

M. leprae- and BCG-induced Chemiluminescence Response of Monocytes from Leprosy Patients and Healthy Subjects: Effects of Gamma-Interferon and GM-CSF¹

Pascal Launois, Laurent Blum, Alioune Dieye,
Jacques Millan, Jean Louis Sarthou, Jean Claude Michel, and
Marie-Anne Bach²

Most *Mycobacterium leprae*-infected subjects develop efficient cell-mediated immunity that results either in subclinical self-resolving infection or in overt leprosy of the tuberculoid (T) type, with a limited number of granulomatous inflammatory lesions and a reduced bacterial load. Some infected subjects fail to mount an efficient *M. leprae*-driven T-cell response; their infection progresses toward lepromatous (L) leprosy with disseminated lesions and a heavy bacterial load (¹⁶). The final outcome of *in vivo* infections by mycobacteria depends both on T-cell ability to develop acquired specific immunity and on infected macrophages to respond to T-cell-mediated activation signals. It is not known yet whether the primary defect of these subjects is at the T-cell level or in the macrophage population or both.

Phagocytes contain a variety of antimicrobial systems that use toxic oxygen metabolites formed by phagocytosis-induced respiratory bursts (^{11, 13}). Previous studies indicated that *M. leprae*, in contrast to BCG, was a poor stimulant of this release of toxic oxygen metabolites by normal human monocytes, even in the presence of interferon-gamma (IFN- γ) (^{7, 10}). In the present

study, we have measured the chemiluminescence (CL) associated with the oxydative phagocyte respiratory burst and the phagocytic activity of human monocytes from either healthy subjects or leprosy patients after stimulation by *M. leprae* or BCG, alone or with IFN- γ or granulocyte monocyte colony stimulating factor (GM-CSF), both lymphokines known for their macrophage-stimulating properties (^{13, 15, 21}).

MATERIALS AND METHODS

Cell isolation. Peripheral blood was obtained by venipuncture using heparinized "Vacutainer" tubes (Becton-Dickinson, Rutherford, New Jersey, U.S.A.) from 21 untreated leprosy patients attending the Institut de Léprologie Appliquée de Dakar, Senegal. These patients were histologically classified as lepromatous (L) or tuberculoid (T) according to the Ridley-Jopling scale (¹⁵). Eighteen healthy subjects served as controls.

Peripheral blood mononuclear cells (PBMC) were isolated over a Ficoll-Hypaque gradient and were suspended in Hanks' solution without phenol red. The percentage of monocytes in the preparation was evaluated on May-Grünwald-Giemsa smears.

Mycobacteria. *M. leprae*, extracted from armadillo tissues, irradiated, and lyophilized, was provided by the World Health Organization (WHO) (batch CD 56 or CD 128). Viable *M. bovis* BCG IP Dakar was obtained in lyophilized samples from the "Service du BCG" (Pasteur Institut, Dakar) and was inactivated by heating for 120 min at 70°C.

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² P. Launois, M.D.; A. Dieye, Biological Pharmacist; J. L. Sarthou, Biological Pharmacist, Immunologie Cellulaire; J. C. Michel, D.Sc., Immunologie Parasitaire, Institut Pasteur de Dakar, B. P. 220, Dakar, Senegal. L. Blum, M.D.; J. Millan, M.D., Director, Institut de Léprologie Appliquée de Dakar, Dakar, Senegal. M.-A. Bach, M.D., Ph.D., Unité de Pathologie de l'Immunité, Faculté Necker Enfants Malades, Paris, France.

Reagents. Luminol (5-amino-2, 3-dihydro-1-4-phthalazinedione) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was dissolved in dimethylsulfoxide (DMSO; Sigma) and used at a final concentration of 10^{-4} M in phosphate buffer. Fluoresceine isothiocyanate (FITC) was purchased from Sigma. Recombinant IFN- γ (r-IFN- γ) was obtained from Amersham (Buckinghamshire, England) and recombinant GM-CSF (r-GM-CSF) from Genzyme (Maidstone, England).

Measurement of CL. We used an automatic photometer and dispenser (1251 Luminometer and 1291 Dispenser; LKB Wallac, Turku, Finland) for measuring chemiluminescence (CL). Briefly, $100 \mu\text{l}$ of a suspension of mononuclear cells ($10^5/\text{ml}$) was transferred to a counting tube previously filled with 1 ml of a suspension of bacteria diluted in phosphate buffer to a ratio of 50 per cell. The tube was placed in a dry tube heater at 37°C under constant agitation, and $200 \mu\text{l}$ of the luminol solution was subsequently added. The tube was then immediately introduced into a photometer-counting chamber ($\lambda = 425 \text{ nm}$). A measurement mode was available for continuous recording during 150 min. Results of reactive oxygen species production were expressed as light intensity in millivolts at the peak of the course per 10^5 mononuclear cells.

Mycobacterial phagocytosis assay. Heat-inactivated BCG and irradiated *M. leprae* were FITC-conjugated by suspending 1 mg of mycobacteria in 1 ml of carbonate-bicarbonate buffer (pH 9.5) with 0.05% Tween 80 and 0.03% FITC for 60 min at room temperature as previously described⁽⁸⁾. The bacteria were pelleted by centrifugation, resuspended in Hanks' solution without phenol red, and then sonicated for 30 sec to disrupt bacteria clumps. Aliquots of $100 \mu\text{g}$ of bacteria were frozen at -20°C until used. Phagocytosis was measured by incubating 10^5 mononuclear cells in 1 ml of Hanks' solution with bacteria at a ratio of 50 per cell. The percentages of monocytes was determined on May-Grünwald-Giemsa smears. After gentle agitation for 4 hr at 37°C , the tubes were centrifuged twice at $300 \times g$ and the supernatants were discarded. The pellets were resuspended in $200 \mu\text{l}$ of Hanks' solution and examined with a $100 \times$

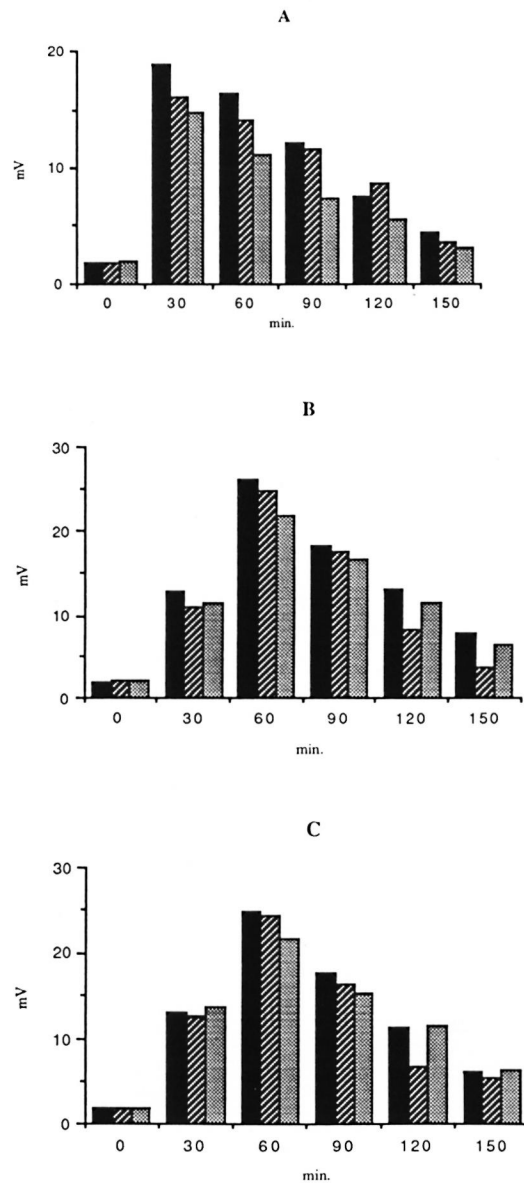


FIG. 1. Kinetics of the effects of r-IFN- γ and r-GM-CSF on the CL response to BCG (ratio 50/1) of mononuclear cells from healthy controls (■) and lepromatous (▨) and tuberculoid (□) leprosy patients. Oxygen-free radical production from not exposed (A), r-IFN- γ (B), and r-GM-CSF (C) stimulated cells was measured.

water immersion lens; 300 to 400 cells were counted and the results were expressed as percentages of monocytes with associated mycobacteria. In some experiments, mycobacterial phagocytosis assays were performed in the presence of a pool of lepromatous patients' sera decanted by heating at 56°C for 30 min.

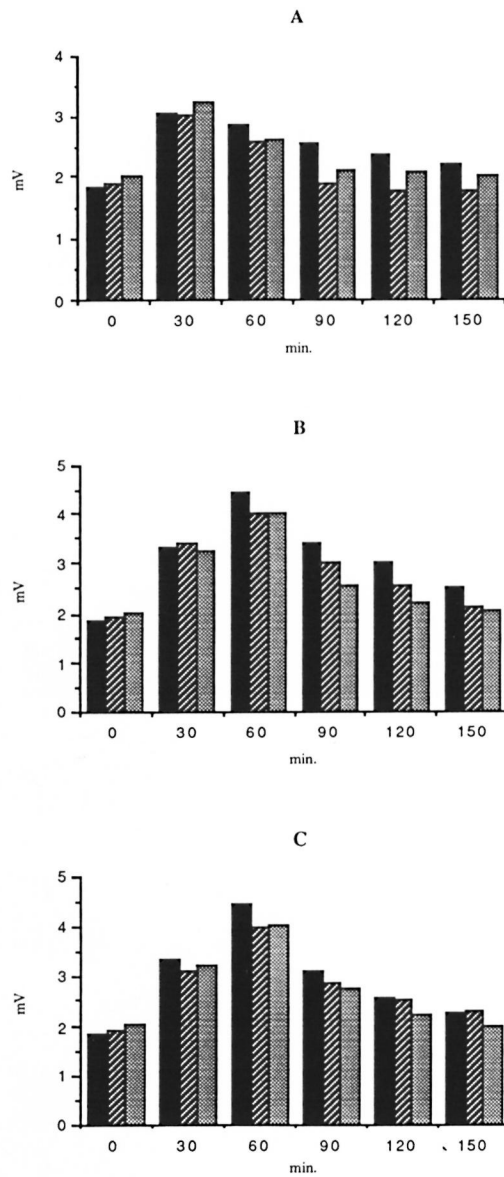


FIG. 2. Kinetics of the effects of r-IFN- γ and r-GM-CSF on the CL response of *M. leprae* (ratio 50/1) of mononuclear cells from all three groups tested. For details, see Figure 1 legend.

Statistics. The Student's *t* test was used to compare the different experimental groups.

RESULTS

Effects of r-IFN- γ and r-GM-CSF on CL response to BCG and *M. leprae*. Preliminary experiments were performed to define

the optimal conditions for GM-CSF to stimulate a maximal CL response to the mycobacteria. Dose-response curve analysis performed in the presence or absence of BCG and *M. leprae* with normal PBMC indicated that the maximal response to mycobacteria at a ratio of 50 per cell could be obtained with 5–10 IU/ml of GM-CSF. Preincubation of PBMC (2–12 hr) with GM-CSF prior to the addition of mycobacteria and CL assay did not improve the CL response (data not show). Thus, subsequent studies were performed with constant concentrations of 10 IU/ml of GM-CSF or of 500 IU/ml of IFN- γ (as determined from our earlier studies¹⁰), added with a ratio of 50 per cell of either mycobacterial suspension.

As previously described¹⁰, even at high concentrations neither viable nor heat-inactivated or irradiated *M. leprae* triggered any CL increase above that recorded for unstimulated cells from healthy subjects. Thus, we used irradiated *M. leprae* in all following experiments.

In all three groups of subjects, the kinetics of CL response of unstimulated cells was different from those observed for IFN- γ - and GM-CSF-stimulated cells. Optimal release was obtained 30 min after exposure to BCG and 60 min after exposure to BCG plus either lymphokines tested. No effects of IFN- γ and GM-CSF on the kinetics of the CL response in the presence of *M. leprae* were shown in any group tested. Mononuclear cells from lepromatous and tuberculoid patients showed the same pattern of response (Figs. 1 and 2). Thus, the CL measurement mode was available for continuous recording during a period of 150 min.

PBMC CL responses to BCG and *M. leprae* were studied in the presence or absence of IFN- γ or GM-CSF in 18 healthy subjects, 11 T patients, and 10 L patients. In the absence of lymphokines, *M. leprae* failed to trigger any significant response in any group of subjects. Indeed, CL response to *M. leprae* was not significantly different from the controls without the bacillus. Conversely, BCG elicited a strong response from all three groups. Both IFN- γ and GM-CSF increased the CL response of PBMC from all subject groups in the absence of mycobacteria. When *M. leprae* were added, no further increase was noted. Conversely, the CL re-

TABLE 1. Chemiluminescence response to lymphokines and mycobacteria of PBMC from healthy subjects and leprosy patients.

Bacteria	Lymphokines	Subjects		
		Healthy controls (N = 18)	T leprosy patients (N = 11)	L leprosy patients (N = 10)
None	None	2.844 ± 0.773 ^a	2.019 ± 0.633 ^b	2.230 ± 0.646
<i>M. leprae</i>	None	3.553 ± 0.756	3.057 ± 0.784	3.235 ± 0.854
BCG	None	18.964 ± 3.279 ^c	<u>16.023 ± 1.769^c</u>	<u>14.697 ± 1.267^c</u>
None	IFN- γ	4.453 ± 0.792 ^d	4.051 ± 0.748 ^d	4.172 ± 0.800 ^d
<i>M. leprae</i>	IFN- γ	4.936 ± 0.851	<u>3.671 ± 0.920</u>	<u>4.130 ± 0.445</u>
BCG	IFN- γ	26.260 ± 4.576 ^c	24.748 ± 2.24 ^c	<u>21.860 ± 1.133^c</u>
None	GM-CSF	4.984 ± 1.162 ^d	4.012 ± 0.619 ^d	4.029 ± 0.702 ^d
<i>M. leprae</i>	GM-CSF	4.980 ± 0.965	<u>3.931 ± 1.058</u>	<u>4.225 ± 0.488</u>
BCG	GM-CSF	24.804 ± 3.699 ^c	24.357 ± 2.472 ^c	<u>21.825 ± 3.541^c</u>

^a mV/10⁵ mononuclear cells (mean ± S.D.).

^b Values significantly different ($p < 0.01$ or $p < 0.05$; unpaired t test) from the corresponding values of healthy controls are underlined.

^c Significantly different from control without bacillus ($p < 0.01$; paired t test).

^d Significantly different from control without lymphokines ($p < 0.01$ or $p < 0.05$; paired t test).

sponse to the costimulation by BCG plus either lymphokine was higher than the sum of these responses obtained with each stimulus alone, suggesting that IFN- γ and GM-CSF exerted more than an additive effect upon the BCG-triggered CL production by human PBMC. No difference could be seen between the CL response triggered by IFN- γ and GM-CSF, neither in the absence nor in the presence of mycobacteria (Table 1). Interestingly, CL responses induced with BCG or without mycobacteria were found slightly but significantly higher in healthy subjects than in either group of leprosy patients (especially L patients) whatever the stimulus. Moreover, no differences were seen in *M. leprae*-induced CL responses in all three groups of subjects.

Effects of anti-*M. leprae* antibodies on *M. leprae*-triggered CL response of mononuclear cells. The influence of anti-*M. leprae* antibodies was evaluated using a pool of lepromatous sera containing high titers (1/2000–1/4000) of anti-*M. leprae*, anti-BCG, and anti-phenolic glycolipid-I (PGL-I) antibodies, as measured in an enzyme-linked immunosorbent assay as previously described (¹) (data not shown). BCG or *M. leprae* at a ratio of 50 per cell were preincubated with lepromatous sera at an optimal dilution of 1/10. Two hours later, mononuclear cells were challenged with this mixture. Table 2 shows that pretreatment of either BCG or *M. leprae* with sera did not enhance the CL responses from mononuclear cells in all three groups.

TABLE 2. Lack of effect of mycobacteria pretreatment by lepromatous patient sera upon CL stimulation.

Bacteria	L patient's serum ^a	Subjects		
		Healthy controls (N = 5)	T leprosy patients (N = 5)	L leprosy patients (N = 5)
None	–	3.057 ± 1.227 ^b	2.412 ± 0.765	2.741 ± 1.123
BCG	–	19.412 ± 3.692	15.525 ± 2.041	15.788 ± 1.857
BCG	+	21.218 ± 4.614	16.218 ± 1.94	16.418 ± 1.612
<i>M. leprae</i>	–	3.658 ± 0.912	3.123 ± 2.018	3.447 ± 1.321
<i>M. leprae</i>	+	3.515 ± 1.013	3.247 ± 1.694	3.515 ± 1.272

^a Serum used at 1/10 dilution: titers (1/2000–1/4000) of anti-*M. leprae*, anti-BCG and anti-PGL-I antibodies. For other details see Table 1.

^b mV/10⁵ mononuclear cells (mean ± S.D.).

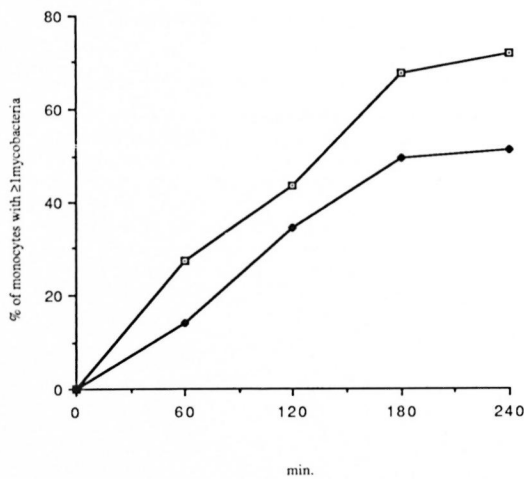


FIG. 3. Time courses of BCG (□) and *M. leprae* (◆) phagocytosis in healthy subjects. Results are expressed as percentile of monocytes with ≥ 1 associated mycobacteria.

Effects of r-IFN- γ and r-GM-CSF on mycobacteria phagocytosis. The failure of *M. leprae* to trigger an efficient CL response of PBMC could result from defective phagocytosis of the bacillus. The number of monocytes in the PBMC from all three groups was identical (28.4 ± 3.7 , 30.4 ± 2.3 , and 27.5 ± 2.6 in healthy controls, L patients, and T patients, respectively). Preliminary results with healthy controls showed that the time course of BCG and *M. leprae* phagocytosis was maximal between 3 and 4 hours. In Figure 3, a representative experiment is shown. Assuming that only monocytes in the preparation of PBMC contain organisms, the number of

FITC-conjugated *M. leprae* associated with monocytes in our phagocytosis assay was indeed significantly less than that of FITC-conjugated BCG in all three groups of patients. Phagocytosis was not altered by the addition of either lymphokine, but there was a significant difference in phagocytosis between healthy controls and tuberculoid subjects (Table 3). In some experiments, percentiles of cells with 1 to 2 or more mycobacteria associated per cell were determined. No statistical difference in the number of bacteria per cell were demonstrated in the three groups of patients (data not shown). Pretreatment of PBMC with the pool of lepromatous sera described above did not increase the number of FITC-conjugated mycobacteria phagocytized (Table 4).

DISCUSSION

The failure of *M. leprae*, as observed in this study and others (^{7,10}), to stimulate the phagocyte oxidative burst in human monocytes may be relevant to its pathogenic role in humans. The mechanism of such a failure of *M. leprae* to trigger an efficient oxydative burst remains unclear. It has been attributed to PGL-I, a unique component of the *M. leprae* cell wall, which acts as an oxygen species scavenger (^{3,14}). However, Vachula, *et al.* (¹⁹) have shown that PGL-I specifically suppressed the generation of superoxide anions by mononuclear cells stimulated by *M. leprae*, but not that triggered by other mycobacteria or by PMA. Thus, an additional specific mechanism for suppressing the metabolic response to *M. leprae* does exist.

TABLE 3. Phagocytosis of mycobacteria by monocytes from healthy subjects and leprosy patients.

Bacteria	Lymphokine	Subjects		
		Healthy controls (N = 13)	T leprosy patients (N = 6)	L leprosy patients (N = 8)
<i>M. leprae</i>	None	59.1 \pm 5.9 ^a	65.9 \pm 7.8 ^d	62.0 \pm 5.8
BCG	None	75.2 \pm 7.9 ^{b,c}	85.8 \pm 6.8 ^b	87.7 \pm 5.7 ^b
<i>M. leprae</i>	IFN- γ	63.5 \pm 7.1	69.0 \pm 7.3	63.2 \pm 6.5
BCG	IFN- γ	76.1 \pm 9.9 ^{b,c}	92.3 \pm 9.6 ^b	84.4 \pm 6.6 ^b
<i>M. leprae</i>	GM-CSF	60.8 \pm 7.84	72.5 \pm 7.6	65.6 \pm 9.9
BCG	GM-CSF	74.5 \pm 11.2 ^{b,c}	91.2 \pm 7.3 ^b	86.2 \pm 9.5 ^b

^a Percent of monocytes associated with mycobacteria (mean \pm S.D.).

^b Significantly different from *M. leprae* ($p < 0.01$; paired *t* test).

^c Significantly different from T leprosy patients ($p < 0.05$; paired *t* test).

^d Significantly different from healthy controls ($p < 0.05$; paired *t* test).

TABLE 4. Lack of effect of mycobacteria pretreatment by lepromatous patient sera upon phagocytosis.^a

Bacteria	L patient sera	Subjects		
		Healthy controls (N = 5)	T leprosy patients (N = 5)	L leprosy patients (N = 5)
<i>M. leprae</i>	–	58.5 ± 6.4 ^b	68.3 ± 2.3	60.6 ± 2.7
<i>M. leprae</i>	+	60.3 ± 3.4	70.4 ± 2.5	61.3 ± 3.4
BCG	–	77.8 ± 4.7	83.1 ± 3.8	84.6 ± 2.9
BCG	+	79.3 ± 2.8	85.3 ± 2.2	83.1 ± 3.6

^a For details, see Table 2.

^b Percent of mycobacteria associated with monocytes (mean ± S.D.).

As shown by the present study, the poor phagocytosis of *M. leprae* by human phagocytes may also contribute to the weak CL phagocyte response. Similar results were reported about *M. lepraemurium*, another poor stimulator of phagocyte respiratory bursts^(2,10) which was less efficiently phagocytosed by murine macrophages than a good stimulator such as *M. microti*⁽²⁾. PGL-I is a part of the lipid capsule surrounding the mycobacteria that could prevent phagocytosis⁽⁵⁾. However, Neil and Klebanoff⁽¹⁴⁾ showed that PGL-I-coated staphylococci were normally ingested—but not killed—by human macrophages, and our previous study could not detect any antiphagocytic function of PGL-I in FITC-conjugated mycobacteria⁽⁹⁾. Moreover, treatment of *M. leprae* and BCG by anti-mycobacterial antibodies, containing anti-PGL-I antibodies, did not modify the capacity of these mycobacteria to stimulate the CL response to *M. leprae* as previously described by Holzer, et al.⁽⁷⁾ and did not increase the phagocytosis of mycobacteria by normal human monocytes. On the other hand, preincubating *M. lepraemurium* with sera from infected mice enhanced phagocytosis and the oxidative burst⁽²⁾. All these data show that the mechanism accounting for the low stimulation of the oxidative burst by certain species of mycobacteria is not unique. Interestingly, neither IFN- γ nor GM-CSF could reverse the defective *M. leprae*-induced CL response; whereas they did synergize with BCG, a mycobacterium of reduced pathogenicity.

In vivo augmentation of the H₂O₂ releasing capacity by the lepromatous macrophages after challenge with PMA, when IFN- γ was injected intradermally, was de-

scribed by Nathan, et al.⁽¹²⁾. According to these data, the responses to PMA from monocytes from all three groups tested in this study were increased (data not shown). Using overnight cultures of adherent human mononuclear cells, small but consistent O₂⁻ production with *M. leprae* stimulation was observed^(8,19). In addition, IFN- γ was able to augment the O₂⁻ production by *M. leprae*-adherent monocytes⁽²⁰⁾. The nature of the effector cells, adherent cells in these studies versus the unseparated mononuclear cells in our work, might explain these conflicting results.

Both GM-CSF and IFN- γ had been shown to activate macrophages and to promote killing of some intracellular pathogens^(13,15,20). However, the role of IFN- γ in the host defense against mycobacteria has been disputed: r-IFN- γ was shown either to enhance^(4,17,18) or to reduce⁽⁶⁾ the *in vitro* growth of mycobacteria within monocytes/macrophages. The role of GM-CSF has not yet been explored. In our study, r-GM-CSF increased the CL response of unstimulated human phagocytes as did r-IFN- γ . BCG stimulation further increased the CL response induced by r-GM-CSF; whereas *M. leprae* stimulation had no detectable effect. These data suggest that the defective activation of the phagocyte oxidative burst by *M. leprae* could not be reversed by either IFN- γ or r-GM-CSF.

SUMMARY

Mycobacterium leprae, in contrast to BCG, failed to trigger any chemiluminescence (CL) response in mononuclear cells from either leprosy patients or healthy subjects, a deficit not reversed by either

interferon-gamma or GM-CSF. Chemiluminescence responses induced without mycobacteria or with BCG were found to be lower in leprosy patients than in controls. *M. leprae* were also less well phagocytosed than BCG. However, there was a significant difference in phagocytosis between healthy and tuberculoid leprosy subjects. Phagocytosis was not altered by the addition of either lymphokine, and no major differences between healthy subjects and patients were observed. Preincubating mononuclear cells with anti-mycobacteria antibodies (lepromatous patients' sera) did not increase the CL response nor the phagocytosis of *M. leprae* or BCG.

RESUMEN

Las células mononucleares de individuos sanos o de pacientes con lepra no generaron quimioluminiscencia (QL) cuando se estimularon con *Mycobacterium leprae* pero sí lo hicieron cuando se estimularon con BCG. El defecto no fue revertido ni por el interferón gamma ni por el GM-CSF. La QL inducida en ausencia de micobacterias o por el BCG, fue menor en los pacientes con lepra que en los controles sanos. El *M. leprae* también fue menos fagocitable que el BCG. Entre los sujetos sanos y los pacientes con lepra tuberculoides hubo una significativa diferencia en la fagocitosis. La fagocitosis no se alteró por la adición de ninguna de las dos linfocinas y no se observaron diferencias importantes entre los sujetos sanos y los pacientes. La preincubación de las células mononucleares con anticuerpos antimicobacterianos (sueros de pacientes lepromatosos) no aumentó la QL de las células ni la fagocitosis del *M. leprae* o del BCG.

RÉSUMÉ

Mycobacterium leprae, par opposition au BCG, ne provoqua aucune réponse de chémoluminescence (CL) au niveau des cellules mononucléaires en provenance soit de patients lépreux, soit de sujets sains; cette déficience n'était pas corrigée par l'interféron-gamma ou le GM-CSF. On a observé que les réactions de chémoluminescence induites sans mycobactérie ou avec le BCG étaient plus faibles chez les patients lépreux que chez les témoins. *M. leprae* était également moins bien phagocyté que le BCG. Cependant, il y avait une différence significative dans la phagocytose entre les sujets sains et les patients présentant une lèpre tuberculoïde. La phagocytose n'était pas altérée par l'addition de lymphokine, et aucune différence importante ne fut observée entre sujets sains et malades. L'incubation préalable des cellules mononucléaires avec des anticorps anti-mycobactérie (sérum de patients lépro-

mateux) n'a pas augmenté la réaction de chémoluminescence ni la phagocytose de *M. leprae* ou du BCG.

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