

Mycobacterial Lipoarabinomannan Inhibits Gamma Interferon-Mediated Activation of Macrophages

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The principal efferent role of the macrophage in acquired resistance to intracellular pathogens depends on activation by T-cell lymphokines, primarily gamma interferon (IFN- γ). However, mouse macrophages that are heavily burdened with *Mycobacterium leprae* are refractory to activation by IFN- γ and are thus severely compromised in their capacity for both enhanced microbicidal and tumoricidal activities. We report here that lipoarabinomannan (LAM), a highly immunogenic lipopolysaccharide that is a prominent component of the cell walls of *M. leprae* and *M. tuberculosis*, was a potent inhibitor of IFN- γ -mediated activation of mouse macrophages in vitro. Inhibition of macrophage activation by LAM required preincubation for approximately 24 h, resulting in uptake of LAM into cytoplasmic vacuoles of macrophages. Intact LAM was necessary to inhibit IFN- γ -mediated activation, as this property was lost when the acyl side chains were removed from LAM by mild alkaline hydrolysis. In addition, LAM was an abundant constituent of macrophages isolated from lepromatous granulomas of *M. leprae*-infected nude mice and likely contributed to the defective activation of granuloma macrophages by IFN- γ .

A conspicuous feature of lepromatous leprosy in humans, which is characterized by anergic T-cell responsiveness (9) and antigen-driven T-cell suppression (19), is the abundance of macrophage-rich granulomas harboring *Mycobacterium leprae* (24). We have recently demonstrated that macrophages isolated from lepromatous granulomas of *M. leprae*-infected nude mice are defective in their responsiveness to lymphokines, including gamma interferon (IFN- γ) (29, 30). One mechanism of reduced macrophage responsiveness to IFN- γ is the induction of prostaglandin E₂ (PGE₂) synthesis by macrophages following uptake of viable *M. leprae* in vitro (L. D. Sibley and J. L. Krahenbuhl, submitted for publication). However, this induction is evidently not the sole factor responsible for the lack of IFN- γ responsiveness, as granuloma macrophages are not restored to normal when PGE₂ production is blocked by indomethacin (30). To further characterize defective macrophage activation in lepromatous leprosy, we have begun examining the effects of purified constituents of the cell walls of *Mycobacterium* spp. on macrophage effector function(s).

Lipoarabinomannan (LAM) is one major constituent of the cell wall that is widely distributed within *Mycobacterium* species and related genera (14, 20) and constitutes the major carbohydrate-containing immunogen recognized by sera from patients with tuberculosis and leprosy (8, 14, 20). Arabinomannan, which lacks the acyl groups, has been extensively studied (14, 20); however, only recently has native LAM been purified from *M. leprae* (LAM_{ml}) and *M. tuberculosis* (LAM_{tb}) (14). Purified LAM contains not only the predominant saccharide units arabinose and mannose, but also lactate, succinate, and phosphatidylinositol that apparently anchor the molecule in the cytoplasmic membranes of the bacilli (14; S. W. Hunter, unpublished results). LAM_{tb} also contains alkali-labile inositol phosphate units that are apparently absent from LAM_{ml} (14; Hunter, unpublished results). Both LAM_{tb} and LAM_{ml} inhibit antigen responsiveness of human peripheral blood leukocytes in an

in vitro assay which depends on macrophage afferent functions (15). In the present report, we examine the ability of LAM to specifically inhibit efferent functions of mouse macrophages activated by IFN- γ .

MATERIALS AND METHODS

Cell culture. Resident peritoneal macrophages were harvested in Hanks balanced salt solution (Bioproducts, Whitaker, Mass.) containing 10 U of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml from adult BALB/c mice and cultured on LUX cover slips (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) or directly in 24-well cluster plates or 4-well Lab-Tek slides (Miles Laboratories). Foot pad granuloma macrophages and peritoneal macrophages were harvested from *M. leprae*-infected HSD *nu/nu* mice (Harlan Sprague Dawley, Indianapolis, Ind.) as previously described (29, 30). Monolayers consisting of 2×10^5 to 5×10^5 macrophages were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS; Hyclone Sterile Systems, Inc., Logan, Utah) supplemented with 2 mM glutamine (Irvine Scientific, Santa Ana, Calif.), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO), and 100 μ g of gentamicin (Sigma) per ml.

LAM treatment. LAM was purified from *M. leprae* (LAM_{ml}) or from *M. tuberculosis* (LAM_{tb}) as previously described (14) and stored lyophilized at 4°C. The purity of LAM preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation followed by periodate-silver staining (14). LAM_{tb} was hydrolyzed with NaOH to remove acyl chains as previously described (14). LAM was reconstituted in sterile phosphate-buffered saline (PBS) by brief sonication and added to macrophage monolayers at doses ranging from 10 to 100 μ g in 1.0 ml of RPMI 1640-10% FBS for 24 h.

Macrophage activation. (i) **Toxoplasmicidal activity.** Control macrophages and macrophages treated with 100 μ g of LAM for 24 h were washed extensively in PBS and activated by culture for 18 h in 1.0 ml of RPMI 1640-10% FBS

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containing 140 U of recombinant mouse IFN- γ (supplied by Michael Shepard, Genentech, Inc., San Francisco, Calif.) supplemented with 1.0 ng of endotoxin (*Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.). Following IFN- γ treatment, the activation state of macrophage monolayers was assessed by the ability to restrict the intracellular growth of *Toxoplasma gondii* (29, 30). Data were expressed as mean numbers of *T. gondii* per infected macrophage at 20 h postchallenge.

(ii) **Tumoricidal activity.** Control macrophages and macrophages treated with 100 μ g of LAM per ml for 24 h were washed extensively in PBS and activated for 6 h with 140 U of IFN- γ in 0.5 ml of RPMI 1640-10% FBS. Following IFN- γ treatment, monolayers were washed and challenged with 1×10^5 ^{51}Cr -labeled EL-4 target cells (200 Ci/g; New England Nuclear Corp., Boston, Mass.) in 1.0 ml of RPMI 1640-10% FBS containing 1.0 ng of endotoxin. At 24 h postchallenge, tumoricidal activity was measured as the increase in release of soluble ^{51}Cr above that observed from ^{51}Cr -labeled target cells cultured in the absence of macrophages (17). Data were expressed as percentages of total ^{51}Cr label released from 1×10^5 EL-4 cells lysed in 0.1% sodium dodecyl sulfate.

Immunofluorescence. Control peritoneal macrophage monolayers, monolayers treated with 100 μ g of LAM per ml, and peritoneal macrophages or foot pad granuloma macrophages from *M. leprae*-infected *nu/nu* mice were cultured on Lab-Tek chamber slides in RPMI 1640-10% FBS. Monolayers were washed in PBS and fixed overnight in 2% paraformaldehyde at 4°C. In addition, some monolayers were washed in PBS at 4°C and remained unfixed prior to staining. Monolayers were stained by indirect immunofluorescence at 4°C as follows. Primary antibodies consisted of normal mouse sera or LAM-specific monoclonal antibody (MAb) 906 (14) diluted 1:10 in PBS-1% FBS. Secondary antisera consisted of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Tago, Inc., Burlingame, Calif.) diluted 1:20 in PBS-1% FBS. Monolayers were washed in PBS, mounted in PBS-glycerol, and examined with an epifluorescence microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.).

Macrophage phagocytosis and metabolism. (i) **Viability.** The viability of macrophage monolayers treated with LAM was evaluated by vital staining with ethidium bromide and acridine orange and examination with an epifluorescence microscope (23).

(ii) **Phagocytosis.** Control and LAM_{tb}-treated macrophage monolayers were cultured on LUX cover slips in 24-well plates. Monolayers were challenged with 1×10^6 heat-killed *Candida albicans* cells suspended in 1.0 ml of RPMI 1640-10% FBS for 1 h at 37°C. Extracellular *C. albicans* cells were removed by rinsing, and monolayers were fixed, stained, and examined microscopically for uptake of *Candida* cells. Data were expressed as mean numbers of *Candida* cells per macrophage.

(iii) **Metabolism.** Control and LAM_{tb}-treated macrophage monolayers were cultured on LUX cover slips and incubated for 24 h at 37°C in RPMI 1640-10% FBS containing 1.0 μ Ci of [4,5- ^3H]leucine (5.0 Ci/mmol; New England Nuclear) or 1.0 μ Ci of [U- ^{14}C]glucose (5.0 mCi/mmol; New England Nuclear). Cover slips were rinsed extensively in PBS, and incorporation was counted by liquid scintillation spectroscopy. In some cases, cover slips were treated with 10% trichloroacetic acid, and incorporation was determined from the trichloroacetic acid precipitate. As values of total disintegrations per minute did not differ by more than 10% from

trichloroacetic acid-precipitable counts, total disintegrations per minute are reported.

(iv) **PGE₂ production.** Control and LAM-treated macrophages were cultured in 24-well cluster plates containing 1.0 ml of RPMI 1640-10% FBS. Supernatants collected after 24 or 48 h of culture were clarified by centrifugation at $10,000 \times g$ for 10 min and stored at -70°C. For analyses, 0.1-ml portions were incubated at 37°C and pH 10.0 for 24 h to convert PGE₂ to its stable end product, bicyclo-PGE₂. Bicyclo-PGE₂ was quantified for triplicate samples by using a radioimmunoassay kit (New England Nuclear) (30).

RESULTS

Macrophage activation. The effects of LAM_{tb} or LAM_{ml} on macrophage activation were assessed by evaluating the enhanced ability of IFN- γ -treated macrophages to restrict the growth of *T. gondii* and to mediate enhanced cytotoxicity for tumor target cells. Normal macrophages treated with IFN- γ demonstrated an enhanced ability to restrict the intracellular growth of *T. gondii* (Fig. 1). In contrast, macrophages that were pretreated with LAM_{tb} or LAM_{ml} were not activated to restrict the growth of *T. gondii* after IFN- γ treatment (Fig. 1). Similarly, whereas normal macrophages treated with IFN- γ demonstrated significant enhancement of cytotoxicity for ^{51}Cr -labeled tumor cell targets, LAM_{tb}- or LAM_{ml}-treated macrophages were not activated by IFN- γ treatments (Fig. 2).

To further explore the molecular basis of inhibition of macrophage activation, LAM_{tb} was treated with alkali to remove the long-chain and short-chain acids, leaving the polysaccharide backbone with attached glycerophosphoinositol and inositol phosphate units intact (14). Macrophages treated with deacylated LAM_{tb} showed normal activation by IFN- γ as measured by cytotoxicity for tumor cells (Fig. 2).

Intracellular localization of LAM. LAM dissolved in culture media was readily accumulated in cytoplasmic vacuoles of mouse macrophages, as detected by indirect immunofluorescence of permeabilized cells specifically labeled with MAb 906 to LAM (Fig. 3a and c). There was no indication of

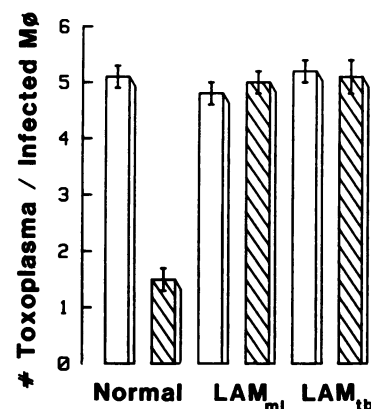


FIG. 1. Failure of LAM_{ml}- or LAM_{tb}-treated macrophages to restrict intracellular growth of *T. gondii* when stimulated with doses of IFN- γ that readily activated normal macrophages. In the absence of IFN- γ stimulation, LAM treatments did not affect the intracellular growth of *T. gondii*. Values represent means \pm standard deviations ($n = 3$) from a representative of five similar experiments. Symbols: □, unstimulated cells; ▨, IFN- γ -stimulated cells. #, Number of; M ϕ , macrophage.

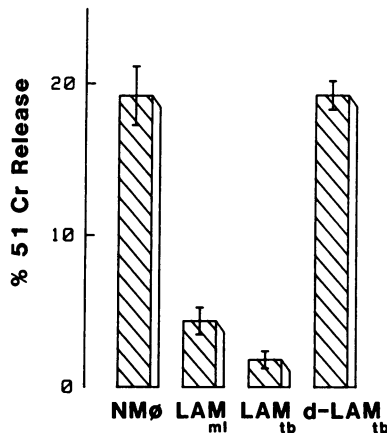


FIG. 2. Failure of LAM_{ml}- or LAM_{tb}-treated macrophages to display enhanced tumoricidal reactivity when treated with doses of IFN- γ that readily activated normal macrophages (NM ϕ). The capability of LAM_{tb} to block IFN- γ -mediated activation depended on the presence of acyl chains and was not observed with deacylated LAM_{tb} (d-LAM_{tb}). Values represent percentages of total ⁵¹Cr release (\pm standard deviations; $n = 3$) from IFN- γ -treated macrophages above that observed from ⁵¹Cr-labeled EL-4 target cells cultured alone ($\leq 22\%$). Data are from a representative of four similar experiments. In the absence of IFN- γ treatments, LAM did not affect the spontaneous rate of ⁵¹Cr release.

surface exposure of LAM on unfixed macrophages analyzed by indirect immunofluorescence with MAb 906 (data not shown). MAb 906 did not label normal macrophages (Fig.

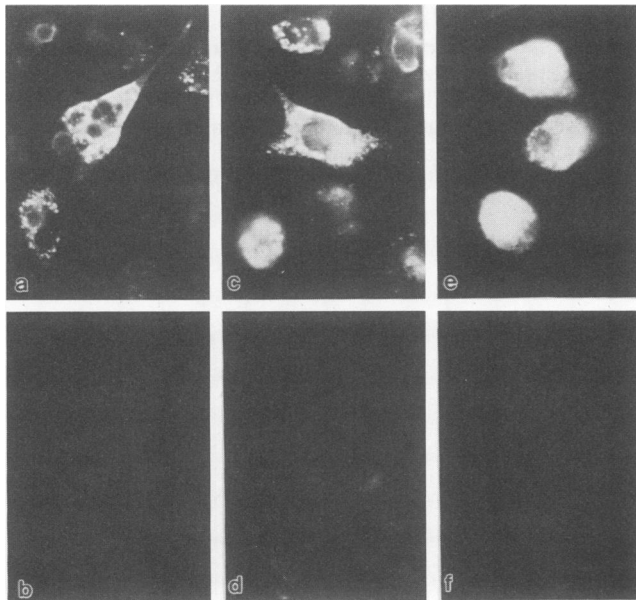


FIG. 3. Immunofluorescence localization of LAM within in vitro-treated macrophages or granuloma macrophages isolated from *M. leprae*-infected nude mice by using LAM-specific MAb 906. LAM was localized within cytoplasmic vacuoles of normal macrophages treated in vitro with either LAM_{ml} (a) or LAM_{tb} (c). High levels of intracellular LAM were also detected within *M. leprae*-gorged macrophages isolated from nude mouse granulomas (e). LAM was not detected in peritoneal macrophages from these same *M. leprae*-infected nude mice (f). Controls were LAM-specific MAb 906 on normal macrophages (b) and normal mouse sera on LAM-treated macrophages (d).

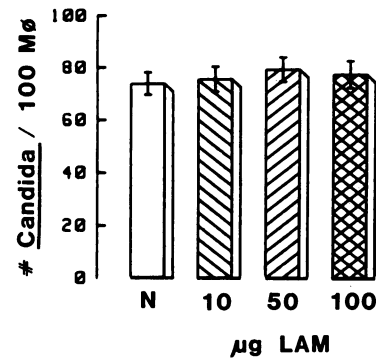


FIG. 4. Viability of LAM_{tb}-treated macrophages as evaluated by capacity to phagocytize heat-killed *Candida* cells. No difference in phagocytosis was observed between normal macrophages (N) and those treated with various doses of LAM_{tb}. Values represent means \pm standard deviations ($n = 3$) from a representative of three similar experiments. #, Number of; M ϕ , macrophages.

3b), and LAM-treated macrophages incubated with normal mouse sera did not show immunofluorescence labeling (Fig. 3d). LAM-specific MAb 906 strongly labeled granuloma macrophages isolated from *M. leprae*-infected *nu/nu* mice, demonstrating the presence of intracellular LAM (Fig. 3e). In contrast, peritoneal macrophages from these same *M. leprae*-infected mice were not labeled by MAb 906 (Fig. 3f).

Effects of LAM on macrophage viability and metabolism. LAM_{tb} treatment also did not affect macrophage viability as determined by vital staining with ethidium bromide and acridine orange (data not shown). LAM_{tb} treatments did not affect the ability of macrophages to phagocytize *C. albicans* (Fig. 4) or the metabolic utilization of [¹⁴C]glucose or [³H]leucine by macrophages (Fig. 5). Unstimulated control and LAM-treated macrophages produced similar levels of ≤ 100 pg of PGE₂ per 10⁶ cells in 24 to 48 h of culture. In comparison, RPMI 1640-10% FBS contained ≤ 20 pg of PGE₂ per ml.

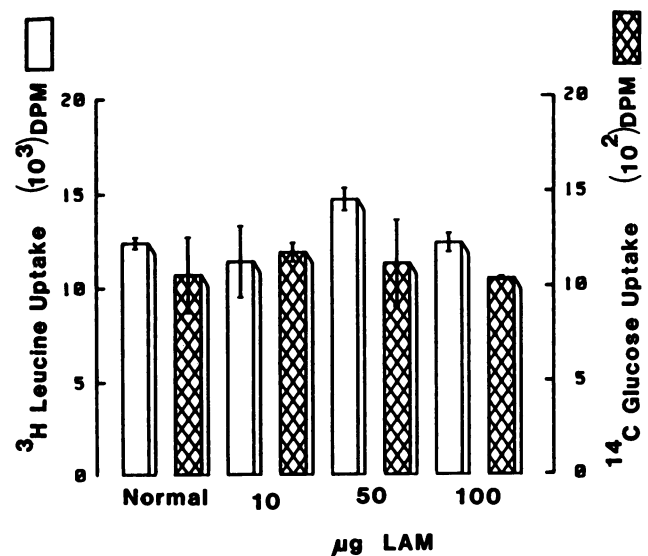


FIG. 5. Inability of LAM_{tb} treatment to affect 24-h metabolic utilization of [³H]leucine (\square) or [¹⁴C]glucose (\boxtimes) by treated macrophages compared with that observed in normal macrophages. Values represent means \pm standard deviations ($n = 3$) from a representative of three similar experiments.

DISCUSSION

The prominent glycolipid-containing capsules of *Mycobacterium* spp. are thought to protect the bacilli from intracellular degradation by forming an indigestible hydrophobic interface between the bacillus and host cell (1, 5). The present report demonstrates that LAM, one major mycobacterial surface component, may contribute directly to intracellular survival by inhibiting IFN- γ -mediated activation. Such inhibition required intracellular accumulation of LAM and was evident only with intact LAM, a finding that stresses the importance of the acyl groups in conferring inhibitory activity. The acyl groups consist of palmitate, succinate, lactate, and 10-methyloctadecanoate (14), which are unlikely to be inhibitory when found as free acids in solution. The precise structural features of LAM that inhibit IFN- γ activation remain to be delineated by further study.

In solution, LAM forms micelles of approximate M_n of 10^6 (14); thus, the extracellular concentration of LAM necessary to block activation was approximately 10^{-7} M. We have subsequently shown that the inhibitory effect of LAM required pretreatment and was not evident when LAM was added simultaneously or following IFN- γ (L. D. Sibley et al., manuscript submitted). Treatment of macrophages with this dose of LAM did not induce PGE₂ production, nor did it affect macrophage viability or metabolism. In contrast, other bacterial lipopolysaccharides are toxic at this dose and dramatically affect macrophage carbohydrate metabolism at low doses (10, 11, 27). While the present study does not directly address the mechanism of LAM inhibition, the study shows that this block of IFN- γ responsiveness evidently involves interruption in the transduction of the IFN- γ signal. The cellular basis of this block is under investigation.

Collectively, our studies of the functional capacity of mouse macrophages harboring *M. leprae* or *M. leprae* products indicate that defective macrophage activation is a prominent feature of lepromatous leprosy that depends on highly localized conditions occurring within macrophage-rich granulomas that contain numerous bacilli (29, 30; Sibley and Krahenbuhl, submitted for publication). It is likely that the specific inhibition of macrophage activation by purified LAM in vitro has important parallels in vivo under localized conditions in which lesions contain numerous *M. leprae*, as LAM is an abundant component of *M. leprae* (15 mg/g; 14). In support of this hypothesis, we have found that *M. leprae*-burdened macrophages isolated from lepromatous granulomas in the nude mouse contain sizable quantities of LAM, a condition contributing to the defective activation of these granuloma macrophages by IFN- γ (29, 30). On the other hand, LAM was not detected in peritoneal macrophages from these same mice, a finding which is consistent with the normal activation of these macrophages by IFN- γ (29, 30). These findings are underscored by recent evidence that when macrophages are preactivated by IFN- γ and then challenged with viable *M. leprae*, they have an enhanced ability to inhibit intracellular metabolism and survival of the bacilli (12, 28; N. Ramasesh et al., manuscript in preparation), consistent with the enhanced nonspecific microbicidal capacity of the macrophages (18, 21, 26). Thus, it is evident that the timing of inhibitory and activating signals, as well as localized conditions, can influence the functional state of the macrophage.

LAM_{tb} and LAM_{ml} inhibit the antigen responsiveness of peripheral blood leukocytes from both lepromatous patients and control subjects (15). Although there is some evidence for a role of T₈⁺ suppressor cells in this system (15), the role

of the macrophage in mediating this suppression was not addressed. However, previous reports have demonstrated that arabinomannan purified from *M. tuberculosis* restricts similar afferent macrophage functions in vitro (7). Consequently, it seems likely that LAM may also inhibit macrophage afferent functions.

The similarity in the mechanisms of IFN- γ -mediated activation of mouse and human macrophages (2, 3, 21) suggests that intracellular accumulation of LAM in human macrophages may lead to defects in macrophage activation similar to those reported here in mouse macrophages. In ongoing studies, we have obtained preliminary evidence that LAM treatment of human monocyte-derived macrophages blocks activation by recombinant human IFN- γ . Although activation by IFN- γ is a prominent mechanism of mouse macrophage-mediated inhibition of mycobacteria, vitamin D analogs seem more effective in activating human monocytes and macrophages to inhibit *M. tuberculosis* (25). Whether LAM also inhibits such alternate pathways of macrophage activation remains to be determined.

Conditions that fulfill the essential features for defective macrophage activation in the experimental mouse model (i.e., granulomatous lesions characterized by high levels of bacilli) are also found in lepromatous leprosy in man (24). Although numerous reports substantiate the finding that peripheral blood monocytes of leprosy patients have normal microbicidal capacity (6) and are responsive to IFN- γ (13, 16, 22), the influence of local conditions on the functional capacity of tissue macrophages from lepromatous leprosy patients remains to be examined. In addition, acute dissemination of opportunistic infections with *M. tuberculosis* and *M. avium-M. intracellulare* in immunocompromised (e.g., AIDS) patients also leads to high tissue burdens of bacilli (4), a condition that may restrict local macrophage function. Consequently, the ability of purified LAM to inhibit both afferent and efferent macrophage functions may be a relevant component in the pathogenesis of such chronic mycobacterial infections in humans.

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