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Contrary to BCG, MLM Fails to Induce the Production of TNFα and NO by Macrophages[1]

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In a previous paper we have qualified *Mycobacterium lepraemurium* as a highly evolved parasite of mouse macrophages ([32]). We based this statement on the following three properties of the bacillus: a) it is a fusiogenic microorganism adapted to live within the harsh environment of the phagolysosome ([5, 11]), b) it is protected by a thick and complex lipid envelope ([12]), and c) it enters the macrophage without triggering the oxidative (microbicidal) response of this cell ([32]). In this communication we offer further evidence on the qualities of *M. lepraemurium* that make this microorganism a highly successful parasite of macrophages. Macrophages possess several powerful microbicidal mechanisms, that acting together help these cells to get rid of the majority of the ingested microorganisms. Within these microbicidal mechanisms of macrophages are the generation of reactive oxygen intermediates (ROI) ([34]) and reactive nitrogen intermediates (RNI) ([22, 23]), as well as the presence of certain pore-forming cationic proteins ([39]). As for the role of the hydrolytic lysosomal enzymes, except for lysozyme, they seem to be mainly digestive rather than microbicidal ([19]).

Although the microbicidal capacity of macrophages is up regulated by the effect of cell mediated immunity exerted mainly, but not solely, through gamma interferon produced by TCD4$^+$ cells ([21, 28]), macrophages themselves are able to produce ROI and RNI, and the RNI-related cytokine tumor necrosis factor alpha (TNFα), in response to the *in vitro* infection with several microorganisms ([40, 42]). Infection of murine macrophages by BCG and other intracellular parasites, leads to production and release of significant amounts of TNFα. This cytokine up-regulates the synthesis of nitric oxide synthase which, in turn, activates the metabolism of arginine to produce nitric oxide ([14, 16, 37]). Both the nitric oxide produced through this pathway and the ROI produced through the respiratory burst pathway ([13, 20, 27, 34]), or a combination of both ([6, 18, 24, 26]), contribute to the killing of BCG and other intracellular microorganisms, as do the free fatty (arachidonic and linolenic) acids produced by macrophages ([1]). In this communication we report the effect of the infection of mouse macrophages with *M. lepraemurium* on the synthesis and release of TNFα and nitric oxide, and compare the results with those observed upon infection of macrophages with *M. bovis* BCG. To our knowledge, this is the first study on the synthesis and release of TNFα and NO, following the *in vitro* infection of non-immune murine macrophages with *M. lepraemurium*.

MATERIALS AND METHODS

Chemicals. Except when indicated, chemicals were from Sigma Chemical Co., St. Louis, Missouri, U.S.A., and cell culture media and supplements were from Invitrogen, Grand Island, New York, U.S.A.

Bacilli. *M. lepraemurium* bacilli were freshly isolated from the spleen and liver of NIH mice bearing a 6-month infection with the microorganism. Purification of bacilli was carried out by following the entire pro-

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TABLE 1. *Twenty-four hr (0–24 hr) and 48-hr (24 hr–48 hr) TNFα production by mouse peritoneal macrophages*[a].

Cells	Exp-1[b]		Exp-2		Exp-3		Mean ± SE[d]		p[e]
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	
Alone (control)	65[c]	20	70	100	100	100	78.3 ± 18.9	73.3 ± 46.1	–
+BCG	1060	540	750	590	320	240	710.0 ± 371.6	456.6 ± 189.2	<0.001
+MLM	90	65	55	35	40	20	61.6 ± 25.6	40.0 ± 22.9	>0.100

[a] Per assay: 0.5×10^6 cells, 25×10^6 bacilli, in 200 µl of MEM-FCS.
[b] Each individual experiment was performed with cell suspensions pooled from six animals.
[c] Average values for each experiment set in triplicate (picograms per 0.5×10^6 cells).
[d] Mean value ± standard error of experiments 1 to 3.
[e] Analysis applied to data at 24 hr: BCG or MLM cultures *versus* control cultures.

cedure of Prabhakharan ([29]) and the Percoll step of Draper's method ([10]) in that order. These methods have been used to isolate bacilli from human or armadillo tissues infected with *M. leprae*. The procedure, as applied to *M. lepraemurium*, has been described in detail elsewhere ([32]). This allowed us to have a very clean suspension of MLM that was adjusted (by using a reference curve prepared with variable amounts of bacilli) to contain 25×10^8 bacilli per ml of PBS (0.01 M phosphate in 0.15 M NaCl, pH 7.4). Bacilli were then collected by centrifugation (6,000 rpm/20 min), suspended to the original volume with tissue culture medium (MEM supplemented with 10% fetal calf serum, amino acids and gentamicin), divided into 1 ml aliquots, and then kept frozen at –20°C until used. Bacilli were used within 60 days of their preparation. *Mycobacterium bovis* BCG (Danish strain) was obtained from the National Institute of Hygiene at Mexico City as a lyophilized vaccine freshly produced. Several ampules of BCG vaccine were reconstituted in pyrogen-free distilled water, diluted with PBS to prepare a suspension containing 25×10^8 bacilli per ml, centrifuged, suspended in MEM-FCS, divided into aliquots and kept frozen at –20°C until used, usually within the following two months.

Macrophages collection and culture. Macrophages were collected from the peritoneal cavity of uninfected, female retired breeder BALB/c mice, intraperitoneally injected with 1.0 ml of sterile mineral oil, 4 days before cell harvesting; this procedure does not seem to metabolically activate macrophages ([32]). The collection of macrophages was carried out as described elsewhere ([32]), and cell suspensions were prepared to contain 5×10^6 mononuclear cells per ml. Cell cultures were prepared by "seeding" 5×10^5 cells in 200 µl of MEM-FCS per well in 96-well culture plates, and the cultures infected with *M. lepraemurium* or BCG, as described below.

Infection of cultures with *Mycobacterium lepraemurium* or BCG. Triplicate culture wells were then treated with 10 µl of culture medium (control cultures), 10 µl of *Mycobacterium bovis* BCG (25×10^6 bacteria per well) or 10 µl of *M. lepraemurium* (25×10^6 bacilli per well). Then cultures were sampled at 24 hrs and 48 hrs to assess the release of tumor necrosis factor (TNF) and nitric oxide (NO). Culture supernatants were removed at 24 hrs and replaced with fresh MEM-FCS medium. Forty-eight-hr supernatants were collected 24 hrs later (representing TNF and NO production 24 hr–48 hr after infection). Some mycobacteria-infected macrophage cultures were stained by the carbol-fuchsin method of Ziehl-Neelsen to estimate the degree of infection with each mycobacteria.

Measurement of TNFα. TNFα secretion was assessed by using a commercial enzyme linked immunosorbent assay (ELISA) kit (RPN 2718 Biotrak, Amersham, England), following the manufacturers' protocol. TNFα concentration in the samples is calculated from standard curves prepared with reference solutions containing known amounts of TNFα (2450 pg/ml, 350 pg/ml and 50 pg/ml).

Measurement of nitric oxide. Nitric oxide was determined by the Griess reaction ([15, 25]) in supernatants of macrophage cultures infected for 24 hrs or 48 hrs with MLM or BCG. Fifty microliters of super-

TABLE 2. *Twenty-four hr (0–24 hr) and 48-hr (24 hr–48 hr) nitric oxide production by mouse peritoneal macrophages*[a].

Cells	Exp-1[b]		Exp-2		Exp-3		Mean ± SE[d]		p[e]
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	
Alone (control)	28[c]	0	32	0	28	0	29.3 ± 2.3	0	–
+BCG	352	244	408	170	214	208	322.6 ± 103	209.3 ± 37.2	<0.001
+MLM	32	8	32	8	28	0	30.6 ± 2.3	5.3 ± 4.6	>0.100

[a] Per assay: 0.5×10^6 cells, 25×10^6 bacilli, in 200 µl of MEM-FCS.
[b] Each individual experiment was performed with cell suspensions pooled from six animals.
[c] Average values for each experiment set in triplicate (ng of NO per 0.5×10^6 cells).
[d] Mean value ± standard error of experiments 1 to 3.
[e] Analysis applied to data at 24 hr: BCG or MLM cultures *versus* control cultures.

natants were admixed with 200 µl of the Griess reagent and the absorbence of the resulting colored product, read in a colorimeter (Multiskan-plus ELISA reader) at 540 nm. Readings were transformed into ng of nitrite by reference to a standard curve ranging from 0 to 100 ng of sodium nitrite (Griess reagent is a volume to volume mixture of 1% sulfanilamide in water and 0.1% N-[1-naphthyl] ethylendiamine·2HCl in 2.5% phosphoric acid).

Analysis of results. Data were subjected to an analysis of variance (ANOVA), and then to the Mann-Whitney test.

RESULTS

Tumor necrosis factor alpha (TNFα). Macrophages released large amounts of TNFα in response to the *in vitro* infection with BCG (710 ± 371.6 picograms [pg] per 0.5×10^6 cells, *versus* 78.3 ± 18.9 pg in the control cells, $p < 0.001$) but did not release this cytokine in response to MLM (61.6 ± 25.6 pg per 0.5×10^6 cells *versus* 78.3 ± 18.9 pg, $p > 0.100$). Most of this cytokine was produced within the 24 hrs following stimulation with BCG (710 ± 371 pg *versus* 456.6 ± 189.2 pg at 48 hrs). The results of three experiments carried out with different animal groups are shown in Table 1.

Nitric oxide. The pattern of release of NO was similar to the pattern of release of TNFα: macrophages responded well to the infection by BCG (322.6 ± 103 nanograms [ng] per 0.5×10^6 cells, *versus* 29.3 ± 2.3 ng in the cells alone, $p < 0.001$) and not at all to the stimulus with MLM (30.6 ± 2.3 ng *versus* 29.3 ± 2.3 ng, $p > 0.100$). Again, most NO was produced within the first 24 hrs following the stimulus with BCG (322.6 ± 103 ng *versus* 209.3 ± 37.2 ng at 48 hrs). The results of three independent experiments are shown in Table 2.

The average simultaneous production of TNFα and NO by macrophages infected by BCG or MLM is illustrated in Fig. 1. The production of TNFα and synthesis of NO seem to be directly related.

Macrophage infection. Macrophages got infected to a similar degree with either MLM or BCG, although a somehow different intracellular distribution of each of the mycobacteria was observed; BCG appeared more clumpy than MLM, either because of the normal clumpy growth of BCG on arti-

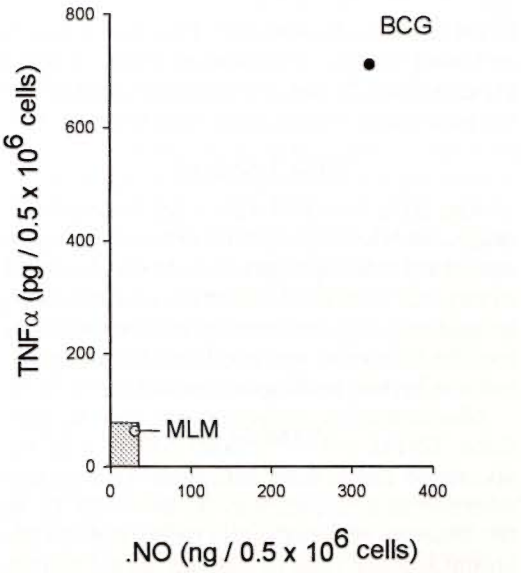

FIG. 1. Simultaneous production of TNFα and NO by macrophages infected *in vitro* with *M. bovis* (BCG) or *M. lepraemurium* (MLM). Only the average levels of these factors are depicted. The shadowed area corresponds to the response in the cultures of macrophages not subjected to infection.

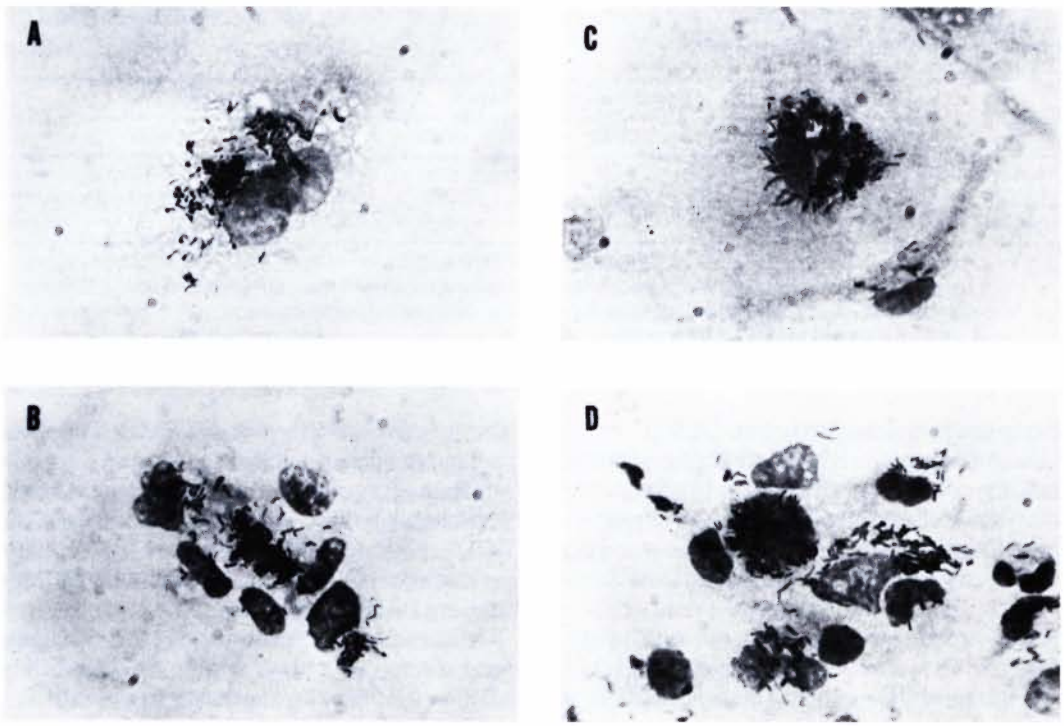

FIG. 2. Cultured macrophages infected for 24-hr with *M. bovis* (BCG) (**A**, **B**) or *M. lepraemurium* (MLM) (**C**, **D**). Infection was performed with a theoretical ratio of 50 bacilli per cell. Notice that although similar numbers of bacilli entered the cells, BCG bacilli appear more clumpy than MLM.

ficial media, or, perhaps, because it has a different entry mechanism into macrophages (Fig. 2). An investigation on this latter possibility is currently underway.

DISCUSSION

The link between IFNγ produced by T cells and NK cells and TNFα and NO produced by macrophages, has been observed in several infectious diseases and has been sought as a key mechanism of macrophages for the killing of intracellular microorganisms, whether pathogenic or not ([37, 14, 38, 35]).

Macrophages, however, are able to produce TNFα ([31, 41, 42]) and NO ([40]) in response to the *in vitro* infection with certain microorganisms, such as *M. bovis* BCG, in the absence of T cells, this ruling out the absolute requirement of cell-mediated immunity for macrophage activation. However, T cell-derived cytokines, namely IFNγ, do indeed improve the microbicidal ability of macrophages by up-regulating the activity of the genes coding for TNFα and nitric oxide synthase (iNOS), a process leading to an overwhelming synthesis of nitric oxide ([14]). Apart from being a chemical mediator, nitric oxide is a key participant in the killing of intracellular microorganisms and is an alternative mechanisms for the killing of ROI-resistant parasites. Those microorganisms that resist the microbicidal mechanisms of macrophages represented by the reactive oxygen-intermediaries (superoxide anion, hydrogen peroxide, hydroxyl radicals), the reactive nitrogen intermediaries (nitric oxide), the combined myeloperoxidase-hydrogen peroxide-halide system, and the non oxygen-dependent hydrolytic and pore-forming lysosomal enzymes, are indeed parasites whose physiological elimination would require the participation of cell mediated immunity, through a plethora of proinflammatory cytokines produced by T lymphocytes and other cells ([7, 8, 9, 17, 28, 30]). Although susceptible to the *in vitro* inhibitory effect of these cytokines, some microorganisms, such as *M. lepraemurium* and *M. leprae*, are, however, "intelligent enough" to prevent overactivation of

macrophages by directly or indirectly suppressing or down-regulating the activity of the mycobacteria-reactive, macrophage activating, T cell clones ([2, 3]).

In this communication, we present evidence on the ability of *M. lepraemurium* to *in vitro* infect mouse macrophages, without triggering a meaningful TNFα/NO-response of these cells. *Mycobacterium bovis* BCG, on the other hand, behaves in a very different manner because it is a good inducer of both TNFα and NO. The particular behavior of these mycobacteria is in tight correlation with their pathogenicity, because it has been seen that as many as 50 million BCG cells produce in NIH mice an early but self-limiting disease. Forty days after infection, only residual, inactive, bacilli-free scar-like lesions are observed in the target (liver and spleen) organs of mice intraperitoneally infected with this high dose of bacilli. On the other hand, as few as 5 million (Rojas-Espinosa, unpublished), or less ([36]), *M. lepraemurium* cells, produced in the mouse a disseminated lepromatous disease that was clearly evident, several months after infection by the same route.

The ability of *M. lepraemurium* to emerge as a very successful parasite of macrophages depends on the development of a series of attributes of the bacteria that include: a) the synthesis of a thick and complex lipid envelope ([12]), b) its ability to survive within the inhospitable intraphagosomal environment, a property probably related to the previous one ([5, 11]), c) its ability to infect macrophages without triggering these cells' oxidative response ([32]), d) its ability to infect macrophages without triggering the release of significant amounts of tumor necrosis factor or nitric oxide (Rojas-Espinosa, *et al.*, this communication), e) its ability to produce a disease in which cell anergy gradually develops ([4]), and f) its ability to produce a disease in which complement is inactivated, thus preventing complement-mediated damage of the host ([33]).

As the degree of macrophage infection by *M. lepraemurium* is apparently similar to the degree of infection by BCG (Fig. 2), the different response of macrophages to the infection with these microorganisms, in terms of TNFα, ROI and RNI release, must have another explanation. Based on preliminary evidence of different protein-phosphorylation patterns in macrophages infected with *M. lepraemurium* or BCG, we believe that the results reflect different entry pathways of the microorganisms. This subject is under research in our laboratory.

SUMMARY

Pathogenic mycobacteria must possess efficient survival mechanisms to resist the harsh conditions of the intraphagosomal milieu. In this sense, *Mycobacterium lepraemurium* (MLM) is one of the most evolved intracellular parasites of murine macrophages; this microorganism has developed a series of properties that allows it not only to resist, but also to multiply within the inhospitable environment of the phagolysosome. Inside the macrophages, MLM appears surrounded by a thick lipid-envelope that protects the microorganism from the digestive effect of the phagosomal hydrolases and the acid pH. MLM produces a disease in which the loss of specific cell-mediated immunity ensues, thus preventing activation of macrophages. *In vitro*, and possibly also *in vivo*, MLM infects macrophages without triggering the oxidative (respiratory burst) response of these cells, thus preventing the production of the toxic reactive oxygen intermediaries (ROI). Supporting the idea that MLM is within the most evolved pathogenic microorganisms, in the present study we found, that contrary to BCG, *M. lepraemurium* infects macrophages without stimulating these cells to produce meaningful levels of tumor necrosis factor alpha (TNFα) or nitric oxide (NO). Thus, the ability of the microorganisms to stimulate in their cellular hosts, the production of ROI and RNI (reactive nitrogen intermediates), seems to be an inverse correlate of their pathogenicity; the lesser their ability, the greater their pathogenicity.

RESUMEN

Las micobacterias patógenas deben poseer eficientes mecanismos de supervivencia para resistir las condiciones agresivas del medio intrafagosomal. En este sentido, *Mycobacterium lepraemurium* (MLM) es uno de los parásitos más evolucionados de los macrófagos murinos: este organismo ha desarrollado una serie de propiedades que le permiten no solo resistir, sino también proliferar dentro del inhóspido medio del fagolisosoma. Dentro de los macrófagos, MLM aparece rodeado de una gruesa envoltura lipídica que lo protege del efecto digestivo de las hidrolasas

fagosomales y del pH ácido de la vacuola. MLM produce una enfermedad en la cual ocurre la pérdida de la inmunidad celular específica, evitando así la activación de los macrófagos. *In vitro*, y probablemente también *in vivo*, MLM infecta a los macrófagos sin estimular la respuesta oxidativa (estallido respiratorio) de estas células, evitando de esta mariera la producción de los intermediarios tóxicos del oxígeno. Además, contrario a lo que ocurre con BCG, *M. lepraemurium* infecta a los macrófagos sin estimular la capacidad de estas células para producir niveles significativos de factor de necrosis tumoral alfa (TNFa) o de óxido nitrico (NO) (esta comunicación). Siendo los metabolitos reactivos del oxígeno, y el óxido nítrico, las armas más letales de los macrófagos, es claro que *M. lepraemurium* está dentro de los parásitos más evolucionados, y por ende, patogénicos de los macrófagos.

RÉSUMÉ

Les mycobactéries pathogènes doivent posséder des mécanismes de survie efficaces pour résister aux conditions hostiles du milieu intraphagosomal. En ce sens, *M. lepraemurium* (MLM) est un des parasites intracellulaires les plus évolués des macrophages des souris. Ce microorganisme a développé une série de propriétés qui lui permettent non seulement de résister, mais aussi de se multiplier dans l'environnement inhospitalier du phagolysosome. Dans les macrophages, MLM apparaît entouré par une épaisse enveloppe lipidique qui le protège d'une digestion par des hydrolases phagosomales et du pH acide. MLM induit un déficit de l'immunité cellulaire en évitant l'action des macrophages. Par ailleurs, *in vitro*, et probablement aussi *in vivo*, MLM infecte les macrophages sans déclencher leur réponse oxydative, prévenant de cette manière la production de dérivés toxiques de l'oxygène. De plus, contrairement à BCG, *M. lepraemurium* infecte les macrophages sans induire la production de quantités significatives de tumeur necrosis facteur alpha (TNFα) ou d'oxyde nitrique (NO) (cf. cet article). Les dérivés de l'oxygène et l'oxyde nitrique étant les armes les plus efficaces des macrophages, il est clair que *M. lepraemurium* est un des parasites les plus évolués et donc les plus pathogènes pour les macrophages.

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