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Antigenic Protein from *Mycobacterium leprae* Released in Macrophages *in vitro* as Indicator of Viability of Bacteria[1]

Ishwari Nair and P. R. Mahadevan[2]

Macrophages are the cells that primarily harbor *Mycobacterium leprae,* the causative organism of leprosy. Macrophages isolated from the peripheral blood of humans and the peritoneal cavity of mice have been shown to phagocytose *M. leprae in vitro.* The ability of macrophages to kill *M. leprae* is generally believed to be poor in leprosy patients, and this is especially so in the cells from lepromatous leprosy patients ([9]). To understand further the host-pathogen interaction in leprosy, we have studied the changes introduced in the host cells, macrophages from humans and mice, by live *M. leprae* in relation to both internal metabolism and membrane structures ([1, 4–6, 8, 12]). It was clear from these observations that in both the leprosy patients and susceptible mice, such as the Swiss white strain, the macrophages were modified both in their structure and function by the presence of live *M. leprae.*

While *M. leprae* were phagocytosed by macrophages *in vitro,* the metabolic ability of *M. leprae* inside such macrophages has been demonstrated primarily by the uptake of precursor molecules, such as thymidine, uracil and hypoxanthine ([3, 11, 14, 15]). But actual multiplication inside the cultured macrophages has not been demonstrated.

In this report, we describe the presence of antigen(s) in *M. leprae*-infected, *in vitro* cultures of mouse macrophages by the affinity of these antigen(s) for antibodies present in lepromatous leprosy patients as demonstrated by an enzyme-linked immunosorbent assay (ELISA). We further show that these antigens are products of the metabolism of *M. leprae* and of the viable bacilli inside the cells.

MATERIALS AND METHODS

M. leprae. *M. leprae* were obtained from tissues such as the spleen and liver from an infected armadillo, and were kindly provided by Dr. E. Storrs, Melbourne, Florida, U.S.A. The tissues were collected under aseptic conditions and transported on dry ice to Bombay, India, within a period of 1 week. The tissues were stored at −90°C, and pieces were taken out as needed.

Such tissues from the *M. leprae*-infected armadillo were processed by washing the pieces in normal saline and chopping them in a sterile petri dish. They were homogenized with a hand homogenizer, and centrifuged at 276 × *g* × 10 min to remove tissue debris. The supernatant was further centrifuged at 3071 × *g* × 30 min, and the pellet suspended in saline. A smear was prepared, stained for acid-fast bacilli (AFB), and quantitated. The bacilli were identified as acid-fast stainable by the Ziehl-Neelsen method, and were free from contaminating fast-growing microbes.

Macrophage cultures. Following the injection of 5 ml of Eagle's minimal essential medium (MEM) + 20% heat-inactivated human serum into the peritoneal cavity of random-bred Swiss white mice, the peritoneal fluid was withdrawn and distributed in sterile 35-mm petri plates (Falcon). The cultures were incubated at 37°C in a 5% CO_2 atmosphere, and the medium of each culture was changed every 24 hr to remove nonadherent cells. After 3 days of culture, esterase-positive phagocytic macrophages were obtained with very few other cell types. *M. leprae* (30 × 10^6/petri dish) were added to these macrophage cultures, incubated at 37°C for 24 hr, and the supernatant removed to delete excess *M. leprae.* Control experiments showed that there were about

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[2] I. Nair, M.Sc., and P. R. Mahadevan, M.Sc., Ph.D., The Foundation for Medical Research, 84-A R. G. Thadani Marg, Worli, Bombay 400018, India.

Reprint requests to Dr. Mahadevan.

1×10^6 macrophages in each dish. By the addition of 30×10^6 *M. leprae,* we get a ratio of 30:1 *M. leprae* to host cell in the culture. In actual experiments, the ratio was 25:1 to 40:1; phagocytosis could vary the number of *M. leprae* per macrophage. The adherent cells that had phagocytosed *M. leprae,* after washing with MEM twice, were scraped off and lysed by ten cycles of freeze-thawing. The resulting suspension was centrifuged at $21,000 \times g \times 30$ min, and the supernatant was filtered through a 0.22 μm Millipore membrane to obtain particle-free, sterile cell lysate. This lysate was the source of the antigens used for testing with the sera from patients. The proteins in the lysate were quantitated by the method of Lowry, *et al.* ([7]).

ELISA. These lysates were used as the source of antigen(s) and coated on 96-well polystyrene plates (Laxboro, Pune, India). An ELISA using human sera for primary antibodies and peroxidase-conjugated antihuman immunoglobulin (IgG) (Miles Laboratories, Slough, U.K.) was carried out as described for *M. leprae* phenolic glycolipid ([2, 16]). The binding of antibodies in the sera to the antigens in the lysate of the macrophages was indicated by the color development during the ELISA, determined as optical density (OD) (492 nm) in the Multiscan ELISA reader (Flow Laboratories, U.K.). The OD intensity indicated the concentration of antibody in the sera to the antigen(s) under test.

Variations in the experiments were those using macrophage lysate prepared after incubating either heat-killed *M. leprae,* irradiated *M. leprae,* or live *M. leprae* in the presence of 5 μg/ml of rifampin (Sigma Chemical Co., U.K.) for 72 hr or with 10 μg/ml of cycloheximide (Sigma, U.K.) for 24 hr. The cells were also exposed to a number of cultivable mycobacteria (30×10^6), and lysates were prepared as described earlier to compare their behavior with sera from leprosy patients.

The sera were prepared from 5 ml of blood obtained from bacillary-positive, untreated or short-term (6 months) treated, lepromatous leprosy patients (B+LL); bacillary-negative, long-term (> 4 years) treated, lepromatous leprosy patients (B−LL); and paucibacillary patients (T). The blood (5 ml) was donated voluntarily by patients while attending the outpatient clinics in Bombay. Blood was also collected from normal healthy individuals (N) who were exposed in varying degrees to *M. leprae* in the city of Bombay. These sera were kept frozen at −20°C until used.

For determining the optimum incubation time, macrophage cultures were infected with *M. leprae* and the cultures were terminated at various time intervals from 24–144 hr. The lysates obtained were tested for their ability to bind to serum antibodies using the ELISA.

Kaolin (Sigma, U.K.) agglutination, based on a method described by Takahashi ([13]), was used to test for competition in the binding ability of serum to antigens induced especially in the presence of *M. tuberculosis.* Acid-washed kaolin powder was used: one part standard kaolin and two parts of the lysate antigen (10 μg) were agitated together and incubated for 30 min at 37°C. Serial dilutions of serum (B+LL) were made at 1:100 and 1:500 such that the total volume was 500 μl. To this was added sensitized kaolin, and the tubes were incubated at 37°C for 30 min, and then centrifuged at $1500 \times g \times 10$ min. The supernatant (the adsorbed serum) was then tested by ELISA against another fresh sample of lysate antigen. The lysate prepared by incubating macrophages with both *M. tuberculosis* and *M. leprae* was coated on kaolin and was used to adsorb B+LL sera. The adsorbed sera was then tested against fresh lysate antigen made either with *M. leprae* or *M. tuberculosis,* and the effect of such modification on the OD level was recorded.

Monoclonal antibodies were obtained from the WHO IMMLEP group and also from the London laboratory of Dr. J. Ivanyi.

Statistical significance was calculated using Student's *t* test. Average values are expressed along with standard deviations (S.D.).

RESULTS

The binding ability of sera from various types of individuals to the lysate antigen produced in the presence of *M. leprae* is depicted as the OD recorded after ELISA in Figure 1A. It is clear that only those sera from bacillary-positive lepromatous leprosy patients (B+LL) show high binding abil-

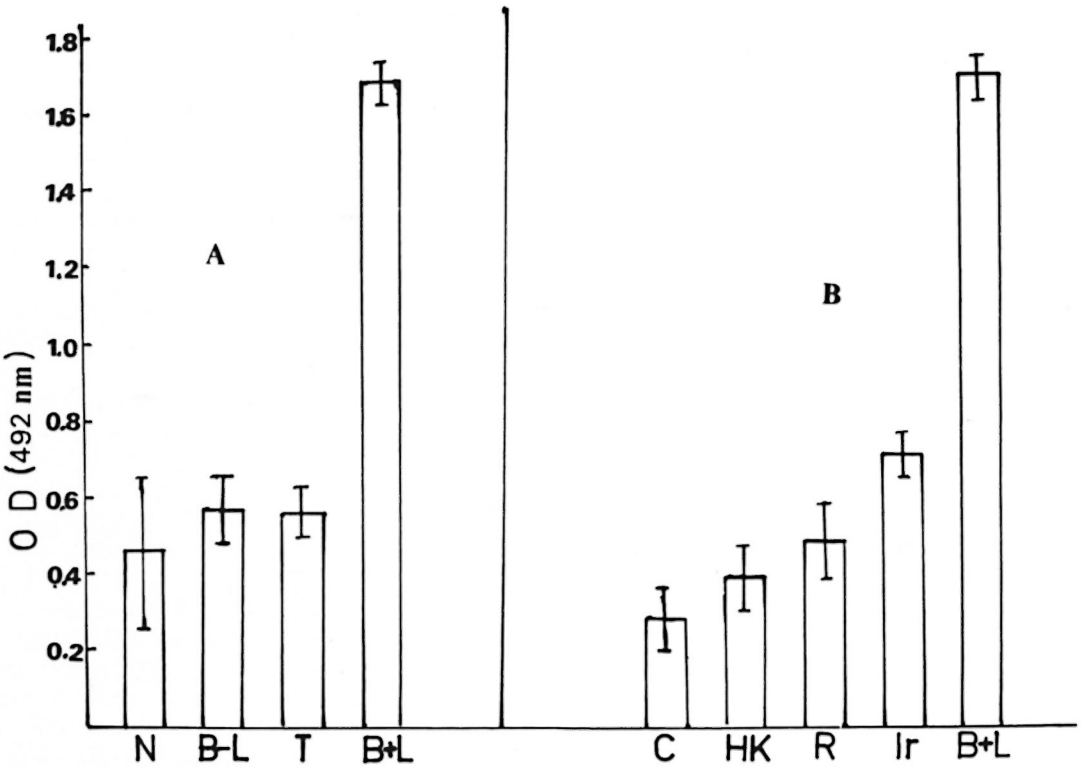

FIG. 1A. ELISA positivity between antigen(s) in the lysate and sera from various categories of individuals: N = sera from 20 normal individuals; B−L = 10 bacteriologically negative lepromatous leprosy patients; T = 10 tuberculoid leprosy patients; B+L = 9 bacteriologically positive lepromatous leprosy patients. Each sample was done in triplicate. B+L bar indicates mean ± 1 S.D., others indicate mean ± 3 S.D. to reveal significant differences between them and B+L. Antigen (protein) concentration was 5 μg; serum dilution, 1:500.

FIG. 1B. ELISA positivity between antigen prepared with various modifications and tested with sera from bacteriologically positive lepromatous leprosy patients. C = macrophage lysate (no addition); HK = with heat-killed *M. leprae* added; R = in presence of *M. leprae* and rifampin; Ir = with irradiated *M. leprae*; B+L = with live *M. leprae* and serum from bacteriologically positive lepromatous leprosy patient. Values are mean ± S.D. Antigen (protein) concentration was 5 μg; serum dilution, 1:500.

ity to the antigen(s). The average OD reading with B+LL sera was quite high compared to that obtained from the other types of sera ($p < 0.0005$). It is also clear that the presence of *M. leprae* and bacteriological positivity was necessary in the patients for the maintenance of the antibody in the serum, since long-term-treated, bacillary-negative, lepromatous patients (B−LL) showed essentially no antibodies in their sera (OD < 0.6).

The variability of the antigen level induced by *M. leprae* inside the cells could be due to the variable percentage of viable bacteria in the samples of *M. leprae* used (28%–42%) as determined by the fluorescein diacetate (FDA) staining method. On the average, 6–10 × 10^6 *M. leprae* were phagocytosed by 10^6 macrophages in all of the experiments. The bacilli were counted after being lysed out from the macrophages after the determination of viability. By using lysate prepared after infecting the peritoneal macrophages with *M. leprae* in separate experiments, one could get large numbers of separate antigenic samples. The data presented in Figure 1B show the binding ability of various antigenic samples obtained using one or more serum samples from B+LL patients.

Neither heat-killed *M. leprae* nor irradiated *M. leprae* were able to induce any antigen(s) inside the macrophages (Fig. 1B). If 5 μg/ml of rifampin was added to the macrophage cultures 72 hr earlier and was present at the time of *M. leprae* entry and during

TABLE 1. *Effect of antigen and antibody levels on ELISA positivity.*

Antibody (serum) dilution (antigen 5 μg/well) (as protein)	OD 492 nm[a] with serum from	
	Normal controls	B+LL patients
1:50	1.3	2.0
1:100	1.1	2.0
1:250	1.0	1.8
1:500	0.78	1.5
1:1000	0.70	1.2
1:2000	0.60	1.3
1:5000	0.34	0.7
Antigen added (μg/well) (as protein)	(1:500 dilution of serum)	
0.5	0.21	0.54
1.0	0.39	0.83
2.0	0.54	1.10
4.0	0.63	1.3
5.0	0.54	1.8
7.0	—	2.0
10.0	0.79	—

[a] Means of five separate experiments.

incubation, no antigen(s) were produced in the macrophage lysate.

Table 1 shows that the ELISA positivity (OD) was proportional to the antibody concentration and also proportionate to the relative concentration of the lysate. The concentration of the lysate as protein was optimum at 1 μg/well, and the antibody at a serum dilution of 1:500 gave good discrimination between the positive sera (B+LL) and negative sera (N). Protease treatment resulted in a loss of antigenicity (data not shown).

Figure 2 shows the time course of the appearance of the antigen(s) inside the macrophages after the entry of *M. leprae.* The rate was slow at the beginning but increased rapidly between 20–24 hr of incubation, was maximum at 24 hr, remained steady until 48 hr, and decreased by 72 hr. There was even less demonstrable antigen by 144 hr of incubation (Fig. 2).

The macrophage lysate was tested for binding to the monoclonal antibodies known to be specific for other *M. leprae* antigens. None of them gave a positive binding to the lysate antigen obtained in these experiments, even at a 1:100 dilution of the monoclonal antibodies (Table 2). No competition assay was carried out. Hence, the macrophage lysate could contain a different protein antigen from those previously described and could be being recorded for the first time now using the sera from leprosy patients.

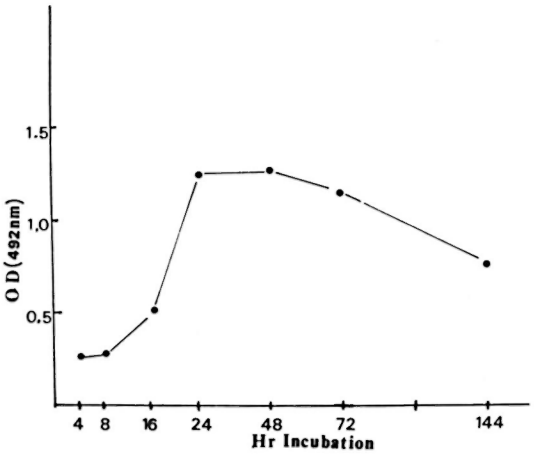

FIG. 2. Antigen concentration in the ELISA lysate as expressed by optical density (OD) in relation to time of incubation of live *M. leprae* inside macrophages.

Preliminary experiments showed that rabbit polyclonal antisera against the macrophage lysate could recognize one faint band of *M. leprae* by Western blot (data not shown), but more detailed studies are needed. Lysate produced by using macrophage cultures exposed to other mycobacteria showed some ELISA positivity with sera from both lepromatous leprosy patients and normal healthy individuals. These lysates could not discriminate between normal sera and B+LL sera. More specifically, these ly-

TABLE 2. *ELISA positivity of the lysate against known specific monoclonal antibodies to* M. leprae.[a]

Monoclonals against proteins	Dilution	
	1:100	1:1000
kD-18[b]	0.081	0.095
kD-36[b]	0.117	0.084
kD-65[b]	0.087	0.089
kD-200[b]	0.088	0.081
Phenolic glycolipid-I[b]	0.104	0.117
MLO_4[c]	0.087	0.092
MLO_6[c]	0.104	0.156

[a] Values are mean of three readings from five separate lysates at two dilutions of the monoclonals. Lysate was used at a level of 1 μg/ml.

[b] Gifts from the WHO IMMLEP group.

[c] Supplied by Dr. J. Ivanyi, against kD-12 and kD-36.

TABLE 3. *ELISA values (OD 492 nm) of bacteriologically positive lepromatous leprosy patients' (B+LL) serum and normal serum to lysate from macrophages infected with other mycobacteria.*[a]

Sera	*M. avium*	*M. intracellulare*	*M. scrofulacem*	*M. smegmatis*	*M. vaccae*	*M. kansasii*	*M. tuberculosis*	*M. leprae*
Normal	0.44	0.47	0.45	0.54	0.87	0.85	1.26	0.35 ± 0.09
B+LL	0.45	0.42	0.49	0.44	0.67	0.78	1.30	1.79 ± 0.10[b]

[a] Values are mean of five readings from two separate lysate preparations using 1 μg of the antigenic preparation.
[b] $p = < 0.001$, Student's *t* test. Significantly higher than *M. leprae*-infected macrophage lysates reacting with normal serum.

sates showed no higher affinity for B+LL sera than for normal sera, while this was readily demonstrable with the *M. leprae*-induced lysate (Table 3). Lysates obtained by incubating macrophages with *M. tuberculosis* gave a positive reaction with sera from B+LL patients, with an equal binding ability to antibodies in normal serum.

B+LL sera adsorbed with kaolin particles coated with lysate proteins produced by *M. tuberculosis*-infected macrophages no longer bound to lysates from *M. tuberculosis*-infected macrophages but still bound to lysates from *M. leprae*-infected macrophages (Table 4). Further, if adsorption of the serum antibody was done with kaolin particles coated with *M. leprae*-induced lysate antigens, the adsorbed serum showed poor affinity (low OD) with *M. leprae*-induced lysate as compared to *M. tuberculosis*-induced lysate (Table 5).

Antigen production was tested in the presence of cycloheximide, a protein synthesis inhibitor of eukaryotic cells. Cycloheximide did not inhibit the production of the lysate antigen, suggesting that it is produced by *M. leprae* in the macrophage (Table 6).

TABLE 4. *B+LL sera after removal of proteins produced by* M. tuberculosis *inside macrophages by kaolin adsorption and tested with lysates produced by both* M. leprae- *and* M. tuberculosis-*infected macrophages (4 separate experiments).*

OD reading (ELISA)	
Lysate from macrophage + *M. leprae*	Lysate from macrophage + *M. tuberculosis*
2.314	0.494
2.104	0.315
1.816	0.424
1.921	0.340

DISCUSSION

The observations show that *M. leprae*-infected macrophages produce antigen(s) that have an affinity for immunoglobulins present in the sera of bacteriologically positive lepromatous leprosy patients. Such antigens are not produced in the presence of dead *M. leprae* nor in the presence of an antileprosy drug such as rifampin. No other sera tested have immunoglobulins that have significant binding ability for these antigen(s).

The lysate produced from mouse macrophages on incubation with live *M. leprae* binds with the sera from bacteriologically positive lepromatous leprosy patients much better (OD 1.7) than it binds with sera from normal healthy individuals (OD 0.45). The antibodies recognized by the *M. leprae*-infected macrophage lysate antigens appear to be primarily of the IgG and not IgM type (data not shown). Long-term-treated lepromatous patients showed a low level of the antibody to the lysate antigens (OD < 0.6), and paucibacillary patients and normal healthy individuals also had very little of the antibody (OD ~ 0.5).

The lysates produced from mouse macrophages on incubation with *M. avium, M. intracellulare, M. scrofulaceum, M. smeg-*

TABLE 5. *B+LL sera after adsorption with kaolin particles coated with* M. leprae-*induced macrophage lysate and tested with lysates produced by both* M. leprae- *and* M. tuberculosis-*infected macrophages (3 separate experiments).*

Lysate from macrophage + *M. leprae*	Lysate from macrophage + *M. tuberculosis*
0.521	>2.000
0.411	1.543
0.132	1.213

TABLE 6. *Lack of antigen production in presence of cycloheximide (Cy) and rifampin (Rf).*

Exper. no.	Test serum	OD (492 nm) ELISA			
		Mϕ [a] only	Mϕ + *M. leprae* + Rf (5 μg/ml)	Mϕ + *M. leprae*	Mϕ + Cy + *M. leprae* (10 μg/ml)
1	Normal	0.178	0.438	0.612	0.446
	B+LL	0.251	0.378	2.312	1.729
2	Normal	0.087	0.190	0.322	0.464
	B+LL	0.335	0.218	1.624	1.829
3	Normal	0.134	0.375	0.304	0.337
	B+LL	0.316	0.353	1.753	1.734
4	Normal	0.180	0.240	0.239	0.372
	B+LL	0.280	0.312	1.934	1.856

[a] Mϕ = macrophage.

matis, M. vaccae, M. kansasii, and *M. tuberculosis* do not preferentially bind with B+LL sera as compared to normal sera. With most of these lysates, there was slightly less binding to B+LL sera than to normal sera, indicating very little overlapping antigenic specificity in the antigens in these lysates compared to the antigens released in the presence of *M. leprae.* In spite of the higher binding ability of lysate with *M. tuberculosis* to B+LL sera, it could not discriminate normal sera from B+LL sera. The higher binding ability to both types of sera may be due to some other antigens not related to those of *M. leprae.* The positive reaction seen with *M. tuberculosis* lysate and B+LL sera may also be due to a different protein, as seen by the kaolin agglutination test. In this test, even after removal of antibodies with affinity to the antigens produced by *M. tuberculosis* by adsorption, the sera samples showed considerable affinity to the *M. leprae*-induced lysate but not to the *M. tuberculosis*-induced lysate.

The presence of live *M. leprae* leads to the introduction of some *M. leprae* products inside the host cells. These products could be identified using antibodies from the sera of leprosy patients. This enabled us to show the appearance of these antigen(s) of *M. leprae* inside a host cell as early as 24 hours after the start of incubation. This activity required live *M. leprae,* as indicated by our observations.

On treating the *M. leprae*-infected macrophages with cycloheximide, the lysate prepared still showed ELISA positivity. This suggested that the protein antigen is either produced by *M. leprae* or that it is a small peptide produced by the host macrophage which is not inhibited by cycloheximide. Since freezing and thawing could release pre-existing *M. leprae* antigens, the experiment was performed with heat-killed and irradiated *M. leprae* incubated with macrophages. Under these conditions, the macrophage lysates showed no antigenic affinity to the B+LL sera. This indicated that pre-existing antigens were not simply being released by the process of freeze-thawing. The *de novo* synthesis of the antigen(s) by *M. leprae* was also indicated by the fact that rifampin could block the production of the antigen(s). This is the first time that an *M. leprae*-related antigen, which seems to be a metabolic product of the bacillus, has been analyzed without extracting it from the bacteria. This opens up a variety of experimental possibilities, since the appearance of the antigen(s) may be related to the viability of *M. leprae* inside the host cells.

In another closely related paper ([10]), we have shown that the antigen production was associated with viable *M. leprae* in the macrophages of Swiss white mice. On the other hand, in cultures of *M. leprae*-infected macrophages from C57BL mice, the viability of the *M. leprae* was reduced, and there was no production of the antigen. The viability of *M. leprae* in *M. leprae*-infected macrophage cultures appears to be genetically controlled in mice. Lysates from cultured human-blood macrophages infected with live *M. leprae* have been studied. If the macrophages were derived from the blood of long-term-treated LL patients, the lysates

contain an antigen reacting with the B+LL serum. This is being studied further.

SUMMARY

Peritoneal macrophages from random-bred, Swiss white mice, when cultured and infected with *Mycobacterium leprae* for 24 hours, are able to show the presence of antigen(s) with binding affinity to antibodies present in the sera of bacteriologically positive, lepromatous leprosy patients. Such antibodies are not seen in sera from normal and healthy persons, tuberculoid leprosy patients, or long-term-treated, bacteriologically negative, lepromatous leprosy patients. The production of the antigen(s) is blocked by the anti-*M. leprae* drug rifampin. Other mycobacteria when incubated with macrophages from mice show very little antigens in the lysate but the antigens have an equal affinity for antibodies in sera from both normal individuals and lepromatous patients. Only the lysates from macrophages exposed to live *M. leprae* could discriminate and could exhibit differential binding to sera from leprosy patients compared to sera from normal individuals. This antigen(s) does not have any binding ability to the monoclonal antibodies available to the antigens of *M. leprae* identified at present and shown to be specific to *M. leprae.* This indicates a separate identity of this product which has potential for further exploitation in exploring host-pathogen interactions related specifically to the leprosy infection and the tolerance of *M. leprae* inside cells.

RESUMEN

Los macrófagos peritoneales de ratones Swiss-white cultivados e infectados con *Mycobacterium leprae* por 24 horas, son capaces de mostrar la presencia de antígenos que reaccionan con anticuerpos presentes en los sueros de pacientes con lepra lepromatosa bacteriológicamente positivos. Tales anticuerpos no se encuentran en los sueros de personas sanas, de pacientes con lepra tuberculoide, o de pacientes lepromatosos con mucho tiempo de tratamiento y bacteriológicamente negativos. La producción de los antígenos se bloquea por la droga antileprosa rifampina. Cuando se incuban otras micobacterias con macrófagos de ratón, se encuentran muy pocos antígenos en el lisado celular que tienen una afinidad igual por anticuerpos presentes tanto en los sueros de individuos normales como en los sueros de pacientes lepromatosos. Sólo los lisados de los macrófagos expuestos al *M. leprae* vivo pudieron discriminar, por su enlazamiento diferencial, a los sueros de los pacientes lepromatosos de aquellos de los individuos normales. Estos antígenos no tienen reactividad con los anticuerpos monoclonales existentes a la fecha que identifican antígenos del *M. leprae.* Esto indica una diferente identidad de estos productos y sugiere su utilidad potencial en el estudio de las interacciones huésped-parásito relacionadas específicamente con la infección y la tolerancia del *M. leprae* en el interior de las células.

RÉSUMÉ

Les macrophages péritonéaux de souris blanches Swiss d'élevage, mis en culture et infectés par *Mycobacterium leprae* pendant 24 heures, peuvent montrer la présence d'antigènes ayant une affinité pour des anticorps présents dans le sérum de lépreux lépromateux ayant une bactériologie positive. De tels anticorps ne sont pas observés dans le sérum de personnes normales en bonne santé, les patients lépreux tuberculoïdes, ou les lépreux lépromateux traités depuis longtemps et bactériologiquement négatifs. La production d'antigène(s) est bloquée par la rifampicine. Les autres mycobactéries, incubées avec des macrophages de souris, montrent très peu d'antigènes dans le lysat, mais les antigènes ont une affinité identique pour les anticorps sériques provenant d'individus normaux et de lépreux lépromateux. Seuls les lysats de macrophages exposés à des *M. leprae* vivants pouvait faire la distinction et présenter une affinité différente pour les sérums de patients lépreux et ceux d'individus normaux. Cet antigène(s) n'a aucune capacité de se lier aux anticorps monoclonaux disponibles vis-à-vis des antigènes de *M. leprae* identifiés à présent, et dont la spécificité pour *M. leprae* a été démontrée. Ceci indique que ce produit a une identité propre, et qu'il mérite d'être utilisé plus avant pour explorer les interactions hôte-pathogène liées spécifiquement à l'infection lépreuse, et la tolérance de *M. leprae* à l'intérieur des cellules.

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