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Phagocytes and Lymphocytes

Mycobacterial lipoarabinomannan affects human polymorphonuclear and mononuclear phagocyte functions differently

ANNA FIETTA, CRISTINA FRANCIOLI, GIULIANA GIALDRONI GRASSI
Laboratorio di Ricerca per le Malattie Respiratorie – Clinic and Institute of Respiratory Diseases, IRCCS Policlinico San Matteo, University of Pavia, Italy

ABSTRACT

Background and Objectives. **The role of mycobacterial lipoarabinomannan (LAM) in regulating the granulomatous response and its effects on cells involved in early responses to tuberculosis have not been clearly defined. The aim of this study was to acquire further evidence about the mechanisms by which LAM takes part in the host response to mycobacterial infections.**

Design and Methods. **We compared the *in vitro* ability of mannosylated LAM (ManLAM) and LAM lacking the terminal mannosyl units (AraLAM) to induce distinct responses in human polymorphonuclear (PMNs) and mononuclear phagocytes [both monocytes and 48-hr monocyte-derived macrophages (MDMs)]. The responses examined were chemotaxis, transient changes in free cytosolic calcium, phagocytosis and metabolic activation.**

Results. **AraLAM and ManLAM affected mononuclear, but not polymorphonuclear, phagocyte functions. Both forms of LAM were chemotactic for monocytes and MDMs. The LAM-induced chemotactic response required new protein synthesis, did not induce a rise in cytosolic free calcium levels and was partially inhibited (about 50%) by genistein, but not by calphostin C or PD 98059. Lastly, at physiologic doses ManLAM significantly reduced phagocytosis of *M. tuberculosis* and zymosan particles by MDMs.**

***Interpretation and Conclusions*.** **Different phagocytic cells can exhibit variable responses to AraLAM and ManLAM. Moreover, LAMs affect cell functions through different mechanisms. Protein synthesis and activation of protein tyrosine kinases are important intermediates in the signal transduction pathway of the chemotactic response of mononuclear phagocytes to AraLAM and ManLAM; whereas ManLAM-induced inhibition of macrophage phagocytic ability could depend on the binding of macrophage mannose receptors and / or the insertion of this molecule into cellular plasma membrane. Together these data highlight the danger of making generalizations regarding the activity of LAMs on immune defenses.**

Key words: AraLAM, ManLAM, PMNs, monocytes, macrophages, chemotaxis, changes in $[Ca^{++}]_i$, superoxide production, phagocytosis

Correspondence: Anna Fietta, M.D., Institute of Respiratory Diseases, Padiglione Forlanini, IRCCS Policlinico San Matteo, via Taramelli 5, 27100 Pavia, Italy. Phone: international+39-0382-422232 – Fax: international +39-0382-422267 – E-mail: tisiolmm@ipv36.unipv.it

The role of mycobacterial products in regulating the granulomatous response has not been clearly defined, although several products, particularly lipoarabinomannan (LAM), have emerged as molecules of potential interest. LAM, which accounts for up to 5 mg/g of bacterial weight, consists of a mannan core with an oligoarabinosyl-containing side chain, that is attached to a phosphatidyl-inositol anchor at one end. Two major chemical forms of LAM have been described, i.e. LAM isolated from *M. tuberculosis* and *M. bovis* BCG, which is capped with mannose residues at the non-reducing arabinofuranosyl termini (ManLAM), and LAM isolated from rapidly growing avirulent strains of mycobacteria which lacks the mannose caps at the arabinofuranosyl ends (AraLAM).[1,2] Results of many *in vitro* studies indicate that LAM is a potent immunomodulator that acts mostly on T-cells and macrophages.[3] Moreover, the presence or absence of terminal mannose residues seems to be of crucial importance in affecting some LAM activities.[4-10] Recently, Riedel and Kaufman[11] demonstrated that AraLAM induces IL-8 and GRO-α in human polymorphonuclear leukocytes (PMNs) at the mRNA and protein levels, suggesting both the presence on these cells of common receptor components for LAM and LPS and the involvement of these cells in the early inflammatory response against mycobacterial infection. It is well established that following ingestion of *M. tuberculosis*, the innate immune response to tuberculosis is predominantly regulated by activated macrophages.[12,13] However, some *in vitro* and *in vivo* evidence has been presented indicating a possible involvement of PMNs in the early host defence mechanisms against mycobacteria.[14–20] Although the mechanisms underlying tuberculosis control in humans are still unknown, complex interactions between PMNs, mononuclear phagocytes, lymphocytes, mycobacteria and/or mycobacterial products at the infection sites are expected to occur and influence the evolution of tuberculous lesions. The present study was designed to acquire further evidence on the role of mycobacterial LAM in the development of the initial host inflammatory response to mycobacterial infection. For this purpose we compared the *in vitro* abilities of ManLAM and AraLAM, in physiologic concentrations, to induce distinct responses in human PMNs and in two monocytic cell populations, i.e. freshly isolated human peripheral blood monocytes and 48-hour monocyte-derived macrophages (MDMs). The responses examined were chemotaxis, fluxes in free cytosolic calcium concentration $[Ca^{++}]_i$, metabolic activation and phagocytosis.

Design and Methods

Reagents, chemicals and micro-organisms

Mannose-capped lipoarabinomannan (ManLAM), isolated from the virulent strain of *M. tuberculosis* H37Rv, and non-capped LAM (AraLAM), isolated from a rapidly growing *Mycobacterium* species, were provided by John T. Belisle (Department of Microbiology, Colorado State University, Fort Collins, USA) under a National Institute of Health contract (NO 1-AI-25147). Endotoxin contamination was 4.38 ng per 1.0 mg of AraLAM and 3.46 ng per 1.0 mg of ManLAM, as determined by the Limulus Amebocyte Assay. Lyophilized mycobacterial LAMs were reconstituted in sterile, pyrogen-free, distilled water and stored at –20°C, until use. Bovine serum albumin (BSA), HEPES, EDTA, NH_4Cl, $KHCO_3$, NaCl, dextran, heparin, Ficoll-Hypaque, Percoll, EGTA, TRIS, *E. coli* lipopolysaccharide (LPS), formyl-methionyl-leucyl peptide (fMLP), phorbol myristate acetate (PMA), nitroblue tetrazolium (NBT), calcium ionophore A23187, calphostin C, genistein, cycloheximide and zymosan A were purchased from Sigma Chemicals Co. IL-8 was obtained from Pepro Tech. Inc; PD 98059 from Calbiochem; while Middlebrook 7H9 broth, ADC, OADC and 7H10 agar were bought from Difco. A pool of sera from healthy, tuberculin skin test non-reactive, donors was used to opsonize bacteria and zymosan particles. After clotting of blood for 30 minutes at room temperature, serum was prepared by centrifugation at 1,100 × g for 20 min, filtered and stored at –80°C. Lyophilized *M. tuberculosis* H37Rv ATCC 25618 (American Type Culture Collection, Rockville, MD, USA) was grown in Middlebrook 7H9 broth enriched with albumin, glucose and catalase (ADC) at 37°C for 3 to 4 weeks. Mid-log-phase cultures were pelleted and resuspended in 7H9 medium. An aliquot of this suspension, diluted in formalin, was counted in a Petroff-Hausser chamber. *Mycobacteria* were then diluted to 1×10^8 cells/mL and frozen at –70°C. Before each experiment, bacteria were thawed, washed in RPMI 1640, pelleted (14,000 × g for 10 min), resuspended in RPMI 1640 and sonicated for 40 s to disrupt clumps. An aliquot of this suspension was plated on 7H10 agar enriched with OADC to determine the number of colony-forming units (CFU) per milliliter. The viability was routinely greater than 50%.

Isolation of peripheral blood PMNs and monocytes

Blood was obtained from healthy adult volunteers who were negative to the purified protein derivative (PPD) skin test for tuberculosis, had no clinical history of tuberculosis and had no known exposure to tuberculosis. Leukocytes were separated over a Ficoll-Hypaque gradient. Erythrocyte-granulocyte pellets were dissolved in NH_4Cl lysis buffer to eliminate erythrocytes and the granulocytes remaining were pelleted, washed three times with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} and finally suspended in RPMI 1640 supplemented with 25 mM HEPES buffer and 2 mM L-glutamine at a density of 1×10^7 cells/mL. Purity and viability of PMN preparations were >95% and no platelets or erythrocytes could be detected by light microscopy or by flow cytometric analysis.

The mononuclear cell (PBMC) layer, removed from the interface, was washed three times in Ca^{2+}-and Mg^{2+}-free HBSS solution. Monocytes were prepared by suspending PBMC (5×10^6 cells/mL) in RPMI medium supplemented with L-glutamine, 10% fetal bovine serum and HEPES buffer and seeded onto the surface of plastic petri dishes for 2 hours at 37°C to let monocytes adhere. After removal of non-adherent cells, the monocyte-enriched population was detached by scraping and suspended at a density of 1×10^7 cells/mL. Monolayers consisted of 86-90% monocytes, as assessed by peroxidase staining.[21] To obtain MDMs, monocytes were cultured for 48 hr in plastic tissue culture plates at a density of 10^6 cells/mL. In one set of experiments, monocytes were prepared on a one-step discontinuous Percoll gradient.[22]

In vitro migration assays

The ability of LAM to induce cell migration was measured by a modified Boyden chamber assay.[23] Briefly, samples containing various concentrations of LAM were placed in the lower wells of the chambers and separated from target cells by a nitrocellulose filter (pore size of 5 and 8 µm, respectively for polymorphonuclear and mononuclear phagocytes). Migration toward 1 µM fMLP was used as positive control. For checkerboard experiments, samples containing LAM were added to lower wells, upper wells, or both in various concentrations. Migration was carried out for 2 h at 37°C in a humidified 5% CO_2 incubator. The filters were labeled, placed on glass slides, clarified and stained with hematoxylin. Migration into the filters was quantified via light microscopy by measuring the distance reached (in µm) of the leading front. At least 10 high-power fields were counted for each sample. Migration was expressed as a percent of spontaneous migration (migration index, control = 100%). All assays were performed in duplicate and at least three experiments were performed with cells from different donors.

Cytosolic free Ca^{++} measurements

To measure changes in $[Ca^{++}]_i$, cells were suspended in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) with added 0.25% BSA, incubated for 30 min at 37°C with 5 µM FURA-2/AM, washed three times by gentle centrifugation (5 min, 300g) and then suspended at the concentration of 2×10^6/mL in PBS-HEPES, supplemented with glucose (1.8 g/L), BSA (0.25%) and $CaCl_2$ (1 mM). Cells loaded with FURA-2/AM were placed in a thermostatically controlled (37°C) cuvette, equipped with a cuvette stirrer, and then stimulated with either fMLP (100nM), AraLAM (1 µg/mL) or ManLAM (1 µg/mL). Changes in $[Ca^{++}]_i$ were continuously recordered using a Perkin-Elmer LS-50B spectrofluorimeter. Excitation of FURA-2/AM was performed at 340 and 380 nm; excitation band widths were set at 5 nm. The ratio of emitted fluorescence signals (510 nm) was used to calculate the cytosolic free Ca^{++} concentration, according to Grynkiewicz *et al.*[24] Fluorescence signals were calibrated adding 0.5% Triton ×100 and 1 mM $CaCl_2$ (maximum), followed by 45 mM TRIS and 50 mM EGTA/TRIS (minimum).

Variations in $[Ca^{++}]_i$ induced by fMLP and LAMs were calculated as the difference (D) between the highest values (peak levels) reached after addition of the agent and mean resting levels (2-min pretreatment values) in each experiment. The lag time between the addition of the agent and the peak attained (time to reach peak, sec) was also calculated.

Oxidative metabolism assay

Superoxide production by adherent and non-adherent leukocytes in response to soluble stimuli LAMs (1 µg/mL), fMLP (10^{-6} M), IL-8 (10^{-6} M), 100 ng/mL PMA and calcium ionophore A23187 (10^{-6} M) was assessed spectrophotometrically and quantified by the O_2^--dependent ferricytocrome-C reduction method.[25] The amount of O_2^- produced was expressed as nanomoles of O_2^- per 10^6 cells per unit of time. All samples were performed in duplicate and at least three experiments for each test were performed with cells from different donors.

Phagocytosis assays

Phagocytosis was measured using plastic-adherent cells in the absence or presence of various concentrations of LAMs. Both serum-treated and non-opsonized zymosan or heat-killed *M. tuberculosis* H37Rv were used as corpusculate stimuli. Phagocytosis of zymosan was measured according to the method described by Preisig and Hitzig, using a ratio of 8 zymosan particles to 1 cell.[26] In order to assess phagocytosis of *M. tuberculosis*, adherent cells and heat killed mycobacteria (ratio 1:8) were incubated for 4 h at 37°C in 5% CO_2. Extracellular bacteria were removed by four washes, then cells were fixed with absolute methanol for 1 min at room temperature. Fixed samples were stained by the cold Kinyoun method to enumerate acid fast bacilli. The number of cells with ingested bacilli was recorded. Results were expressed as a phagocytosis frequency (number of phagocytosing cells ×100/total cells) and/or a phagocytosis index (mean number of particles/cell). All samples were performed in duplicate and at least three experiments for each test were performed with cells from different donors.

Data presentation and statistical analysis

Results are expressed as mean values ± standard deviations (SD) for each series of experiments. The number of experiments is indicated in the tables and figures. Statistical significance of the data was determined by the two-tailed Student's t-test. Differences were considered significant if the *p* value was less than 0.05.

Results

Induction of cell migration

LAM was tested for its ability to induce *in vitro* migration of PMNs, monocytes and MDMs. We observed that both AraLAM and ManLAM, in the range of concentrations from 0.01 to 20 µg/mL, failed to induce a migratory response in human PMNs, the migration index being between 104±9 and 115±11% of control (unstimulated) migration. In contrast, LAM induced migration of monocytes (Figure 1) and MDMs (data not shown) in a dose-dependent manner. The migratory index was significantly

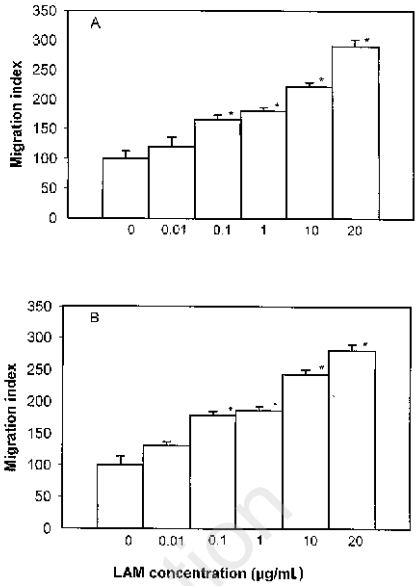

Figure 1. Migration of human monocytes in response to purified LAM. The migratory responses of monocytes to AraLAM (A) and ManLAM (B) in absence of human serum are shown. Migration assays were performed for 2 h in a modified Boyden chamber and data are expressed as migration index (percent of control migration in medium alone) ± SD (n, 6). The chemotactic response to 1 µM fMLP was 389 ± 45% of control migration. Asterisks denote statistically significant differences in migration relative to unstimulated cells ($p < 0.05$).

different ($p < 0.05$) from that of unstimulated mononuclear phagocytes (leading front measure: 11.2±2.5 µm) in the presence of concentrations equal or superior to 0.1 µg/mL of both forms of LAM. The maximal responses were observed using the highest concentration of LAMs (20 µg/mL) and ranged between 280 and 300% of control migration. LAM molecules at this concentration showed a potency equal to about 75% of that showed by 1 µM fMLP, a well-known bacteria-derived chemotactic oligopeptide. In fact, we found that in the same experimental conditions the chemotactic response of human phagocytic cells to 1 µM fMLP was 389.12±45% of control unstimulated migration (n, 5). Checkerboard experiments were performed using either AraLAM or ManLAM to determine whether the migratory response was chemotactic or chemokinetic. As shown in Table 1, significant monocyte migration was observed only when a concentration gradient of LAM was established across the filter membrane. When LAM was present in only the upper wells of the modified Boyden chamber, or when LAM was present in both the upper and the lower wells, no significant migration occurred. Similar responses were observed when MDMs were used (data not shown). The results of our study indicate that migration of mononuclear phagocytes to AraLAM and ManLAM is a chemotactic response, characterized by cell migration in response to a concentration gradient. Migration of

Table 1. LAM is chemotactic for human monocytes.

Lower chamber	Upper chamber None	0.1 µg/mL	1.0 µg/mL	10 µg/mL
ManLAM				
None	100 (±15)	98 (±13)	102 (±18)	97 (±10)
0.1 µg/mL	160* (±10)	97 (±15)		
1.0 µg/mL	190* (±12)		101 (±16)	
10 µg/mL	250* (±21)			98 (±12)
AraLAM				
None	100 (±13)	101 (±11)	95 (±10)	99 (±9)
0.1 µg/mL	150* (±11)	99 (±12)		
1.0 µg/mL	180* (±15)		96 (±11)	
10 µg/mL	230* (±19)			110 (±14)

Checkerboard analyses were performed using either ManLAM or AraLAM. The table shows the migratory responses of monocytes to various concentrations of ManLAM and AraLAM present in the lower, upper or both wells of modified Boyden chambers. Migration assays were performed for 2 h as described in the text, and results are expressed as migration index ± SD (n, 3). Asterisks denote statistically significant differences in migration relative to control cells that were not exposed to lipoarabinomannans ($p < 0.05$).

mononuclear phagocytes to LAMs was not due to cell activation during the monocyte preparation, since additional experiments performed with Percoll purified monocytes gave similar results (data not shown). Migration to LAMs was not due to contaminating LPS, because we found that LPS alone failed to induce mononuclear cell migration, even at concentrations as high as 10 µg/mL (migration index with respect to unstimulated control samples was 119±16 and 110±13%, respectively for monocytes and MDMs). Moreover, the endotoxin contamination of the LAM preparation was 4.38 ng and 4.46 ng per 1 mg of AraLAM and ManLAM, respectively, as assessed by the Limulus Amebocyte assay. All these assays were performed under serum-free conditions, consequently the migratory response of monocytes to LAMs was serum independent. Experiments were performed to assess whether LAM could compete with the classical chemotactic agonist, fMLP. Monocytes were pre-treated for 15 min with various concentrations of LAMs before being tested in a chemotactic assay. Exposure of monocytes to LAM in the range of concentrations from 0.1 to 2.5 µg/mL resulted in less than a 5% average reduction in stimulated migration towards 1 µM fMLP (data not shown). The percent migration of LAM-treated cells to fMLP showed a slight (about 15%), but not significant ($p > 0.05$), reduction with respect to the chemotactic response of non LAM-treated cells, only when monocytes were pre-exposed to high LAM concentrations (≥ 10 µg/mL) (Table 2). We subsequently demonstrated that neither was the migratory response of PMNs and MDMs to fMLP altered by LAM treatment (data not shown).

Effect of LAMs on intracellular [Ca^{++}] fluxes

Since AraLAM and ManLAM induced a chemotactic response in human mononuclear phagocytes similar to that induced by the classical chemotactic agonist fMLP and since intracellular mobilization of Ca^{++} is one of the earliest responses to chemoattractants,[27] we examined the ability of LAMs to induce intracellular calcium signals. For these experiments, cells were loaded with the intracellular fluorescent probe FURA-2/AM and then stimulated with either fMLP (100 nM), AraLAM (1 µg/mL) or ManLAM (1 µg/mL). Changes in $[Ca^{++}]_i$ were continuously recorded using a Perkin-Elmer spectrofluorimeter and the ratio of emitted fluorescent signals at 510 nm was used to calculate cytosolic free Ca^{++} concentrations, as described in the *Design and Methods* section. As shown in Table 3, the mean ± SD $[Ca^{++}]_i$ under standard conditions was 100.4±19.1, 128.6±38.8 and 101.1±9.1 nM respectively for PMNs, fresh monocytes and MDMs (n, 3). The addition of 100 nM fMLP induced a rapid and transient rise of intracellular Ca^{++} concentrations in all types of cells. The maximal rise (Δ peak) in PMNs was 127.4±48 nM and was reached in about 12 sec (11.9±3.6 sec). The maximal $[Ca^{++}]_i$ rise in fresh monocytes and MDMs was reached in about the same time (9.1±0.8 and 13.3±1.3 sec, respectively) but was significantly lower (55.8±12.6 and 72.0±38 nM, respectively) than that measured in PMNs. Neither AraLAM nor ManLAM at physiologic relevant concentrations (≤ 1 µg/mL) induced significant rises in intracellular Ca^{++} concentrations in these cells, $[Ca^{++}]_i$ fluxes being in

Table 2. Effect of LAM treatment on chemotactic response of human monocytes to 1 µM fMLP.

Treatment with	Migration index to fMLP
Nil	319±37%
AraLAM (10 µg/mL)	270±31%
ManLAM (10 µg/mL)	275±29%

Monocytes were pretreated for 15 min with LAMs before assessing chemotaxis versus 1 µM fMLP. Results represent the means ± SD from 3 experiments. Differences were not significant ($p > 0.05$).

Table 3. $[Ca^{2+}]_i$ responses to AraLAM or ManLAM in PMNs, fresh monocytes and MDMs.

Stimulus	$[Ca^{++}]^i$ (nM) (Δ peak) PMNs	Monocytes	MDMs
FMLP (100 nM)	127.4±48.0	55.8±12.6	72.0±4.0
AraLAM (1 µg/mL)	-2.9±1.8	2.7±8.5	10.4±4.6
ManLAM (1 µg/mL)	-1.6±1.1	-6.5±8.3	4.1±1.2

Cells were loaded with the fluorescent probe and then stimulated with either fMLP (100 nM), AraLAM (1 µg/mL) or ManLAM (1 µg/mL). Changes in $[Ca^{2+}]_i$ were continuously recorded by a Perkin-Elmer LS-50B spectrofluorimeter. The ratio of emitted fluorescence signals at 510 nm was used to calculate cytosolic free Ca^{++} concentration. Values are means±SD of 3 experiments performed with cells obtained from different donors. Δ indicates the maximal difference versus respective resting levels. $[Ca^{++}]_i$ resting levels for PMNs, fresh monocytes and MDMs were 100.40±19, 128.60±39 and 101.1±9.10 nM, respectively.

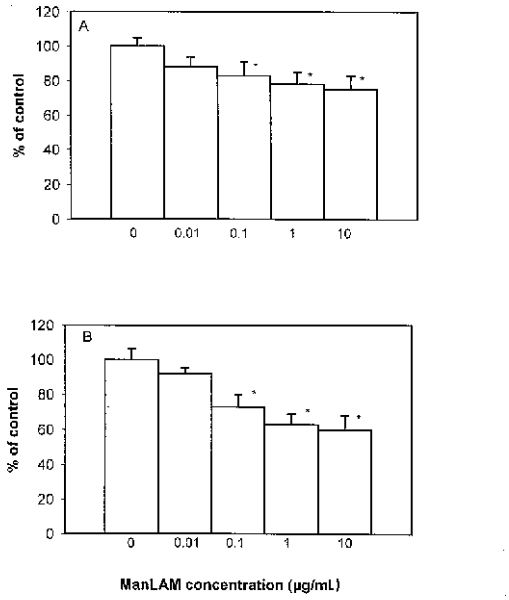

Figure 2. Concentration-dependent inhibitory effect of ManLAM on phagocytosis of zymosan particles by MDMs. Monocyte-derived macrophages were incubated for 2 hr at 37°C, 5% CO_2, in medium alone (control), or in the presence of AraLAM or ManLAM (from 0.01 to 10 µg/mL) and then assayed for phagocytosis of zymosan particles. Values are means ± SD (n=6) and are expressed as percentage of phagocytosis frequency (A) and index (B) with respect to control values. Control phagocytosis frequency and index were respectively 65.5±5.39% and 2.08±0.60. AraLAM did not affect zymosan phagocytosis in the range of concentrations from 0.01 to 10 µg/mL. Asterisks denote statistical significant differences in phagocytosis relative to control cells that were not exposed to ManLAM ($p < 0.05$).

the range of those measured in resting polymorphonuclear and mononuclear phagocytes. In some experiments, MDMs seemed to respond partially to LAM, demonstrating a very small and slow increase of free cytosolic Ca^{++} concentrations above those found in resting cells (data not shown). The possible modulation of fMLP responsiveness in cells exposed to LAMs was assessed. Cells were incubated without and with 1 µg/mL LAMs for 0.5 and 2 hrs, stimulated with fMLP, and then $[Ca^{++}]_i$ fluxes were measured. Incubation with LAM did not affect the $[Ca^{++}]_i$ rise induced by fMLP in either PMNs, monocytes or MDMs (data not shown).

Effect of LAM on cell metabolism and phagocytosis

Besides cell migration, classical chemotactic agonists may induce other biological activities, e. g. metabolic activation and phagocytosis. Consequently, we tested the *in vitro* effect of LAMs on superoxide production and phagocytosis. The exposure of monocytes and MDMs, as well as that of PMNs, for 2 hours to LAMs, in a range of concentrations from 0.01 to 10 µg/mL, did not induce superoxide production (less than 5% average variation with respect to control cells) nor significantly affect superoxide production induced by different stimuli, i.e. PMA, fMLP, IL-8, calcium ionophore A23187 (data not shown). Phagocytosis of both zymosan particles and *M. tuberculosis* H37Rv was measured after incubation of adherent cells without (control) and with LAM in a range of concentrations from 0.01 to 10 µg/mL. We found that none of the tested concentrations of ManLAM and AraLAM affected phagocytosis of either serum-opsonized or non-opsonized zymosan particles when monocytes or PMNs were exposed for two hours to LAM. The percentages of phagocytosis frequency and index ranged between 99.8 (±6.5) and 106.5 (±7.1) of control (LAM-non-treated) phagocytosis. The effect of LAM on phagocytosis of non-opsonized zymosan particles by MDMs is shown in Figure 2. AraLAM did not influence phagocytosis at any of the tested concentrations, while ManLAM significantly reduced ($p < 0.05$) both phagocytosis frequency and index, in a dose-dependent manner. The inhibitory effect was shown when ManLAM concentrations equal to or greater than 0.1 µg/mL were used. Moreover, results obtained using serum opsonized zymosan particles, as phagocytic challenge, were similar (data not shown). The influence of ManLAM on phagocytosis of heat killed *M. tuberculosis* H37Rv is shown in Table 4. Plastic adherent PMNs, monocytes and MDMs were exposed for four hours to bacteria (ratio 1:8) in the absence or in the presence of various concentrations of H37Rv ManLAM and the percentage of cells with ingested mycobacteria was determined as described in the *Design and Methods* section. None of the tested concentrations (from 0.01 to 10 µg/mL) of ManLAM influenced *M. tuberculosis* H37Rv phagocytosis by human PMNs but did significantly affect ($p < 0.05$) that by mononuclear phagocytes. Inhibition of *M. tuberculosis* uptake by fresh monocytes was observed only in the presence of high concentrations of ManLAM (≥ 5 µg/mL), while the inhibitory effect on *M. tuberculosis* phagocytosis by MDMs was significant ($p<0.05$) also at physiologic ManLAM concentrations (≤ 1 µg/mL).

Role of protein synthesis and protein kinase activation in LAM-induced migration

Since chemotaxis elicited by LAM was not mediated by classical signals, it remained possible that the chemotaxis required synthesis of unidentified proteins. To examine this possibility we evaluated the effect of cycloheximide (CHX) on chemotaxis elicited by AraLAM and ManLAM. Monocytes were treated with CHX (10 µg/mL) for 15 min before exposure to 1 µg/mL LAM and continued to be exposed to the drug throughout the assay. Exposure of cells to CHX alone for about 2 h resulted in less than an 8% average reduction in unstimulated migration, indicating that this drug did not affect the capacity of monocytes to migrate. However, stimulation of monocyte migration by LAM was significantly reduced (75.3±10.2% inhibition), suggesting that cell migration to LAM requires new protein synthesis. Moreover, the capacity of selected protein kinase (PK) inhibitors, i.e. calphostin C, genistein and PD 98058, to affect monocyte migration in response to LAM was assessed. Cells were treated for 15 min with each

Table 4. Effect of ManLAM treatment on phagocytosis of *M. tuberculosis* H37Rv.

Cells	LAM concentrations (µg/mL) None	0.01	0.1	1.0	5.0
PMNs	23.4±5.8	24.4±7.7	23.9±5.8	23.4±5.3	21.12±6.3
Monocytes	30.76±7.6	27.6±5.6	27.7±6.2	27.5±7.1	21.45±5.7*
MDMs	35.9±8.6	30.7±5.2	28.8±5.6	24.3±5.8*	20.90±6.3*

Adherent cells were incubated at 37°C for 4 h with heat-killed M. tuberculosis *H37Rv (ratio 1:8) in the absence or presence of ManLAM. Results represent the mean percentage±SD (n, 7) of cells which had phagocytosed acid fast bacilli. *p < 0.05.*

inhibitor (1-10 µM) before being exposed to LAM and continued to be exposed to PK inhibitors throughout the assay. Inhibitors alone did not affect unstimulated migration at the concentration used (migration index 99±11%). The PKC inhibitor, calphostin C, and the MEK inhibitor, PD 98059, did not significantly influence LAM-induced migration. The PTK inhibitor genistein reduced monocyte migration by 50-60% (Figure 3). These results suggest that activation of tyrosine kinases, but not that of protein kinase C and ERK kinases, could be partially involved in the chemotactic response of monocytes to LAM.

Discussion

The mechanisms involved in the control of the evolution of tuberculous infection in humans are still unknown, nevertheless complex interactions between mononuclear phagocytes, PMNs, lymphocytes and mycobacteria and/or mycobacterial products at the infectious site are expected to occur and influence the evolution of tuberculous lesions. Of several mycobacterial products which could be involved in regulating the granulomatous response, LAM has emerged as a molecule of potential interest. Besides being a potent modulator of T-cell and macrophage activities,[3] LAM is involved in the non-opsonic interaction of *M. tuberculosis* with macrophages[28,29] and *in vitro* and *in vivo* findings indicate that LAM could be released in the infectious site by infected macrophages, thus influencing local immune response. Vesicles containing LAM are released from phagosomes following macrophage ingestion of *M. tuberculosis*[30,31] and anti-LAM antibodies are present in the sera of patients with tuberculosis.[32]

In this paper we report that LAMs, at physiologic concentrations, modulate mononuclear, but not polymorphonuclear, phagocyte activities. Purified AraLAM and H37 RV LAM induce migration of human monocytes and MDMs, under serum-free conditions *in vitro*, showing a potency equal to about 75% of that shown by the chemotactic peptide fMLP. This activity is dependent on the presence of a concentration gradient, demonstrating that LAMs are chemotactic for these cells. These results are partially discordant with a report indicating that AraLAM, but not Erdman LAM, induced a chemotactic response in mononuclear phagocytes *in vitro*[33] but are

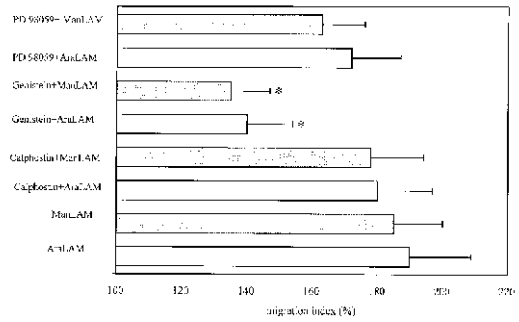

Figure 3. Effects of protein kinase inhibitors on LAM-induced monocyte chemotaxis. The migratory responses of monocytes to 1 µg/mL of AraLAM (□) and H37Rv LAM (■) were measured in the presence of the PKC inhibitor, calphostin; the PTK inhibitor, genistein, and the MEK inhibitor, PD 98059. Cells were treated with kinase inhibitors (10 µM) throughout the assay. Migration assays were performed for 2 h as described in the text and data are expressed as migration index ± SD. Inhibitors alone did not affect unstimulated migration at the concentration used (migration index 97±8). Asterisks denote statistically significant differences in migration relative to cells that were not exposed to inhibitors ($p < 0.05$).

in agreement with those obtained using human T-cells.[34] Minor differences in the degree of mannosylation of ManLAM obtained from different strains of *M. tuberculosis* could influence the biological activity of this molecule to a high degree. Differences in the structures of LAMs from different strains of *M. tuberculosis* at their non-reducing termini have been found to affect the ability of these molecules to induce macrophage activation.[5] Moreover, ManLAM from *M. bovis* BCG did not exhibit chemoattractant activity for T-cells in contrast to LAM from H37 RV and Erdman *M. tuberculosis* strains.[34] Directional migration in response to a gradient of chemoattractant argues strongly for receptor-ligand interaction. Despite the well-documented biological activities of LAMs, detailed investigation into the mechanisms by which LAMs mediate these effects at the plasma membrane level has not yielded clear elucidation. Multiple receptors, among which CD14, complement receptors, and macrophage mannose receptors (MRs), may serve as signaling receptors for LAMs on different cells.[13,28,33] Moreover, LAMs may integrate directly into the host cell plasma membrane through their GPI anchor, without apparent involvement of surface receptors.[35] As a consequence, LAMs could perturb short-range interactions between surface molecules and/or modulate events in transmembrane signaling pathways. Binding of LAMs to CD14 could be involved in LAM-induced chemotactic response as suggested also by Bernardo *et al*.[33] However LPS does not share the chemotaxis-inducing activity of LAMs and LAMs did not induce PMN chemotaxis. One possibility is that a CD14-associated signal-transducing molecule is required for LAM-induced chemotaxis. Since we found that inhibition of translation by cycloheximide significantly reduced the chemotactic response toward LAM, an additional protein(s) may be necessary to effect a signal trans-

duction event. Activation of G protein-coupled receptors by chemoattractants, i.e. fMLP, leukotriene B4, C4a and IL-8, induces not only direct motility but also other biological functions, such as the respiratory burst and phagocytosis.[36] Results of this study indicate that LAMs, in contrast to classical chemoattractants, failed to induce production of O_2^- or phagocytosis. The most well-defined classical signal transduction pathway through G protein-coupled receptors is the activation of phospholipase C resulting in the release of diacylglycerol and inositol 1,4,5-triphosphate, which in turn act as second messengers to activate protein kinase C and elevate cytosolic $[Ca^{++}]_i$, respectively.[36] We found that calphostin, a specific protein kinase C inhibitor, did not significantly affect the chemotactic response of monocytes to LAMs. Moreover, we demonstrated that LAMs neither mobilized $[Ca^{++}]_i$ nor altered the chemotactic response induced by fMLP. Protein phosphorylation is a common mechanism of intracellular signaling in virtually all types of cells, involving beside protein kinase C, tyrosine phosphorylation[36] and the rapid elevation of the enzymatic activity of a family of closely related serine-threonine kinases, known as MAP kinases.[37] We found that genistein, a PTK inhibitor, but not the MEK-1 inhibitor, PD 98059, significantly affected monocyte chemotaxis toward LAMs. These results, in agreement with those obtained with T-cells,[34] suggest that the chemotactic response of monocytes to LAMs depends, at least in part, on the activation of protein tyrosine kinases. These enzymes in turn may activate enzymes of the MAP kinase signal transduction cascade, other than MEK-1.

As far as concerns phagocytosis, we found that ManLAM, at physiologic concentrations, inhibited in a dose-dependent manner phagocytosis of *M. tuberculosis* and zymosan particles by MDMs. The receptor-ligand interactions in *M. tuberculosis* and zymosan phagocytosis for macrophages are complex and require co-operation between different receptor types, among which complement receptors and mannose receptors.[28,29,38,39] The binding of ManLAM to macrophage MRs could be the mechanism by which ManLAM inhibits macrophage phagocytosis. However, we cannot exclude that insertion of LAM into plasma membrane could alter membrane fluidity and perturb both cellular functions and receptor expression. Results of this study are consistent with findings obtained in other *in vitro* systems indicating that LAMs could participate in the early immune response to mycobacteria mainly through their ability to modulate mononuclear phagocyte activities. In particular, the chemoattractant activity of LAMs for human mononuclear phagocytes may represent an important event *in vivo* for the formation and maintenance of tuberculous granuloma. Moreover, the inhibition of macrophage phagocytosis by ManLAM may act synergistically with other anti-inflammatory capabilities of this molecule in order to favor the survival of *M. tuberculosis* and the establishment of a chronic infection.

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AF was responsible for the conception of the study and its design; she wrote the article with CF, and both were responsible for the data interpretation. CF died suddenly on March 1999 before the revision of this manuscript was concluded. GGG indicated the area of research, made a fundamental contribution to the analysis and interpretation of the data, revised the manuscript and gave final approval of the version sent for submission. The order of the authors is related to their contribution. The authors thank Marco Manstretta for his technical assistance; Dr. Simona Cattaneo for help with intracellular calcium measurements; Dr. John T. Belisle (Colorado State University, Fort Collins, USA) for generously providing LAM preparations.

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Disclosures

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Potential implications for clinical practice

- These findings provide some insights into the current understanding of the immunopathogenesis of tubercolosis, indicating that LAM of the mycobacterial cell wall has an important modulating role in host-bacterium interactions and may be considered a key pathogenetic factor.
- The identification of specific mycobacterial constituents and the mechanism by which they favor the entry of *Mycobacterium tuberculosis* into phagocytes and/or modulate cell functions will lead to the development of novel strategies that may ultimately constitute a basis for preventing and/or treating tuberculosis.
- At present, however, no clinical indications can be inferred from the results so far obtained.

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