INFECTION AND IMMUNITY, May 1991, p. 1755–1761
0019-9567/91/051755-07$02.00/0

Vol. 59, No. 5

Lipoarabinomannan, a Possible Virulence Factor Involved in Persistence of *Mycobacterium tuberculosis* within Macrophages

JOHN CHAN,[1] XUEDONG FAN,[2]† SHIRLEY W. HUNTER,[3] PATRICK J. BRENNAN,[3] AND BARRY R. BLOOM[2]*

Department of Medicine, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103[1]; Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461[2]; and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523[3]

Received 26 November 1990/Accepted 26 February 1991

***Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively, produce large quantities of lipoarabinomannan (LAM), a highly immunogenic, cell wall-associated glycolipid. This molecule has been previously reported to be a potent inhibitor of gamma interferon-mediated activation of murine macrophages. Studies of the mechanism by which this mycobacterial glycolipid down-regulates macrophage effector functions provide evidence that LAM acts at several levels and that it can (i) scavenge potentially cytotoxic oxygen free radicals, (ii) inhibit protein kinase C activity, and (iii) block the transcriptional activation of gamma interferon-inducible genes in human macrophage-like cell lines. These results suggest that LAM can inhibit macrophage activation and triggering and cytocidal activity and that it may represent a chemically defined virulence factor contributing to the persistence of mycobacteria within mononuclear phagocytes.**

Over a century ago, Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis (TB) (20). At the time, TB was rampant, causing one-seventh of all deaths in Europe and one-third of deaths among productive young adults (6). Today, TB remains a global health problem of enormous dimension. It is estimated that there are 1 billion persons infected worldwide, with 8 million new cases and 3 million deaths per year (33). In the United States, the steady decline of TB incidence since 1953 has recently reversed (3). This increase in the number of persons with disease caused by *M. tuberculosis* is primarily the result of the human immunodeficiency virus (HIV) epidemic. Available statistics indicate that a close association exists between AIDS and TB (3). Therefore, further spread of HIV infection among populations with a high prevalence of TB infection is resulting in dramatic increases in TB, particularly in Africa.

Regrettably, little is currently understood about the mechanisms of the pathogenicity of mycobacteria. It remains paradoxical that *M. tuberculosis* and *Mycobacterium leprae* can survive and replicate inside mononuclear phagocytes that are specialized to contain potent cytocidal mechanisms and constitute the major effector system in cellular immunity (13, 24). The best-understood cytocidal mechanism of activated macrophages is the oxygen-dependent system that provides reactive oxygen intermediates, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and singlet oxygen (17, 19, 29). We and others have recently provided evidence that microbial glycolipids may play a role in the pathogenicity and virulence of intracellular pathogens by scavenging toxic oxygen radicals (4, 25). Our results demonstrated that phenolic glycolipid-I and lipophosphoglycan (LPG), the major glycolipids of two intracellular pathogens, *M. leprae* and *Leishmania donovani*, respectively, scavenged oxygen free radicals effectively (4). Recent advances made in understanding the highly complex chemistry of the cell wall of *M. tuberculosis* have afforded us the opportunity to test this principle with lipoarabinomannan (LAM), a major cell wall-associated glycolipid produced by *M. tuberculosis* and *M. leprae* in large amounts (15 mg/g of bacteria) (16). LAM is a complex glycolipid consisting of repeating saccharide units of arabinose and mannose linked to a phosphatidylinositol moiety. This phospholipid moiety, with palmitate and tuberculostearate (10-methyloctadecanoate) as the major acyl groups, apparently attaches LAM to the cytoplasmic membrane of the bacilli (16). In the present study, we provide evidence that LAM may represent a virulence factor for the pathogenic mycobacteria that can provide protection at several levels from the antimicrobial activities of mononuclear phagocytes.

MATERIALS AND METHODS

Glycolipids. LAM and deacylated LAM (d-LAM) were prepared from *M. tuberculosis* as previously described (16).

Cell culture. The human macrophage-like cell line U937, derived from cells of a patient with histiocytic lymphoma (34), was provided by N. Hogg (Imperial Cancer Research Fund, London). U937 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml), and nonessential amino acids (100 μM). THP-1 cells, derived from a patient with monocytic leukemia (35), were obtained from the American Type Culture Collection (Rockville, Md.). THP-1 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml).

Reagents. H7 [1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine dihydrochloride] was obtained from Seikagaku America (St. Petersburg, Fla.). Recombinant gamma interferon (IFN-γ), generously provided by Genentech (South San Francisco, Calif.), had a specific antiviral titer of 2×10^7 to 4×10^7 units/mg against encephalomyocarditis (EMC) virus in human lung carcinoma A549 cells. Phosphatidylserine, di-

* Corresponding author.
† Present address: Schering Plough Research, Bloomfield, NJ 07003.

olein, histone (type III), *Escherichia coli* lipopolysaccharide (LPS), soybean L-α-phosphatidylinositide, xanthine, xanthine oxidase, dimethyl sulfoxide (DMSO), cytochrome *c*, superoxide dismutase, and catalase were purchased from Sigma. The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Aldrich. DMPO was dissolved in distilled-deionized water (final concentration, 1 M), purified, and stored as previously described (30). Iron-free phosphate-buffered saline (pH 7.8), glassware, and pipette tips were prepared as previously described (30).

Generation of hydroxyl radical (OH·). OH· was generated by the iron-catalyzed Haber-Weiss-Fenton reaction, in the presence of DMSO, as previously described (4, 12). DMSO (10%, vol/vol) was included in the radical-generating reactions because it reacts with the short-lived OH· to form the more stable carbon-centered methyl radical (CH_3·) (28). In all experiments, xanthine, xanthine oxidase, and Fe^{2+} were used at concentrations of 0.3 mM, 0.08 unit/ml, and 0.05 mM, respectively.

ESR studies. Electron spin resonance (ESR) spectroscopy and spin trapping were used to measure OH· as previously described (4). Since DMSO was included in the OH·-generating reaction, the relative concentrations of OH· were measured as a function of the control ESR signal of the spin adduct DMPO/CH_3·. The reactions generating OH· were carried out at room temperature in a 1.2-mm-bore capillary tube at a final volume of 50 μl. The spin trap DMPO was used at a final concentration of 0.05 M. ESR spectra were recorded with a Varian E-9 ESR spectrometer at room temperature 2 and 10 min after initiation of the reaction by the addition of xanthine oxidase. The 10-min spectra were compared. Preliminary experiments showed that the peak height of the ESR spectrum was proportional to the area under the curve. Since the latter reflects the amount of radicals present, all signal intensities were estimated from peak-to-peak amplitudes. In all experiments, the height of the first peak was chosen for comparisons. The instrument parameters were as follows: field set, 3,430 G; scan range, 200 G; modulation frequency, 100 kHz; modulation amplitude, 1 G; receiver gain, 3.2×10^3; time constant, 1 s; microwave frequency, 9.557 GHz; microwave power, 20 mW; scan time, 16 min.

Spectrophotometric measurement of superoxide anion (O_2^-). O_2^- generated by the xanthine-xanthine oxidase system was measured by the superoxide dismutase-inhibitable cytochrome *c* reduction assay as described previously (4). At 35 s after initiation of the reaction at room temperature by the addition of xanthine oxidase, the A_{550} was recorded every second for at least 20 min by using a double-beam spectrophotometer.

Uric acid measurements. Uric acid production from xanthine was measured with the Sigma diagnostic kit (4).

Assay of PKC activity. Cytosolic preparations of protein kinase C (PKC) were prepared from U937 cells (7). PKC activity was assayed as previously described (21), with modification. A typical reaction is carried out for up to 15 min at room temperature, in a final volume of 130 μl. The reaction mixture contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 60 μg of phosphatidylserine per ml, 6 μg of diolein per ml, 5 mM dithiothreitol, 1 mg of histone type III per ml, 100 μM ATP, 5 μCi of [γ-^{32}P]ATP (specific activity, 5,000 Ci/mmol), and an appropriate amount of PKC (determined by preliminary standardization experiments). Reactions were initiated by the addition of [γ-^{32}P]ATP. At various time points, 40 μl of reaction mix was pipetted onto Whatman p-81 phosphocellulose paper. The paper was then washed with distilled water and assayed for radioactivity in 3 ml of Liquisint. PKC activity was measured in terms of the amount of [γ-^{32}P]ATP incorporated into the substrate histone, via the kinasing property of PKC.

Transcriptional activation of IFN-γ-regulated genes. U937 cells were cultured (5×10^5 cells per ml) at 37°C for 24 h in the presence of 100 to 150 μg of LAM or d-LAM per ml. Control cultures contained no glycolipids. Cells were then exposed to 10 units of IFN-γ per ml for 4 h in the continued presence of LAM or d-LAM. The dose of, as well as the time of exposure to, IFN-γ chosen for the induction of gene γ.1 was based on kinetics experiments done previously (7). RNA was then isolated by the guanidine isothiocyanate method (5), electrophoresed (25 μg per lane) on 1.2% agarose gels containing 1 M formaldehyde, and transferred to nylon filters. The blots were hybridized with nick-translated γ.1- and actin-specific cDNAs (specific activity, 2×10^8 to 3×10^8 cpm/μg at 2×10^6 cpm/ml), washed, and then subjected to autoradiography with Kodak XAR film as previously described (7). The effect of LAM and d-LAM on the IFN-γ induction of HLA-DRβ mRNA in THP-1 cells was analyzed in a similar manner.

RESULTS

Ability of LAM to scavenge oxygen free radicals in vitro. ESR spectroscopy and spin trapping (using the spin trap DMPO) were used to determine whether LAM could function as a scavenger of oxygen radicals. Our results (Fig. 1A) indicated that LAM effectively attenuates the control ESR signal of the DMPO/CH_3· spin adduct generated from OH· in the presence of DMSO in a dose-dependent manner. LAM attenuated 52 and 100% of the control ESR signal at concentrations of 4.8×10^{-4} M and 9.6×10^{-4} M, respectively (Fig. 1A). By way of comparison, benzoate, a commonly used OH· scavenger, attenuated only 28% of the control ESR signal at a much higher concentration, 0.05 M (data not shown). d-LAM, obtained by mild alkaline hydrolysis of LAM (16), which contains the basic polysaccharide but lacks the aliphatic hydrocarbon side chains, also attenuates the control ESR signal effectively (Fig. 1B), indicating that this property of LAM is likely to be a function of the carbohydrate moiety. Interestingly, d-LAM appears to be a better OH· scavenger than LAM when compared at equal molar concentrations (Fig. 1).

The ability of LAM to attenuate the ESR signal could be due to any of the following mechanisms. (i) LAM is a potent scavenger of OH·. (ii) LAM is a potent scavenger of O_2^-. (iii) LAM inhibits the oxygen radical-generating enzyme xanthine oxidase. The last possibility was formally excluded when we observed no decrease in uric acid production by the xanthine-xanthine oxidase system in the presence of LAM at a concentration that attenuated the control ESR signal by 50% (data not shown), indicating that the glycolipid had no effect on the radical-generating enzyme system. To examine whether LAM scavenges O_2^-, we measured the effect of LAM on superoxide dismutase-inhibitable cytochrome *c* reduction by O_2^-. Results (Fig. 2) indicate that LAM indeed scavenges O_2^- effectively. Since O_2^- is required for the formation of OH· in the Haber-Weiss-Fenton reaction, our data do not indicate whether LAM scavenges OH· directly or indirectly through its ability to scavenge O_2^-. In keeping with the results obtained with OH· scavenging, removal of the acyl groups did not affect the ability of LAM to scavenge

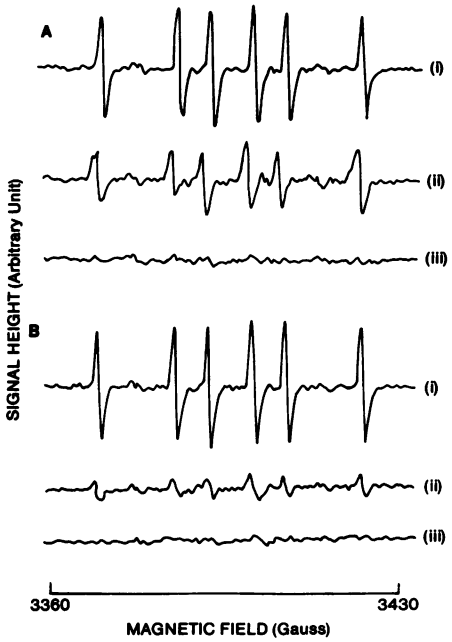

FIG. 1. Scavenging of OH· by LAM. (A) Control ESR spectrum (i) of the DMPO/CH_3· adduct generated by spin trapping CH_3· produced by the Haber-Weiss-Fenton reaction in the presence of 10% (vol/vol) DMSO, by using the spin trap DMPO. ESR spectra of the DMPO/CH_3· adduct in the presence of 4.8×10^{-4} M (ii) and 9.6×10^{-4} M (iii) LAM show 52 and 100% attenuation of the control signal, respectively. The spectra shown are representative of two experiments. (B) Scavenging of HO· by d-LAM. Control ESR spectrum (i) obtained as described for spectrum i in panel A. ESR spectra of the DMPO/CH_3· adduct in the presence of 2.4×10^{-4} M (ii) and 4.8×10^{-4} M (iii) d-LAM.

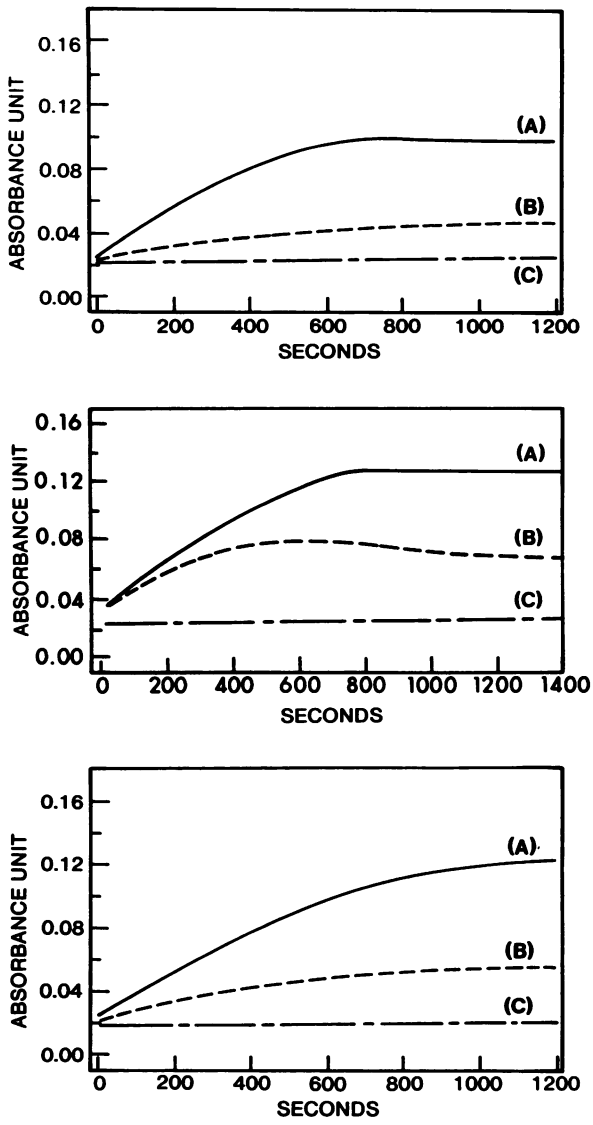

FIG. 2. LAM and d-LAM scavenge O_2^- in the cytochrome *c* reduction assay. Results from three experiments are shown here. (A) Superoxide dismutase-inhibitable cytochrome *c* reduction in a control reaction containing 0.1 mM xanthine, 0.02 unit of xanthine oxidase per ml, 1 mg of cytochrome *c* per ml, 10 μg of catalase per ml, 1.2 μg of superoxide dismutase per ml, and 10% (vol/vol) DMSO in phosphate-buffered saline. (B) Reaction with LAM (4.8×10^{-4} M). (C) Reaction with d-LAM (4.8×10^{-4} M). LAM and d-LAM attenuated the maximum A_{550} of control reactions by 62 ± 5% and 95.3 ± 1.5%, respectively.

O_2^-; indeed, d-LAM scavenges O_2^- more effectively than LAM does.

Two lines of evidence indicate that the ability of LAM to scavenge oxygen radicals is a specific property of this glycolipid. (i) *E. coli* LPS, the microbial glycolipid used as controls in these studies, failed to exhibit any O_2^--scavenging ability at concentrations ranging from 1 ng/ml to 7 mg/ml (Fig. 3). (ii) The scavenging activity of LAM is structure dependent and can be localized to the carbohydrate moiety of the glycolipid.

Ability of LAM to inhibit PKC activity in vitro. PKC has recently been shown to be an important regulatory element in the signal transduction pathway involved in macrophage activation (14). In particular, PKC is believed to be critical for activating the respiratory burst in phagocytic cells (10, 11, 26, 32) and for mediating the effects of IFN-γ in activating macrophages (7, 14, 15). Recently, work from this laboratory and others has suggested that the LPG of *L. donovani*, a molecule structurally similar to LAM, may play an important protective role for the parasite within the host macrophages by being a potent inhibitor of PKC (22). Consequently, we tested the ability of LAM to inhibit macrophage-derived PKC in an in vitro assay. The PKC inhibitor H7 was used as a positive control in these assays. Our data indicate (Fig. 4) that LAM effectively inhibits PKC activity in a dose-dependent manner, producing 80% inhibition at a concentration of 270 μM. This PKC-inhibiting activity of LAM is not likely to be a nonspecific property of glycolipids in general, because *E. coli* LPS (at concentrations ranging from 1 ng/ml to 1 mg/ml) and soybean L-α-phosphatidylinositide (at concentrations ranging from 30 to 270 μM) produced no inhibitory effect (data not shown). Of particular interest was the finding that, in contrast to the oxygen radical-scavenging activity of LAM, which is completely independent of the acyl functions, removal of the acyl groups of LAM completely abolished its ability to inhibit PKC (Fig. 4).

Effect of LAM on the transcriptional activation of IFN-γ-regulated genes. IFN-γ, a product of activated T-lymphocytes, is regarded as the principal lymphokine for inducing

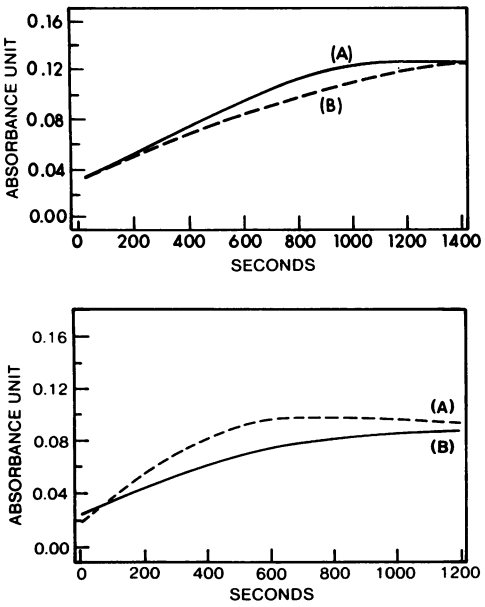

FIG. 3. *E. coli* LPS is ineffective in scavenging O_2^-. Results from two experiments are shown here. (A) Control reaction set up as described in the legend to Fig. 2. (B) Reaction in the presence of 7 mg of LPS per ml. The ineffectiveness of LPS in scavenging O_2^- was also demonstrated at concentrations of 3 mg/ml, 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, and 1 ng/ml.

enhanced competence of macrophages to destroy microbes and tumor cells (1). Recent data have shown that IFN-γ modulates PKC activity in the activation of murine peritoneal macrophages (15). In addition, we have shown that PKC plays a key role in the signal transduction pathway leading to transcriptional activation of IFN-γ-regulated genes of cells of the mononuclear phagocyte lineage (7). Because our data indicated that LAM is a potent PKC inhibitor in vitro, we examined the effects of LAM in vivo on the transcriptional activation of a recently described macrophage- and endothelial cell-specific, IFN-γ-regulated gene (γ.1) in U937 cells after treatment with IFN-γ (8). LAM

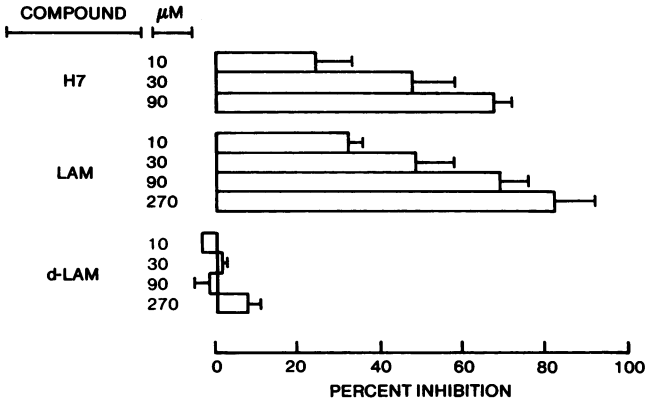

FIG. 4. LAM inhibits PKC activity in vitro. The experiments were repeated twice for LAM and once for d-LAM. Results shown here are means with standard errors. Percent inhibition of PKC activity was expressed as follows: {1 − ([^{32}P]ATP incorporated in control reactions in counts per minute)/([^{32}P]ATP incorporated in reactions with LAM or d-LAM)} × 100.

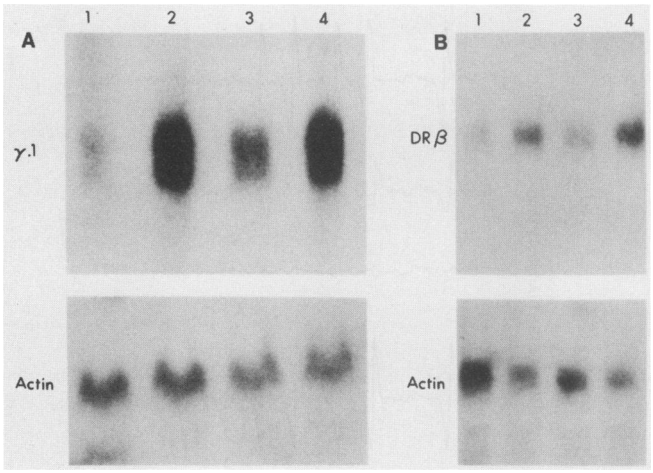

FIG. 5. (A) Effect of LAM on the transcriptional activation of γ.1 in the human macrophage-like cell line U937. Lanes: 1, RNAs from unstimulated cells; 2, RNAs from IFN-γ-stimulated cells; 3 and 4, RNAs from IFN-γ-stimulated cells in the presence of LAM and d-LAM, respectively. Data shown are representative of two experiments. (B) Studies on the effect of LAM on the induction of HLA-DRβ mRNAs in THP-1 cells confirm the inhibitory effect of LAM on the transcriptional activation of IFN-γ-inducible genes. Lane assignment is the same as that in panel A.

markedly restricted the IFN-γ-activated transcription of γ.1 (Fig. 5A). This effect cannot be attributed merely to a toxic effect of LAM on macrophages, because the viabilities of cells with and without exposure to LAM were comparable (>95%). In other studies, LAM did not affect the viability of human peripheral mononuclear cells (18) or the viability and metabolism (phagocytosis, leucine and glucose utilization, and prostaglandin E_2 production) of mouse peritoneal macrophages (31). Finally, this inhibitory effect of LAM on the transcriptional activation of γ.1 is specific since it does not inhibit the synthesis of actin message (Fig. 5). Consistent with its PKC-inhibiting activity, the ability of LAM to restrict transcriptional activation of γ.1 in U937 cells was completely dependent on the presence of the acyl groups (Fig. 5A). In contrast to the inhibitory effect of LAM, our studies done on the transcriptional regulation of γ.1 mRNA showed that endotoxin LPS from *E. coli* enhances the expression of this message in U937 cells (Fig. 6).

To learn whether the ability of LAM to inhibit IFN-γ-

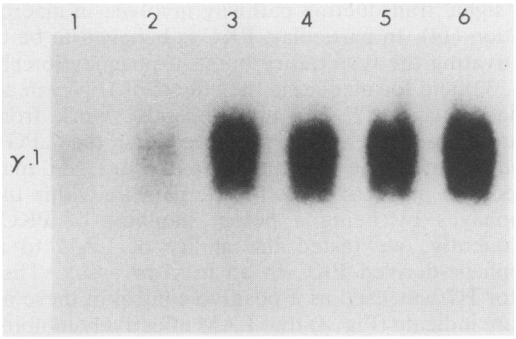

FIG. 6. Effect of LPS on the induction of γ.1 mRNA in U937 cells. Lane 1, RNAs from unstimulated cells; lanes 2 to 6, RNAs from cells treated with LPS at concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml, respectively.

induced transcriptional activation of γ.1 has broader implications in the regulation of other genes inducible by IFN-γ, we studied the effect of LAM on the induction of HLA-DRβ, which can be expressed in another human macrophage cell line, THP-1. We chose to study the activation of HLA-DRβ because it is a major histocompatibility gene known to be regulated by IFN-γ and plays a pivotal role in antigen presentation to T cells. Results in Fig. 5B indicate that LAM inhibits the induction of HLA-DRβ mRNA by IFN-γ. These results further suggest that the inhibitory effects of LAM on macrophages demonstrated by the present study are not merely nonspecific properties of any microbial glycolipid but rather may be important functional attributes of this specific mycobacterial glycolipid.

DISCUSSION

The mechanisms by which intracellular pathogens in general, and pathogenic mycobacteria in particular, evade the microbicidal effector functions of mononuclear phagocytes are poorly understood. Although the cell wall of the tubercle bacillus has long been implicated in the virulence and pathogenicity of this global pathogen (for a review, see reference 2), direct evidence for cell wall involvement has not been forthcoming because of the considerable difficulties in elucidating the highly complex cell wall architecture of the mycobacterium. The recent identification, characterization, and purification of a variety of mycobacterial cell wall components now permit the beginnings of a systematic evaluation of the possible role of these compounds in the persistence of *M. tuberculosis* and *M. leprae* inside macrophages. One hopes that these chemical and in vitro approaches will soon be supplemented with genetic approaches in producing defined mutations to allow analysis of pathogenicity in vivo.

LAM, a recently identified cell wall-associated glycolipid of *M. tuberculosis* and *M. leprae*, is produced in prodigious amounts and secreted by the bacilli and is highly immunogenic in patients with TB and leprosy (16). The present study provides evidence that this molecule effectively inhibits effector functions of macrophages by (i) scavenging toxic oxygen free radicals, (ii) inhibiting PKC activity, and (iii) restricting transcriptional activation of the IFN-γ-inducible genes, γ.1 and HLA-DRβ. While objections have been raised (23) that the suppressive effects of LAM on T-cell proliferation may be secondary to the presence of bacterial LPS contaminating the LAM preparation, polymyxin B treatment failed to adsorb all the suppressive activity of *M. leprae* soluble filtrate and did not affect the suppressive activity of LAM. These data suggest that LAM must have suppressive activity in the absence of contaminating LPS (23). In the present study, *E. coli* LPS was used as a control and consistently failed to scavenge oxygen radicals and to inhibit PKC activity. Moreover, in contrast to the inhibitory effect of LAM on the transcriptional activation of γ.1, an IFN-γ-inducible gene, LPS actually enhanced transcription of this gene.

The ability of LAM to scavenge oxygen free radicals is in keeping with the finding that mouse peritoneal macrophages heavily burdened with *M. leprae*, which is known to produce LAM, have a significantly decreased capacity to generate O_2^- upon stimulation with IFN-γ (32). Also, the inability of IFN-γ-stimulated macrophages from both humans and mice to effectively kill *M. tuberculosis* in vitro (9, 27) may be secondary to this oxygen radical-scavenging property of LAM. An unexpected finding was that d-LAM was a better oxygen radical scavenger than LAM. Although the exact mechanism(s) for this observation is presently unknown, it could be due to the fact that LAM (estimated M_r, 20,000), because of its fatty acyl functions, readily forms micelles in aqueous solution, with an approximate M_r of 10^6 (16), thus lowering the actual effective concentration of the glycolipid in radical scavenging. In addition, deacylation of LAM also results in the removal of the short-chain acids, succinate and lactate, that are directly attached to the carbohydrate portion of the glycolipid (16). Thus, it is conceivable that substitution of the short-chain acyl functions in the polysaccharide moiety of the glycolipid with a hydroxyl group renders d-LAM a more potent oxygen radical scavenger than LAM.

The ability of LAM to inhibit PKC activity and to restrict IFN-γ induction of cellular genes implies that the effects of LAM on human macrophages are likely to be complex, because (i) IFN-γ is the principal factor responsible for initiating enhanced competence of macrophages to perform numerous effector functions (1), (ii) PKC has a regulatory role in the various signal transduction pathways involved in macrophage activation (14), and (iii) HLA-DRβ, a member of the major histocompatibility complex, plays a crucial role in antigen presentation to T cells in the immune response. Sibley and Krahenbuhl (31, 32) have recently reported that resident mouse peritoneal macrophages, either infected with *M. leprae* or treated with LAM, were not responsive to IFN-γ activation as assessed by microbicidal and tumoricidal activities, O_2^- production, and surface Ia antigen expression. This phenomenon could indeed be explained by the ability of LAM to scavenge oxygen radicals, inhibit PKC activity, and restrict IFN-γ induction of macrophage gene expression at the transcriptional level. In addition, the ability of LAM to inhibit the transcriptional activation of major histocompatibility complex class II genes in macrophages could, in part, explain the well-known nonspecific inhibitory effect of LAM on T-cell proliferation by interfering with antigen presentation (18).

Of particular interest from the point of view of structure and function of the lipid molecules are the data demonstrating that while the ability of LAM to scavenge oxygen free radicals is entirely independent of the acyl groups of the phosphatidylinositol moiety, the latter are crucial to the inhibition of PKC activity as well as the transcriptional activation of IFN-γ-inducible genes. Sibley et al. have recently reported that the ability of LAM to inhibit IFN-γ activation of macrophages is also dependent on the acyl functions (31). Like LAM from *M. tuberculosis*, LPG, a glycolipid of *L. donovani* that has been suggested to play an important role in the pathogenesis of this protozoal parasite (4, 22), is also a complex glycolipid with a glycosyl phosphatidylinositol component. These findings suggest that the latter may play an important role in the pathogenic capacity of specific glycolipids of intracellular parasites in inhibiting macrophage effector functions.

Taken together, we believe that the data presented here provide substantive evidence that LAM could play an important role in the pathogenesis of leprosy and tuberculosis by down-regulating macrophage effector functions. If these data can be extrapolated to the situation in natural infection, one would predict that LAM would have the following abilities: (i) to block macrophage activation by lymphokines such as IFN-γ, which is likely to involve PKC-mediated transcriptional activation of a number of cellular genes (7, 14, 15); (ii) to inhibit the triggering of the respiratory burst upon phagocytosis, which also involves PKC as a regulatory

element in the signal transduction pathway (7, 10, 11, 26, 36); and (iii) if either of those processes were incompletely inhibited, to scavenge potentially toxic oxygen radicals with the carbohydrate moiety. If the ability of this molecule to protect *M. tuberculosis* or *M. leprae* from the oxygen-dependent microbicidal mechanisms of mononuclear phagocytes were totally efficient, then one might justifiably wonder how one ever recovers from mycobacterial infections. We offer two conjectures. First, free radical cytocidal reactions occur very quickly, and the complex lipids require days to be synthesized and secreted in significant amounts. We speculate that many mycobacteria are rapidly killed in healthy individuals immediately upon infection of macrophages but that those not killed immediately are likely to be resistant to the oxidative cytocidal mechanisms and persist. These speculations are consistent with two well-known observations. (i) Only a fraction of individuals converting to tuberculin positivity develop clinical TB. (ii) TB in older individuals most commonly derives from "secondary breakdown" of a primary infection that occurred decades previously. Second, we have evidence to suggest that non-oxygen radical-dependent cytocidal mechanisms of macrophages, such as nitric oxide (unpublished data), may be important for resistance to professional intracellular pathogens. We hope that these studies will encourage further research on the pathogenic roles of complex carbohydrates and lipids of intracellular pathogens and provide some insights into the pathogenesis of TB.

ACKNOWLEDGMENTS

We thank Philip Aisen for allowing us to use the Varian E-9 ESR spectrometer and Cynthia Bozic for the preparation of glycolipids.

This work was supported by grants AI 07118, AI 26491, AI 23545, and AI 05074 from the National Institutes of Health, the Rockefeller Foundation, the Foundation of the University of Medicine and Dentistry of New Jersey, the World Health Organization (WHO) Special Programmes for Research and Training in Tropical Diseases, and the WHO Special Programme for Vaccine Development.

REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1987. Molecular transductional mechanisms by which IFN-γ and other signals regulate macrophage development. Immunol. Rev. **97**:5–27.
2. **Brennan, P. J.** 1989. Structure of mycobacteria: recent developments in defining cell wall carbohydrates and proteins. Rev. Infect. Dis. **11**:S420–S430.
3. **Centers for Disease Control.** 1989. Tuberculosis and human immunodeficiency virus infection: recommendation of the advisory committee for the elimination of tuberculosis (ACET). Morbid. Mortal. Weekly Rep. **38**:236–250.
4. **Chan, J., T. Fujiwara, P. J. Brennan, M. McNeil, S. J. Turco, J.-C. Sibille, M. Snapper, P. Aisen, and B. R. Bloom.** 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. USA **86**:2453–2457.
5. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
6. **Daniel, T. M.** 1982. Robert Koch, tuberculosis, and the subsequent history of medicine. Am. Rev. Respir. Dis. **125**(Pt. 2):1–3.
7. **Fan, X.-D., M. Goldberg, and B. R. Bloom.** 1988. Interferon-gamma-induced transcriptional activation is mediated by protein kinase C. Proc. Natl. Acad. Sci. USA **85**:5122–5125.
8. **Fan, X.-D., G. R. Stark, and B. R. Bloom.** 1989. Molecular cloning of a gene selectively induced by gamma interferon from human macrophage cell line U937. Mol. Cell. Biol. **9**:1922–1928.
9. **Flesch, I., and S. H. E. Kaufmann.** 1987. Mycobacterial growth inhibition by interferon-γ-activated bone marrow macrophages and differential susceptibility among strains of Mycobacterium tuberculosis. J. Immunol. **138**:4408–4413.
10. **Gavioli, R., S. Spisani, A. Giuliani, and S. Traniello.** 1987. Protein kinase C mediates human neutrophil cytotoxicity. Biochem. Biophys. Res. Commun. **148**:1290–1294.
11. **Gennaro, R., C. Florio, and D. Romeo.** 1985. Activation of protein kinase C in neutrophil cytoplasts. FEBS Lett. **180**:185–190.
12. **Haber, F., and J. Weiss.** 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. London Ser. A **147**:332–351.
13. **Hahn, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
14. **Hamilton, T. A., and D. O. Adams.** 1987. Molecular mechanisms of signal transduction in macrophages. Immunol. Today **8**:151–158.
15. **Hamilton, T. A., D. L. Becton, S. D. Somers, P. W. Gray, and D. O. Adams.** 1984. Interferon-γ modulates protein kinase C activity in murine peritoneal macrophages. J. Biol. Chem. **260**:1378–1381.
16. **Hunter, S. W. H., H. Gaylord, and P. J. Brennan.** 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. **261**:12345–12351.
17. **Iyer, G. Y. N., M. F. Islam, and J. H. Quastel.** 1961. Biochemical aspects of phagocytosis. Nature (London) **192**:535–541.
18. **Kaplan, G., R. R. Gandhi, D. E. Weinstein, W. R. Levis, M. E. Patarroyo, P. J. Brennan, and Z. A. Cohn.** 1987. *Mycobacterium leprae* antigen-induced suppression on T cell proliferation in vitro. J. Immunol. **138**:3028–3034.
19. **Klebanoff, S. J.** 1980. Oxygen intermediates and the microbicidal event, p. 1105–1141. *In* R. Van Furth (ed.), *Mononuclear phagocytes, functional aspects*, part 2. Martinus Nijhoff, Dordrecht, The Netherlands.
20. **Koch, R.** 1932. Die Aetiologie der Tuberculose (a translation by B. Pinner and M. Pinner, with an introduction by A. K. Krause). Annu. Rev. Tuberc. **25**:285–323.
21. **Le Peuch, C. J., R. Ballester, and O. M. Rosen.** 1983. Purified rat brain calcium- and phospholipid-dependent protein kinase phosphorylates ribosomal protein S6. Proc. Natl. Acad. Sci. USA **80**:6858–6862.
22. **McNeely, T. B., and S. J. Turco.** 1987. Inhibition of protein kinase C activity by the *Leishmania donovani* lipophosphoglycan. Biochem. Biophys. Res. Commun. **148**:653–657.
23. **Molloy, A., G. Gaudernack, W. R. Levis, Z. A. Cohn, and G. Kaplan.** 1990. Suppression of T-cell proliferation by Mycobacterium leprae and its products: the role of lipopolysaccharide. Proc. Natl. Acad. Sci. USA **87**:973–977.
24. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49**:298–337.
25. **Neill, M. A., and S. J. Klebanoff.** 1988. The effect of phenolic glycolipid-I from Mycobacterium leprae on the antimicrobial activity of human macrophages. J. Exp. Med. **167**:30–42.
26. **Pontremoli, S., E. Melloni, F. Salamino, B. Sparatore, M. Michetti, O. Sacco, and B. L. Horecker.** 1986. Activation of NADPH oxidase and phosphorylation of membrane proteins in human neutrophils: coordinate inhibition by a surface antigen-directed monoclonal. Biochem. Biophys. Res. Commun. **140**:1121–1126.
27. **Rook, G. A. W., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis; comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology **59**:333–338.
28. **Samuni, A., A. J. Carmichael, A. Russo, J. B. Mitchell, and P. Riesz.** 1986. On the spin trapping and ESR detection of oxygen-derived radicals generated inside cells. Proc. Natl. Acad. Sci. USA **83**:7593–7597.
29. **Sbarra, A. J., and M. L. Karnovsky.** 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. **234**:1355–1362.
30. **Sibille, J.-C., K. Doi, and P. Aisen.** 1987. Hydroxyl radical

formation and iron-binding protein. J. Biol. Chem. **262**:59–62.
31. **Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl.** 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infect. Immun. **56**:1232–1236.
32. **Sibley, L. D., and J. L. Krahenbuhl.** 1988. Induction of unresponsiveness to gamma interferon in macrophages infected with Mycobacterium leprae. Infect. Immun. **56**:1912–1919.
33. **Styblo, K., and A. Rouillon.** 1981. Estimated global incidence of smear-positive pulmonary tuberculosis: unreliability of officially reported figures on tuberculosis. Bull. Int. Union Tuberc. **56**:118–126.
34. **Sundstrom, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U937). Int. J. Cancer **17**:565–577.
35. **Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, and T. Konno.** 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer **16**:171–176.
36. **Wilson, E., M. C. Olcott, R. M. Bell, A. H. Merrill, Jr., and J. D. Lambeth.** 1986. Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. J. Biol. Chem. **261**:12616–12623.