INTERNAL RADIOLABELING OF MYCOBACTERIAL ANTIGENS
AND USE IN MACROPHAGE PROCESSING STUDIES

THESIS

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*Mycobacterium avium* complex serovars 4 and 20 were cultured in the presence of $[^3H]$-fucose, $[^3H]$-methionine, and $[^3H]$-mannose to specifically radiolabel the oligosaccharide of the glycopeptidolipid (GPL) antigens. Distribution of radioactivity in lipid was determined by thin-layer chromatographic methods. Examination of acid hydrolysates from radiolabeled antigens revealed that $[^3H]$-methionine incorporated into methylated sugars in polar and apolar GPL components, whereas $[^3H]$-mannose incorporated exclusively into the oligosaccharide of polar GPL antigens. Least incorporation of radiolabel into antigens was observed with $[^3H]$-fucose. Use of radiolabeled serovar 4 antigens in macrophage uptake studies revealed maximum uptake to be slightly above 250 $\mu$g/3.2 x $10^5$ cells. Timed experiments demonstrated that GPL antigens were relatively inert to degradation by resident peritoneal macrophages.
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CHAPTER I

INTRODUCTION

Mycobacteria have infected man for thousands of years. Mummified bodies from the Old and the New World have exhibited organisms quite similar to present day mycobacteria. Proof of mycobacterial invasion of the bones and joints can even be encountered in skeletal remains from prehistorical man (38). In modern times, mycobacteria continue to be agents of many infections. For example, people infected with the new condition known as Acquired Immune Deficiency Syndrome (AIDS) have developed serious infections caused by a specific group of mycobacteria, the *Mycobacterium avium* complex (*M. avium* complex) (38).

The *M. avium* complex was not considered a "complex" until recently. The first organism to eventually be included in the *M. avium* complex was *M. avium*, initially recognized as the cause of tuberculosis in chickens (3, 45). The classical *M. avium* was highly pathogenic for chickens and rabbits but not guinea pigs (45). In 1949, Cuttino and McCabe described a disseminated disease caused by an organism similar to *M. avium* which they initially named "Nocardia intracellularis" and later renamed *Mycobacterium intracellulare* (45). Runyon proposed that the strains virulent for chickens and rabbits be referred to as *M. avium*, and those strains that were similar to the avium strain but were not virulent for chickens and rabbits be known as "Battey bacilli" (45). The unclassified mycobacteria were subclassified by Runyon according to the effect of light on colonial growth.
production and according to rate of growth, as follows: Group I. Photochromogens-- little or no pigment when grown in the dark, pigmented after exposure to light; Group II. Scotochromogens-- pigmented when grown in the dark, more reddish if grown continuously in light; Group III. Nonphotochromogens-- little or no pigment when grown in the dark or light; and Group IV. Rapid growers-- little or no pigment but colonies appear after two to four days of growth (31). Species which now comprise the *M. avium* complex were classified as being in Group III of Runyon's groups.

The two species, *M. avium* and *M. intracellulare*, are so similar that they can not be distinguished by common lab tests (37, 45). *M. avium* is an organism which grows slowly on standard mycobacterial media. Its generally smooth colonies, lack of cord formation, lack of niacin production, and marked polymorphism help to distinguish *M. avium* from *Mycobacterium tuberculosis* but not from other nontuberculous non-pigmented mycobacteria of Runyon's group III (45). Characteristics such as unimpaired growth at 45°C and absence of arylsulfatase (26) are helpful in distinguishing *M. avium* from the otherwise similar *M. intracellulare* but are not entirely reliable criteria for differentiating between other mycobacterial species (45). Accurate identification of the *M. avium* complex thus depends upon other procedures (19).

Until recent years, animal inoculation was the most reliable way of identifying members of the *M. avium* complex. Later, the agglutination reaction was widely used as a method for identification and classification of bacteria, but this test was not of value in the identification of members of the *M. avium* complex because hydrophobic properties of these organisms made it difficult to obtain stable suspensions (35). Therefore in 1965, Schaefer described an
agglutination test which differentiated the various mycobacteria of Runyon's groups I and III (19, 24, 37) into serovars (serotypes) where each serovar is distinguished by a type-specific antigen (35, 37). Of these three methods used to classify mycobacteria, Schaefer's seroagglutination typing appears to be a more accurate means of identifying nontuberculous mycobacteria (19, 36) and was responsible for the recognition of 31 serovars within \textit{M. avium} (serovars 1-3), \textit{M. intracellulare} (serovars 4-28), and \textit{Mycobacterium scrofulaceum} (serovars 41-43) (41). However, the rough colony variants do not have the type-specific antigens and are therefore unsuitable for the seroagglutination test (3, 37, 41).

In a search of other identifiable features characteristic of nontuberculous mycobacteria, Marks and colleagues recognized an array of closely related polar and apolar lipids shared by smooth colony strains in the \textit{M. avium} complex (23, 28). The introduction of thin-layer chromatography (TLC) for the analysis of lipophilic substances provided a potentially simple and inexpensive technique enabling Marks and Szulga in 1965 to investigate lipid patterns of opportunistic mycobacteria (8, 38). Thin-layer chromatography was designed to supplement the more conventional biochemical and seroagglutination tests (9). Initially, two-dimensional analysis was utilized (22, 28) and as methods were improved, they were applied to the study of \textit{M. avium} complex members and similar organisms (22, 28). Eventually unidimensional runs of a number of strains in parallel, using a variety of developing solvents, established specific lipid patterns for many mycobacterial species including members of the \textit{M. avium} complex (22).

Lipid extracts from \textit{M. avium} complex members yield characteristic TLC patterns of the polar antigens to allow reliable identification of individual \textit{M. avium} complex serovars by
TLC (8). The specific lipid patterns from *M. avium* complex serovars depend upon the relative positions of one or more spots and particularly on the colors of these spots (22). A marked feature of the lipids is a vivid, long-lasting yellow-gold coloration with orcinol in sulfuric acid (8, 9, 11) in contrast to the apolar variety, which assume a pink hue (11). This yellow-gold coloration of the spots reflects the large quantities of 6-deoxyhexoses in the *M. avium* complex antigens, due to the fact that 6-deoxyhexoses produce this yellow-gold response to orcinol spray (11).

Lipid analysis by TLC showed that *M. avium*, *M. intracellulare*, and *M. scrofulaceum* are closely related and that there is correspondence between serotype and lipid pattern (23, 28). Lipid patterns obtained by TLC methods may be used to identify species if reference strains are examined in parallel (28). The relationship between *M. avium*, *M. intracellulare*, and *M. scrofulaceum* was initially controversial, however, Tsukamura and his colleagues in 1969 (42) and Marks, Jenkins, and Schaefer in 1971 (28) supported the idea that *M. avium*, *M. intracellulare*, and *M. scrofulaceum* belonged to a single entity (23, 28, 42).

Finally, a compromise resulting in the formation of the *M. avium* complex was achieved among the members of the International Working Group on Mycobacterial Taxonomy (IWGMT) and the Tuberculosis Panel of the United States-Japan Cooperative Medical Science Program (46). The grouping of the three separate species (*M. avium*, *M. intracellulare*, and *M. scrofulaceum*) was abandoned in favor of the designation "*M. avium* complex." Twenty numbers in the scheme were reserved after the former *M. intracellulare* group (serovars 4-20) for possible new types related to this group or *M. avium* (46). The designation for *M. scrofulaceum* strains began with 41 (41, 46). In 1983, Tsang, Drupa,
Goldberg, et al. developed a collection of reference strains (41) containing at least one strain of serovars 1 through 27 and 41 through 43 and described the characteristic lipid profiles for each of the referenced serovars (41).

Members of the *M. avium* complex have been found in soil (11), dust (11), and grasses or plants used for food or animal bedding (29, 33). The serovars 4 through 11 have been cultured from animal water tanks, from water wells, from frogs and toads, and from swamp water (4, 24, 33, 44, 45). Sawdust has also been found to be a favorable medium for the *M. avium* complex serovar 8 (33). Serovars found in water include in the order of frequency: 8, 14, 42, 18, and 1; in raw milk: 9, 13, and 19; and in soil and dust: 14, 7, 16, 20, and 43 (45).

Animal disease caused by *M. avium* was described approximately one century ago, and *M. avium* was portrayed as the causative agent of avian tuberculosis in 1890 by Maffucci (37). However, the *M. avium* organism was not recognized as a human pathogen until 1943 (47) and even then *M. avium* disease was rare in man (47). Today, members of the *M. avium* complex commonly cause mycobacterial infections in man (47).

The pathogenicity of nontuberculous mycobacteria differs from *M. tuberculosis* in several respects. Even though person-to-person transmission generally does not occur by *M. avium* complex members, environmental sources are thought to be important in the infection process (34, 47). The pathogenic potential of members of the *M. avium* complex varies for humans which is demonstrated by the fact that few people infected with members of the *M. avium* complex become diseased (34, 47). Because of the low pathogenicity of nontuberculous mycobacteria for humans, the existence of a predisposing condition frequently is required for
tissue invasion by these organisms. Also patients with malignancies and immunosuppression, and in particular those with AIDS are at an increased risk of developing a wide spectrum of disease manifestations including infections caused by members of the M. avium complex (47).

In 1980, M. avium complex was second only to M. tuberculosis as the most frequently isolated mycobacterial isolate in the United States (47). However in 1985, members of the M. avium complex were isolated from more patients suffering from mycobacteriosis than M. tuberculosis (47), with slightly over half of the M. avium complex isolates being cultured from patients suffering from AIDS (47). In a comprehensive study of the frequency of opportunistic infections among 446 patients suffering from AIDS, nontuberculous mycobacteriosis occurred in 15% of those cases (32). Other researchers, within the context of their own AIDS patient populations, have identified mycobacterial isolates in up to 50% of the cases (32). General agreement among investigators supports the idea that over 80% of the nontuberculous mycobacteria isolates from people suffering from AIDS are members of the M. avium complex (32).

Serovars 1, 2, 9, 14, 16, 41, 42, and 43 were the M. avium complex serovars that Schaefer isolated most commonly at National Jewish Hospital and Research Center in 1965 (11, 30). In a later study performed by Schaefer at the same lab, the following frequency in the M. avium complex serovars had changed to the following: 19, 1, 13, 9, 16, 8, 14, 42, and 12 (24, 30). Of the isolated M. avium complex serovars from the second study by Schaefer, 84% were from patients inflicted with pulmonary infections (30). A third survey published in 1981 for the years 1976 to 1978 revealed that the frequency of M. avium
complex serovars had changed to the following order: 8, 16, 4, 19, and 9 (24). It is interesting to note that in the first two studies serovar 4 was not a major isolate but in the later study this serovar had become the third most common \textit{M. avium} complex isolate (24).

Good reported that of 146 \textit{M. avium} isolates from patients suffering from AIDS, 63 [43\%] were serovar 4 and 22 [15\%] were serovar 8 (32). McNeil, Tsang, and Brennan reported that in 1987 the majority of \textit{M. avium} isolates from people from the eastern part of the USA suffering from AIDS was serovar 4 (32).

The historical significance of the glycopeptidolipid (GPL) antigens in the classification of the members of \textit{M. avium} complex has been amply documented. It was born of two distinct observations, thought to be unconnected at the time. Schaefer had observed that most nontuberculous mycobacteria were endowed with highly immunogenic species- or type-specific antigens (35). Meanwhile, Marks, Jenkins, and their colleagues recognized that whole-lipid extracts derived from \textit{M. avium} complex serovars contained an individualistic array of glycolipids (11). In what has proved to be a key study, Brennan and Goren in 1979 demonstrated that the Schaefer typing antigens and the Marks-Jenkins lipids were synonymous (11) and were in fact polar C-mycosidic GPLs in which small oligosaccharides were attached to the allo-threonine substituent (5, 8, 11) of a common fatty acyl-peptidyl-O-(3,4-di-O-methylrhamnose) "C-mycoside" core:

\[
\text{Fatty acyl-Phe-}\text{alloThr-Ala-Alaninol-}(3,4\text{-di-O-Me-Rha})\text{6-deoxyTalose}
\]
The C-mycosides were described by French investigators as type-specific glycolipids characteristically found in *M. avium* (43). Brennan and Goren (8) and Brennan, Souhrada, et al. (11) demonstrated that individual serovars of the *M. avium* complex contain two classes of C-mycosides, which were called apolar C-mycosidic GPLs and polar C-mycosidic GPLs (4). Of the two classes of GPLs described, the apolar variety is similar to the C-mycosides (4, 8). The polar GPLs differ from the apolar C-mycosides in that the polar antigens have an oligosaccharide attached to the D-allothreonine instead of the single 6-deoxytalose (4). Because identical apolar GPLs based on TLC patterns occur in most *M. avium* complex serovars, the apolars are not type-specific (8). On the other hand, the mobility of the polar GPLs is markedly different from one serovar to another (5, 8) and these polar antigens are the definitive lipids among the *M. avium* complex serotypes (8). Each serovar is characterized by its complement of serologically active polar GPLs (5).

In 1982, Barrow and Brennan reported that pure cultures propagated in the laboratory under certain conditions displayed rough morphology (3). Thin-layer chromatography of lipid extracts from these rough variants in solvents designed to resolve both the apolar and the polar GPLs demonstrated the absence of both classes (3). However, the normal complement of other lipids, among them phospholipids and neutral lipids, was retained in the rough variants (3).
In order to assume the role of specific serological factors throughout all serovars, the polar GPLs must exhibit much greater structural variability than is possible for the apolar GPLs \((5, 8)\). The fatty-acyl portion of the polar GPLs is chromatographically indistinguishable and the sugar \(3,4\text{-di-O-Me-rhamnose}\) is always associated with the lipopeptide \((5, 8, 10)\). Therefore, the structural difference and serological specificity of the polar antigens resides in small variable oligosaccharides which modify the relatively invariant fatty acyl-peptidyl core \((5, 7, 8, 10)\).

The oligosaccharide moieties of the GPL antigens from all \(M.\ avium\) complex serovars examined to date contain an invariant region comprised of 6-deoxytalose and L-rhamnose and a variable region containing a set of sugars unique to each serovar \((5)\). These two basal sugars are always at the internal, "reducing" end of the oligosaccharide, and the linkage between these two basal sugars is alpha-1,2 \((5, 6, 14)\). The sugar, 6-deoxytalose, occupies the reducing end of the oligosaccharide and is the link to the peptide moiety while rhamnose is always penultimate to 6-deoxytalose \((7, 10, 14)\). The outer one or two sugar residues of the oligosaccharide renders the individually distinctive features required for type specificity of each polar GPL \((5, 6, 14)\). The general structure of the polar GPL is as follows:

\[
\text{Fatty acyl-Phe-}\text{alloThr-Ala-Alaninol-(3,4-di-O-Me-Rha)} \\
\text{|} \\
\text{O} \\
\text{|} \\
\text{6-dTal-Rha-(6-deoxyhexose)_2}
\]

(Abbreviations: Phe: D-Phenylalanine; alloThr: D-alloThreonine; Ala: D-Alanine; Me-Rha: Methyl-Rhamnose; dTal: deoxyTalose; Rha: Rhamnose) \((8)\)
Brennan, et al. reported in 1981 (10) that the oligosaccharide moieties of the \textit{M. avium} complex GPL antigens contain mostly an array of naturally O-methylated and non-methylated 6-deoxyhexoses (5, 10) and these moieties are small to medium in size (5). Trisaccharides or tetrasaccharides are the minimum size of the oligosaccharide moieties among the polar GPL antigens of \textit{M. avium} complex members (10). In addition, a characteristic of all oligosaccharide moieties is that some or all of the free hydroxyl groups are acetylated (4). Thus, Collins has suggested that the structures of the polar GPL antigens resemble the blood group glycosphingolipids (14), more so than the enterobacterial O-antigens as previously suggested by Brennan, et al. (10).

The sugars contained in the oligosaccharide moiety of serovar 20 are present as a tetrasaccharide (4). The outer two sugars contained in the moiety have been identified as 2-O-methyl-fucose and 2-O-methyl-rhamnose with the latter sugar being the outermost sugar (4). The serovar 4 oligosaccharide moiety is also a tetrasaccharide and is identical to the oligosaccharide of serovar 20 except for the presence of 4-O-methyl-fucose rather than 2-O-methyl-fucose (32).

In 1974, Draper demonstrated that the mycosides of \textit{M. avium} and \textit{Mycobacterium lepraemurium} are similar (16). \textit{M. avium} and \textit{M. lepraemurium} are severe pathogens in their respective hosts and the "natural" environment of both species is the phagolysosome of the host cell (16). Immunological evidence, both from serology and immunization experiments, suggest that these species are closely related (16). Apparently the organisms protect themselves from the environment with similar layers of mycosides (16). Draper and Rees in 1973 (17) described a protective zone of "parallel fibrils wrapped longitudinally around the
bacteria" (17) when M. avium complex members were encased within vacuoles in phagocytic cells (4, 8). This capsule has been referred to as the L1 layer by Barksdale and Kim (1, 2, 39).

Barrow, et al. in 1980 described their investigation of M. avium serovar 20 that supported the presence of a superficial sheath surrounding certain nontuberculous mycobacteria (4). Moreover, analysis of this superficial material revealed that the most superficial L1 layer described by Barksdale and Kim consisted predominately of the serologically active polar GPLs (2, 4). Because serovar 20 was a member of the M. avium complex, Barrow, et al. suggested that all serovars of the M. avium complex contain the serologically specific polar GPLs antigens within the L1 layer (4).

The surface location of the polar GPL antigens and their capability as a capsular protective shield against the phagolysosomal environment (2, 8, 39) was strongly suggestive of a role for these glycopeptidolipids in pathogenesis (8). This protective capability of nontuberculous mycobacteria within phagosomes may be parallel to that exhibited by the O-antigenic lipopolysaccharides of the virulent strains of Salmonella typhimurium (8).

In 1982, Brennan and Barrow described methods utilized for the production of antibodies to the polar GPL antigens of a member of the M. avium complex (2). Although the polar GPL antigens alone did not produce an immune response, when complexed with methylated bovine serum albumin (MBSA), the antigens were able to generate antibodies detectable by gel diffusion, seroagglutination, and indirect fluorescent-antibody techniques (2). From that study, it was concluded that the polar GPLs are not immunogens but haptens (2).

Tereletsy and Barrow in 1983 developed immunocytochemical techniques to study the
distribution of polar GPL antigens in the superficial $L_1$ layer and the role of the antigens in nontuberculous mycobacterial pathogenicity (39). Through these techniques, Tereletsky and Barrow discovered that the $L_1$ layer of serovar 20 was uniformly distributed around the entire surface of the cell, further supporting the results of other investigations concerning the $L_1$ layer of nontuberculous mycobacteria (39). Ferritin granules disclosed the superficial distribution of the GPL antigens within the $L_1$ layer and clearly demonstrated intraphagosomal location of the mycobacterial cells within the macrophage (39). The fact that the $L_1$ layer completely encases the phagocytosed mycobacteria strongly implies that this superficial layer may very well protect the mycobacterial cell from degradation by lysosomal enzymes (39).

Hooper, et al. described the process of internally radiolabeling of GPL antigens found within the $L_1$ layer of $M. avium$ complex serovar 20 and the utilization of these radiolabeled antigens in macrophage uptake and retention studies (20). By complementing the immunocytochemical techniques with radioisotope procedures, a more reliable evaluation of macrophage processing of GPL antigens was obtained (20). Lipid radiolabeled with tritiated-alanine and -phenylalanine demonstrated incorporation of the label into the invariant fatty acyl-peptide core at the phenylalanine, alanine, and alaninol moieties (20). The fact that the antigens were labeled in the fatty acyl peptide core, which is shared by all members of the $M. avium$ complex, implies that the internal radiolabeling procedures would also be applicable for radiolabeling the GPL antigens of other members of the $M. avium$ complex (20).

Purified radiolabeled GPL antigens were utilized by Hooper, et al., to determine the
maximum uptake and retention in murine peritoneal macrophages (20). The level of maximum antigen uptake by the murine macrophages occurred between 200 and 250 μg GPL per 3.4 x 10⁵ cells. In retention studies, murine peritoneal macrophages which were pulsed with 200 μg of GPL antigen, demonstrated gradual release of antigens throughout a four day incubation period (20). Examination of spent medium and macrophages throughout the 4 days after the antigen pulse revealed that the radioactivity was primarily associated with the intact GPL antigens, indicating that very little degradation had taken place, not even deacetylation (20).

Even though the majority of the radioactivity from the chloroform-extractable material from retention studies performed by Hooper, et al., was associated with the polar GPL antigens, 2-3% of the radioactivity was exhibited in the upper portion of the plate (20). This suggests that removal of the oligosaccharide may have occurred, leaving a degradation product similar to the apolar GPL components that migrate in that region of the plate (20). Therefore, development of techniques for radiolabeling the oligosaccharide became necessary to examine this latter possibility.

This investigation will determine whether the oligosaccharide moiety of the GPL antigen of M. avium complex serovar 4 can be specifically radiolabeled by culturing mycobacteria in the presence of [³H]-fucose, [³H]-mannose, or [³H]-methionine. Following extraction, the lipid will be examined by TLC procedures to determine the distribution of radioactivity within the GPL antigen. Monosaccharides obtained from hydrolysis of purified GPL antigens will be examined by TLC procedures to determine specific sugars that incorporated the radiolabel. The internally radiolabeled antigens will be utilized in macrophage uptake and timed studies
to examine the fate of the serologically specific portion of the GPL antigen.
CHAPTER II

MATERIALS AND METHODS

Mycobacteria. *Mycobacterium avium* complex serovars 4 and 20 were used in this investigation. Serovar 4 was obtained from National Jewish Hospital and Research Center (Denver, CO) through Dr. Darrel Gwinn, National Institute of Allergy and Infectious Diseases (NIH, Bethesda, MD). *Mycobacterium* sp. NQ was a gift from L. Barksdale, New York University School of Medicine. Previous seroagglutination identified this mycobacterium as serovar 20 (2, 4).

Mice. Male and female C57Bl/6Hsd mice were purchased from Harlan-Sprague-Dawley, Indianapolis, Ind., and raised in small groups on a diet of mouse chow and water. Both male and female mice, six to twelve weeks, were used in the macrophage uptake and timed studies.

Growth of Mycobacteria. Mycobacterial cultures were cultivated in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 2 ml of glycerol. Media was dispensed into 250-ml Kontes side-arm flasks for the 100 ml cultures and 2800 ml flasks for the liter cultures and autoclaved. Prior to bacterial inoculation of the media, oleic acid-albumin-dextrose (OADC) supplement (Difco) (ten ml per 90 ml of 7H9) was added to
each flask and the flasks were incubated overnight to check for contamination (4, 20, 39). Media was inoculated with mycobacteria to achieve a reading of 20 to 30 Klett units on a Klett-Summerson photoelectric colorimeter (Arthur H. Thomas Company, Philadelphia, PA) using a #42 filter. Cultures were incubated at 35°C on a shaker incubator (New Brunswick Scientific, Edison, NY) set at 125 rpm. D-Fucose [6-3H] (30 Ci/mMol) ([3H]-Fuc); D-Mannose [2-3H] (30 Ci/mMol) ([3H-Man]); and L-Methionine [methyl-3H] (50 Ci/mMol) ([3H-Meth]) (ICN Radiochemicals, Inc., Irvine, CA) were added individually to the mycobacterial cultures in early exponential phase (100 to 150 Klett units) in concentrations of 25, 50, and 100 μCi/100 ml. After mycobacteria reached late stationary phase (500 to 550 Klett units), cultures were autoclaved and cells harvested by centrifugation (Beckman Model J-21C Centrifuge, Beckman Instruments, Inc., Palo Alto, CA). Cells were then lyophilized and stored at -20°C until lipids were extracted.

**Lipid Extraction.** Native lipid was extracted from lyophilized mycobacterial cells according to the Folch procedure (2, 39). Briefly, lyophilized cells were suspended in chloroform-methanol (2:1, v/v) at a volume equivalent to 32 ml/g of cells and incubated overnight in a 50°C water bath. Cell debris was removed by filtration through a Buchner funnel with Whatman number 1 filter paper and rinsed with chloroform-methanol (2:1, v/v). The filtrate was then mixed with phosphate buffered saline (PBS) (0.001 M NaPO₄ and 0.015 M NaCl, pH 7.2-7.4) to a ratio of chloroform-methanol-PBS (8:4:3, by vol) and allowed to separate in a separatory funnel overnight or until the layers cleared. The lower lipid containing chloroform layer was drained off and the upper aqueous phase was
discarded. The chloroform layer was concentrated on a rotary evaporator (Brinkman Instruments, Westbury, NY), rinsed with chloroform-methanol (2:1, v/v) and chloroform, then transferred to a weighed tube, dried under nitrogen and dessicated in vacuo overnight. Following reconstitution with chloroform, native lipid was stored at -20°C. Total incorporation of radioactivity into native lipid was determined by placing lipid into Ecolite scintillation fluid (WestChem, San Diego, CA) for counting on a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL.).

**Deacetylation.** For deacetylation, native lipid was treated with 0.2 N methanolic-NaOH (8) for 30 minutes at 37°C, neutralized with glacial acetic acid, and dried under nitrogen. The residue was partitioned by means of centrifugation in chloroform-methanol-water (3:2:1, by vol) and the upper aqueous layer was discarded. The lower chloroform layer, containing the GPLs, was dried under nitrogen, then reconstituted in chloroform and stored at -20°C. Radioactivity of deacetylated lipid was determined as described above.

**Chromatography.** Distribution of radioactivity in native and deacetylated radiolabeled lipid was determined by thin layer chromatographic (TLC) methods. Radiolabeled lipid fractions were applied to silica gel TLC plates (250 µ thickness) (Whatman, Inc., Clifton, NJ) in concentrations ranging from 100 to 300 µg. Chromatography tanks were equilibrated with solvent and plates were developed separately in either chloroform-methanol-water (60:12:1, by vol) (solvent A) or chloroform-methanol (11:1, v/v) (solvent B). After separated lipid components were visualized with iodine, centimeter sections were scraped
into scintillation vials containing four ml of Ecolite and counted as above. Glycopeptidolipid antigens were detected by spraying developed TLC plates with orcinol-sulfuric acid reagent (0.1% orcinol in 40% H₂SO₄), a procedure that turns the GPL polar antigens a yellow-gold color (due to the 6-deoxyhexoses) upon heating at 100⁰C for 10 to 15 min (4). Appropriate lipid standards were run in parallel with the radiolabeled lipids to confirm the location of GPL antigens.

**Purification of Lipids.** Pure preparation of both native and deacetylated polar GPL lipids were obtained by fractioning native lipid extracts on a column (Kontes Scientific Glassware/ Instruments, Vineland, NJ) of type H silica gel (Sigma Chemical Company, St. Louis, MO) (15). Sixty grams of activated silica gel was mixed with 300 ml of chloroform to form a slurry which was used to prepare a column with dimensions of 2.5 cm x 20 cm. Lipid samples were dissolved in chloroform and loaded onto the column under pressure. The column was eluted first with chloroform, then solutions of increasing percentages of methanol-chloroform. Samples were collected in three ml fractions on a LKB 2211 SuperRac fraction collector (LKB Instruments, Inc., Gaithersburg, MD) and GPL antigens were eluted in chloroform-methanol (93:7, v/v). Fractions were monitored with silica gel TLC plates using solvent A, and GPL antigens were detected with orcinol-sulfuric acid spray (15). Fractions containing GPL antigens were pooled and transferred to weighed tubes for drying under nitrogen and desiccation. Purified antigens were reconstituted to 100μg/10μl with chloroform and stored at -20⁰C.
Confirmation of Radiolabel Incorporation into Oligosaccharide. Native and deacetylated pure radiolabeled GPL antigens from serovars 4 and 20 were hydrolyzed by two different methods. In the first method, lipid was hydrolyzed with Killiani's reagent (2.5 ml HCl, 13.7 ml water, 8.7 ml acetic acid) (1 mg lipid/1 ml Killiani's reagent) for six hours at 100°C in a multi-bloc heater (Lab-Line Instruments, Inc., Melrose Park, IL.) (20). The sample was extracted three times with 0.5 ml of hexane to remove fatty acids, then dried under a stream of nitrogen. The sample was reconstituted in aqueous 50% ethanol and dried again several times to remove acid. In the second method, GPL antigens were hydrolyzed with 2M trifluoroacetic acid for two hours in a sealed tube using a hot oil bath at 100°C. Hydrolysate was extracted with hexane to remove fatty acids under a stream of nitrogen. Radioactivity in samples was determined by adding a portion of the sample to Ecolite and counting in a scintillation counter.

The phenol-sulfuric acid test was used to determine the percent carbohydrate in the hydrolysates (18). Each carbohydrate sample was diluted to a total of 200 µl with water. To each sample was added 200 µl of 5% phenol (5.5 ml 90% phenol in 94.5 ml water) and 1 ml of reagent grade sulfuric acid. A reference blank was prepared with 200 µl of water plus the phenol and sulfuric acid. Rhamnose standards were used as controls. Absorbance of each sample was measured on a Gilford Spectrophotometer 250 (Gilford Instrument Laboratories, Inc., Oberlin, OH) at 490 nm.

Resulting sugars from both hydrolysis procedures were separated by development on cellulose TLC plates (Whatman) in butanol-ethanol-water (32:4:40, by vol) (solvent C) (8) and detected by spraying plate with aniline-oxalate (90% oxalic acid in water and 0.89 ml
aniline) and heating at 100-105° C for fifteen minutes (21). Distribution of radioactivity was determined by scraping half centimeter sections of the cellulose plate into vials containing Ecolite and counting as described above. Appropriate sugar standards were derived from serovar 20 (20) or obtained commercially.

**Isolation and Cultivation of Mouse Peritoneal Macrophages.** Nonelicited peritoneal macrophages were obtained from either female or male C57Bl/6 mice using procedures described previously (8, 12, 20) with either NCTC medium 109 (Difco) or RPMI-1640 medium (Whittaker M. A. Bioproducts, Inc., Walkersville, MD). Before use, L-glutamine (1 ml glutamine/100 ml medium or 2mM), penicillin G (1 ml penicillin/ 100 ml medium or 100 units/ml), and ten percent sodium bicarbonate were filtered sterilized and added separately to the medium.

To obtain nonelicited peritoneal macrophages, mice were sacrificed by cervical dislocation and peritoneal cells removed by irrigation of the peritoneal cavity with two ml of ice-cold medium containing 5U of Heparin (Sigma)/ml of medium using a 3cc sterile syringe with a 23 gauge needle. One to 1.5 ml of lavaged fluid was aspirated per mouse and pooled in a centrifuge tube chilled on ice.

Viability of peritoneal cells was determined by trypan blue exclusion to be always >97% (20). Briefly, one part 5X saline (4.25% NaCl, w/v in water) and four parts trypan blue (0.2% w/v in water) were added to equal parts of cell suspension and counted microscopically using an American Optical Bright-Line hemacytometer (American Optical, Buffalo, NY). The number of cells per ml of cell suspension was determined by analysis on
a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Five drops of lysing agent (Scientific Products, McGaw Park, IL), 40 μl of cell suspension and 20 ml saline were combined in a plastic Coulter vial and analyzed on the Coulter. Following dilution to $2 \times 10^6$ cells/ml with collection media (without heparin), the cell suspension was added in 1.0 ml volumes to 24-well tissue culture plates (Falcon, Becton Dickinson Labware, Oxnard, CA). Cultures were incubated for two hours at $37^\circ$C under 5% carbon dioxide to allow for macrophage adherence, after which nonadherent cells were removed by washing monolayers with RPMI. In some experiments, cultures were incubated overnight under the same conditions. Following a PBS rinse and fixation with methanol, control monolayers were stained using rapid Wright-Geimsa stain kit (Scientific Products) and enumerated under 20X magnification with a Whipple micrometer reticle (American Optical).

**Pulsing of Macrophages with GPL Antigens.** GPL antigens were routinely kept at -20°C in chloroform and transferred to sterile glass vials before use in pulsing experiments. Antigens preparations were dried under a stream of nitrogen by passing through a 0.22 μm filter. Before addition of antigen suspension to adherent cells, antigen preparations were reconstituted in chloroform-methanol (2:1, v/v) several times and dried under nitrogen. Complete dryness was accomplished by desiccation *in vacuo* for 45 minutes. Before pulsing, dried antigen preparations were suspended in medium 109 or RPMI by sonication in ultrasonic bath (Branson Cleaning Equipment Company, Shelton, CN) for 1.5 - 2.0 h and refluxed through 27 gauge needle to obtain a uniform suspension (20).

Adherent cells were pulsed with the GPL antigen suspension for 2 h at $37^\circ$C under 5%
carbon dioxide. Each monolayer received the appropriate amount of antigen suspended in a total of 1 ml of media. For uptake studies, GPL antigen concentrations ranged from 10 μg to 250 μg GPL/ml medium. At appropriate times, adherent cells and spent media were assayed for radioactivity by counting in Ecolite. Adherent cells were removed by adding 1 ml of 0.05% Triton X-100 (Scientific Products) in PBS and scraping with a Teflon policeman. To determine radioactivity in lipid components, adherent cells and spent media were extracted with chloroform-methanol-water (8:4:3, by vol) and chloroform-extractable material assayed by TLC for distribution of radioactivity (20).

To determine the percent of cell types in peritoneal fluids, lavage fluid was stained by a nonspecific esterase staining procedure (27). Smears were prepared on methanol-rinsed slides by mixing 170 μl of cell suspension with 30 μl fetal calf serum and allowing to air-dry. Smears were fixed with cold fixative (100 mg KH₂PO₄, 20 mg Na₂PHO₄, 30 ml distilled water, 45 ml acetone, and 25 ml 30% formaldehyde) for thirty seconds and then rinsed with three changes of water. Slides were incubated for 45 min at room temperature in a filtered mixture of 47.5 ml 0.15M Sorenson buffer (pH 6.3), 0.25 ml hexazotized pararosaniline, and 2.5 ml alpha-naphthyl butyrate substrate (1 g alpha-naphthyl butyrate and 50 ml ethylene glycol monomethyl ether). The hexazotized pararosaniline was prepared by dissolving 0.1 grams of NaNO₃ in 2.5 ml water and adding 0.5 ml pararosaniline stock (1 g pararosaniline hydrochloride and 25 ml 2N HCl) and filtering before use. After incubation in the above mixture, slides were rinsed with distilled water and counterstained with 0.5% methyl green for 1.0 min. Finally, slides were rinsed with distilled water and air-dried, after which macrophages were quantitated microscopically by enumerating cells which were
positive for esterase activity.
CHAPTER III

RESULTS

Incorporation of Radiolabel into Native Lipid. After mycobacteria had been cultured in the presence of individual radiolabeled components, native lipid was extracted and amount of radioactivity reported as $[^3H] \text{cpm/mg lipid}$ (Table 1). Examination of $[^3H] \text{cpm/mg lipid}$ shown in Table 1 revealed that use of $[^3H]-\text{Fuc}$ to radiolabel the GPL antigens of serovars 4 and 20 resulted in the least incorporation into native lipid. Because of the low incorporation of radioactivity into native lipid (Table 1), $[^3H]-\text{Fuc}$ was not considered for further use as a probe in subsequent macrophage uptake studies. However due to limitations associated with TLC, the distribution of $[^3H]-\text{Fuc}$ in native lipid was difficult to determine. Use of $[^3H]-\text{Man}$ and $[^3H]-\text{Meth}$ resulted in higher incorporations of radioactivity into native lipid of serovar 4 and 20 (Table 1) with $[^3H]-\text{Meth}$ providing the highest $[^3H] \text{cpm/mg}$ (Fig 1).

Separation of Apolar and Polar GPL Component. When a TLC plate is developed in a polar solvent system like solvent A, the polar GPL antigens migrate toward the middle of the plate (Fig 2). However, in an apolar solvent system like solvent B, the polar antigens migrate in the bottom 2-3 cm of the plate.

The apolar GPL components differ from the polar antigens in that a single 6-deoxytalose is present instead of an oligosaccharide moiety. Following development of plate in the polar
Table 1. Incorporation of $[^3\text{H}]-\text{Fucose}, -\text{Mannose}, \text{or-Methionine}$ into native lipid of serovar 4 and 20.
### Serovar 4

<table>
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<tr>
<th>Radiolabel</th>
<th>Amt. added (µCi)/100ml culture</th>
<th>Amt. incorporated (cpm x 10^3)/mg lipid</th>
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<tbody>
<tr>
<td>[6-³H]-L-fucose</td>
<td>25</td>
<td>7.2</td>
</tr>
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<td></td>
<td>50</td>
<td>9.0</td>
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<tr>
<td></td>
<td>100</td>
<td>10.0</td>
</tr>
<tr>
<td>[2-³H]-D-mannose</td>
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<td>12.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>19.7</td>
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<tr>
<td></td>
<td>100</td>
<td>24.5</td>
</tr>
<tr>
<td>[methyl-³H]-L-methionine</td>
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<td>88.9</td>
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<td></td>
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<tr>
<td></td>
<td>100</td>
<td>1,081.5</td>
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</table>

### Serovar 20

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<th>Radiolabel</th>
<th>Amt. added (µCi)/100ml culture</th>
<th>Amt. incorporated (cpm x 10^3)/mg lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>[6-³H]-L-fucose</td>
<td>25</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<tr>
<td></td>
<td>100</td>
<td>230.3</td>
</tr>
</tbody>
</table>
Figure 1. Structure of GPL antigens showing incorporation of various radiolabeled components.
$[^3\text{H}]$-phenylalanine \[\text{fatty acyl-D-phenylalanine-D-allo-threonine-D-alanine-L-alaninol-R}_1\]

$[^3\text{H}]$-fucose

$[^3\text{H}]$-mannose $\rightarrow \rightarrow R_2 \quad \leftarrow [\text{methyl-}^3\text{H}]-\text{methionine}$

$R_1$: -3,4-di-O-methyl-rhamnose

$R_2$: \text{Apolar GPL}

-6-deoxytalose

\text{Serovar 4} 
-6-deoxytalose-rhamnose-2-O-methyl-fucose-4-O-methyl-rhamnose

\text{Serovar 20} 
-6-deoxytalose-rhamnose-2-O-methyl-fucose-2-O-methyl-rhamnose
Figure 2. TLC migration patterns of polar and apolar GPL components in polar and apolar solvent systems.
Solvent A, the apolar counterparts run above the polar GPL antigens but in the apolar solvent system, the apolar components migrate toward the middle of the plate. The primary reason for developing TLC plates in the apolar solvent system is due to the fact that this system allows for easier observation of the apolar GPL components.

**Distribution of Radiolabel in Native and Deacetylated Lipid.** Thin-layer chromatographic analysis of lipid radiolabeled with [³H]-Fuc resulted in a wide distribution of radioactivity throughout the spectrum of native lipid components, for both serovar 4 (Fig 3A) and 20 (Fig 3B), indicating incorporation in both polar GPL antigens and their apolar counterparts. Examination of radiolabeled native and deacetylated lipid by TLC in solvent A revealed that successful incorporation of radiolabel into GPL antigens was accomplished with [³H]-Meth and [³H]-Man for both serovars 4 and 20 (Figs. 4-7). Lipid radiolabeled with [³H]-Meth analyzed by TLC procedures in solvent A exhibited radioactivity in the area of the plate associated with the polar antigens and the apolars in both serovar 4 (Fig 4A) and 20 (Fig 5A). Tritiated-mannose labeled native lipid examined by TLC procedures resulted in a distribution of radioactivity concentrated in the area coinciding with the polar GPL antigens for both serovar 4 (Fig 6A) and 20 (Fig 7A).

To further confirm that the radioactivity in radiolabeled lipid corresponded with the antigens, native serovar 4 and 20 lipid radiolabeled with either [³H]-Man or [³H]-Meth was treated with mild alkali to deacetylate the antigens to facilitate their identification on TLC. Following deacetylation, the polar antigens migrate as one component on TLC because of the removal of acetyl groups attached to the individual sugars of the oligosaccharide moiety.
Figure 3. TLC distribution of radioactivity in native lipid from $^{3}$H-Fucose labeled (A) serovar 4 and (B) serovar 20. Lipid was applied at a concentration of 200 µg and the plate was developed in solvent A. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar GPL component (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
Examination by TLC of deacetylated lipid labeled with $[^{3}H]$-Man or -Meth from serovar 4 and 20 revealed that distribution of radioactivity was still aligned with the GPL antigens (Figs. 4B, 5B, 6B and 7B). The deacetylated lipid labeled with $[^{3}H]$-Meth exhibited cpm in the area of the plate associated with the apolar components as well as the antigens in serovar 4 (Fig 4B) and serovar 20 (Fig 5B). However, $[^{3}H]$-Man labeled deacetylated lipid from serovar 4 (Fig 6B) and serovar 20 (Fig 7B) displayed radioactivity only in the area of the plate associated with the antigens.

To demonstrate that $[^{3}H]$-Man had exclusively radiolabeled the antigens and not the apolar components of serovar 4, native lipid radiolabeled with either $[^{3}H]$-Man or -Phe/Ala was examined by TLC methods in solvent B (Fig 9). The $[^{3}H]$-Phe/Ala labeled native lipid demonstrated radioactivity in area of the plate corresponding to both the polar and the apolar components (Fig 9A). However, the native lipid labeled with $[^{3}H]$-Man revealed $[^{3}H]$ cpm/mg only in the area of the plate corresponding to the polar antigens (Fig 9B). Lipid from serovar 20 labeled with $[^{3}H]$-Man was also examined in solvent B demonstrating radioactivity in the area of the plate associated with polar antigens (Fig 7C). Lipid radiolabeled with $[^{3}H]$-Meth from both serovars 4 and 20 was examined by TLC methods in solvent B. The $[^{3}H]$-Meth-labeled native lipid exhibited radioactivity in areas of the plate corresponding to both polar and apolar components for both serovar 4 (4C) and 20 (Fig 5C).

**Purification of Native and Deacetylated Lipid.** Native and deacetylated radiolabeled lipid from serovar 4 and 20 were purified by a one-step chromatographic procedure (15).
Figure 4. TLC distribution in (A) native lipid developed in solvent A, (B) deacetylated lipid developed in solvent A, and (C) native lipid developed in solvent B from [³H] Methionine-labeled serovar 4. Lipid was applied at a concentration of (A) 100 μg, (B) 200 μg, and (C) 100 μg. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar GPL components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
Figure 5. TLC distribution in (A) native lipid developed in solvent A, (B) deacetylated lipid developed in solvent A, and (C) native lipid developed solvent B from $^3$H] Methionine-labeled serovar 20. Lipid was applied at concentration of (A) 300 µg, (B) 200 µg, and (C) 100 µg. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar GPL components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
A

GPL=18%

aGPL=29%

cm from origin

B

dGPL=16%
aGPL=20%

C

GPL=27%
aGPL=27%
Figure 6. TLC distribution in (A) native lipid developed in solvent A, (B) deacetylated lipid developed in solvent A, and (C) native lipid developed in solvent B from $[^3$H]$\text{Mannose}$-labeled serovar 4. Lipid was applied at a concentration of (A) 200 $\mu$g, (B) 300 $\mu$g, and (C) 200 $\mu$g. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar GPL components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
Figure 7. TLC distribution in (A) native lipid developed in solvent A, (B) deacetylated lipid developed in solvent A, and (C) native lipid developed in solvent B from [3H] Mannose-labeled serovar 20. Lipid was applied at concentration of (A) 300 μg, (B) 200 μg, and (C) 300 μg. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
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### Chart A

- **GFL = 52%**
- **kcpm** vs. **cm from origin**
- Peaks at 5, 10, and 15 cm.

### Chart B

- **dGFL = 59%**
- **kcpm** vs. **cm from origin**
- Peak at 5 cm.

### Chart C

- **GFL = 85%**
- **kcpm** vs. **cm from origin**
- Peaks at 5, 10, and 15 cm.
Figure 8. TLC distribution of radioactivity in native lipid from (A) [3H] Phenylalanine/Alanine and (B) [3H]-Mannose labeled serovar 4. Lipid was applied at a concentration of (A) 100 µg and (B) 200 µg and plate developed in solvent A. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
Figure 9. TLC distribution of radioactivity in native lipid from (A) $[^3]$H Phenylalanine/Alanine and (B) $[^3]$H-Mannose labeled serovar 4. Lipid was applied at a concentration of (A) 100 µg and (B) 200 µg and plate developed in solvent B. The radioactivity was located by counting sections in Ecolite. GPL antigens and apolar components (aGPL) were detected by their migration with respect to verified standards and characteristic color reaction to orcinol-sulfuric acid.
Following elution of pigments and apolar GPL antigens in chloroform-methanol (96:4, v/v) respectively, the solvent system was changed to chloroform-methanol (93:7, v/v) and fractions collected at 10 minute intervals. Fractions were monitored by TLC in solvent A and those fractions containing GPL antigens were pooled. The GPL antigens routinely came off after 500-1000 ml of 7% methanol had eluted. The yield of pure GPL antigens averaged 14%.

**Distribution of Radiolabel in the Oligosaccharide Moiety.** Purified deacetylated lipid radiolabeled with either $[^3]H$-Man or $[^3]H$-Meth from both serovar 4 and 20 was hydrolyzed with trifluoroacetic acid to determine which sugar or sugars had incorporated the radiolabel. The monosaccharides produced from the hydrolysis procedure of lipid from each serovar labeled with each radiolabel were examined by TLC methods for the distribution of radioactivity. Hydrolyzed lipid labeled with $[^3]H$-Man displayed cpm in the area of the plate corresponding to the sugar 2-O-Me-fucose in both serovar 4 (Fig 10B) and 20 (Fig 11B). Tritiated-methionine-labeled hydrolyzed lipid from serovars 4 (Fig 10A) and 20 (Fig 11B) demonstrated radiolabel in the area of the plate corresponding to the methylated sugars, 2-O-Me-fucose, 2-O-Me-rhamnose or 4-O-Me-rhamnose, and 3, 4-di-O-Me-rhamnose.

**Uptake of Radiolabeled GPL Antigens by Mouse Macrophages.** Unelicited peritoneal cell counts from the C57BI/6 mice averaged $4.49 \times 10^6$ cells/ml (standard deviation, ± 1.3, n=4). The area of each tissue culture well was 2 cm$^2$, and the addition of $2 \times 10^6$ peritoneal cells to each well resulted in an average monolayer of $3.17 \times 10^5$ adherent cells.
Figure 10. Cellulose TLC distributions of monosaccharides obtained from hydrolyzed (A) \(^{3}\text{H}\)-Methionine and (B) \(^{3}\text{H}\)-Mannose labeled serovar 4 antigens. Monosaccharides were applied at a concentration of (A) 49 \(\mu\)g and (B) 21 \(\mu\)g, and plate developed in solvent C. The radioactivity was located by counting sections in Ecolite. Monosaccharides were detected with an aniline-oxalate spray and identified by their migration with respect to sugar standards obtained from serovar 20.
Figure 11. Cellulose TLC distributions of monosaccharides obtained from hydrolyzed (A) $[^3H]$-Methionine and (B) $[^3H]$-Mannose labeled serovar 20 antigens. Monosaccharides were applied at a concentration of (A) 51 μg and (B) 29 μg, and plate developed in solvent C. The radioactivity was located by counting sections in Ecolite. Monosaccharides were detected with an aniline-oxalate spray and identified by their migration with respect to sugar standards obtained from serovar 20.
A

3,4-di-Me-Rha

2-0-Me-Rha

2-0-Me-Fuc

6-dTal

Rha

B

2-0-Me-Fuc

6-dTal

Rha

2-0-Me-Rha

3,4-di-Me-Rha

kcpm vs. cm from origin
Concentration of GPL antigen necessary for maximum uptake of the GPL antigens by macrophage was determined by pulsing macrophage monolayers with a concentration of [3H]-Phe/Ala-labeled GPL antigens (1.54 x 10^5 cpm/mg) ranging from 10 to 250 μg/3.17 x 10^5 cells. Following 2 hour pulse with antigen, macrophages were assayed for radioactivity, and data was expressed as [3H] cpm per 10^5 cells. Maximum antigen uptake by the macrophages occurred slightly above a concentration of 200 to 250 μg of GPL/ml of media (Fig 12).

**Distribution of Radioactivity of Chloroform-Extractable Material.** The possible degradation of the GPL antigens by mouse peritoneal macrophages was examined by [3H]-Man-labeled antigen (4.6 x 10^4 cpm/mg) in a timed experiment. To be consistent with previous experiments (28), an antigen concentration of 200 μg GPL/ml media was used to pulse monolayers (3.07 x 10^5 adherent cells) for 0, 24, 48, and 96 h. To determine distribution of radioactivity within macrophage cultures from the timed experiment, combined spent medium and macrophages from 0, 24, 48, and 96 h following antigen pulse were extracted with chloroform-methanol-water (8:4:3, by vol). The mean cpm in the aqueous layer was 8.6 cpm/ml (standard deviation, ± 3.9, n=4) and the mean cpm in the chloroform fraction was 7173 cpm/mg (standard deviation, ± 400, n=3). The percent total radioactivity remaining in the combined spent media and macrophages was 99.8%, 99.3%, 99.5%, and 99.4% for 0, 24, 48, and 96 h, respectively (standard deviation, ± 0.2, n=4).

Examination by TLC of chloroform-extractable material revealed that all of the GPL
Figure 12. Uptake of radiolabeled GPL antigens by mouse peritoneal macrophages. Macrophage monolayers were pulsed for 2 h with [3H]-Phenylalanine/Alanine radiolabeled serovar 4 antigen suspended in RPMI at concentration ranging from 0-250 μg of GPL per ml medium. Activity was $1.54 \times 10^5$ [3H] cpm/mg for radiolabeled antigens. Each point represents the mean cpm $3.2 \times 10^5$ cells ± standard deviation of triplicate assays.
antigens of serovar 4 were present in the chloroform-extractable material throughout the four days following antigen pulse. Following development of TLC plates in solvent A, the GPL antigens were identified by their characteristic color reaction when sprayed with orcinol-sulfuric acid, and their location on the TLC plate with respect to purified GPL standards. Further examination of the chloroform-extractable material by TLC revealed that radioactivity was concentrated in the area of the plate corresponding to GPL antigens. This pattern was observed at 0, 24, 48, and 96 h (Fig 13).

Macrophage Degradation Products. In summary, Fig 14 lists the possible degradation products of the polar GPL antigens following contact with resident peritoneal macrophages. Because the majority of the cpm were in the lower chloroform layer, the antigens from serovar 4 appear to be relatively inert to degradation under these conditions.
Figure 13. TLC distribution of radioactive chloroform-extractable components from spent media and macrophages obtained from C57Bl/6 peritoneal macrophages cultures at 0 h (■) and 96 h (▲) after antigen pulse with [3H]-Mannose-labeled serovar 4 GPL antigen. Lipid was applied at a concentration of 300 μg and plate was developed in Solvent A. Radioactivity was located by counting sections in Ecolite. Antigens were located by their characteristic yellow-gold reaction to orcinol-sulfuric acid reagent.
Figure 14. Possible degradation products of polar GPL antigens following contact with macrophages.
\[
\text{CH}_3 \text{CO-N} \rightarrow \text{NO} [^5\text{H}] \\
\text{CH}_3 \text{CO-N} \rightarrow \text{NO} \\
\text{CH}_3 \text{CO-N} \rightarrow \text{NO} \\
\text{CH}_3 \text{CO-N} \rightarrow \text{NO} \\
\]
Information regarding the elements of pathogenesis for nontuberculous mycobacteria in the *M. avium* complex is restricted. Even though members of the *M. avium* complex are facultative intracellular parasites, no substantial reason for their survival in the phagolysosomal compartments of host macrophages has been documented. Draper and Rees have suggested that the superficial network of fibrillar lipid components known as the \( L_1 \) layer may function as a protective capsule (17). A later report exposed the fact that the GPL antigens of *M. avium* complex were the predominant elements composing the \( L_1 \) layer (4).

Previous investigations by Tereletsky and Barrow strove to monitor the relationship between the GPL antigens and murine peritoneal macrophages following phagocytosis by using immunocytochemical techniques (39). As a consequence of the fact that these attempts had problems in monitoring the exact intracellular location of the GPL antigens, new radiolabeling techniques were developed to more accurately evaluate the postphagocytic events (39).

Hooper, *et al.* described radiolabeling techniques used to monitor the degradation of the GPL antigens in mouse peritoneal macrophages (20). From that study, it was concluded that GPL antigens are relatively inert to macrophage degradation (20). Those results represented the first confirmation supporting the suggestions of Draper and Rees with respect to the
inertness of the L₁ layer and indicated that the GPL antigens of *M. avium* complex may indeed play a significant role in pathogenicity (20).

In the study by Hooper, et al. (20), it was revealed that four days after antigen pulse, most of the total radioactivity associated with macrophages was affiliated with intact GPL antigens. However, they reported that 2-3% of the radioactivity corresponded to elements that migrated to the upper portion of the TLC plate, a finding which suggested that degradation of the oligosaccharide determinant of the GPL antigens had occurred (20). To examine this latter possibility, it became necessary to develop techniques to radiolabel the oligosaccharide determinants of the GPL antigens.

This investigation was designed to develop procedures to radiolabel the oligosaccharide determinant of the polar GPL antigens of the *M. avium* complex. Serovar 4 was chosen because it represents the most frequent *M. avium* complex serovar that is isolated from AIDS patients (40). Ability to radiolabel the oligosaccharide determinant would enable those radiolabeled GPL antigens to be used in macrophage processing studies to examine the fate of the determinant portion of these potentially important mycobacterial components. Choice of radiolabeled components was made depending upon the commercial availability, cost effectiveness, and previous studies on biochemical pathways. Therefore, tritiated-L-fucose, D-mannose, and L-methionine were employed to radiolabel the oligosaccharide. Fucose is found within the oligosaccharide moiety as the third sugar from the attachment point of the oligosaccharide moiety to the fatty acyl-peptide (Fig. 1) (32). Touster reported that mannose converts to fucose (40) and this sugar is found in the oligosaccharide of serovar 4 (32). Methionine is a donor of methyl groups and has been used in a previous study to radiolabel
the methylated sugars found in the oligosaccharide (4).

With the exception of [³H]-mannose, incorporation of radiolabeled components into GPL antigens was least successful with serovar 20 than serovar 4. This probably resulted from the fact that serovar 20 is more lab adapted than serovar 4 and therefore had a different rate of metabolism. Additional studies will be necessary to determine the optimum time for addition of radiolabeled components to growing cultures of each serovar.

Due to the fact that methionine is a donor of methyl groups, incorporation of radioactivity into the GPL antigens and their apolar counterparts can be justified. It was expected that each of the methylated sugars in the oligosaccharide determinant and the 3,4-di-O-Me-rhamnose attached to alaninol in the peptide core would be radiolabeled with the tritiated-methyl groups from methionine. Mannose, on the other hand, could have been incorporated as rhamnose and/or fucose in the oligosaccharide moiety of the GPL antigens or 3,4-di-O-Me-rhamnose on the fatty acyl-peptide core.

To more accurately and definitively determine whether mannose was incorporated into the apolar counterparts of the GPL antigens, [³H]-mannose-labeled serovar 4 lipid was re-examined by TLC procedures along with serovar 4 lipid radiolabeled with [³H]-phenylalanine and [³H]-alanine. The [³H]-Phe/Ala label incorporates into the peptide portion of the GPL structure (20) and as a result will radiolabel the apolar GPL components as well as the GPL antigens in all serovars (Fig. 1). Development of TLC plates in both polar (i.e. Solvent A) and apolar (i.e. Solvent B) solvent systems made it possible to conclude that [³H]-mannose did not radiolabel the apolar GPL components as had [³H]-phenylalanine and [³H]-alanine, indicating that [³H]-mannose had not been incorporated into either 3,4-di-O-
methyl-rhamnose or 6-deoxytalose (Fig. 1). Examination of \[^{3}\text{H}]\)-methionine-labeled serovar 4 lipid by TLC in Solvent B further confirmed that the apolar components were radiolabeled with methionine.

Following hydrolysis of purified radiolabeled GPL antigens from each serovar, cellulose plates were developed in Solvent C to establish the distribution of radioactivity within the sugars comprising the oligosaccharide moiety. Serovar 4 and 20 lipid radiolabeled with mannose exhibited cpm in the area of the plate associated with the sugar 2-O-Me-fucose (Figs. 10B and 11B). Examination of other biosynthetic pathways for sugars indicates that mannose can be converted to fucose (40). This can explain the fact that mannose was being incorporated into the fucose molecule of the oligosaccharide moiety. As expected from previous studies (4), \[^{3}\text{H}]\)-methionine-labeled lipid from serovars 4 and 20 demonstrated radioactivity in the area of the plate associated with only the methylated sugars-- 2-O-Me-fucose, 2-O-Me-rhamnose or 4-O-Me-rhamnose, and 3,4-di-O-Me-rhamnose (Figs. 10A and 11A). Thus, it can be concluded from this investigation that \[^{3}\text{H}]\)-mannose is incorporated specifically into the oligosaccharide of both serovar 4 and 20 at the penultimate position (i.e. 2-O-Me-fucose), while the methyl groups from \[^{3}\text{H}]\)-methionine are incorporated into the oligosaccharide and fatty acyl-peptide portion of the GPL antigens from both serovar 4 and 20.

Glycopeptidolipid antigen structures for serovars 8, 9, 16, and 25 have been established as follows:
Serovar 8
4,6-(1'-carboxyethylidene)-3-O-Me-glucose-rhamnose-6-deoxytalose (7, 10)

Serovar 9
2,3-di-O-Me-fucose-2,3-di-O-Me-fucose-rhamnose-6-deoxytalose (7, 10)

Serovar 16
4-O-Me-rhamnose-rhamnose-6-deoxytalose (10)

Serovar 25
2-O-Me-fucose-2-O-Me-fucose-rhamnose-6-deoxytalose (7, 10)

Based on the present work, speculations can be made regarding radiolabeling of the oligosaccharide moiety of the GPL antigens from the above four serovars. The fact that $[^3H]$-methionine incorporated methyl groups into the methylated sugars of serovar 4 and 20 implies that lipid from serovars 8, 9, 16, and 25 can also be radiolabeled at sites coinciding with methylated sugars-- 3-O-Me-glucose, 2,3-di-O-Me-fucose, 4-O-Me-rhamnose, and 2-O-Me-fucose, respectively, as well as the 3,4-di-O-Me-rhamnose of the peptide core. The monosaccharides 2,3-di-O-Me-fucose and 2-O-Me-fucose which are found in serovar 9 and 25, respectively, should incorporate $[^3H]$-mannose.

The results of the macrophage pulsing experiments with $[^3H]$-phenylalanine/alanine labeled antigens from serovar 4 indicated that the maximum level of antigen uptake by murine peritoneal macrophages takes place somewhere slightly above the 200-250 μg GPL per ml media (Fig 13) observed with serovar 20 in a previous study (20). Following extraction of lipid from spent media and macrophages at the four time periods, $[^3H]$-cpm/mg was
calculated to determine percent cpm in the upper and lower phases of the Folch wash. Suggestions of the location of the radiolabeled antigen whether intact or in degradation fragments can be determined by analyzing each Folch wash fraction for radioactivity. All but 1% of the $^{3}$H-cpm from the Folch layers were expressed in the lower chloroform fraction suggesting that the GPL antigens were not degraded by the macrophages. Degradation products absent of the fatty acyl-peptide core would have separated out in the upper aqueous Folch layer causing $^{3}$H-cpm to accumulate in this layer. The presence of intact GPL antigen would result in $^{3}$H-cpm located in the chloroform fraction of the Folch wash. Analysis of the spent medium and macrophages by TLC throughout the four days of antigen pulsing disclosed that most of the total radioactivity was exhibited in the area of the plate associated with the GPL antigens. The presence of cpm in the antigens suggested the fact that little degradation occurred and that serovar 4 antigens are relatively inert to phagocytosis.

In summary, $^{3}$H-methionine gave the highest incorporation of radioactivity into the native lipid while $^{3}$H-fucose exhibited the least incorporation. The $^{3}$H-methionine incorporated as a methyl group into the methylated sugars: 2-O-Me-fucose, 2-O-Me-rhamnose or 4-O-Me-rhamnose, and 3, 4-di-O-Me-rhamnose. This results in radiolabeling of both the polar and the apolar components of the GPL antigen. The sugar moiety, 2-O-Me-fucose, was radiolabeled with $^{3}$H-mannose achieving internal radiolabel specifically in the oligosaccharide determinant of both serovar 4 and 20. Maximum uptake of serovar 4 $^{3}$H-GPL antigen was found to be slightly higher than that observed for GPL antigens from serovar 20. Glycopeptidolipid antigens from serovar 4 appear to be relatively inert to degradation by nonelicited murine peritoneal macrophages.
BIBLIOGRAPHY


