

Mycobacterium leprae-Burdened Macrophages Are Refractory to Activation by Gamma Interferon

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Mycobacterium leprae grows to enormous numbers in the *nu/nu* mouse footpad, producing granulomas resembling those of lepromatous leprosy in humans. Footpad granuloma cells gorged with *M. leprae* were established in primary cell culture to examine their functional capabilities. These cells were classified as macrophages by the following criteria: positive staining for nonspecific esterase, reduction of Nitro Blue Tetrazolium during phagocytosis of *Candida albicans*, possession of Fc receptors, and possession of Mac-1 antigen. Footpad macrophages also phagocytized and supported the intracellular growth of *Toxoplasma gondii*. However, unlike peritoneal macrophages, footpad macrophages could not be activated to kill or inhibit *T. gondii* by macrophage-activating factor produced by mitogen-stimulated spleen cells or by recombinant gamma interferon. Thus, although the lepromatous macrophages appeared to be normal in many of their functions, they were defective in response to macrophage-activating signals.

Mycobacterium leprae is an obligate intracellular pathogen which resides chiefly within cells of the mononuclear phagocyte system. In the skin of lepromatous leprosy patients, large granulomas comprising heavily infected macrophages are evidence of the prolific growth of *M. leprae*. Thus, although the underlying mechanism(s) of defective cell-mediated immunity in leprosy is not clearly understood (7, 11; B. Bloom, Editorial, *J. Immunol.* 137:i-x, 1986), the failure of the macrophage to kill or inhibit *M. leprae* is a conspicuous characteristic of the lepromatous form of the disease. It therefore seems apparent that the ability of the macrophage to cope with *M. leprae* is an issue central to an understanding of the mechanisms of host resistance to the leprosy bacillus.

Multiplication of *M. leprae* in the footpads of conventional mice represents a means of quantitating the growth of *M. leprae* isolates (23) but is a poor animal model for the human disease. However, in footpads of athymic (*nu/nu*) mice, *M. leprae* replicates extensively, producing large granulomas that resemble those of lepromatous leprosy in humans (3). In the present study, cells from the footpad granulomas of *M. leprae*-infected *nu/nu* mice were isolated in primary culture, characterized, and tested for their capacity to respond to the lymphokines macrophage-activating factor (MAF) and recombinant gamma interferon (IFN- γ).

MATERIALS AND METHODS

Mice. HSD *nu/nu* mice and *nu/++* littermates were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. The *nu/nu* mice used had been inoculated with 10^6 *M. leprae* cells in the hind footpad 12 to 14 months previously. BALB/c mice were locally bred.

Cell culture. (i) **Peritoneal macrophages.** Resident peritoneal cells were harvested as described previously (13). Adherent cells were plated on LUX cover slips (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) in 24-well plates (Corning Laboratories, Corning, N.Y.) in RPMI 1640 medium (GIBCO Laboratories, Grand Island,

N.Y.) supplemented with fresh glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 100 U of penicillin (GIBCO) per ml, and 20% heat-inactivated fetal calf serum (FCS) (Hyclone; Sterile Systems, Inc., Ogden, Utah). None of the tissue culture media used contained endotoxin detectable by the *Limulus* Amoebocyte Assay (Sigma Chemical Co., St. Louis, Mo.). For some experiments, adherent peritoneal macrophages were treated for 2 h at 37°C with collagenase and DNase at concentrations equivalent to those used to separate granuloma cells (see below).

(ii) **Footpad macrophages.** To harvest *M. leprae*-infected footpad macrophages from *nu/nu* mice, the enlarged footpad was scrubbed with Betadine, dipped in ether, and removed. The granuloma was digested enzymatically by a modification of the technique described by Russell and McIntosh (21). Briefly, the epidermis of the footpad was aseptically removed, and the remaining footpad tissue was gently minced with sharp scalpels in Hanks balanced salt solution (GIBCO). The footpad cells were dispersed by treatment with collagenase (15 U/ml; Cappel-Worthington Biochemicals, Malvern, Pa.), and DNase (25 μ g/ml; Calbiochem-Behring, La Jolla, Calif.) in 10 ml of RPMI 1640-0% FCS. Dispersion was performed at 37°C for 1 h and was repeated twice with fresh enzyme solutions. The cell suspensions produced by each digestion were washed in Hanks balanced salt solution and held at 4°C. Pooled cell suspensions (6.0 ml) were layered on 6.0 ml of Nycodenz (Accurate Chemical and Scientific Corp., Westbury, N.Y.) and centrifuged at $300 \times g$ for 25 min. A band of cells (>95% viable) distinct from the debris at the tube bottom was removed from the Nycodenz gradient and washed three times in Hanks balanced salt solution. The cells were suspended in RPMI 1640-20% FCS at 2×10^6 /ml, and 0.5 ml was plated on LUX cover slips in 24-well plates. After 2 h, nonadherent cells were removed by washing and the adherent cells were cultured in RPMI 1640-20% FCS.

(iii) **Cell viability.** The viability of cells isolated from the footpad granulomas was tested by using the ethidium bromide-acridine orange stain described by Parks et al. (20).

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TABLE 1. Characteristics of footpad granuloma cells and peritoneal macrophages from *M. leprae*-infected *nu/nu* mice

Characteristic ^a	Presence of characteristic ^b		
	Peritoneal macrophages	Footpad granuloma cells	
		Fibroblasts (type 1)	Macrophages (type 2)
Intracellular acid-fast bacilli	—	—/(+)	++++
Adherence	++++	+	++++
Replication	—	+	—
Trypsin removed	—	+	—
Esterase	+	—	+
Phagocytosis	+	—	+
NBT reduction	+	—	+
Fc receptors	+	—	+
MAC-1 antibody	+	—	+

^a *C. albicans* was used to determine phagocytosis and NBT reduction, and rabbit anti-SRBC IgG was used to determine the presence of Fc receptors.

^b Symbols: —, characteristic not observed; +, characteristic observed; + + + +, characteristic observed to a remarkable degree.

Macrophage phenotypic markers. (i) Nonspecific esterase stain. Monolayers were examined for nonspecific esterase activity by using alpha-naphthol butyrate as the substrate and the procedures described by Koski et al. (12).

(ii) MAC-1. Monoclonal antibody to MAC-1, a surface macrophage marker (9), was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Cell monolayers were fixed briefly in 2% Formalin, washed in phosphate-buffered saline (PBS), and incubated for 30 min at 37°C in MAC-1 antibody diluted 1:10 in PBS. Control serum consisted of supernatant medium from NS-1 hybridoma cells. Binding of MAC-1 antibody was visualized by using an avidin-biotin complex goat anti-mouse immunoglobulin G (IgG) kit (Vector Labs, Inc., Burlingame, Calif.).

(iii) Phagocytosis and NBT reduction. Heat-killed *Candida albicans* cells were suspended in RPMI 1640-20% FCS containing 0.2 mg of Nitro Blue Tetrazolium (NBT) (Sigma) per ml and used to challenge peritoneal and footpad monolayers at a ratio of two yeast cells per macrophage. After 30 min, extracellular yeast cells were rinsed off and the monolayers were fixed in 50% methanol, counterstained with 2% safranin, and examined for intracellular formazan precipitate indicative of NBT reduction.

(iv) Fc receptors. Sheep erythrocytes (SRBC) were coated with a 1:100 dilution (in PBS) of rabbit anti-SRBC IgG (Cappel-Worthington), incubated 1 h at 37°C, and washed three times in PBS. IgG-coated SRBC and normal SRBC were incubated with mouse peritoneal macrophage and footpad macrophage monolayers for 30 min at room temperature. Nonadherent cells were removed by extensive rinsing in PBS, and the cover slips were stained with hematoxylin and eosin and examined for Fc rosette formation.

(v) Trypsin removal. Adherent footpad cells and, in certain experiments, peritoneal macrophages were washed and treated for 10 min with 0.05% trypsin (three-times crystallized; Cappel-Worthington).

Lymphokine activation and *Toxoplasma gondii* microbicidal assay. Live *T. gondii* RH tachyzoites were freshly harvested from peritoneal cavities of 2-day-infected mice and purified by filtration through 3- μ M polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.).

MAF was produced from BALB/c mouse spleen cell cultures stimulated with concanavalin A for 48 h at 37°C as

previously described (24). Control MAF was produced from cultures of unstimulated spleen cells. Recombinant murine IFN- γ (Mu IFN- γ) was obtained from H. M. Shepard, Genentech Inc., South San Francisco, Calif.

Activation of peritoneal or footpad macrophages was evaluated by measurement of the capability of MAF or Mu IFN- γ to induce an enhanced microbicidal capacity for *T. gondii*. Macrophages cultured on LUX cover slips were incubated with MAF or control MAF diluted 1:4 or with various dilutions of Mu IFN- γ in RPMI 1640-20% FCS. All lymphokine preparations also included 2 ng of endotoxin (*Escherichia coli* O111:B4; Sigma) per ml. After 18 h of stimulation, this medium was removed and cell monolayers were challenged with *T. gondii*, as previously described (24). Evaluation of *T. gondii* survival was based on counts of the percentage of macrophages infected after 1 or 20 h of culture postinfection. Replication of *T. gondii* was evaluated by counting the mean number of *T. gondii* cells per infected macrophage at 20 h postinfection on three cover slips per sample for each experiment. For some experiments, macrophages were incubated with doses of Mu IFN- γ for 72 h before challenge with *T. gondii*. In these experiments the initial dose of Mu IFN- γ applied on day 1 was replaced with fresh Mu IFN- γ at 48 h postinfection.

RESULTS

Enzyme dispersion of *M. leprae*-infected footpad granulomas liberated 2×10^7 to 3×10^7 viable cells. These suspensions yielded two types of adherent cells, present in approximately equal numbers. Characteristics of the granuloma cells tested in parallel with peritoneal macrophages from *M. leprae*-infected *nu/nu* mice are presented in Table 1. Type 1 footpad cells were fibroblastlike, were capable of proliferation in culture, and contained few acid-fast bacilli. After 5 to 6 days these type 1 cells completely overgrew the cover slips. The second cell type was tightly adherent to plastic within 1 h of plating and contained numerous acid-fast bacilli, but showed no evidence of replication in culture. Although more than one-half the total number of cells was lost by centrifugation through a Nycodenz gradient, the purified suspension was highly enriched for the type 2 cells. Adherent type 1 cells and type 2 cells gorged with *M. leprae* are shown in Fig. 1A. As shown in Table 1, the fibroblastlike cells were removed by a 10-min treatment with trypsin, whereas peritoneal macrophages and footpad granuloma macrophages were not. Nonspecific esterase activity was detected in peritoneal macrophages, as well as in footpad macrophages, but not in the footpad fibroblasts. When challenged with *C. albicans*, fibroblasts failed to engulf the yeast cells; however, the yeast cells were readily phagocytized by the peritoneal macrophages and footpad macrophages. Phagocytosis of *C. albicans* was accompanied by a reduction of NBT cells in peritoneal macrophages and footpad macrophages (70% of yeast cells) but not fibroblasts. Study of the ability to form rosettes with IgG-coated SRBC revealed that footpad granuloma macrophages expressed normal numbers of Fc receptors comparable to those of peritoneal macrophages. However, IgG-coated SRBC were not bound by mouse footpad fibroblasts. Finally, the constitutive monoclonal antibody macrophage surface marker MAC-1 was present on peritoneal macrophages and footpad macrophages but not on the fibroblasts.

Despite a heavy load of intracellular *M. leprae* cells, footpad macrophages supported intracellular *T. gondii* growth at a rate similar to that observed in normal peritoneal

macrophages (Fig. 1B). Direct evaluation of macrophage microbicidal capacity on the leprosy bacillus could not be tested due to the inability to establish the viability of intracellular *M. leprae* cells. Therefore, the response of peritoneal macrophages and footpad granuloma macrophages to macrophage-activating signals was measured indirectly by evaluating their functional capacity to kill or inhibit the multiplication of *T. gondii*.

Peritoneal macrophages from BALB/c *nu/++* heterozygotes and *nu/nu* mice were readily activated by treatment with MAF in the presence of endotoxin but not by control MAF. Activation was characterized by a significant reduction in the percentage of macrophages which remained infected with *T. gondii* at 20 h postchallenge (Fig. 2A). This difference was not due to different rates of uptake or initial levels of infection since the percentage of macrophages containing *T. gondii* at 1 h postchallenge was identical in both groups (data not shown). The ability of activated peritoneal macrophages to kill or inhibit the growth of *T. gondii* is clearly evident by comparing the numbers of *T. gondii* cells which survived at 20 h (Fig. 2B). In contrast to peritoneal macrophages, footpad granuloma macrophages were not activated by similar doses of MAF and the growth of *T. gondii* was unrestricted in these cells (Fig. 2A and B).

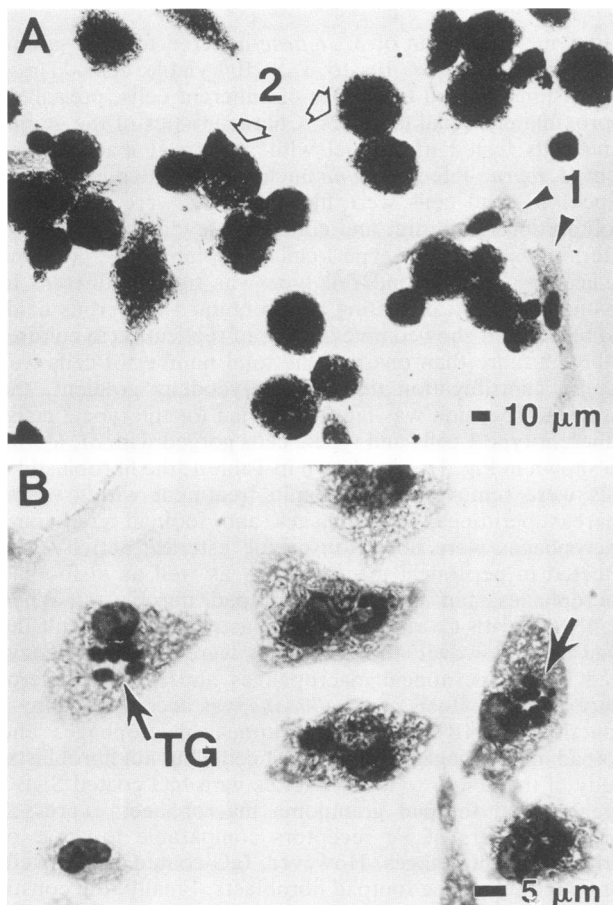


FIG. 1. (A) Explant cell cultures from *M. leprae*-infected *nu/nu* mouse footpad granulomas. 1, Type 1 fibroblastlike cell containing a few *M. leprae* cells; 2, type 2 macrophagelike cell gorged with *M. leprae* cells. Acid-fast/hematoxylin stain. (B) Growth of *T. gondii* (TG) in type 2 footpad granuloma cells gorged with *M. leprae* cells. Acid fast/hematoxylin stain.

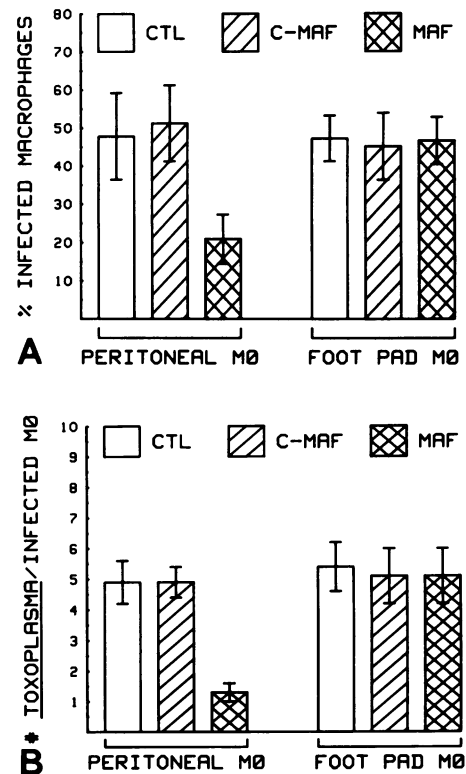


FIG. 2. Effects of treatment of peritoneal macrophages and footpad granuloma macrophages with MAF or control MAF on the intracellular growth of *T. gondii* 20 h postchallenge. CTL, Control medium; C-MAF, control MAF; MAF, concanavalin A-stimulated MAF. The data are expressed as the mean \pm standard deviation for three cover slips from each of three or more experiments.

To further evaluate the dose required for and the kinetics of the activation response of macrophages, recombinant Mu IFN- γ was used rather than MAF. In the experiments for which results are shown in Fig. 3, a broad dose range of Mu IFN- γ was used. A threshold dose of 125 U of Mu IFN- γ was required to achieve significant activation of normal peritoneal macrophages (Fig. 3A and B). In contrast, activation was not observed in footpad granuloma macrophages at doses of Mu IFN- γ which greatly exceeded 125 U (Fig. 3A and B). Failure of footpad granuloma macrophages to become activated was also observed when Mu IFN- γ incubation was extended to 72 h before challenge with *T. gondii* (Table 2). The inability of footpad granuloma macrophages to respond to lymphokines was not a consequence of the enzyme digestion procedures since monolayers of peritoneal macrophages treated for 1 h with collagenase and DNase were readily activated to kill *T. gondii* by Mu IFN- γ (data included with that for control macrophages in Fig. 2 and 3).

DISCUSSION

Our studies of mouse lepromatous granuloma macrophages should prove relevant to an understanding of the lack of host response to *M. leprae* in lepromatous leprosy. That the lepromatous macrophage is unable to control the intracellular growth of *M. leprae* is clearly evident. Most theories about defective cell-mediated immunity in lepromatous leprosy are concerned with the failure of the specifically sensitized T cell to respond to *M. leprae* antigens (7, 14, 16;

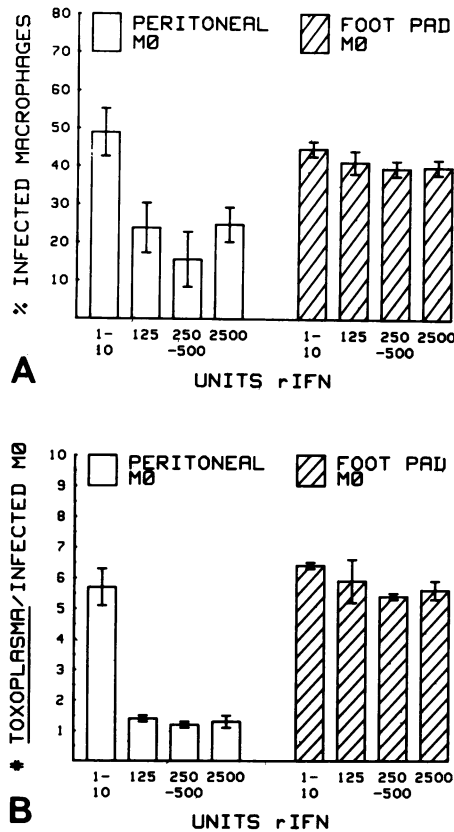


FIG. 3. Effects of treatment of peritoneal macrophages and footpad granuloma macrophages with recombinant Mu IFN- γ on intracellular growth of *T. gondii* 20 h postchallenge. The data are expressed as the mean \pm standard deviation for three cover slips from each of three or more experiments.

Bloom, Editorial) rather than the inability of the macrophage to perform its effector function. Postulated roles for defective macrophage function in leprosy have usually been concerned with the failure to process and present antigen (8, 17).

In this report, we describe the isolation of footpad granuloma macrophages that were tightly adherent and had the capacity to phagocytize, reduce NBT, and retain macrophage characteristics, including Fc receptors. The strong reactivity of these footpad granuloma macrophages with the universal macrophage monoclonal antibody marker MAC-1, suggests that they also possess characteristic C3BI receptors (9). Over the 2 months that these granuloma cells were isolated and cultured, these characteristics remained unchanged, in contrast to the heterogeneous granuloma cells described by Amsden and Baros (1). Others have cultured granuloma cells from lepromatous lesions of human patients and obtained low numbers of large adherent esterase- and peroxidase-positive cells containing *M. leprae*, although functional studies were not performed (15).

The hypothesis explored in the present study was whether the microbicidal capacity of lepromatous macrophages could be enhanced by macrophage-activating lymphokines. *T. gondii* killing was used as an indicator of the microbicidal function of lepromatous macrophages since a direct method of measuring intracellular *M. leprae* viability is not available. Whereas *T. gondii* cells survive in normal macrophages, they are readily killed by macrophages activated with MAF

TABLE 2. Effects of prolonged treatment (72 h) with various doses of Mu IFN- γ on microbicidal capacity of peritoneal macrophages and footpad granuloma macrophages from *M. leprae*-infected *nu/nu* mice

Macrophage type	Dose (U) of Mu IFN- γ	% Infected macrophages (mean \pm SD) ^a	No. of <i>T. gondii</i> cells/infected macrophage (mean \pm SD) ^a
Peritoneal	0 (control)	37.31 \pm 4.08	5.11 \pm 0.20
	125	14.88 \pm 0.79	1.22 \pm 0.08
	250	11.98 \pm 3.45	1.22 \pm 0.20
	2500	10.90 \pm 2.94	1.14 \pm 0.04
	500	10.90 \pm 2.94	1.14 \pm 0.04
Footpad	0 (control)	43.0 \pm 5.27	5.72 \pm 0.12
	125	38.35 \pm 3.56	5.49 \pm 0.26
	250	41.44 \pm 7.43	5.29 \pm 0.37
	2500	37.68 \pm 1.68	5.45 \pm 0.44
	500	37.68 \pm 1.68	5.45 \pm 0.44

^a Done in triplicate at 20 h postchallenge.

or Mu IFN- γ (2, 18). Failure of lymphokine-treated footpad macrophages to restrict the growth of intracellular *T. gondii* in the present study, albeit in mice, suggests that although lepromatous macrophages appear to be normal in many of their characteristics and functions, they appear to be defective in their response to macrophage-activating signals.

A number of studies have concluded that there is no intrinsic defect in the microbicidal capacity of macrophages from leprosy patients (6, 10, 11). However, these studies were performed with peripheral blood monocytes and did not examine tissue macrophages from the lepromatous granuloma. An exception to these findings is the recent work of Nathan et al. (19), who reported that the monocytes from lepromatous patients were deficient in their ability to release hydrogen peroxide, an important microbicidal component in the respiratory oxidative burst. Interestingly, this blood monocyte defect was restored within a few days after intradermal injection of human IFN- γ into lepromatous lesions. Moreover, preliminary observations suggested that local IFN- γ treatment also resulted in detectable clearance of bacilli from the lesion. In previous *in vivo* human studies, Convit et al. (4, 5) showed that intradermal injection of a mixture of *Mycobacterium bovis* and killed *M. leprae* cells resulted in the development of a local epithelioid granuloma in lepromatous patients and the elimination of leprosy bacilli from the site. Neither of these studies made a distinction between activation of local granuloma macrophages and recruitment of fresh circulating monocytes. Differences in methodology preclude direct comparison between these *in vivo* studies and the present work. However, the demonstration that mouse lepromatous macrophages are refractory to Mu IFN- γ activation suggests that a similar defect may exist in the macrophages found in the lesions of lepromatous leprosy in humans. Finally, since IFN- γ appears to be the active molecule in MAF-containing T-cell supernatant media (22), these studies suggest that it is important to focus on the macrophage-IFN- γ interaction, as well as on T-cell deficiencies, in lepromatous leprosy. We are presently examining whether these granuloma macrophages lack the ability to receive the activating signal or whether they have a defect in their microbicidal mechanisms.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Julie Loesch and the clerical assistance of Myran Booker and Penne Cason.

These studies were supported by Public Health Service grants AI22442 and AI22492 from the National Institute of Allergy and Infectious Diseases.

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