cultivable AFB isolated from lepromatous nodules. Supplied by C. V. Bapat, Cancer Research Institute, and E. Borges Marg, Parel, Bombay 400012, India. M. intra, *M. intracellulare*; M. hab, *M. habana*; M. scrof, *M. scrofulaceum*; M. smeg, *M. smegmatis*; H37ra, *M. tuberculosis* H37ra (avirulent strain); lepromin, heat-killed *M. leprae*; Mϕ, macrophage.

1). In macrophage cultures from bacteriologically positive lepromatous patients, the amount of EA rosetting was reduced in the control cultures, and so no additional decrease was evident upon in vitro *M. leprae* challenge.

Infection of macrophages from lepromatous patients with other mycobacteria. Treated lepromatous patients (BI-ve) were used in this study to see if their macrophages also behaved abnormally with other mycobacteria. Our data demonstrate that reduced EA rosetting was not observed in response to any of the other mycobacteria tested. Interestingly, autoclaved *M. leprae* also failed to reduce the percentage of rosetting cells (Fig. 2).

Dose response. Various doses of *M. leprae* were added to macrophages from normal subjects and from leprosy patients (Fig. 3). In lepromatous cultures, a reduction in EA rosetting was seen with 10^4 *M. leprae* cells per culture tube, and upon an additional increase to 2×10^7 *M. leprae* cells per culture tube, the rosetting was completely abrogated. However, in cultures from normal individuals, there were no significant decreases observed in rosetting ability even upon the addition of 3×10^7 *M. leprae* cells per culture. Macrophages of tuberculoid patients had an intermediary threshold dose and demonstrated a 45% decrease in rosetting at a dose of 20×10^6 *M. leprae* cells per culture.

The drop in rosetting levels of macrophages from lepromatous patients with such small numbers of bacilli can be visualized better from the data in Fig. 4. Macrophages from infected cultures have been divided into two populations,

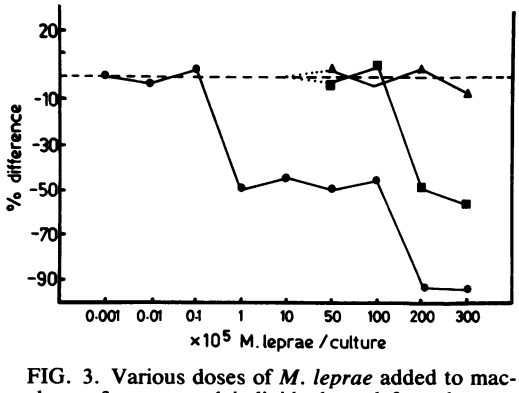

FIG. 3. Various doses of *M. leprae* added to macrophages from normal individuals and from leprosy patients. The results are expressed as the mean of two experiments. Symbols: ●, Macrophages from lepromatous patients; ■, macrophages from tuberculoid patients; ▲, macrophages from normal individuals. The rosetting values of macrophages cultures not infected in vitro with *M. leprae* have been considered as baseline (0% difference). Percent difference is calculated between the EA rosetting levels of the control uninfected culture and the *M. leprae*-infected cultures.

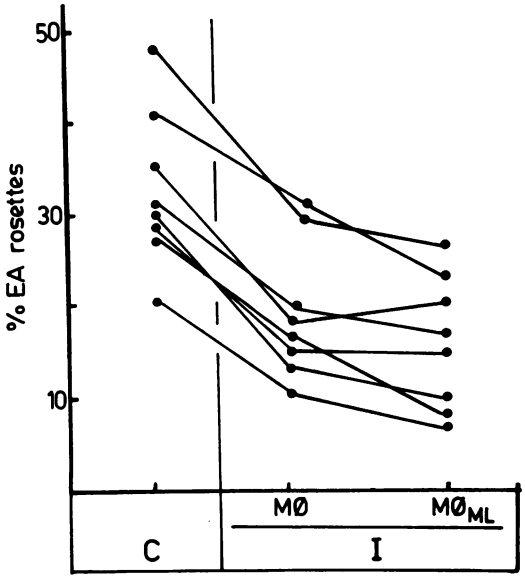

FIG. 4. EA rosetting of macrophages from lepromatous (BI-ve) patients in the presence of viable *M. leprae*. C, Control, uninfected macrophage culture; I, *M. leprae*-infected macrophage culture; Mϕ, macrophages with no intracellular *M. leprae*; $M\phi_{ML}$, macrophages with intracellular *M. leprae*. Control versus Mϕ $P < 0.005$; Mϕ versus $M\phi_{ML}$, not significant.

macrophages containing intracellular *M. leprae* and macrophages not containing intracellular *M. leprae*. The possibility of a soluble factor that affects the macrophages with no intracellular *M. leprae* is discussed below.

Effect of neuraminidase and Tween 80. Neuraminidase and Tween 80 had no significant effects on the *M. leprae*-induced reduction in EA rosetting (Fig. 5a and b), although with neuraminidase treatment the percentage of rosetting cells increased in both control and *M. leprae*-infected cultures; however, the *M. leprae*-induced decrease was still evident (Fig. 5a).

Effect of trypsin. Both concentrations of trypsin used were able to nullify the effect of *M. leprae* on macrophages and return the percentage of EA rosetting to normal levels (Fig. 6a and b). This reversal was, however, temporary, since further incubation of the cultures after trypsin had been washed off reduced the rosetting level again (Fig. 6c). The moiety affected by the interaction between the lepromatous macrophage and the pathogenic *M. leprae* and responsible for the alteration in Fc levels was extremely trypsin sensitive, since 0.05% trypsin (Fig. 6a) could also nullify its effect while not having any direct effect on the Fc receptors themselves.

Effects of cytochalasin B and colchicine. The *M. leprae*-induced alteration in EA rosetting was abolished when cultures were treated with

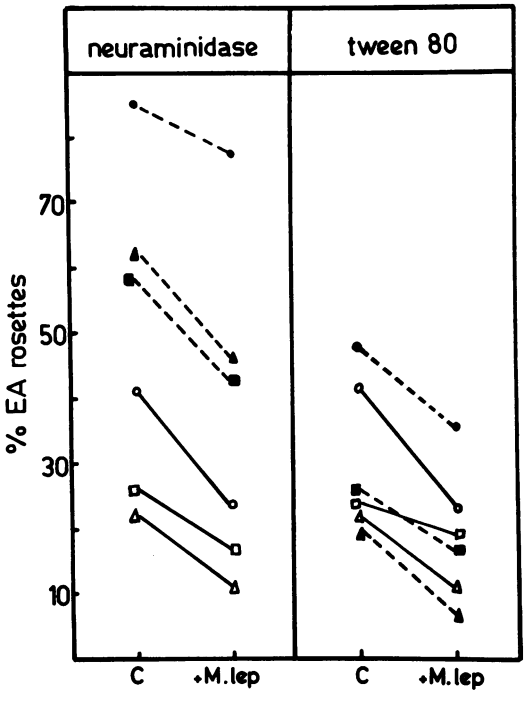

FIG. 5. Effect of treatment with neuraminidase or Tween 80 on macrophages from lepromatous (BI-ve) patients. Each symbol represents the results obtained with macrophage cultures from a lepromatous patient; ○, untreated macrophage culture; ●, macrophage culture treated with neuraminidase or Tween 80; C, uninfected macrophage culture; +M.lep, *M. leprae*-infected macrophage culture.

colchicine ($P < 0.005$) (Fig. 7a), but not with cytochalisin B (Fig. 7b). Thus, microtubule, but not microfilament, involvement is indicated in the reduction of the rosetting ability of lepromatous macrophages containing *M. leprae*.

Effects of cycloheximide and rifampicin. It is evident from the data presented in Fig. 8 that the levels of EA rosetting were restored if cycloheximide was added together with *M. leprae* in both macrophage populations, i.e., macrophages containing intracellular *M. leprae* and macrophages without intracellular bacilli. However, if *M. leprae* was added 48 h before the addition of cycloheximide, only the macrophages with no intracellular bacilli showed improved levels of rosetting (Fig. 8). In uninfected cultures treated with cycloheximide, there was no deviation from control values.

If *M. leprae* metabolism was blocked with rifampicin, a known antileprosy drug, the reduction in the amount of EA rosetting seen in *M. leprae*-infected macrophage cultures without rifampicin was not exhibited (Fig. 9).

This suggests that metabolically active macrophages and *M. leprae* was required for the reduction in the EA rosetting ability of the macrophages.

DISCUSSION

Knowledge of the organization and dynamics of the membrane is a prerequisite for any useful attempt to build models on how various inflammatory signals can be transduced through the membrane. Such dynamic processes could be studied by the expression of receptors on the membrane.

The data of Amsden and Boros (2) revealed marked differences in cell kinetics and the display and specificity of Fc receptors of macrophages obtained from various granulomas. These differences seemed related to the etiology of the granuloma and to the intensity and duration of the inflammatory signals that prevailed in the lesions. Similar studies have been carried out for leprosy by Ridley et al. (11). The lowered levels of Fc receptors have also been implicated in the pathogenesis of Sjögren's disease (4).

This study demonstrates that macrophages from lepromatous patients respond abnormally by a reduction in EA rosetting values when challenged with viable *M. leprae*. Long-term-treated lepromatous patients who are bacteriologically negative have normal numbers of rosetted macrophages which are once again reduced upon exposure to *M. leprae* in vitro. These results stress the importance of classifying the lepromatous patients according to their bacteriological status when conducting immunological studies and also stress the importance of studying so-called cured patients.

The characteristic pattern of reduced levels of EA rosetting in lepromatous macrophages emerged only with viable *M. leprae* challenge and not with lepromin (heat-killed *M. leprae*) challenge. It could therefore be reasoned that the effect of *M. leprae* on the membrane was perhaps via an *M. leprae*-derived metabolic product acting on the macrophage.

Additional confirmation was obtained by using rifampicin, a known bactericidal drug for *M. leprae*. In the presence of rifampicin, *M. leprae* did not reduce the amount of EA rosetting of lepromatous macrophages. However, host cell metabolism was of equal importance since simultaneous addition of *M. leprae* and cycloheximide, an inhibitor of protein synthesis in eucaryotes, to lepromatous macrophage cultures did not reduce the amount of EA rosetting.

The fact that in vitro *M. leprae* challenge brought about a reduction in the EA rosetting values from a near-normal level in lepromatous (BI-ve) individuals indicated that such a character formed an intrinsic trait of susceptible macrophages. To test this, 24 familial contacts of

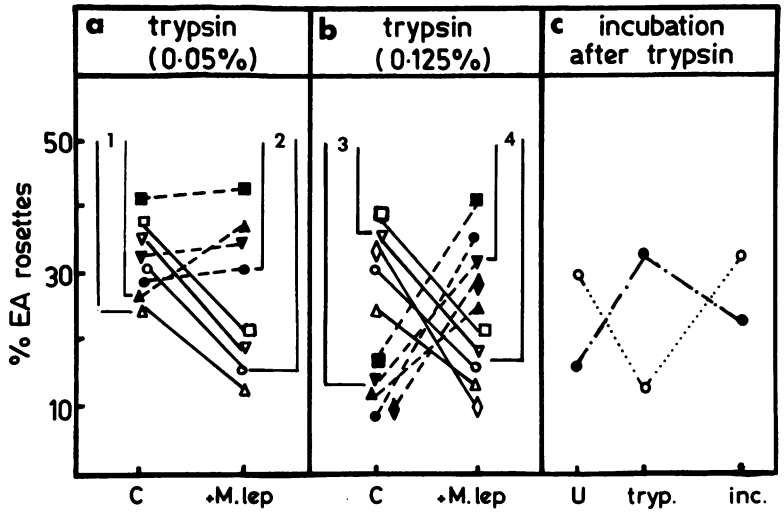

FIG. 6. Effect of trypsin on EA rosetting of macrophages from lepromatous (BI-ve) patients. (a) Treatment of control and *M. leprae*-infected cultures with trypsin (concentration, 0.05%). Closed symbols, EA rosetting of cultures treated with trypsin; open symbols, EA rosetting of cultures not treated with trypsin; C, uninfected macrophage culture; +M.lep, *M. leprae*-infected macrophage culture; 1, not significant; 2, $P < 0.005$; 3, $P < 0.005$; 4, $P < 0.005$. (b) Treatment of control and *M. leprae*-infected cultures with trypsin (concentration, 0.125%). Closed symbols: EA rosetting of cultures treated with trypsin; open symbols, EA rosetting of cultures not treated with trypsin. (c) Effect of prolonged incubation of infected cultures after removal of trypsin from culture medium. Symbols and abbreviations: ●, trypsin-treated infected cultures; ○, infected cultures not treated with trypsin; U, EA rosetting in untreated infected cultures; tryp., EA rosetting in trypsin-treated infected cultures; inc., EA rosetting on prolonged incubation (6 days) after removal of trypsin.

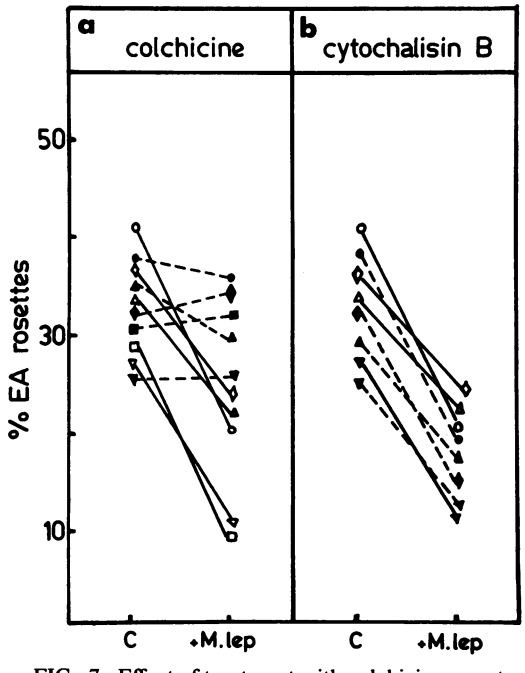

FIG. 7. Effect of treatment with colchicine or cytochalasin B on macrophages from lepromatous (BI-ve) patients. ■, ◆, ▲, ▼, EA rosetting in uninfected cultures treated with colchicine or cytochalisin B; □, ◇, △, ▽, EA rosetting in infected cultures not treated with colchicine or cytochalisin B; ○, untreated macrophage culture; ●, macrophage culture treated with colchicine or cytochalasin B; C, uninfected macrophage culture; +M.lep, *M. leprae*-infected macrophage culture.

lepromatous leprosy patients were tested for their ability to express Fc receptors in control cultures and in cultures infected with *M. leprae*. Macrophages from six contacts showed an ability to react to *M. leprae* similar to macrophages from lepromatous patients (unpublished data). The association between genetic markers and susceptibility to lepromatous leprosy has also been demonstrated by other workers (6, 9).

That an inhibitory factor (protein) does result from an lepromatous macrophage-*M. leprae* interaction is evident from Fig. 4 and 8. The results with cycloheximide show that the inhibitory factor is produced in the early stages of the interaction, since a lapse of 48 h between antigen addition and cycloheximide addition does not result in an increase in rosetting activity of cells harboring AFB. However, cells not harboring intracellular AFB do not show any depression. The results, therefore, implicate two steps that cause suppression. The first is a stable, early-interaction product produced intracellularly by the macrophage in the presence of *M. leprae*

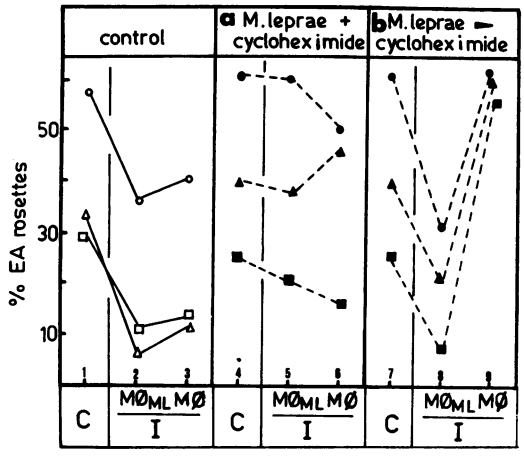

FIG. 8. Effect of cycloheximide on EA rosetting. Abbreviations and symbols: C, uninfected macrophage cultures; I, *M. leprae*-infected macrophage cultures; Mϕ, macrophages without intracellular *M. leprae*; $M\phi_{ML}$, macrophages with intracellular *M. leprae*; □, △, ○, untreated cultures. ■, ▲, ●, cultures treated with cycloheximide. Each symbol in all three panels denotes a single patient. 1 versus 4, Not significant; 5 versus 6, not significant; 8 versus 9, $P < 0.005$; 4 versus 5, not significant; 7 versus 8, $P < 0.005$.

(12). The second mediates amplification, since macrophages containing intracellular *M. leprae* secrete a soluble product which suppresses the rosetting ability of macrophages without intracellular bacilli. Additional evidence that such a factor exists is being published elsewhere.

Although the sites of action of colchicine and trypsin are different, temporary reversibility of the depression of EA rosetting remained the final outcome of treatment with these compounds. This lends support to the probability that the receptors are present and that their synthesis is not blocked by intracellular *M. leprae* but that the receptors are not available. Colchicine may unmask these receptors by depolymerizing the microtubules (8), which in turn affects the mobility of the receptors, whereas trypsin may digest some external proteins, which allows for the reexpression of the receptors.

Unless there is in vivo conditioning by a blocking antibody, it is difficult to imagine a blocking antibody playing an effector role since the long culture time would certainly result in antibody dissociation from the cell membrane. Also, the depression in macrophage EA rosetting is manifested only by the addition of *M. leprae* in vitro, exclusive of the presence of any specific antibody.

Although at present it is difficult to visualize the exact mechanism involved, we have preliminary indications that Fc receptor expression alone is not altered and that there are alterations in other membrane-bound moieties (concanavalin A receptors, HLA-D/DR antigens [common determinants]), indicating a more widespread membrane change.

A dose-induced immune response threshold operates in the lepromatous patient. Perhaps a genetic- or otherwise-controlled disposition may not allow their macrophages to function normally if confronted with a dose of 10^4 *M. leprae* per culture. The same dose, however, would be highly immunogenic for a normal or tuberculoid individual, each group possessing its own threshold dose. This defect in lepromatous patients may be genetic, operating with environmental factors such as the route of infection (13).

So far, *M. leprae* has only been characterized by means of ambiguous markers, such as morphology and staining properties, and by cumbersome experimental procedures such as footpad growth curves. Monitoring of Fc receptors on lepromatous macrophage cell membranes can be used as an identification marker for pathogenic *M. leprae* or for identifying cultivated *M. leprae*, because of the unusual degree of specificity of this technique.

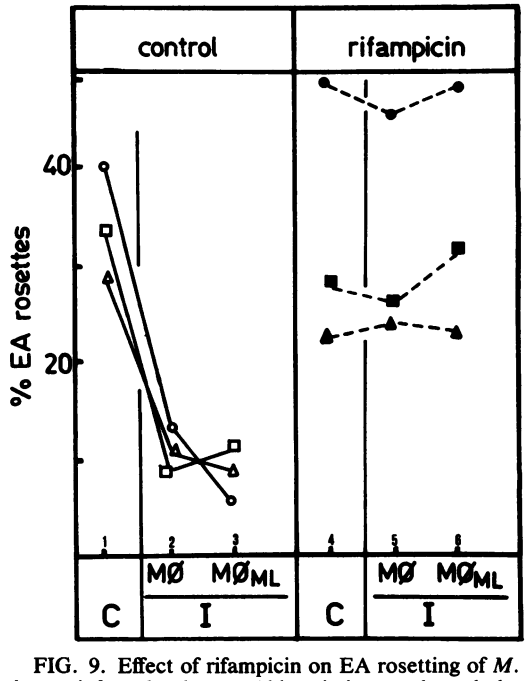

FIG. 9. Effect of rifampicin on EA rosetting of *M. leprae*-infected cultures. Abbreviations and symbols: C, uninfected macrophage cultures; I, *M. leprae*-infected macrophage cultures; Mϕ, macrophages without intracellular *M. leprae*; $M\phi_{ML}$, macrophages with intracellular *M. leprae*; □, △, ○, untreated cultures; ■, ▲, ●, cultures treated with rifampicin. Each symbol in both panels denotes a single patient. 1 versus 4, Not significant; 3 versus 6, $P < 0.005$.

In conclusion, it appears that *M. leprae*, upon entering lepromatous macrophages, initiates the production of a protein(s) which acts via the microtubules to alter membrane topography. This alteration is extremely trypsin sensitive. The infected macrophage also releases a factor into the environment which is responsible for the amplification of the defect in other macrophages. It is possible that the altered membrane prevents effective macrophage-lymphocyte interaction. This could be one of the mechanisms by which cell-mediated immunity is suppressed in lepromatous leprosy. Macrophages have also been implicated in the reduced cell-mediated immunity found in lepromatous leprosy.

The importance of monitoring macrophage receptors in other infectious disease remains hypothetical until additional data are gathered. However, it would be attractive to imagine sequestration of receptors as a distinctive character of a "suppressor macrophage" or as a measure of specific susceptibility, or both. More generally, it may serve to unify suppressor interactions in a group of related infectious diseases.

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