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Immunomodulatory Spectrum of Lipids Associated with *Mycobacterium avium* Serovar 8

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Lipid fractions obtained from *Mycobacterium avium* serovar 8 were assessed for the ability to affect various immune functions of human peripheral blood mononuclear cells (PBM). Lipids included a total lipid fraction and fractions eluted from silicic acid column separation of that total lipid fraction, using chloroform and chloroform-methanol combinations. Lipid fractions were assayed for total carbohydrate and total 6-deoxyhexose content and were assessed for the ability to influence human macrophage function and the capacity to induce secretion of prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha in PBM. The total lipid and serovar-specific glycopeptidolipid (GPL) fractions both induced significant levels of tumor necrosis factor alpha, as well as PGE_2, in PBM exposed to a sublethal concentration of 100 μg lipid per 2×10^6 cells. In addition, the same concentrations of the 5 to 7% and GPL fractions induced significant levels of leukotriene B4 in PBM. Comparison of carbohydrate and 6-deoxyhexose contents of each fraction suggested a relationship to carbohydrate content and ability of fractions to induce immune modulator secretion. Analysis of GPL fractions from *M. avium* serovars 4 and 20 revealed that those GPL lacked the ability to induce PGE_2. These results are explained by considering the difference in the carbohydrate residues of the oligosaccharide moieties.

Recent interest in the *Mycobacterium avium* complex has resulted in numerous publications regarding the pathogenic nature of this AIDS-associated opportunistic pathogen. Several studies have suggested that factors contributing to *M. avium* pathogenicity are associated with that organism's cell envelope, as reviewed recently (38). Because components of the mycobacterial cell envelope are the initial factors that affect host responses, it is important to identify and characterize these specific components in order to fully understand the pathogenic nature of the *M. avium* complex. Our previous studies have focused upon the serovar-specific glycopeptidolipid antigens (GPL) that are noncovalently associated with the outer portion of the cell envelope (3) and are slowly degraded substances (31, 56) that accumulate postphagocytically as the mycobacteria grow within host macrophages (16, 41). The GPL are unique to the *M. avium* complex and represent a major portion of the *M. avium* cell envelope.

Recently, we demonstrated that a total lipid fraction extracted from *M. avium* serovar 4 is immunosuppressive and can induce significant amounts of prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) in human peripheral blood mononuclear cells (PBM) (2). We also demonstrated that a purified GPL fraction from *M. avium* serovar 4 can induce secretion of TNF-α in mouse spleen cells (36) and human PBM (2) but is only somewhat immunosuppressive to mouse splenic cells (36) and not to human PBM (2). The chemically derived lipopeptide core (i.e., β-lipid) of the GPL, however, is immunosuppressive (50) and does not induce TNF-α but induces significant levels of PGE_2 in human PBM (2). The oligosaccharide moiety is not immunosuppressive, nor does it induce TNF-α or PGE_2 (2). Those results have led us to suspect that *M. avium* lipids have a variable effect on host responses, depending upon their chemical composition and their inherent ability to interact with host cells.

In an effort to expand on our previous studies, we have examined the immunomodulatory properties of lipids extracted from *M. avium* serovar 8, another clinically significant serovar frequently isolated from human immunodeficiency virus (HIV)-infected patients (58). A total lipid fraction was extracted from serovar 8 by a Folch extraction procedure, after which various lipid fractions were obtained by adsorption column chromatographic separation techniques. The lipid fractions were then assessed for the ability to affect the function of human macrophages and for the ability to induce the secretion of immunomodulators important in host responses to *M. avium*. Our findings substantiate previous studies with *M. avium* lipids and further define some of the immunomodulatory properties of lipids associated with the *M. avium* cell envelope.

MATERIALS AND METHODS

Mycobacteria. *M. avium* complex serovar 8 (TMC 1468) was provided by the National Jewish Hospital and Research Center (Denver, Colo.) through Darrel Gwinn, National Institute of Allergy and Infectious Diseases (Bethesda, Md.). Mycobacteria were cultivated in 7H9 Middlebrook broth or agar (Difco Laboratories, Detroit, Mich.) which had been supplemented with glycerol and oleic acid-albumin-dextrose (Difco) as described previously (2).

Extraction and purification of lipids. Total lipids were extracted from lyophilized mycobacteria with chloroform-methanol (2:1) and processed by means of the Folch procedure as previously described (2, 50, 57). Individual lipid fractions were purified by means of a short column chromatographic procedure as previously described (14). Lipid fractions were examined by development in chloroform-methanol-water (60:12:1; solvent A) or chloroform-methanol-water (65:25:4; solvent B), and GPL were identified by reaction with orcinol-sulfuric acid (2). Mycobacterial fractions (at equivalent concentrations used in this study) were tested for endotoxin activity by means of a Pyrogent Plus kit (Whittaker).

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All samples contained ≤0.03 endotoxin unit/100 μg of lipid. The same lipid fractions were used in all of the experiments. The GPL represent about 14.3 ± 3.4% (n = 7-liter batches of *M. avium*) of the total extractable lipid from the mycobacteria under normal shake culture conditions. This does not take into account the amount of GPL released into the medium, which represents about 0.6% of the cell mass (3). From previous results obtained by using a comparison of turbidimetric readings and viable plate counts (i.e., Klett reading of 50 = 4.6×10^7 viable units/ml) (51), plus an assessment of total lipid extracted from *M. avium* (i.e., 100 ml of an *M. avium* culture with a Klett reading of 50 contains approximately 4.6×10^9 mycobacteria from which approximately 1.84 mg of GPL can be obtained), we estimate that the amount of GPL used in this study (100 μg/2×10^6 mononuclear cells) would be equivalent to a ratio of approximately 125 mycobacteria per mononuclear cell. This does not, however, take into account the GPL that accumulates during prolonged growth in culture (3), nor does it take into account the large amount of GPL that accumulates in the host (41) because of the resistance to degradation that these lipids demonstrate (31, 56).

In some experiments, radiolabeled lipids were purified by the same procedure in order to monitor the contents of lipid samples. Those radiolabeled lipids were assayed by means of high-pressure liquid chromatography (HPLC) to confirm the presence or absence of GPL in various fractions (2). Lipids were separated on a Beckman Ultrasphere 5-μm spherical 80-Å (8-nm) pore SI analytical column (4.6 by 250 mm), using Beckman System Gold for computer analysis, a model 166 variable UV detector (257 nm), and a model 171 solid system radioisotope detector, as described previously (2, 4, 57). Samples were separated in a mobile phase of 100% chloroform for 10 min followed by separation in a 40-min gradient of 0 to 10% methanol in chloroform, at a flow rate of 1.0 ml/min. Following the gradient step, 10% methanol in chloroform was maintained for 10 min and then returned to 100% chloroform over 10 min (2, 50, 57).

Analysis of HPLC data analysis. Beckman System Gold was used for computer analysis, after which graphs were produced by a procedure described by Burrier (12). Sample files for each HPLC analysis were analyzed and subsequently translated to .DIF files, using the File Management utility in System Gold (12), as described previously (2). To facilitate printing, portions of HPLC runs were deleted on the graph. These portions of the analysis do not contain any radiolabeled lipid components.

Human PBM. Human PBM were derived from the buffy coat fraction of whole blood from normal donors as described previously (2, 39). Viability was determined by means of the trypan blue dye exclusion test to be ≥97%. For some TNF induction experiments, platelets were removed by centrifugation over fetal calf serum (FCS) in a manner similar to that described by Merino et al. (34). Aliquots of the PBM suspension at 2×10^7/ml in RPMI 1640 were layered over threefold volumes of FCS and centrifuged at 125 × g for 15 min. The cell pellet was then resuspended in RPMI 1640 with 10% heat-inactivated FCS (RPMI complete), and the cell count was adjusted to 10^6 viable cells per ml by trypan blue exclusion.

Application of lipids. Glass coverslips (PGC Scientifics, Gaithersburg, Md.) were washed in a 2% solution of Citranox (Alconox, New York, N.Y.) to remove endotoxin and then rinsed with endotoxin-free distilled water followed by methanol. Coverslips were then heat sterilized at 180°C for 4 h and inserted in the bottom of wells of a 12-well tissue culture plate. Lipids were dissolved in chloroform, applied to the sterile coverslips, and then allowed to dry prior to addition of human cells. Appropriate controls included coverslips to which equivalent amounts of solvent had been applied.

Intracellular growth experiments. Human macrophage monolayers were infected by *M. avium* serovar 2 as described previously (2, 39). The macrophages were allowed to phagocytize the bacteria for 4 h at 37°C, after which all of the extracellular bacilli were thoroughly washed away with Hanks balanced salt solution. The number of phagocytized bacteria was determined by lysing the macrophages with 0.25% (wt/vol) sodium dodecyl sulfate (SDS) and plating the lysates on 7H11 agar medium for viable count determinations (2, 39). Addition of 0.25% sodium dodecyl sulfate does not lower the bacterial viable counts (39). After phagocytosis, viable bacteria were also enumerated by lysing the macrophages at various time intervals and plating the bacteria onto 7H11 agar medium. The results were compared with the growth of bacteria in the control culture (untreated macrophages). In experimental studies, macrophages were first exposed to lipid fractions for 2 h prior to infection (2).

Viability assay. Viability assays were conducted as described previously by applying lipids dissolved in chloroform onto glass coverslips which had been placed in cell culture wells (2, 50). In all cases, glass coverslips were treated in parallel with equivalent amounts of solvent to obtain appropriate control values for data compilation. Human mononuclear cells were added to individual wells to achieve actual concentrations ranging from 5 to 100 μg of the fraction being tested per 4×10^5 cells. This is equivalent to 25 to 500 μg per 2×10^6 cells, which is the number of actual cells used for the various assays in this study. Following 2, 24, and 48 h of exposure, viability of cells was determined by trypan blue dye exclusion as described previously (50).

PGE_2 and LTB4 assay. PGE_2 was measured by means of a radioimmunoassay (Pasteur Diagnostics, Paris, France) as described previously (2, 46) and also with a PGE_2 enzyme immunoassay kit purchased from Cayman Chemical Company (Ann Arbor, Mich.) Leukotriene B4 (LTB4) was assayed with a LTB4 enzyme immunoassay kit purchased from Cayman Chemical Company. Supernatants from treated and nontreated cells were derived from culture wells as described previously (2).

TNF-α assay. A two-site sandwich enzyme-linked immunosorbent assay for TNF-α was developed by using a monoclonal capture antibody (Upstate Biotechnology, Inc.) and a polyclonal immunoglobulin G fraction of rabbit antisera (Sigma). This procedure was described in a previous publication (2) and was used here to quantitate TNF-α. A TNF-α bioassay, using the L929 mouse target cell line developed from studies by Valone et al. (54) and Flick and Gifford (24), was used to confirm biological activity of TNF-α induced by the various lipid fractions. This procedure was described in a previous publication (2).

Statistical analysis. A one-way analysis of variance (ANOVA) was used to determine the significance of differences between groups, and a correction for multiple comparisons was done by means of the Bonferroni method (InStat software; GraphPad Software, San Diego, Calif.).

RESULTS

Mycobacterial fractions. Five mycobacterial lipid fractions were used in this investigation and are defined here as total lipid, chloroform, 3%, 5 to 7%, and purified GPL fractions. The total lipid fraction contains the total lipid extractable by means of the Folch procedure and includes noncovalently linked lipids uncontaminated with nonlipid components such as free sugars and amino acids (2, 32). The other fractions were obtained by column adsorption chromatographic purification involving the use of chloroform and various concentrations of methanol in chloroform (14). The chloroform fraction contained neutral lipids, such as hydrocarbons, carotenoid pigments, glycerides, waxes, fatty alcohols, and fatty acids (32). Following the elution of the chloroform fraction (as judged by elution of pigments), lipids were eluted with various concentrations of methanol in chloroform. The various fractions are depicted by the HPLC pattern obtained by analyzing [^{14}C]Phe-radiolabeled lipids (Fig. 1). The 3% fraction contained lipids eluted in 3% methanol in chloroform, and the 5 to 7% fraction contained lipids eluted in 5 to 7% methanol in chloroform. The 3% fraction would contain nonglycosylated lipopeptides. One of these lipopeptides has previously been identified by us in both rough and smooth colony variants of *M. avium* serovar 4 and contains phenylalanine, alanine, and isoleucine but no carbohydrate (4). Likewise, similar lipopeptides have been identified in *M. avium* serovar 2 (6). Examples of lipids in the 5 to 7% fraction would be the apolar GPL that have a structure similar to that of the GPL, with the exception that the oligosaccharide is replaced with 6-deoxytalose (Fig. 1) (9, 10, 27). The purified GPL were obtained by further elution with 8 to 10% methanol in chloroform (Fig. 1) (14). Structural analysis for the GPL of serovar 8 has been reported previously to contain an oligosaccharide of the following structure: 4,6-(1′-carboxyethylidene)-3-*O*-Me-D-Glc*p*-(β1-3)-L-Rha*p*-(α1-2)-6d-L-Tal (8). The lipid fractions were also assayed by thin-layer chromatography (TLC), the results of which are given in Fig. 2. The GPL can be identified by their characteristic yellow-gold color reaction to orcinol following TLC separation (Fig. 2, lanes A and E).

The GPL from serovars 4 and 20 were purified by column chromatography using procedures that have been described previously (3, 14, 11). Structural analysis for the GPL of serovar 4 has been reported previously to contain an oligosaccharide of the following structure: 4-*O*-Me-L-Rha*p*-(α1-4)-2-*O*-Me-L-Fuc*p*-(α1-3)-L-Rha*p*-(α1-2)-6d-L-Tal (33), and analysis of the GPL from serovar 20 has been reported to be 2-*O*-Me-D-Rha*p*-(α1-3)-2-*O*-Me-L-Fuc*p*-(α1-3)-L-Rha*p*-(α1-2)-6d-L-Tal (3).

Effects of lipids on viability of human mononuclear cells. Cells were exposed to individual lipid fractions for 24 and 48 h, after which cell viability was determined by trypan blue exclusion. Viability for nontreated cells was 89% ± 2.4% and 90% ± 4.5% at 24 and 48 h, respectively. At 24 h, the viability for

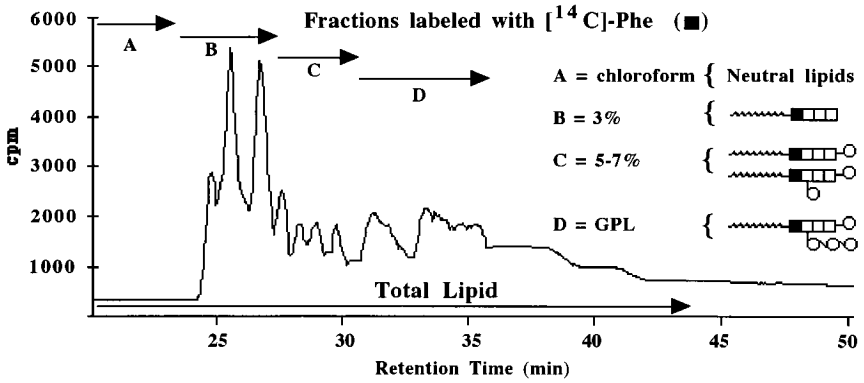

FIG. 1. Representation of HPLC separation of *M. avium* serovar 8 lipid fractions. Lipids had previously been internally radiolabeled with [^{14}C]Phe. Areas which coincide with fractions eluted by silicic acid adsorption column chromatography are given as chloroform (A), 3% methanol in chloroform (B), 5 to 7% methanol in chloroform (C), and glycopeptidolipid (GPL) (D). Structures of representative lipids that have previously been identified in each fraction are given as ∿∿∿■□□□, representing lipopeptides, ∿∿∿■□□□—○, representing partially glycosylated lipopeptides, ∿∿∿■(○)□□□—○, representing apolar GPL, and ∿∿∿■(○\○\○\○)□□□—○, representing GPL containing complete oligosaccharide. HPLC conditions for separation are described in the text.

the various lipid fractions ranged from 90% ± 3.2% (total lipid) to 97% ± 0.7% (GPL). At 48 h, the viability ranged from 83% ± 6.0% (5 to 7% fraction) to 94% ± 2.1% (GPL). Thus, there was no significant decrease in viability following 48 h of treatment with the various lipid fractions.

Effects of mycobacterial fractions on intracellular growth of *M. avium*. Human PBM were added to wells that contained coverslips to which lipids had previously been applied. One hundred micrograms of each lipid fraction was applied to each coverslip, and 2×10^6 cells were added to each well. Exposure of human macrophages to the chloroform lipid fraction resulted in a marked decrease in the ability of the cells to restrict the growth of mycobacteria. This is represented as an increase in the number of intracellular viable mycobacteria obtained from the infected macrophages after 7 days (Fig. 3). All other lipid fractions affected the ability of the macrophages to control the intracellular growth of mycobacteria, but to a lesser degree than the chloroform lipid fraction (Fig. 3). Analysis by an ANOVA, with a Bonferroni multiple-comparison test, revealed that there was no significant difference between the means of the nontreated and the treated groups, except for the cells treated with the chloroform fraction, at day 7 ($P < 0.01$).

PGE_2 release from cells exposed to mycobacterial lipids. Two sets of experiments were conducted with regard to PGE_2 release from PBM. In one experiment, PBM were obtained from healthy donors in Paris, France. Release of PGE_2 was assayed by means of a radioimmunoassay previously described

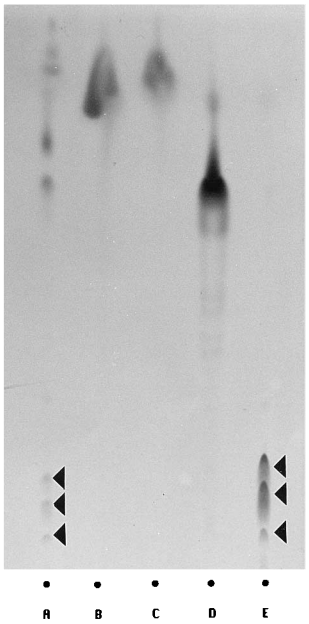

FIG. 2. TLC separation of *M. avium* serovar 8 lipids. Lipids were applied to silica gel plates in concentrations of 100 μg and developed in solvent A. Lipids were identified by spraying plate with orcinol-sulfuric acid solution and heating. GPL were identified by their characteristic yellow-gold color after heating of the TLC plate and are marked by arrowheads. Fractions: A, total lipid extracted from *M. avium* serovar 8; B, chloroform fraction; C, 3% fraction; D, 5 to 7% fraction; E, GPL fraction.

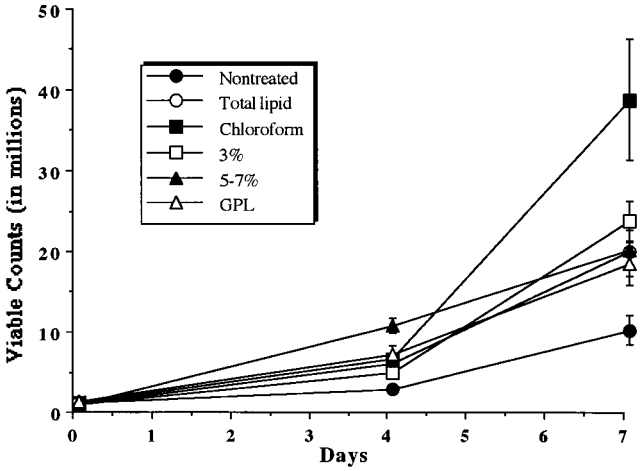

FIG. 3. Intracellular growth of *M. avium* serovar 2 in human peripheral blood macrophages following exposure of macrophages to lipid fractions obtained from *M. avium* serovar 8. Controls (Nontreated) were exposed to coverslips to which an equivalent amount of solvent had been applied. Macrophages were lysed with SDS, and mycobacteria were plated on Middlebrook 7H10 agar to determine the number of viable cells at 0, 4, and 7 days following infection. Final concentrations of lipid fractions were 100 μg of lipid per 2×10^6 macrophages. The results are presented as means ± SEM for three assays from one healthy donor.

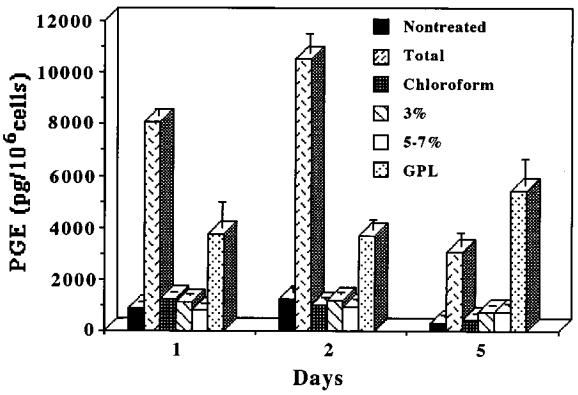

FIG. 4. Secretion of PGE_2 from human PBM exposed to lipid fractions obtained from *M. avium* serovar 8. Cells were exposed to lipids at a concentration of 100 μg of lipid per 2×10^6 cells for 1, 2, and 5 days prior to the PGE_2 assay of supernatants. Controls (Nontreated) consisted of nontreated cells exposed to coverslips previously treated with chloroform. Results are reported as means ± SEM for two experiments.

(2). In the second experiment, PBM were obtained from healthy donors in Fort Worth, Tex., and PGE_2 release was assayed by means of an immunoassay using a commercial kit obtained from Cayman Chemical Company. In both experiments, PBM were exposed to the same concentration of lipids as used for the phagocytic assay, i.e., 100 μg of lipid per 2×10^6 cells.

In the first experiment, PGE_2 was assayed following 1, 2, and 5 days of exposure to lipid fractions. PBM from two donors were examined, and the results are reported as the means of both experiments. In these experiments, it was observed that both the total lipid fraction and the purified GPL fraction induced significant levels of PGE_2 after 1 day of exposure (Fig. 4). Levels of PGE_2 remained elevated during the 5-day exposure period. None of the other lipid fractions induced significant levels of PGE_2 during the 5-day exposure period. These results were similar to those observed previously in human PBM exposed to equivalent concentrations of total lipid extracted from *M. avium* serovar 4 (2). However, in contrast to that previous study, GPL from serovar 8 also induced significant levels of PGE_2 secretion throughout the exposure period (2). Previously we had observed that GPL from serovar 4 did not induce PGE_2 secretion in PBM (2). As a result, we repeated the experiments and assayed PGE_2 release by means of an immunoassay and also examined the ability of GPL from serovars 4 and 20 to induce the immunomodulator.

In these experiments, PBM were obtained from four individual healthy donors in Fort Worth, Tex. As before, exposure of PBM to total lipid extracted from serovar 8 produced significant levels of PGE_2 following 24 h of exposure to total lipid and purified GPL (Table 1). In addition, GPL from serovar 8 also induced significant levels of PGE_2 following 24 h of exposure (Table 1). PBM from two of the donors were also exposed to GPL from serovars 4 and 20; however, no significant levels of PGE_2 were detected following 24 h of exposure. These results confirm our previous study in which we observed no significant levels of PGE_2 following exposure of PBM to GPL from serovar 4 (2). It is not yet clear why GPL from serovar 8 induced significant levels of PGE_2, whereas GPL from serovars 4 and 20 did not. However, a possible explanation is given in Discussion.

LTB4 release in PBM exposed to lipids. In an effort to search for other possible immunomodulators, we also examined the ability of the lipid fractions to induce secretion of LTB4. PBM were assayed for LTB4 following 24 h of exposure to lipid fractions (100 μg of lipid per 2×10^6 cells). Fractions obtained from PBM exposed to the chloroform fractions were not assayed for LTB4 because previous analysis revealed a lack of PGE_2. It was observed that the 5 to 7% and GPL fractions were able to induce 29.2 ± 10.3 (standard error of the mean [SEM]) and 24.1 ± 6.6 (SEM) pg of LTB4 per ml, respectively, in PBM obtained from four healthy patients, whereas the nontreated cells only produced 12.8 ± 1.3 (SEM) pg of LTB4 per ml. These results were judged to be significant by a repeated-measures ANOVA and a Bonferroni multiple-comparison posttest. None of the other fractions induced LTB4 in significant levels compared with the nontreated cells. The total lipid fraction induced almost a twofold increase in LTB4 (23.1 ± 5.8 [SEM] pg/ml); however, the results were not found to be significant when tested by a repeated-measures ANOVA and a Bonferroni multiple-comparison posttest. However, using a less conservative posttest (e.g., the Student-Newman-Keuls multiple-comparison posttest), the results for the total lipid fraction were also found to be significant ($P < 0.05$).

TABLE 1. Levels of PGE_2 induced in human PBM following a 24-h exposure to 100 μg of lipid per 2×10^6 cells[a]

Fraction	PGE_2 (ng/ml)	*P* value
Nontreated	3.3 ± 0.3	
Total lipid	62.8 ± 7.9	<0.001
Chloroform	4.9 ± 1.2	NS
3%	6.1 ± 1.8	NS
5–7%	10.4 ± 4.2	NS
GPL	76.9 ± 14.8	<0.001

[a] PGE_2 was assayed by means of an immunoassay described in the text. Values represent means obtained from four patients ± SEM. Statistical significance was determined by a one-way ANOVA and a Bonferroni multiple-comparison posttest. NS, not significant. Lipid fractions are described in the text.

TNF-α release from PBM exposed to mycobacterial lipids. PBM in the second experiment described above were also assayed for TNF-α following 24 h of exposure to lipids. Results obtained from all four donors are given in Table 2. Treatment with either total lipid or GPL, at a concentration of 100 μg of lipid per 2×10^6 cells, resulted in significant amounts of TNF-α secretion in PBM (Table 2). Analysis of PBM (2×10^6 cells) exposed to 50 μg of either the chloroform or the GPL fraction revealed a level of 276 ± 43 (SEM) or 194 ± 27 (SEM) pg of TNF-α per ml, respectively, indicating a dose response in the PBM from all four patients to those two fractions. Higher doses of 250 μg of lipid per 2×10^6 cells were not used because these have been shown to approach toxic levels in PBM (2). Although not significant, a threefold increase in

TABLE 2. Levels of TNF-α induced in human PBM following a 24-hour exposure to 100 μg of lipid per 2×10^6 cells[a]

Fraction	TNF-α (pg/ml)	*P* value
Nontreated	19.8 ± 3.2	
Total lipid	332 ±59.8	<0.001
3%	33.5 ± 13.2	NS
5–7%	56.0 ± 24.6	NS
GPL	229 ± 67.0	<0.01

[a] TNF-α was assayed by means of an immunoassay described in the text. Values represent means obtained from four patients ± SEM. Statistical significance was determined by a repeated-measures ANOVA and a Bonferroni multiple-comparison posttest. NS, not significant. Lipid fractions are described in the text.

TABLE 3. Percent carbohydrate and 6-deoxyhexose contents of serovar 8 lipid fractions compared with their ability to induce TNF-α, PGE_2, and LTB4

Lipid fraction	% Carbohydrate (mean ± SE)[a]	% 6-Deoxyhexose (mean ± SE)[b]	Response[c]		
			TNF-α	PGE_2	LTB4
Total lipid	13 ± 0.8	10 ± 0.5	++++	++++	+ (NS)
Chloroform	<5	NT[d]	−	−	NT
3%	<5	NT	−	−	−
5–7%	46 ± 3.5	41 ± 3.5	++ (NS)	++ (NS)	++
GPL	35 ± 1.8	22 ± 1.2	++++	++++	+

[a] Total carbohydrate content was determined as described in the text (17).
[b] Total 6-deoxyhexose content was determined as described in the text (15).
[c] Quantitation of immunomodulators was performed by immunoassay procedures described in the text. Response is arbitrarily given as − to ++++, depending on the amount of immunomodulator produced. Each + indicates a onefold increase over normal values. In some cases, responses indicated as ++++ were actually more than fourfold increases. NS, not significant. Results are reported as positive here because some activity was noted (twofold increase), even though evaluation by an ANOVA and posttest indicated lack of significance.
[d] NT, not tested because of negative carbohydrate assay.

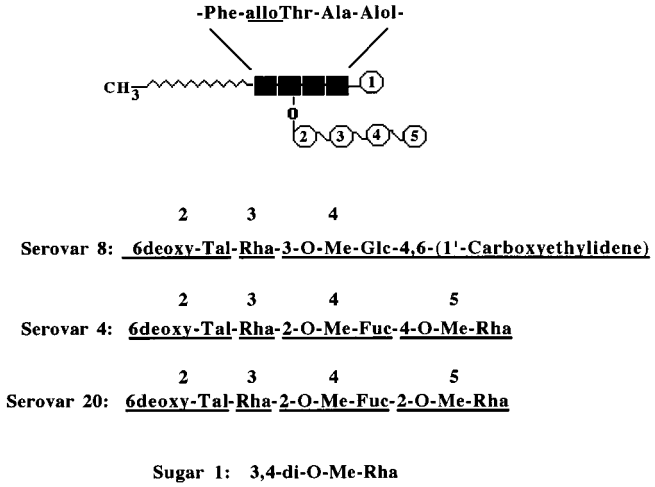

FIG. 5. Structural representation of the oligosaccharide moieties from the GPL of *M. avium* serovars 4 (33), 8 (8), and 20 (3).

TNF-α secretion was induced in cells exposed to the 5 to 7% fraction (Table 2). This result possibly suggests that further purification of the fraction might result in a component(s) that has the ability to induce secretion of significant levels of TNF-α.

Ability of GPL from serovar 20 to induce TNF-α release was also examined in PBM from two of the donors in experiment 2 described above. The increase in levels of TNF-α was observed to be only 29.0 ± 6.4 (SEM) pg/ml ($P > 0.05$, not significant). Levels of TNF-α secretions from PBM exposed to equivalent concentrations of serovar 4 GPL have been reported previously as 262 ± 71 pg/ml (SEM), levels similar to those reported in the current study for serovar 8 (2). These findings indicate a variability in the capacity of different GPL to induce TNF-α.

Carbohydrate and 6-deoxyhexose content of lipid fractions. Lipid fractions were assayed for total carbohydrate by using a phenol-sulfuric acid colorimetric method (17) and for total 6-deoxyhexose content by using a colorimetric method described by Dische and Shettles (15) for methylpentoses. Each fraction was assayed in triplicate. The percent total carbohydrate and 6-deoxyhexose contents for the lipid fractions are given in Table 3, along with a rating regarding the ability of each fraction to induce secretion of various immunomodulators. With regard to the carbohydrate and 6-deoxyhexose contents, it is important to note the following. The carbohydrate moieties that would be detected by these procedures would include the 6-deoxyhexoses commonly associated with the GPL. These sugars would give a positive reaction for either of the assay procedures. Therefore, it is not surprising to see that of the three fractions showing carbohydrate content, all but one is equivalent with the value obtained for the 6-deoxyhexose assay. The equivalent values for the total lipid and 5 to 7% fractions can be explained by the presence of apolar GPL, which contain a 3,4-di-*O*-methylrhamnose linked to alaninol on the terminal end of the peptide moiety (sugar 1; Fig. 5) and a 6-deoxyhexose linked to *allo*-threonine in the peptide moiety. Both of these sugars would give a positive reaction for the carbohydrate and 6-deoxyhexose assays.

The total carbohydrate and 6-deoxyhexose values for the GPL fraction were not equivalent. This can be explained by considering the previous structural studies conducted on the GPL of the *M. avium* complex (8). As discussed in Material and Methods, the structures for the GPL oligosaccharide moieties of serovars 4, 8, and 20 have been reported previously. An evaluation of the sugars indicates that of the four sugars associated with the GPL of serovar 8 (Fig. 5), all would give a positive reaction in the carbohydrate assay but only three would react in the 6-deoxyhexose assay. Sugar 4 (Fig. 5) is not a 6-deoxyhexose and would therefore not react in that assay procedure. This would account for the difference in the values obtained for those two tests. When the GPL from serovars 4 (personal observation) and 20 (3) are assayed, the values for carbohydrate and 6-deoxyhexose are equivalent. This is to be expected because all five sugars associated with those GPLs are 6-deoxyhexoses and would therefore react in both assay procedures.

DISCUSSION

Concepts of mycobacterial pathogenicity take on a different perspective when one considers the *M. avium* complex. As discussed in a previous publication, it is more reasonable to assume that *M. avium* pathogenicity results from multiple factors that are "subtle in nature" and promote virulence by "either protecting the organism from the destructive properties of the host's macrophages and/or by modifying the immunological response of the host" (1). This is in contrast to *Mycobacterium tuberculosis*, which has a higher degree of pathogenicity, as suggested by the reduced ability of an HIV-infected host to control that infecting organism compared with *M. avium*. Patients infected with HIV can generally maintain a status quo with an *M. avium* infection until the CD4 lymphocyte counts are depleted to $<100/mm^3$, something that occurs much later in the spectrum of the HIV infection (19). With *M. tuberculosis*, this loss of immunological effectiveness is frequently seen quite a bit earlier in the course of the HIV infection, sometimes before other opportunistic infections are observed (55). Although a complete explanation for mycobacterial pathogenicity cannot be given as yet, there is evidence to suggest that pathogenicity of both *M. tuberculosis* and *M. avium* is related to the ability of these organisms to induce various cytokines and other immunomodulators.

Regarding *M. avium* pathogenicity, several studies have demonstrated the ability of that organism, or its products, to modulate host responses. From the intracellular growth experiments reported here, it was observed that all the lipid fractions

markedly affected the ability of the macrophages to control intracellular growth; however, only the chloroform fraction was able to do so in a significant manner. Lack of correlation with the ability of the various lipid fractions to induce immunomodulators and their ability to alter macrophage function lead us to suspect that the lipids are acting in a nonspecific manner with regard to their effect on macrophage function. The effects that we observed on macrophage function are probably associated with the lipid's chemical ability to nonspecifically interact with the membranes of those cells and not the result of their specific ability to induce immunomodulators via receptor mechanisms. This is apparently not the case for induction of various immunomodulators, however.

As discussed in previous publications (2, 38), reports on the ability of *M. avium* to induce PGE_2 secretion are varied. Some investigators report the immunosuppressive capacity of *M. avium* and show evidence for PGE_2 release by reversing the effects with indomethacin (13, 18, 43). Others have reported on partial removal of suppression by indomethacin and implied other mediators in addition to PGE_2 (37, 53), while others have reported that indomethacin was not effective at removing suppression, indicating that PGE_2 was not involved (52).

In our previous publications, we have demonstrated the immunosuppressive effects of *M. avium* lipid components (11, 30, 50) and reported that exposures of human PBM to a total lipid fraction and the β-lipid fragment of the serovar-specific GPL from *M. avium* serovar 4 are capable of inducing secretion of PGE_2 (2). Because we also obtained varying results when indomethacin was used (36, 37), we suggested that perhaps other mediators of immunosuppression also play a role in *M. avium* pathogenicity (2, 36).

As a confirmation of our previous studies, we have further demonstrated the ability of lipids from another *M. avium* serovar to induce PGE_2 secretion in human PBM. Total lipid extracted from serovar 8 was able to induce secretion of PGE_2 throughout a 5-day exposure period and was consistently observed in several donors. In addition, the GPL from serovar 8 was capable of inducing significant levels of PGE_2 in human PBM. Interestingly, the GPL from *M. avium* serovars 4 and 20 were not capable of inducing significant levels of PGE_2 secretion in these cells (reference 2 and this report). It is not clear why these results occurred, but as discussed below, we feel that it may be somehow related to the different oligosaccharide moieties attached to the GPL of these serovars.

In this study, we also observed that exposure of human PBM to the 5 to 7% lipid fraction and GPL fraction resulted in the secretion of significant levels of LTB4. None of the other lipid fractions were capable of inducing significant levels of LTB4, suggesting that GPL components (apolar GPL are present in the 5 to 7% fraction) are important in stimulating this immunomodulator. Although the total lipid fraction was able to induce LTB4, the levels were not statistically significant, as judged by a repeated-measures ANOVA and the Bonferroni multiple-comparison test. However, when a less conservative multiple-comparison test (i.e., Student-Newman-Keuls test) was used, the response to the total lipid fraction was significant. This may have resulted because even though the GPL components were present in the total lipid fraction, their ability to induce LTB4 was probably diluted by other lipids which are not active in this capacity.

LTB4 is in a class of leukotrienes which are potent immunomodulators derived from the metabolism of arachidonic acid (7, 42). In addition to a variety of different functions, LTB4 has been reported to be able to augment macrophage and monocyte cytotoxic activities, which enhances production of interleukin-1 (IL-1) and TNF-α (25, 26) and also IL-6 (40). LTB4, at concentrations of 10^{-14} to 10^{-8}, significantly enhanced TNF-α activity in human monocytes by 15 to 75% in 24 h, with the maximal enhancement being observed at 10^{-10} to 10^{-8} M LTB4 (25). The levels of LTB4 that were observed in our study are well within this range (significant levels of LTB4 in our study were 10^{-11} to 10^{-10} M). In studies with human monocytes, it has been determined that treatment with LTB4 induces the transcription of the IL-2 receptor (IL-2R) alpha gene by 30 min, resulting in augmented expression of IL-2R alpha gene transcripts by 3 h (47). In addition, treatment with LTB4 can stimulate the expression of IL-2R beta and, with the augmented expression of both alpha and beta chains of IL-2R, causes an enhanced sensitivity of monocytes to IL-2 with regard to TNF-α production (47). LTB4 has also been shown to induce the transformation of human T lymphocytes into suppressor T cells, resulting in suppression of mitogen-induced cell proliferation (45). This may be one explanation for the varied ability of other investigators to completely inhibit immunosuppression by using indomethacin, a drug that inhibits PGE_2 but not LTB4 (see discussion above).

TNF-α is another cytokine that has been associated with mycobacterial pathogenicity. In two previous studies, we reported that a total lipid fraction and a purified GPL fraction obtained from *M. avium* serovar 4 were capable of inducing secretion of TNF-α in human PBM (2, 36). Of the various lipid fractions obtained from serovar 8 in the current study, only the total lipid and GPL fractions were capable of inducing secretion of significant levels of TNF-α in PBM. The results with regard to TNF-α secretion were therefore similar to those reported by us previously for *M. avium* serovar 4. The ability of serovar 8 lipid fractions to stimulate TNF-α secretion was clearly related to the carbohydrate content, which, as indicated by the 6-deoxyhexose content, was most likely due to GPL and/or GPL-related structures. It was also observed that the 5 to 7% fraction was able to induce TNF-α levels almost three times that of nontreated cells; however, when the results from all four patients were analyzed together by means of an ANOVA, they were not found to be significant. We feel that this finding indicates that the 5 to 7% fraction may contain lipids that have the ability to induce TNF-α, particularly since some individual responses for this fraction were statistically significant when computed alone (data not shown). Further purification will be necessary to define their potential for this activity. It is interesting to note that GPL from serovar 20 did not induce significant levels of TNF-α, a finding which may suggest a receptor mechanism as an explanation for this variation.

It has been demonstrated that TNF-α can stimulate arachidonic acid metabolism in vivo (23) and in tissue cultures of intestinal epithelial cells (28) and L929 cells (29). This may be one explanation for PGE_2 secretion in our study. PGE_2 generally mediates inhibition of TNF-α (21, 22); however, there are instances in which levels of PGE_2 and TNF-α are elevated together. For example, thermal injury results in altered macrophage function and is associated with a loss of PGE_2-dependent down-regulation of TNF-α synthesis which results in elevated levels of both PGE_2 and TNF-α (35). It has been reported that the glycocalyx of *Staphylococcus epidermidis* (at 10 to 100 μg/ml) can inhibit phytohemagglutinin-stimulated proliferation of human PBM as the result of monocyte production of PGE_2 (48). When the supernatants from those cells are examined, it is revealed that the glycocalyx also activates monocyte production of TNF-α and IL-1, in addition to PGE_2 (48). It has been reported that delayed-type hypersensitivity-initiating factors (oxazolone and picryl chloride) can induce cytokine release (IL-1, IL-6, and TNF-α) from murine macro-

phages in addition to PGE_2 synthesis, cytokine mRNA accumulation being observed at 2 to 4 h, with maximal production of PGE_2 at 8 h (20). In another study involving patients with sepsis, it was observed that total monocyte TNF-α levels were "massively" increased even though there was also a "massive" concomitant increase in PGE_2 levels as well (49). Thus, increased levels of both PGE_2 and TNF-α can be observed under the same experimental conditions.

In a recent publication, it was reported that colonial morphotypes of *M. avium* are important in determining cytokine expression in human monocytes. Shiratsuchi et al. (44) reported that the less virulent smooth-dome colony type of *M. avium* is able to induce higher levels of IL-1α, IL-1β, and TNF-α than the virulent smooth-transparent colony type in human monocyte cultures. However, Northern (RNA) blot analysis revealed that levels of expression of mRNA for IL-1 and TNF-α were comparable for the two colonial morphotypes, suggesting that the translational capacity for the expression of some cytokines may be reduced by the smooth-transparent colony type (44). These results also indicate that there may be different factors associated with the colony morphotypes that contribute to their ability to induce various cytokine events. In that regard, it is interesting to note that Belisle and Brennan (5) have reported that when the GPL contents of smooth-transparent and smooth-opaque (i.e., dome) colony morphotypes of *M. avium* are compared, it is found that the smooth-opaque morphotype always produces more total GPL than the smooth-transparent morphotype. Therefore, it is very likely that variability in the composition of the *M. avium* cell envelope plays an important role in the inherent ability of that organism to modulate immune responses in a host and that there are a multiplicity of factors that contribute to *M. avium* pathogenicity.

In conclusion, it is important to remember the structural difference between the oligosaccharide moiety of the serovar-specific GPL components studied in this investigation. Although the GPL from serovars 4, 8, and 20 are similar, the terminal sugar in the oligosaccharide moiety is different for each of them. As discussed previously (see Results), in the case of serovars 4 and 20, the terminal sugars are 4-*O*-methylrhamnose and 2-*O*-methylrhamnose, respectively. For serovar 8, the terminal sugar is 4,6-(1′-carboxyethylidene)-3-*O*-Me-D-Glc*p*. The difference in these terminal sugars may, in part, be responsible for the variability in our results regarding the various immunomodulators.

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