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Structural Basis of Capacity of Lipoarabinomannan To Induce Secretion of Tumor Necrosis Factor

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The results of this study show that lipoarabinomannans (LAM) isolated from a virulent strain and from an avirulent strain of *Mycobacterium tuberculosis*, which have recently been shown to differ markedly in terms of the structures of their nonreducing termini, also differ markedly in the capacity to induce the secretion of tumor necrosis factor from murine macrophages. It was found that LAM from the avirulent H37Ra strain was 100-fold more potent at inducing tumor necrosis factor secretion than LAM from the virulent Erdman strain, thus leading us to hypothesize that the structure of LAM from a given mycobacterial isolate may directly influence its ability to elicit, or avoid, cytokine-mediated mechanisms of host resistance.

Tumor necrosis factor alpha (TNF) has been implicated in the regulation of immune responses to a wide range of infectious pathogens and in the mediation of the manifestations of disease caused by these organisms. Intravenous administration of TNF causes physiologic and metabolic derangements characteristic of septic shock due to infection with gram-negative bacteria (21), and elevated levels of TNF in serum have been found with a number of infectious diseases, including human immunodeficiency virus infection, malaria, and leishmaniasis. In these infections, the highest concentrations of TNF have been found in sera of patients with more severe manifestations of disease, suggesting an immunopathologic role for this cytokine. On the other hand, TNF enhances microbicidal activity against bacteria such as *Salmonella* and *Listeria* species, fungi such as *Torulopsis* and *Candida* species, and protozoan parasites, indicating that TNF may be involved in host defenses against a wide variety of pathogens (8, 10, 18–20).

TNF appears to have direct antimycobacterial properties and inhibits mycobacterial growth in vitro (2). Studies with animals suggest that TNF may inhibit the extent of mycobacterial infection by mediating granuloma formation in vivo (13). Elevated concentrations of TNF have been found in sera of patients with leprosy and in pleural fluids of patients with tuberculous pleuritis (1, 24), suggesting a role for TNF in the human immune response against mycobacteria. In addition to eliminating organisms, TNF may contribute to the immunopathology of these diseases by causing fever, weight loss, and tissue necrosis (27, 28).

A comprehensive understanding of the role of TNF in the pathogenesis of human infectious disease requires definition of the biochemical components of microbial pathogens which induce TNF release. In this respect, it is now known that mycobacteria contain their own lipopolysaccharides (LPS), lipoarabinomannan (LAM) and lipomannan (LM), with physical properties analogous to those of O-antigenic LPS (3). There is now appreciable evidence that LAM can interact directly with cells of the immune system, thus possibly modifying the expression of acquired resistance to mycobacterial diseases. Such modifications may involve the inhibition of blastogenesis by T lymphocytes (12, 16), probably as a result of the secretion of large amounts of TNF by macrophages (1, 17, 28), by interference with gamma interferon-mediated macrophage activation (25), or indirectly by scavenging toxic oxygen intermediates produced by mycobacterium-infected host cells (4).

We now report that in the course of examining the activity of LAMs from different mycobacteria to evoke TNF secretion by mouse mononuclear cells, a marked difference between avirulent (H37Ra) and virulent (Erdman) *Mycobacterium tuberculosis* was observed. This observation is discussed in the light of our recent delineation of marked structural differences in the nonreducing termini of LAMs from these two sources (5, 6), as are speculations on the structural basis of the biological activity of LAM and on the basis of the capacity of mycobacterial strains to give rise to persistent disease within the infected host.

To prepare mycobacterial glycolipids, cultures of *M. tuberculosis* Erdman TMC 107 were grown for 6 to 8 weeks in glycerol-alanine salts medium, autoclaved, filtered, and washed. The bacterial cells (~100 g [wet weight]) were resuspended in phosphate-buffered saline containing 0.5% Triton X-100 and 0.02% NaN_3 (200 ml) and sonicated; the sonicate was passed through a French pressure cell at 20,000 lb/in^2. This material was then centrifuged at 27,000 × *g* for 45 min. Distilled acetone was added to the precooled supernatant to a final concentration of 90% acetone to precipitate LAM, LM, and PIM (phosphoinositolmannoside), which were collected by centrifugation and air dried. The dry acetone precipitate (1 g) was suspended in 6 M guanidine HCl in 10 mM Tris HCl, pH 7.4, by brief sonication and applied to a Sephacryl S-400 column (1.5 by 150 cm). Fractions were collected and monitored by polyacrylamide gel electrophoresis (PAGE). Final purification of LAM was achieved by applying a mixture of LAM, LM, and PIM to a Sephacryl S-200 column with a buffer (23) containing 10 mM Tris HCl (pH 8.0), 0.2 M NaCl, 0.5 M EDTA, and 0.25% deoxycholate. Fractions containing pure LAM, LM, and PIM, which were resolved at this stage, were pooled and dialyzed. To ensure that minimal LPS contamination was present in preparations, lyophilized LAM, LM, and PIM were redissolved in sterile, pyrogen-free water, filtered through a polytetrafluoroethylene filtration unit (pore size, 0.45 μm), passed through a 2-ml Detoxi-Gel column (Pierce Chemical, Rockford, Ill.), and refiltered through a sterile filter (pore size, 0.20 μm); the filtrate was collected into a sterile, pyrogen-free vial and dried. Levels of contaminating

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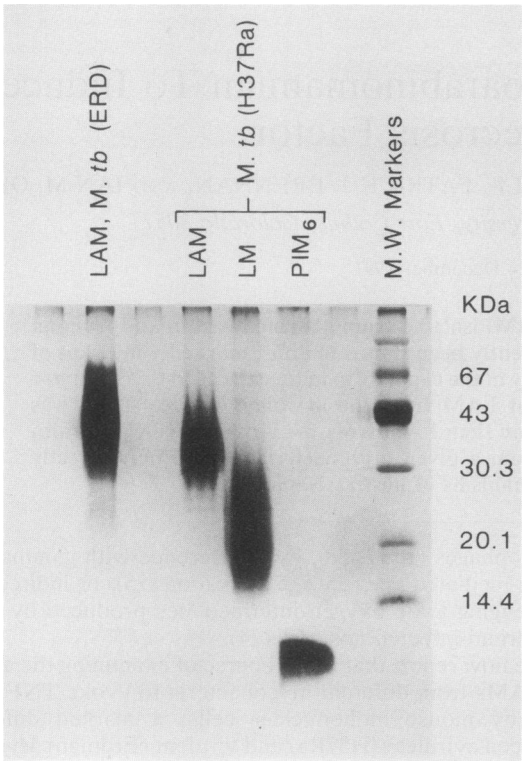

FIG. 1. Sodium dodecyl sulfate-PAGE of purified LAM, LM, and PIM from *M. tuberculosis* H37Ra and LAM from Erdman. (About 1.5 μg of each component was loaded on the gel.) The gels are 15% acrylamide cross-linked with 0.8% bisacrylamide and electrophoresed at 15 mA per gel in a constant environment. The gels were stained with a silver stain including periodic acid. Abbreviations: M. *tb*, *M. tuberculosis*; PIM_6, PIM containing six mannosyl residues; M. W., molecular mass.

LPS endotoxin in stocks of LAM, LM, and PIM were determined by using a *Limulus* amebocyte lysate commercial assay (E-Toxate, Sigma kit no. 210; Sigma Chemical Co., St. Louis, Mo.).

Procedures for the purification of LAM, LM, and PIM from *M. tuberculosis* H37Ra, which predate the procedures described above, have been described previously (11).

Experiments were performed with female C57BL/6 mice, 6 to 8 weeks old, which were purchased from the Jackson Laboratory, Bar Harbor, Maine. Peritoneal macrophages were harvested from animals inoculated intraperitoneally with 2 ml of a 3.5% suspension of casein (Kodak, Rochester, N.Y.) 72 h earlier. Cells were obtained from normal mice and from mice primed by infection with 10^5 *M. tuberculosis* Erdman organisms 10 days earlier. Harvested macrophages were washed twice in Hanks' balanced salt solution (Sigma Chemical Co.) and resuspended to 10^7 cells per ml in Dulbecco's modified eagle medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Cells were dispensed in 100-μl volumes into 96-well tissue culture plates containing suspensions of LAM or its relatives and incubated at 37°C in 5% CO_2 for 24 h. Similar studies were performed with bone marrow-derived cultured macrophages. Cells obtained from femur washouts were cultured in media as described above; media were supplemented with 10% L-929 fibroblast-conditioned media for 8 to 10 days in 96-well plates at a density of approximately 10^6 cells per well. In some experiments, LPS, polymyxin B (Sigma Chemical Co.), or the specific LPS inhibitor diphosphoryl lipid A (26) was added to culture wells. Supernatants were then removed, and their contents of TNF were assayed by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Boston, Mass.).

PAGE (Fig. 1) of purified LAMs from the Erdman and H37Ra strains of *M. tuberculosis* and of LM and PIM (which apparently have communal structures in all strains) shows typical diffuseness but evidence of considerable purity.

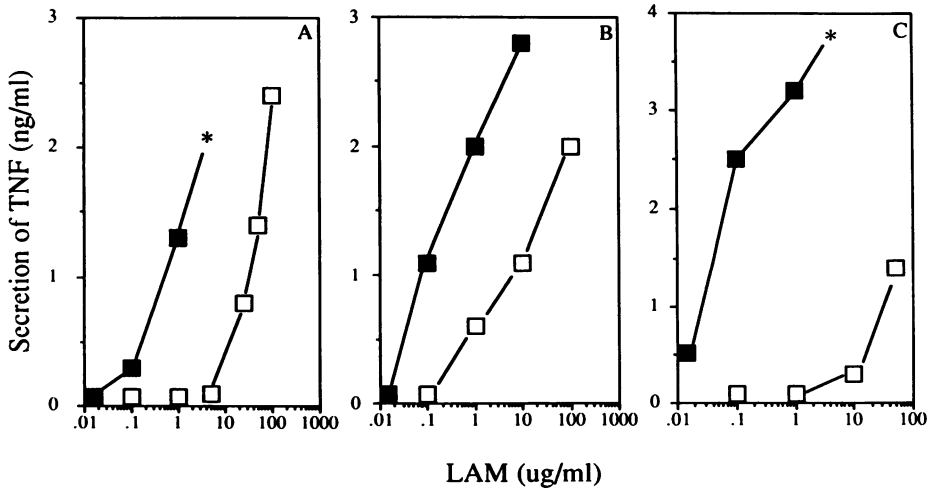

FIG. 2. Capacity of H37Ra LAM (■) and Erdman LAM (□) to trigger macrophages to secrete TNF. Sources of these cells were casein-elicited peritoneal exudate macrophages from normal mice (A) or from *M. tuberculosis*-infected mice (B) and bone marrow-derived macrophages from normal mice (C). Data are expressed as accumulation of TNF over a 24-h culture period (mean value, $n = 3$; standard errors of the means were <15%). Asterisks denote values above that of the sensitivity range of the ELISA method (>3.2 ng/ml). Background TNF by elicited macrophages was <0.07 ng/ml. In each case, cells were pooled from three donor mice.

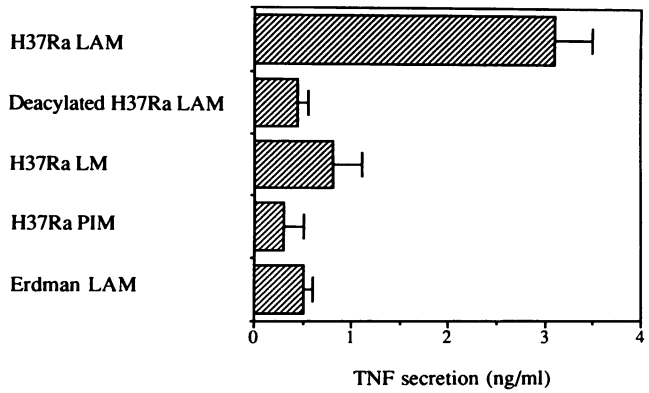

FIG. 3. Capacity of H37Ra LAM and related molecules to elicit secretion of TNF from peritoneal exudate macrophages. Data are expressed as means ± standard errors of the means (n = 3).

Following isolation, LAMs from the H37Ra and Erdman strains of *M. tuberculosis* were compared for the ability to induce TNF secretion from peritoneal or bone marrow-derived macrophages (Fig. 2). While LAMs from both strains elicited TNF release in a dose-dependent manner, approximately 100-fold more of the Erdman-derived LAM was required to evoke levels of TNF secretion similar to those induced by H37Ra LAM, regardless of the type of macrophage studied. In further experiments, the intact H37Ra LAM was compared with purified LM, PIM, and deacylated LAM from the same strain (Fig. 3). All three materials showed a substantially reduced capacity to induce TNF secretion. Indeed, the levels of TNF activity produced by these materials were comparable to that of Erdman LAM. Loss of function following deacylation of LAM has been noted previously (16).

In a final series of experiments, a variety of controls was run to exclude the possibility that the observations described above could not be fully or partially explained by contamination of the LAM preparations by LPS endotoxin. In Fig. 4A, intact H37Ra LAM was compared with a corresponding level of LPS contamination (determined to be 80 ng per 100 μg of LAM); as shown, LPS was responsible for only very low levels of TNF secretion compared with LAM. In a second experiment (Fig. 4B), LAM was cocultured in the presence of two LPS inhibitors, polymyxin B and diphosphoryl lipid A. Neither material significantly inhibited the capacity of LAM to elicit TNF secretion.

In a second series of control experiments, bone marrow-derived macrophages from an LPS-responsive mouse strain (C3H/HeN) were compared with those from an LPS-nonresponsive strain (C3H/HeJ). In both cases, it was reproducibly observed that LAM elicited TNF secretion while LPS did not (Fig. 5). A noticeable finding, however, was that the LPS-nonresponsive strain (C3H/HeJ) macrophages were poorly responsive, eliciting only about 0.6 to 0.9 ng of TNF per ml in various experiments when stimulated with 100 μg of LAM per ml. This finding may suggest strain variations in the response of mouse macrophages to LAM; this possibility is currently under investigation.

Thus, the results of this study demonstrate a surprising relationship between the structures of the nonreducing terminal regions of LAMs, recently elucidated in this laboratory (5, 6), and the capacity of LAMs to elicit TNF secretion by macrophages. Simply stated, LAM from the avirulent H37Ra strain of *M. tuberculosis*, characterized as it is by extensive arabinan side chains (5), was extremely potent at inducing TNF secretion, even at very low concentrations in vitro. In contrast, LAM from the virulent Erdman strain, in which the arabinan side chains were recently found to be extensively masked by short mannan segments (6), had approximately 100-fold less activity. LM and PIM devoid of arabinose showed consistency in eliciting low levels of TNF release similar to those induced by the Erdman LAM. These results are surprising in that upon discovery that LAM and LM were prokaryote versions of the important family of glycosylphosphatidyl inositols, we had predicted that biological activity would be attributable to the presence of a phosphatidyl inositol "membrane anchor" (15). Thus, the

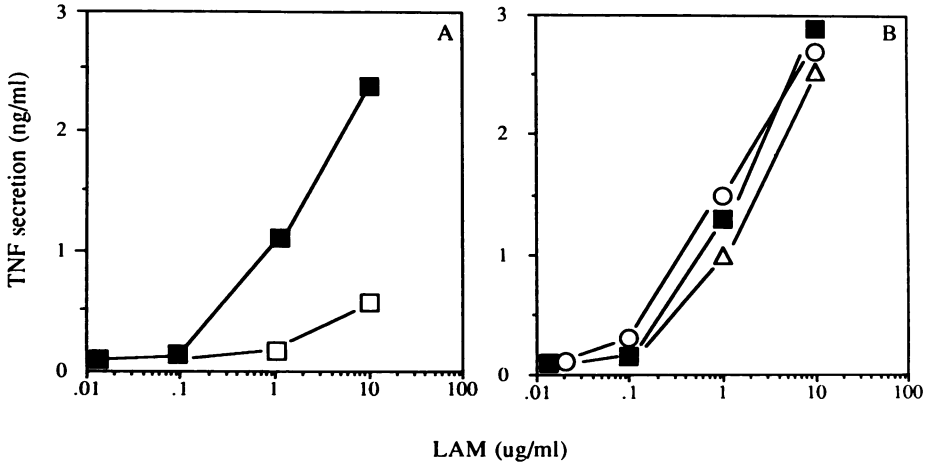

FIG. 4. (A) TNF secretion by bone marrow-derived macrophages stimulated with H37Ra LAM (■) or LPS (□). The amount of LPS present at each datum point was the corresponding level of LPS contamination determined by a Limulus assay to be present in the LAM stock solution (found to be 80 ng of LPS per 100 μg of LAM). Thus, for test wells stimulated with 1 μg of LAM, parallel control wells were stimulated with 0.8 ng of LPS, etc. Data are expressed as mean values (n = 3; standard errors of the means were <20%). (B) Bone marrow macrophages stimulated with LAM alone (■) or in the presence of the LPS inhibitors polymyxin B (○, 20 μg/ml) or diphosphoryl lipid A (△, 1 μg/ml). Data are expressed as mean values (n = 3; standard errors of the means were <20%).

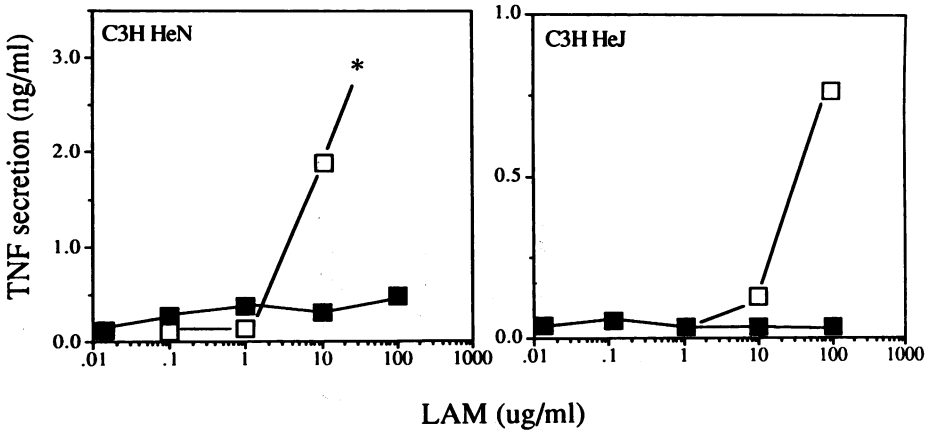

FIG. 5. Capacity of bone marrow-derived macrophages from LPS-responsive (C3H/HeN) and LPS-nonresponsive (C3H/HeJ) mice to secrete TNF in response to H37Ra LAM (□) or LPS (■). The amount of LPS present reflected the contaminating level in the LAM stock, as described in the legend to Fig. 4. Data are expressed as mean values (n = 3; standard errors of the means were <10%). *, TNF level >3.2 ng/ml.

paradigm of the glycosylphosphatidyl inositols seems not to apply to LAM.

These findings also have other implications. The first is that the capacity of the LAM possessed by a given strain of *M. tuberculosis* to elicit TNF secretion by the infected macrophage may directly influence the subsequent ability of the organism to survive and replicate within the host. Accordingly, it is possible that the H37Ra strain, which does not replicate in infected animals and which at best may give rise to a low-level chronic disease state (7), is unable to generate a productive infection because LAM rapidly triggers the release of TNF (and probably other cytokines) from the infected macrophages. As a result, these infected cells may acquire enhanced bacteriostatic activity, and other mononuclear phagocytes may accumulate, resulting in rapid granuloma formation. In this regard, the role of TNF in the recruitment and formation of such granulomas is now well established (13).

In contrast, the lesser potency of the LAM from the Erdman strain to elicit TNF activity may be central to the ability of this particular organism to proliferate in an unrestrained manner within infected host macrophages prior to the subsequent generation of T-cell-mediated immunity (22). If this hypothesis is correct, the precise structure of the LAM from a given mycobacterial species may directly influence the capacity of the organism to survive and give rise to active infection. In addition, it is possible that the expression of terminal mannose units which effectively mask the biologically active arabinan side chains within the Erdman LAM structure may reflect an adaptive evolutionary change which may allow the organism to survive within the infected host. Such an adaptation may also be regarded as a form of molecular mimicry, given the structural similarity to the mannose-rich segments of many mammalian glycoproteins (14).

With regard to the precise nature of the interaction of the LAM molecule with the macrophage cell surface, there is currently no information in the literature to suggest that macrophages possess receptors that specifically bind arabinose and hence could explain the potent ability of the H37Ra LAM to trigger TNF secretion in a specific manner by such cells. In contrast, it is well established that these cells possess a mannose receptor (29). This receptor has been structurally defined and has been found to be a complex molecule in that there are at least eight protein regins formed into domains by disulfide bonding within the overall molecule in which carbohydrate can be bound (9). It is therefore theoretically possible that the receptor can be promiscuous and bind ligands other than mannoses. Which ligands are bound and the number of domains occupied may influence the type of signaling transmitted into the cell itself and hence may influence the physiological response of the cell in terms of secretion of various mediators.

Finally, it is intriguing that recent structural information and recent evidence of the biological activity of mycobacterial LAM, when viewed collectively, indicate that biological functions of LAM are endowed at both ends of the molecule. Thus, as shown above, masking of arabinose terminal units by mannose dramatically reduces the biological activity of LAM. However, a similar loss of activity can also be obtained by the removal of fatty acyl groups associated with the phosphoinositol core of the LAM molecule, as shown here and elsewhere (17). This may indicate that anchoring of the LAM molecule into the target cell membrane, mediated by the fatty acyl groups, is an event that must occur before sugar ligands at the nonreducing termini of the molecule can interact with their target receptors.

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