Radiolabelling of *Mycobacterium avium* Oligosaccharide Determinant and Use in Macrophage Studies

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Internal radiolabelling procedures were used to radiolabel the oligosaccharide determinant of the glycopeptidolipids (GPL) from serovars 4 and 20 of the *Mycobacterium avium* complex. Mycobacteria were cultured in the presence of [6-³H]fucose, [2-³H]mannose or [methyl-³H]methionine, after which radiolabelled native lipid was extracted and distribution of radioactivity in native and deacetylated lipid was determined by thin-layer chromatographic methods. Incorporation of radiolabel was confirmed by examining acid hydrolysates of purified GPL for ³H-labelled sugars on cellulose thin-layer plates. Least incorporation of radiolabel into GPL was observed with [6-³H]fucose, whereas better incorporation was obtained with [2-³H]mannose and [methyl-³H]methionine. Use of [methyl-³H]methionine resulted in the radiolabelling of the methylated sugars in both the oligosaccharide determinant and the 3,4-di-*O*-methylrhamnose located at the terminus of the peptide core. Use of [2-³H]mannose resulted in the incorporation of radioactivity into the oligosaccharide determinant as 2-*O*-methylfucose, found in the GPL of both serovars 4 and 20. GPL radiolabelled with [2-³H]mannose were subsequently examined in macrophage cultures and found to be relatively inert to degradation by those phagocytic cells. These results substantiate earlier findings with the GPL of serovar 20 and indicate that these mycobacterial components may play a role in pathogenesis.

INTRODUCTION

Currently, disseminated infections caused by members of the *Mycobacterium avium* complex are a major consideration in the management of individuals suffering from AIDS (Fainstein *et al.*, 1982; Greene *et al.*, 1982; Kiehn *et al.*, 1985; Masur, 1982). Disseminated *M. avium* complex infections are difficult to treat in such patients because of their impaired cellular immunity and also because members of the *M. avium* complex are resistant to most of the antitubercular drugs (Davidson *et al.*, 1981; Rosenzweig, 1976; Yeager & Raleigh, 1973).

The *M. avium* complex is composed of some 31 individual but related serovars that are differentiated on the basis of an array of closely-related polar glycopeptidolipid (GPL) antigens (Brennan & Goren, 1979) which allow for identification by serological (Schaefer, 1965) and thin-layer chromatographic (TLC) (Brennan *et al.*, 1978) procedures. The general structure of the polar GPL antigens is depicted as a fatty acyl-peptide-sugar core, which is common to all serovars, and an oligosaccharide determinant, which varies from serovar to serovar (Fig. 1) (Brennan & Goren, 1979). Individual serovars also contain apolar counterparts (aGPL) of the polar GPL antigens which do not possess the oligosaccharide determinant but instead contain only a single 6-deoxytalose which is linked to the peptide moiety of the fatty acyl-peptide-sugar

Abbreviations: Ala, alanine; Fuc, fucose; GPL, glycopeptidolipids; aGPL, apolar glycopeptidolipids; dGPL, deacetylated GPL; Man, mannose; Met, methionine; Phe, phenylalanine; Rha, rhamnose; TLC, thin-layer chromatography; TFA, trifluoroacetic acid.

Fig. 1. Structure of apolar GPL components and GPL of serovars 4 and 20, showing incorporation of various radiolabelled components. Incorporation of [^3H]phenylalanine and [^3H]alanine was reported in a previous publication (Hooper *et al.* 1986), and incorporation of [^3H]fucose, [^3H]mannose and [$\text{methyl-}^3\text{H}$]methionine are reported in this paper.

core (Fig. 1) (Brennan & Goren, 1979). The GPL antigens are unique to the *M. avium* complex and represent a major superficial component which is evenly distributed on the surface of the mycobacterial cell (Barrow *et al.*, 1980; Tereletsky & Barrow, 1983).

Because the GPL antigens represent major constituents of the outermost layer of mycobacteria in the *M. avium* complex, it has been suggested that they play a role in pathogenesis (Draper & Rees, 1970). For that reason, previous studies in this laboratory have been designed to examine the fate and distribution of the GPL antigens following phagocytosis by host macrophages (Hooper *et al.*, 1986; Tereletsky & Barrow, 1983). By using radiolabelled GPL antigens, it was determined that the GPL are relatively inert to macrophage degradation (Hooper *et al.*, 1986); however, in that investigation the GPL antigens were radiolabelled with [^3H]alanine and [^3H]phenylalanine, which places the radioactivity in the fatty acyl-peptide moiety that is common to all serovars (Fig. 1). An examination of the postphagocytic handling of the serovar-specific oligosaccharide moiety would be more informative. The purpose of this investigation was to specifically radiolabel the oligosaccharide moiety of the GPL so that the radiolabelled lipids might be used in macrophage degradation studies to examine the fate of the determinant portion of these potentially important mycobacterial components.

METHODS

Mycobacteria. *Mycobacterium avium* complex serovar 4 (TMC #1463) was obtained from the National Jewish Hospital and Research Center (Denver, Colorado, USA) through Dr Darrel Gwinn, National Institute of Allergy and Infectious Diseases (NIH, Bethesda, Maryland, USA). *M. avium* serovar 20 has been described previously (Barrow & Brennan, 1982; Barrow *et al.*, 1980). Mycobacteria were cultivated in 7H9 Middlebrook medium (Difco) supplemented with glycerol and oleic acid-albumin-dextrose (OADC) supplement (Difco) (Brownback & Barrow, 1988; Hooper *et al.*, 1986) until they reached early exponential phase (100–150 Klett units; Klett–Summerson spectrophotometer, no. 42 filter), after which [$6\text{-}^3\text{H}$]-L-fucose (specific activity 25 Ci mmol $^{-1}$, 925 GBq mmol $^{-1}$) ([^3H]Fuc), [$2\text{-}^3\text{H}$]-D-mannose (specific activity 30 Ci mmol $^{-1}$, 1110 GBq mmol $^{-1}$) ([^3H]Man), and [$\text{methyl-}^3\text{H}$]-L-methionine (specific activity 50 Ci mmol $^{-1}$, 1850 GBq mmol $^{-1}$) ([^3H]Met) (ICN Radiochemicals) were added individually to cultures at concentrations ranging from 0.25 to 1.0 $\mu\text{Ci ml}^{-1}$. For batch cultures (1000 ml), [^3H]Man was added at a concentration of 0.5 $\mu\text{Ci ml}^{-1}$.

Some mycobacterial cultures were cultivated in the presence of [$2,3\text{-}^3\text{H}$]-L-alanine (specific activity 50 Ci mmol $^{-1}$, 1850 GBq mmol $^{-1}$) ([^3H]Ala) and [$\text{side chain-}^3\text{H}$]-L-phenylalanine (specific activity 25 Ci mmol $^{-1}$, 925 GBq mmol $^{-1}$) ([^3H]Phe) (ICN) at a concentration of 0.3 $\mu\text{Ci ml}^{-1}$ each (Hooper *et al.*, 1986). Mycobacteria were allowed to grow until they reached stationary phase at 500–550 Klett units, which was equivalent in dry cell weight to 4.4 mg ml $^{-1}$ (SD, ± 0.07 , $n = 4$) and 4.5 mg ml $^{-1}$ (SD, ± 0.09 , $n = 4$) for serovars 4 and 20, respectively. Cells were then autoclaved, harvested by centrifugation and lyophilized (Hooper *et al.*, 1986). Lyophilized cells were stored at -20°C until use.

Chemical procedures. Native lipid was extracted from lyophilized cells with chloroform/methanol (2:1, v/v) at 50 °C as described in previous publications (Hooper *et al.*, 1986; Tereletsky & Barrow, 1983). Total incorporation of radioactivity was determined by measuring the radioactivity of native lipid in EcoLite scintillation fluid (WestChem, San Diego, California, USA). Distribution of radioactivity in radiolabelled lipid was determined by TLC procedures. Briefly, radiolabelled lipids were applied to silica gel TLC plates and developed in either chloroform/methanol/water (60:12:1, by vol.) (solvent A) or chloroform/methanol (11:1, v/v) (solvent B). Following development, 1 cm sections were transferred to scintillation vials and their radioactivity measured in EcoLite scintillation fluid. Location (cm from origin) of GPL was determined by spraying TLC plates with orcinol/sulphuric acid and heating (Barrow *et al.*, 1980; Brennan & Goren, 1979).

In some procedures, deacetylated GPL (dGPL) were also examined by TLC. To obtain dGPL, native lipid was treated with 0.2 M-methanolic NaOH, as described by Brennan & Goren (1979). Distribution of radioactivity in dGPL was determined by TLC as described above.

Purification of both native and deacetylated GPL was accomplished by a one-step adsorption chromatographic procedure described previously (Dimitrijevic *et al.*, 1986). For analysis of sugars, purified GPL were hydrolysed in 2 M-trifluoroacetic acid (TFA) (0.2 ml per mg lipid) at 100 °C for 2 h. Fatty acids were extracted with hexane and sugars were separated on cellulose thin-layer plates in butanol/ethanol/water (32:8:40, by vol.) (solvent C) and detected with aniline/oxalate spray (Horrocks & Manning, 1949). Distribution of radioactivity was determined by measuring the radioactivity of 1 cm sections in EcoLite scintillation fluid and identification of sugars was accomplished by using known standards originating from GPL antigens of serovar 20 (Hooper *et al.*, 1986).

Animals. Female and male C57BL/6NHsd mice were purchased from Harlan-Sprague-Dawley, Indianapolis, Indiana, USA, and raised in groups of 10–20 on a diet of mouse chow and water. Mice 5–12 weeks old were used.

Preparation of macrophage monolayers. Resident peritoneal macrophages were obtained from mice by irrigation of the peritoneal cavity with 2.0 ml ice-cold NCTC medium 109 (Difco) containing 5 U heparin (Sigma) per ml of solution, as described in previous publications (Hooper *et al.*, 1986; Tereletsky & Barrow, 1983). Cell viability was determined by trypan blue exclusion to be >97%. After dilution to 2×10^6 cells ml⁻¹, peritoneal cells were added to 24-well tissue culture plates (Falcon Plastics) in 1.0 ml volumes and incubated for 2 h at 37 °C under 5% CO₂-in-air. Nonadherent cells were removed by washing with medium 109 and plates allowed to incubate overnight to achieve macrophage monolayers as described previously (Hooper *et al.*, 1986).

Addition of radiolabelled GPL to macrophages. Prior to use in macrophage experiments, GPL were maintained in chloroform at -20 °C. To prepare GPL suspensions, samples were transferred to sterile glass vials and dried under a stream of nitrogen as described previously (Hooper *et al.*, 1986). Medium 109 was added to the dried GPL preparations, followed by sonication in an 80 W ultrasonic bath (Branson) for 30–60 min at room temperature and refluxing through a 27 gauge needle (Hooper *et al.*, 1986). Each monolayer was treated with an appropriate amount of GPL suspended in a total of 1.0 ml medium 109 for 2 h at 37 °C under 5% CO₂-in-air. Following treatment, adherent cells and spent media were assayed for distribution of radioactivity in EcoLite scintillation fluid as described previously (Hooper *et al.*, 1986).

Distribution of radioactivity in chloroform-extractable material. To examine the fate of GPL in macrophage cultures, adherent cell monolayers were treated with 200 µg GPL per 3.2×10^5 cells in 1.0 ml medium 109. At each appropriate time interval, spent medium from three culture wells was removed and added to a 60 ml separating funnel. Macrophages from the respective three wells were lysed and removed by adding 1.0 ml 0.05% Triton X-100 (Sigma), as described previously (Hooper *et al.*, 1986). Macrophage lysate was added to the spent medium in the separating funnel and chloroform, methanol and water were added to obtain a solvent ratio of 8:4:3 (by vol.). Mixtures were allowed to stand until two phases had formed, after which the lower chloroform phase was removed, dried under nitrogen, and its radioactivity measured in EcoLite to determine c.p.m. mg⁻¹. The volume of the upper methanol/water phase was measured in a graduated cylinder; 1.0 ml samples were removed and their radioactivity measured in EcoLite to determine the total c.p.m. ml⁻¹.

RESULTS

Incorporation of radioactivity into native lipid

Preliminary studies were conducted on a small scale to determine if sufficient incorporation of radiolabel into GPL could be accomplished with [³H]Fuc, [³H]Man or [³H]Met. Mycobacteria were cultured in 100 ml volumes in the presence of each radiolabelled component at concentrations of 0.25, 0.5 and 1.0 µCi (ml culture)⁻¹. Native lipid was extracted and the amount of radioactivity determined as c.p.m. (mg lipid)⁻¹; the distribution of radioactivity was examined to determine whether the radiolabelled component was appropriate for use in subsequent macrophage studies involving the GPL.

Fig. 2. Distribution on TLC of radioactivity in native lipid (a) and deacetylated lipid (b) from [^3H]Met-radiolabelled serovar 4. Plates were developed in solvent A, and GPL, deacetylated GPL (dGPL) and apolar GPL (aGPL) components detected by spraying with orcinol/sulphuric acid. Percentage distribution of radioactivity ($\pm$ SD) was determined as: (a) GPL = 40%, $n = 2$; aGPL = 37%, $n = 2$ and (b) dGPL = 22% $\pm$ 2.6, $n = 3$; aGPL = 24% $\pm$ 2, $n = 3$. The absolute value corresponding to 100% was (a) 1.5×10^5 c.p.m. $n = 2$ and (b) $5.9 (\pm 0.9) \times 10^4$ c.p.m., $n = 3$.

Examination of radiolabelled lipid indicated that use of [^3H]Fuc resulted in the least incorporation into native lipid. Incorporation of [^3H]Fuc into native lipid of serovar 4 amounted to 7.2×10^3 , 9.0×10^3 and 10.0×10^3 c.p.m. (mg lipid) $^{-1}$ and 1.5×10^3 , 2.0×10^3 and 2.7×10^3 c.p.m. (mg lipid) $^{-1}$ for serovar 20 when concentrations of 0.25, 0.5 and $1.0 \mu\text{Ci ml}^{-1}$ were used. Examination of the distribution of radioactivity in the [^3H]Fuc-labelled lipid from serovars 4 and 20 by TLC revealed that only a minimal amount was incorporated into the GPL. Because of the low incorporation of radioactivity into native lipid, [^3H]Fuc was not considered for further use as a probe in subsequent macrophage uptake studies involving radiolabelled GPL.

Using [^3H]Man, the mean incorporation into native lipid from serovar 4 was 10.4×10^3 , 17.3×10^3 and 20×10^3 c.p.m. (mg lipid) $^{-1}$ ($n = 2$), and for serovar 20 it was 13.1×10^3 , 28.6×10^3 and 52.4×10^3 c.p.m. (mg lipid) $^{-1}$ (one experiment), for concentrations of 0.25, 0.5 and $1.0 \mu\text{Ci ml}^{-1}$, respectively. Use of [^3H]Met resulted in incorporation into native lipid from serovar 4 of 88.9×10^3 , 529×10^3 and 1080×10^3 c.p.m. (mg lipid) $^{-1}$, and for serovar 20, incorporation of 74.9×10^3 , 124×10^3 and 230×10^3 c.p.m. (mg lipid) $^{-1}$ (one experiment), for concentrations of 0.25, 0.5 and $1.0 \mu\text{Ci ml}^{-1}$, respectively.

Distribution of radiolabel in native lipid

Analysis of serovar 4 [*methyl*- ^3H]Met-radiolabelled lipid by TLC in solvent A (Fig. 2a) revealed that radioactivity was distributed over a wide area, with 40% ($n = 2$) and 37% ($n = 2$) of the radioactivity localized in the area corresponding to GPL and aGPL, respectively. Examination of deacetylated serovar 4 [*methyl*- ^3H]Met-radiolabelled lipid by TLC in solvent A (Fig. 2b) confirmed that 22% (SD, ± 2.6 , $n = 3$) of the radioactivity was localized in an area

Fig. 3. Distribution on TLC of radioactivity in native lipid (a, c) and deacetylated lipid (b) from [³H]Man-radiolabelled (■) and [³H]Phe/Ala-radiolabelled (▨) serovar 4. Plates were developed in solvent A (a, b) or solvent B (c), and GPL, deacetylated GPL (dGPL) and apolar GPL (aGPL) components detected by spraying with orcinol/sulphuric acid. Percentage distribution of radioactivity ($\pm$ SD) was determined as: (a) GPL = $57\% \pm 6.7$, $n = 3$; (b) dGPL = 64% , $n = 2$ (■) and 15% , $n = 2$ (▨); aGPL = 20% , $n = 2$ (▨); (c) GPL = 85% , $n = 2$ (■) and 25% , $n = 2$ (▨); aGPL = 27% , $n = 2$ (▨). The absolute value corresponding to 100% was 2.9×10^3 c.p.m., $n = 2$ (■) and 5.2×10^3 c.p.m., $n = 2$ (▨).

corresponding to only the dGPL and 24% (SD, ± 2 , $n = 3$) in an area corresponding to the aGPL components. Examination of the deacetylated lipid is more accurate because the dGPL can be confined to a single 1 cm section, thus allowing for a more precise assessment of radioactivity distribution. Similar findings were observed for serovar 20 (data not presented) and are consistent with findings reported previously in which it was determined that [*methyl*-³H]Met radiolabelled the methylated sugars of the GPL antigens (Barrow *et al.*, 1980).

Examination of [³H]Man-radiolabelled lipid from serovar 4 by TLC in solvent A (Fig. 3a) revealed that the majority (57%; SD, ± 6.7 , $n = 3$) of the radioactivity was localized in an area corresponding to only the GPL and not the aGPL. Lack of incorporation into the aGPL was established by comparing the TLC distribution of radioactivity from deacetylated [³H]Man-radiolabelled lipid with that of deacetylated [³H]Phe/Ala-radiolabelled lipid in solvent A (Fig. 3b). In the case of the [³H]Man-radiolabelled lipid, radioactivity was localized almost entirely in the area corresponding to the dGPL (mean 64%; $n = 2$) and not the aGPL components (Fig.

3b). Likewise, when native lipid from serovar 4 was developed in solvent B, for better separation of aGPL components, it was further demonstrated that radioactivity was detected in the area corresponding to the aGPL for only lipid radiolabelled with [^3H]Phe/Ala (mean 27%; $n = 2$) and not [^3H]Man (Fig. 3c). Use of [^3H]Phe/Ala has been shown to lead to incorporation of radiolabel into the peptide core which is common to both the GPL and the aGPL (Hooper *et al.*, 1986). Although a certain percentage of radioactivity was observed in the upper region of the plate (i.e. cm 15, Fig. 3b, c), this was not associated with the aGPL components. The identity of that component is the subject of another investigation.

Purification of native and deacetylated GPL

Native and deacetylated radiolabelled lipids from serovars 4 and 20 were purified by a one-step chromatographic procedure (Dimitrijevič *et al.*, 1986). Results for purification of GPL antigens from serovar 20 have been reported previously (Dimitrijevič *et al.*, 1986). For serovar 4, lipid was first eluted in chloroform/methanol (96:4, v/v) to remove pigments, followed by elution in chloroform/methanol (93:7, v/v) to remove the GPL. Yield of pure GPL from serovar 4 averaged 14% of total lipid.

Distribution of radiolabel in sugars from purified GPL

To further confirm the incorporation of radiolabel into the GPL, purified deacetylated radiolabelled GPL from serovars 4 and 20 were hydrolysed in 2 M-TFA and hydrolysates examined by development on cellulose TLC. Sugars in the TFA hydrolysates were identified by development on cellulose TLC along with standards obtained from serovar 20 (Barrow *et al.*, 1980). Analysis of the hydrolysate from [*methyl*- ^3H]Met-radiolabelled GPL of serovar 4 revealed that radioactivity was localized in the area corresponding to 2-*O*-methylfucose (2-*O*-MeFuc), 4-*O*-methylrhamnose (4-*O*-MeRha), and 3,4-di-*O*-methylrhamnose (3,4-di-*O*-MeRha) (data not shown). Analysis of sugars in the TFA-hydrolysate of purified [^3H]Man-labelled serovar 4 GPL revealed that only 2-*O*-MeFuc was radiolabelled (data not shown). This identical pattern was also observed for GPL antigens from serovar 20, with the exception that 2-*O*-MeRha replaced 4-*O*-MeRha. Therefore, for specific incorporation of radiolabel into the oligosaccharide moiety of the GPL, [^3H]Man was determined to be the radiolabelled component of choice because of its ability to be incorporated into the 2-*O*-MeFuc moiety of the oligosaccharide determinant, both for serovar 4 and serovar 20 (Fig. 1).

Uptake of radiolabelled GPL by mouse peritoneal macrophages

Unelicited peritoneal cell counts from C57BL/6 mice averaged 4.2×10^6 cells ml^{-1} (SD, $\pm 1.4 \times 10^6$, $n = 7$). The area of each tissue culture well was 2 cm^2 , and the addition of 2×10^6 peritoneal cells to each well resulted in an average macrophage monolayer of 3.2×10^5 cells (SD, $\pm 0.7 \times 10^5$, $n = 7$).

Uptake of GPL by peritoneal macrophages was determined by pulsing adherent cells with varying concentrations of serovar 4 GPL (10–250 μg GPL ml^{-1} per 3.2×10^5 cells). GPL used in the uptake studies were radiolabelled with [^3H]Phe/Ala and their ^3H radioactivity was 1.5×10^5 c.p.m. mg^{-1} . The concentration for maximum uptake appeared to be slightly above 250 μg GPL ml^{-1} (Fig. 4). The mean percentage uptake ($\pm$ SD) of [^3H]Phe/Ala-radiolabelled GPL was 17 ± 1.2 , 19 ± 2.0 , 19 ± 1.1 , 19 ± 1.4 , 19 ± 0.6 and 20 ± 1.3 ($n = 3$), for GPL concentrations of 10, 20, 50, 100, 200 and 250 μg ml^{-1} , respectively. This is in contrast to the percentage uptake for serovar 20 GPL antigens, which ranged from 60 to 76% for GPL concentrations of 10–250 μg ml^{-1} (Hooper *et al.*, 1986). To be consistent with previous studies involving GPL antigens from serovar 20, a concentration of 200 μg GPL ml^{-1} was chosen as the optimum concentration for macrophage degradation studies to follow.

Distribution of radioactivity in chloroform-extractable material from macrophage cultures

Degradation of GPL by mouse peritoneal macrophages was examined by using [^3H]Man-radiolabelled GPL. As compared to the [^3H]Phe/Ala-radiolabelled serovar 20 GPL antigens used in our previous study (Hooper *et al.*, 1986), the [^3H]Man-radiolabelled GPL from serovar 4

Fig. 4

Fig. 5

Fig. 4. Uptake of radiolabelled GPL by mouse peritoneal macrophages. Macrophage monolayers were pulsed for 2 h with [³H]Phe/Ala-radiolabelled GPL (radioactivity 1.5×10^5 ³H c.p.m. mg⁻¹) from serovar 4. Bars represent SD ($n = 3$).

Fig. 5. Distribution on TLC of chloroform-extractable components from spent media and macrophages obtained from peritoneal macrophage cultures at 0 h (■) and 96 h (▨) following pulse with purified [³H]Man-radiolabelled serovar 4 GPL. Plates were developed in solvent A and GPL detected by their yellow-gold reaction to orcinol/sulphuric acid reagent.

had a reduced amount of total radioactivity and exhibited a lower percentage of macrophage uptake. Therefore, it was decided that macrophage monolayers would be exposed to [³H]Man-radiolabelled GPL and subsequently examined for degradation products without washing the monolayers following the initial pulse, as had been done previously (Hooper *et al.*, 1986). A concentration of 200 µg GPL ml⁻¹ (4.6×10^4 ³H c.p.m. per mg GPL) was added to monolayers (3.1×10^5 adherent cells) for time periods of 0, 24, 48 and 96 h, after which combined spent medium and macrophages were extracted with chloroform/methanol/water (8:4:3, by vol.) and radioactivity in upper (methanol/water) phase and lower (chloroform) phase was determined. It was observed that 99% (SD, ± 0.2 , $n = 4$) of the radioactivity was extractable with chloroform at 0, 24, 48 and 96 h. These results are comparable to those obtained with the GPL antigens from serovar 20 in our previous study (Hooper *et al.*, 1986).

Examination by TLC of chloroform-extractable material revealed that all of the serovar 4 GPL were present in the chloroform-extractable material throughout the 96 h following addition of GPL. Following development of TLC plates in solvent A, GPL were identified by their characteristic colour reaction when sprayed with orcinol/sulphuric acid, and their location with respect to purified GPL standards. Examination of the chloroform-extractable material by TLC in solvent A revealed that radioactivity was localized in an area corresponding to GPL (Fig. 5). This same pattern was observed at 0, 24, 48 and 96 h.

DISCUSSION

Because the GPL antigens may play a role in pathogenesis, they have been the focus of research in this laboratory during the last several years (Hooper *et al.*, 1986; Tereletsky & Barrow, 1983). Using immunocytochemical and radiolabelling techniques, we have precisely defined the superficial location and distribution of the GPL antigens on the mycobacterial cell wall (Tereletsky & Barrow, 1983) and demonstrated that these mycobacterial components are relatively inert to macrophage degradation (Hooper *et al.*, 1986). These findings represent the first direct evidence that supports previous suggestions regarding the inertness of the superficial L₁ layer (Draper & Rees, 1970) and indicate that the GPL are important in pathogenesis. In addition, more recent studies in this laboratory have revealed that the GPL, or their metabolites, have the ability to alter lymphocyte function (Brownback & Barrow, 1988).

In an effort to better examine macrophage degradation of GPL, we have developed procedures for radiolabelling the oligosaccharide portion of these potentially important

mycobacterial components. The choice of radiolabelled fucose, mannose and methionine to radiolabel the oligosaccharide was based upon their commercial availability and cost effectiveness, and the results of previous studies (Barrow *et al.*, 1980; Ginsberg, 1960; Hooper *et al.*, 1986; Touster, 1962).

Under the conditions of this study, [^3H]Fuc gave the least incorporation of radiolabel into native lipids for serovars 4 and 20. Examination of the distribution of radioactivity in the [^3H]Fuc-labelled lipids from serovars 4 and 20 by TLC revealed that only a minimal amount was incorporated into the GPL. Comparison of these results with previous studies (Hooper *et al.*, 1986) indicated that incorporation of [^3H]Fuc was not sufficient for macrophage degradation studies, therefore its use in this investigation was discontinued. Use of [^3H]Man and [^3H]Met proved to be more favourable for incorporation into native lipid and GPL; of particular importance was the ability of [^3H]Man to radiolabel 2-*O*-MeFuc, which is present in the oligosaccharide determinant of serovars 4 and 20. This latter accomplishment will not only increase our ability to examine degradation of GPL by various enzymic systems, but it will also allow for a more efficient way of exploring the biosynthetic pathway of these mycobacterial lipids.

Based on the present findings, speculations can be made regarding radiolabelling of the oligosaccharide moiety of the GPL from other serovars in the *M. avium* complex. The fact that [^3H]Met incorporated methyl groups into the methylated sugars of serovars 4 and 20 further substantiates the observation that GPL from other serovars in the *M. avium* complex can be radiolabelled because of the presence of 3,4-di-*O*-MeRha on the fatty acyl-peptide core (Brennan *et al.*, 1981 *a, b*; Brennan & Goren, 1979). In addition, serovars such as 8, 9, 16, and 25 can be radiolabelled at sites coinciding with the methylated sugars 3-*O*-methylglucose, 2,3-di-*O*-methylfucose, 4-*O*-methylrhamnose and 2-*O*-methylfucose, which have been identified as a part of their oligosaccharide moiety (Brennan *et al.*, 1981 *a, b*). Likewise, the monosaccharides 2,3-di-*O*-MeFuc and 2-*O*-MeFuc, which are found in the oligosaccharide determinant of serovars 9 and 25 (Brennan *et al.*, 1981 *a, b*), should be radiolabelled with [^3H]Man.

Because [^3H]Man allowed for specific incorporation of radiolabel into the oligosaccharide determinant, it was chosen as the radiolabel for subsequent macrophage degradation experiments in an effort to extend our previous studies involving GPL antigens and their possible role in pathogenesis (Hooper *et al.*, 1986). Although use of [^3H]Met resulted in a higher degree of radiolabel incorporation into the GPL antigens, it was not specific for the oligosaccharide determinant, but instead resulted in the radiolabelling of 3,4-di-*O*-MeRha, which is the terminal sugar on the fatty acyl-peptide unit (R_1 , Fig. 1).

The macrophage pulsing experiments indicate that the maximum level of serovar 4 GPL association with murine peritoneal macrophages takes place somewhere slightly above the 200–250 $\mu\text{g ml}^{-1}$ level observed for GPL antigens from serovar 20 (Hooper *et al.*, 1986). Although the maximum uptake of serovar 4 GPL was not determined in this study, it was decided to use a concentration of 200 $\mu\text{g ml}^{-1}$ per 3.2×10^5 cells to be consistent with our previous study involving serovar 20 GPL antigens (Hooper *et al.*, 1986). The reason for the low level of serovar 4 GPL association with macrophages was not determined; however, the explanation may have to do with the difference in the GPL structure of the two serovars. From previous studies, it appears that the oligosaccharide determinant of the GPL from serovars 4 and 20 is very similar, differing only in the terminal sugar of the oligosaccharide moiety; serovar 4 contains 4-*O*-MeRha (McNeil *et al.*, 1987), whereas serovar 20 has 2-*O*-MeRha (Barrow *et al.*, 1980). Although this difference is minor, it may account for the variability in GPL association properties observed in the studies reported here. We have noted in our studies that the GPL from serovar 4 do not disperse as well in the tissue culture medium as do the GPL from serovar 20. This may have contributed to the differences in macrophage association for GPL from serovars 4 and 20.

Because of the reduced amount of radioactivity incorporated with the use of [^3H]Man and the reduced percentage uptake of the serovar 4 GPL as compared to that of the serovar 20 antigens (Hooper *et al.*, 1986), the previous experimental design was modified in this investigation. Potential GPL degradation was assessed by examining combined macrophage and spent

medium pools. Following extraction of lipid from spent medium and macrophage monolayers after exposure to [^3H]Man-radiolabelled GPL for periods up to 96 h, it was observed 99% of the radioactivity from the macrophage cultures remained in the chloroform-extractable material, suggesting that little degradation of the GPL of serovar 4 had occurred. Because these results were consistent with previous findings (Hooper *et al.*, 1986) and indicate that little, if any, GPL degradation had occurred, the delineation of antigen products in spent medium and macrophage monolayers was not attempted. These results, in addition to previous findings (Hooper *et al.*, 1986), further substantiate that the GPL are slowly metabolized mycobacterial components and indicate that their accumulation in host tissue may be a real consideration with respect to infections involving members of the *M. avium* complex.

It should be pointed out that these studies are limited by the fact that only resident peritoneal macrophages were utilized in the GPL degradation studies. It is unlikely that the GPL are completely inert to degradation in an intact host system. Indirect evidence for this conclusion is derived from our recent observation that GPL as well as their metabolites may act as immunomodulators (Brownback & Barrow, 1988). Thus, the unelicited macrophages used in the current and previous (Hooper *et al.*, 1986) study may not have possessed the enzymic capability to degrade the GPL. Now that we have methods for radiolabelling specific sites on the GPL structure, it will be possible to examine whether other macrophage populations or enzymic systems have the capability to degrade these mycobacterial components.

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