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Macrophage Uptake and Retention of Radiolabeled Glycopeptidolipid Antigens Associated with the Superficial L_1 Layer of *Mycobacterium intracellulare* Serovar 20

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Glycopeptidolipid (GPL) antigens which are associated with the superficial L_1 layer of *Mycobacterium intracellulare* serovar 20 were labeled with radioisotopes by means of internal labeling techniques and used in macrophage uptake and retention studies. The use of tritiated alanine and phenylalanine allowed the incorporation of label into the GPL invariant fatty acyl peptide core, which is common to all members of the *Mycobacterium avium-M. intracellulare* complex. Radiolabeled GPL antigens were then purified by a one-step column chromatographic procedure and subsequently used to determine the maximum uptake and retention in peritoneal macrophages isolated from C57BL/6 and CBA/J mice. Maximum uptake for peritoneal macrophages from both strains of mice occurred at a concentration between 200 and 250 μg of antigen per ml of medium when 3.4×10^5 cells were pulsed. Timed experiments demonstrated that approximately 20% of the antigens remained associated with the macrophages up to 4 days after a pulse of 200 μg of GPL, and examination of chloroform-extractable components from both macrophages and spent medium revealed that 98% or more of the radioactivity corresponded to intact GPL components. The ability of the GPL antigens to become associated with macrophages is demonstrated by these results, which strongly suggest that these potentially important mycobacterial antigens are inert to degradation by those cells.

With the appearance of acquired immune deficiency syndrome (AIDS) it became apparent that our knowledge of certain opportunistic pathogens is critically lacking. One such group of opportunistic pathogens which has been associated with life-threatening infections in patients with AIDS is the class of nontuberculous mycobacteria collectively referred to as the *Mycobacterium avium-Mycobacterium intracellulare* serocomplex (27, 33). Although the *M. avium-M. intracellulare* complex has previously been recognized as a group of clinically significant pulmonary pathogens (33), very little is known regarding the pathogenic aspects of these intracellular parasites or antigenic determinants which might possibly be important in cellular immunity. This lack of information is crucial in view of the fact that members of the *M. avium-M. intracellulare* complex are resistant to antituberculous drugs, and, as a result, individuals infected with them do not respond well to therapy (7, 26, 30). Prognosis becomes even worse in the case of individuals suffering from AIDS because of an impaired cellular immunity (12, 13, 28).

An important feature of the mycobacterial disease process is the ability of the bacteria to survive within host macrophages (25). Evidence indicates that postphagocytic survival by some nontuberculous mycobacteria may be due to the presence of a protective capsule which shields the organism from phagolysosomal digestion (9–11). More recently, this capsule has been referred to as the L_1 layer (1), and although it has been suggested that the L_1 layer acts as an inert protective sheath for mycobacteria within macrophages, this has not been demonstrated specifically.

Previous investigations have determined that the major components of the L_1 layer of *M. intracellulare* serovar 20 are glycopeptidolipid (GPL) antigens (3) which are the basis for serotyping of mycobacteria in the *M. avium-M. intracellulare* complex (4). Because the GPL antigens are associated with the superficial L_1 layer, it is possible that they play some role in pathogenesis and also represent a group of antigens that could prove to be important in protective immunity. To investigate these possibilities, prior immunocytochemical studies in this laboratory have attempted to examine the fate of GPL antigens after the uptake of intact serovar 20 by host macrophages (29). It was felt that by complementing the immunocytochemical procedures with radioisotope techniques, a more accurate assessment of macrophage processing of the GPL antigens could be obtained.

This paper describes the internal labeling of GPL antigens associated with the superficial L_1 layer of *M. intracellulare* serovar 20 and the use of those radiolabeled antigens in macrophage uptake and retention studies. The use of tritiated alanine and phenylalanine allowed the incorporation of label into the invariant fatty acyl peptide core, a segment of the GPL antigens which is common to all members of the *M. avium-M. intracellulare* serocomplex (4). Purified radiolabeled GPL antigens were used to determine the maximum uptake and retention in peritoneal macrophages obtained from both C57BL/6 and CBA/J mice. The ability of the GPL antigens to become associated with mouse peritoneal macrophages was demonstrated by the results, which strongly suggest that these potentially important mycobacterial antigens are inert to degradation by those cells.

MATERIALS AND METHODS

Cultivation of serovar 20. Initially, *M. intracellulare* serovar 20 was cultivated in 7H9 Middlebrook medium (Difco Laboratories, Detroit, Mich.) with the addition of glycerol and OADC (Difco) supplement. Growth was moni-

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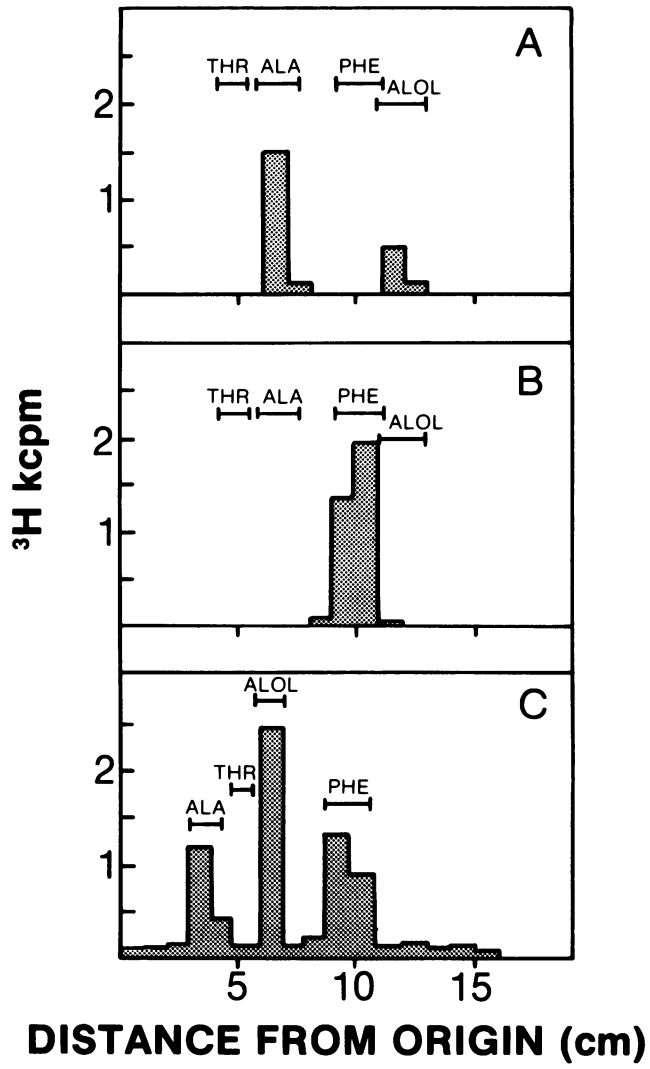


FIG. 1. Cellulose TLC distribution of amino acids obtained from hydrolysis of radiolabeled GPL antigens internally labeled with the following: A, [^{3}H]Ala (0.5 μCi/ml); B, [^{3}H]Phe, (0.5 μCi/ml); C, [^{3}H]Ala and [^{3}H]Phe (0.3 μCi/ml each). TLC plates were developed in either solvent D for A and B or solvent E (C), and amino acids were detected with 0.5% ninhydrin in acetone. Radioactivity was located by counting sections in Beta Gel. Appropriate standards included alanine (ALA), alaninol (ALOL), threonine (THR), and phenylalanine (PHE).

tored by using a Klett-Summerson spectrophotometer with a no. 43 filter, as described previously (29). For some experiments involving internal labeling, the OADC supplement was eliminated. After cultures had reached early exponential phase (100 to 150 Klett units), L-[2,3-^{3}H]alanine (50 Ci/mmol) ([^{3}H]Ala) and L-[side chain-^{3}H]phenylalanine (25 Ci/mmol) ([^{3}H]Phe) (ICN Radiochemicals, Inc., Irvine, Calif.) were each added at a concentration of 0.2, 0.3, or 0.5 μCi/ml. Cells were autoclaved and harvested when the mid-stationary phase had been achieved (500 to 550 Klett units); they were then lyophilized and stored at −20°C until the lipids were extracted.

Animals. Male and female C57BL/6NHsd and CBA/JHsd mice were purchased from Harlan-Sprague-Dawley, Indianapolis, Ind., and raised in small groups on a diet of mouse chow and water. Male and female mice, 5 to 12 weeks old, were used in this investigation.

Extraction and purification of lipids. Lipids were extracted from lyophilized mycobacteria with $CHCl_3$-CH_3OH (2:1) as described in previous papers (3, 29). Lipid antigens were purified from resulting lipid fractions by previously described silicic acid-Celite-DEAE-cellulose column chromatography (3) or a one-step column chromatographic procedure developed for purification of the GPL antigens (8). Purification was determined by means of thin-layer chromatography (TLC) with chloroform-methanol-water (60:12:1) (solvent A) (3) for one-dimensional chromatographic separation and chloroform-methanol-water (65:15:1) (solvent B) and chloroform-methanol-acetone-acetic acid-water (65:10:20:10:3) (solvent C) for two-dimensional separation (18). GPL antigens were detected by spraying developed TLC plates with orcinol-sulfuric acid reagent, a procedure that turns the GPL antigens a yellow-gold color upon heating (4). Purified antigens were stored at −20°C in chloroform until use.

To determine the distribution of radioactive material, radiolabeled lipid fractions were spotted on silica gel TLC plates and developed in solvent A. Plates were scored, and 1-cm sections were scraped into scintillation vials containing Beta Gel scintillation fluid (WestChem, San Diego, Calif.) for counting in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Appropriate lipid standards were run in parallel with the radiolabeled lipids and sprayed with orcinol-sulfuric acid reagent to confirm the locations of the GPL antigens.

Chemical procedures. Initially, lipids were treated with 0.2 N methanolic-NaOH for 30 min at 37°C (4) to facilitate the localization of the GPL antigens. It has been demonstrated previously that such treatment deacetylates GPL antigens I through IV found in the native lipid and results in only one detectable GPL component that has an R_f value similar to that of antigen IV in the native lipid (3). This occurs because

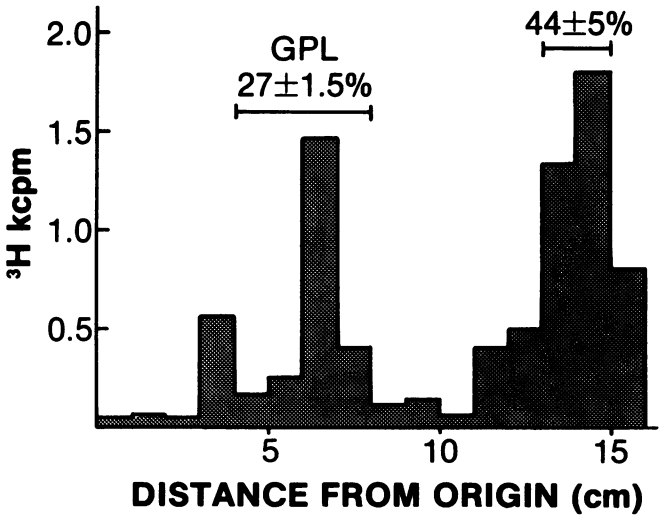


FIG. 2. TLC distribution of radioactivity in native lipid from serovar 20 cultured in the presence of [^{3}H]Ala and [^{3}H]Phe (0.3 μCi/ml each). Lipid was applied at a concentration of 100 μg, and the plate was developed in solvent A. The mean percent distribution (standard deviation) of radioactivity was determined by counting sections in Beta Gel from three separate analyses. GPL antigens were detected by their migration with respect to verified GPL standards and their characteristic color reaction to orcinol-sulfuric acid.

the four GPL antigens differ only in the number of acetyl groups attached to the carbohydrate moiety of the antigen structure (see Fig. 3), and antigen IV has the lowest number of acetyl groups in its native state (3). In subsequent studies reported here, GPL antigens were not deacetylated but were used in their native state.

To confirm the incorporation of radiolabeled amino acids, purified GPL antigens were hydrolyzed in a Killiani solution (52.2 ml of HCl, 13.8 ml of water, and 35 ml of glacial acetic acid) as described previously (4). Fatty acids were extracted from the hydrolysate with hexane, and amino acids were recovered by elution on a small Dowex 50 H^+ (×8) column with 3 M ammonium hydroxide (4). Resulting amino acids were then separated by development on cellulose TLC plates in either *n*-propanol-ammonium hydroxide (70:30) (solvent D) or butanol-acetic acid-water (40:10:10) (solvent E) and detected by staining with 0.5% ninhydrin acetone solution (4). Distribution of radioactivity was determined by scraping sections of the cellulose TLC plates into vials containing Beta Gel scintillation fluid and counting as described above. Appropriate amino acid standards were run in parallel to confirm the locations of radiolabeled amino acids in GPL hydrolysates.

Isolation and cultivation of mouse peritoneal macrophages. Nonelicited peritoneal macrophages were obtained from either male or female C57BL/6 and CBA mice by procedures described previously (29). Briefly, mice were sacrificed by cervical dislocation, and peritoneal cells were removed by irrigation of the peritoneal cavity with 2.0 ml of ice-cold NCTC medium 109 (Difco) containing 5 U of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml of solution. Viability of cells was determined by trypan blue exclusion to be always >97%.

Peritoneal cells were diluted to 2×10^6 cells per ml and added in 1.0-ml volumes to 24-well tissue culture plates (Falcon Plastics, Oxnard, Calif.). Cultures were allowed to incubate for 2 h at 37°C under 5% CO_2, after which nonadherent cells were removed by washing monolayers with medium 109. Additional overnight incubation and washing were necessary to achieve monolayers consisting of approximately 98% macrophages, as determined by a nonspecific esterase staining procedure (20). Resulting macrophage monolayers were maintained during pulsing experiments as described previously (29). Control monolayers were stained by using a rapid Wright-Geimsa method (Diff-Quick; Scientific Products, McGraw Park, Ill.) and enumerated under ×200 magnification by using a Whipple micrometer reticle (American Optical Corp., Buffalo, N.Y.).

Pulsing of peritoneal macrophages with GPL antigens. GPL antigens were routinely kept at −20°C in chloroform and transferred to a sterile glass vial before use in pulsing experiments. Antigen preparations were dried under a stream of nitrogen by passing the gas through a 0.22-μm filter

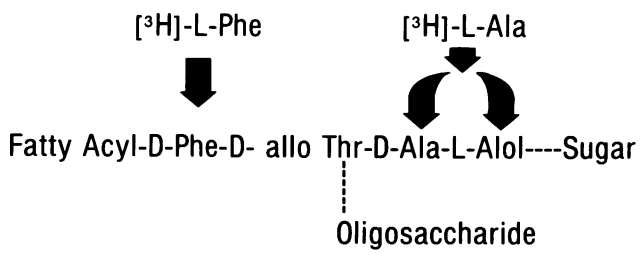


FIG. 3. Structure of GPL antigens from serovar 20 (3), representing the incorporation of radiolabeled amino acids.

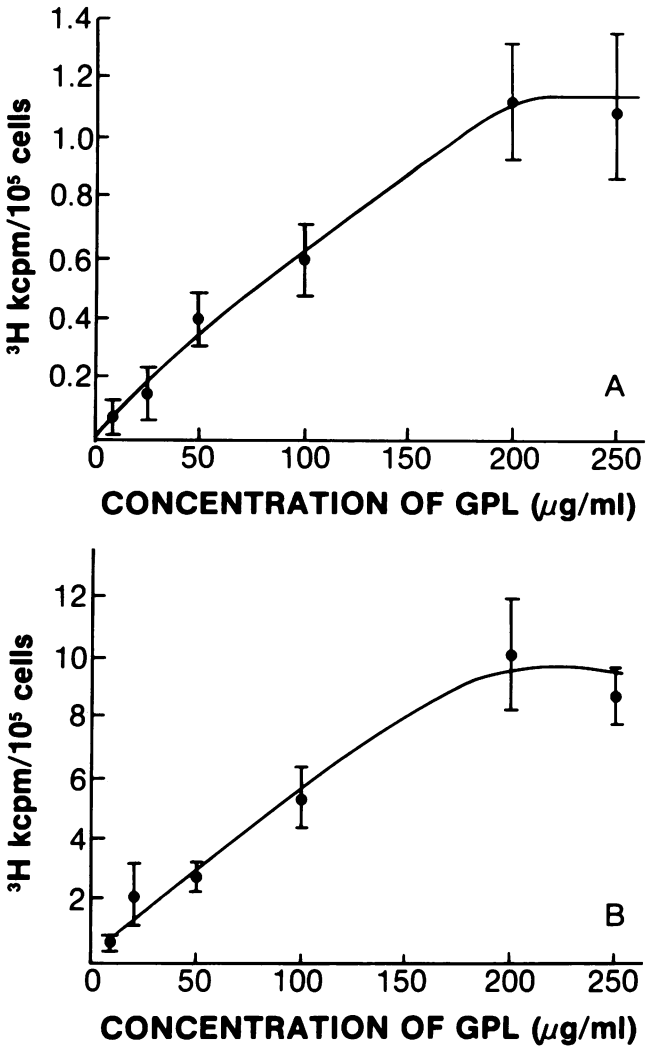


FIG. 4. Uptake of radiolabeled GPL antigens by mouse peritoneal macrophages. Macrophage monolayers, obtained from either C57BL/6 (A) or CBA/J (B) mice, were pulsed for 2 h with radiolabeled GPL antigens suspended in medium 109 at concentrations ranging from 10 to 250 μg of GPL per ml of medium. Activity was 4.9×10^4 and 2.8×10^5 cpm/mg for the radiolabeled GPL antigens used to pulse the C57BL/6 and CBA/J macrophages, respectively. Each point represents the mean counts per minute per 10^5 cells and the standard deviation of four separate experiments in which macrophage monolayers were assayed in triplicate.

(Gelman Sciences, Inc., Ann Arbor, Mich.) to ensure sterility. Before addition of antigen suspensions to adherent cells, antigen preparations were reconstituted in chloroform: methanol (2:1) several times and dried under nitrogen. Complete dryness was accomplished by desiccation in vacuo for 1 to 2 h. Before pulsing, dried antigen preparations were suspended in medium 109 by sonication in an 80-W ultrasonic bath (Branson Cleaning Equipment Co., Shelton, Conn.) for 30 to 60 minutes and refluxed through a 27-gauge needle to obtain a uniform suspension. Adherent cells were pulsed with the GPL antigen suspension for 2 h at 37°C under 5% CO_2, washed, and incubated under the same conditions until assayed. Each monolayer (3.4×10^5 cells) received the appropriate amount of antigen suspended in a total of 1.0 ml of medium 109.

At the appropriate times, adherent cells and spent medium

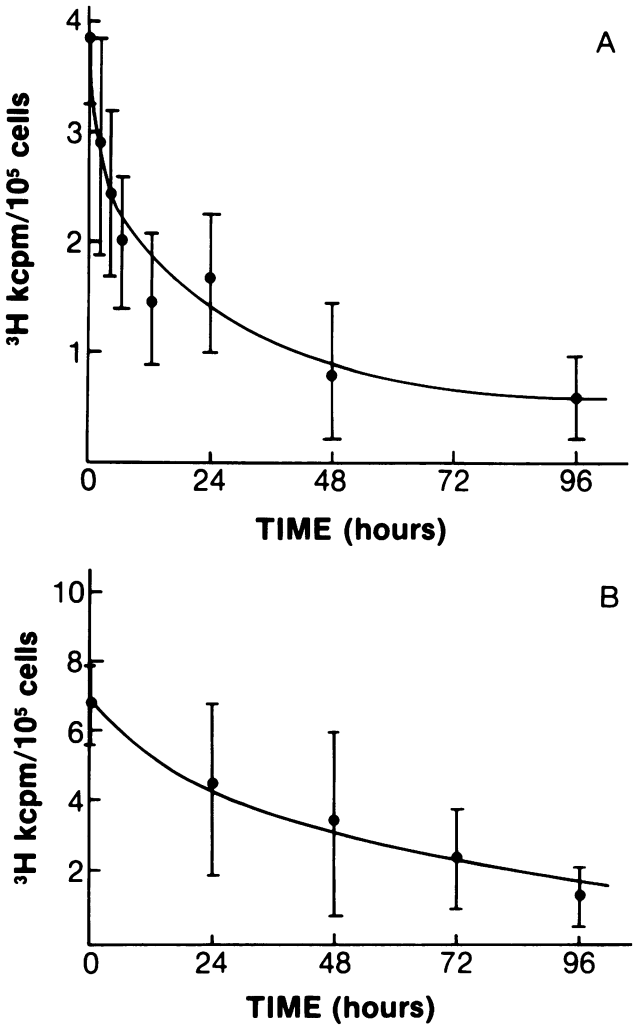


FIG. 5. Retention of radiolabeled GPL antigens by mouse peritoneal macrophages, obtained from either C57BL/6 (A) or CBA/J (B) mice. Monolayers were pulsed for 2 h with radiolabeled GPL antigens suspended in medium 109 at a concentration of 200 μg of GPL per ml of medium. After pulse monolayers were assayed for radioactivity in Beta Gel at intervals ranging from 0 to 96 h. The activity of the GPL antigens was 2.8×10^5 cpm/mg. Each point represents the mean counts per minute per 10^5 cells and the standard deviation of four separate experiments in which macrophage monolayers were assayed in triplicate.

were assayed for distribution of radioactivity by counting in Beta Gel scintillation fluid. Adherent cells were removed by adding 1.0 ml of 0.05% Triton X-100 in phosphate-buffered saline and scraping with a Teflon policeman. After removal, the adherent cells were extracted with chloroform-methanol-water (8:4:3), and the chloroform-extractable material was assayed for distribution of counts by TLC. In a similar fashion, spent medium was also assayed.

Immunofluorescent labeling of GPL-pulsed peritoneal macrophages. Macrophages, previously pulsed with GPL antigens, were treated with 1% paraformaldehyde in phosphate-buffered saline overnight and then labeled with either preimmune or anti-GPL immune rabbit serum as described previously (29). Macrophages were then treated with goat anti-rabbit immunoglobulin G (IgG) fluorescein conjugate (Miles Laboratories, Inc., Naperville, Ill.) and observed on a Zeiss photomicroscope III, using incident-light excitation for fluorescein isothiocyanate (FITC) fluorescence. Photomicrographs were taken with Ektachrome 400 film (Eastman Kodak Co., Rochester, N.Y.) (29).

RESULTS

Internal labeling of GPL antigens. Initially, serovar 20 was grown in the presence of individual amino acids to confirm their incorporation into the GPL antigen structure. Because it has previously been reported that the fatty acyl peptide core of the GPL structure contains D-alanine, D-phenylalanine, and D-threonine (3, 4), the radiolabeled L-forms of these amino acids were used to label the GPL antigens of serovar 20. The use of L-[^{3}H]threonine to label the peptide portion resulted in low incorporation (unpublished observation); therefore, subsequent cultures were grown in the presence of only [^{3}H]Ala and [^{3}H]Phe.

It has been demonstrated that deacetylation greatly facilitates the separation of the GPL antigens (3, 4). Therefore, native lipid obtained from serovar 20 which had been grown in the presence of single radiolabeled amino acids was first deacetylated before the GPL antigens were purified.

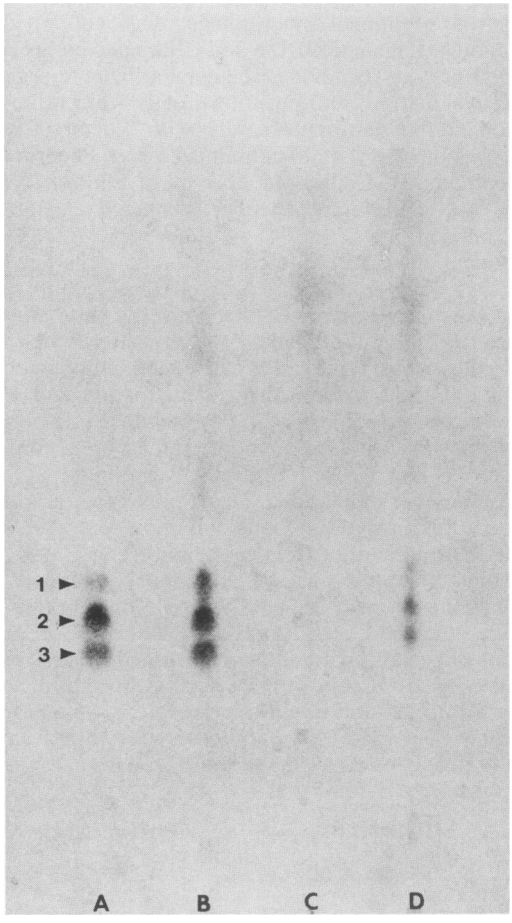


FIG. 6. TLC distribution of GPL antigens I through III (1, 2, 3) (A) and chloroform-extractable components from spent medium 48 h after a pulse of 200 μg GPL (B), macrophage monolayers not previously pulsed with GPL antigens (C), and macrophage monolayers 48 h after a pulse of 200 μg of GPL antigens (D). TLC plates were developed in solvent A, and the GPL antigens were identified by their migration with respect to verified GPL standards and their characteristic color reaction to orcinol-sulfuric acid.

Deacetylated GPL antigens obtained from serovar 20 which had been cultured in the presence of [^{3}H]Ala (0.5 μCi/ml) had counts of 4.5 × 10^4 cpm/mg, whereas deacetylated antigens obtained from serovar 20 cultured in the presence of [^{3}H]Phe (0.5 μCi/ml) had counts of 4.3 × 10^4 cpm/mg. When deacetylated GPL antigens were hydrolyzed in 6 M Killiani solution, cellulose TLC analysis of the amino acids in the hydrolysate revealed that radioactivity corresponded to alanine and alaninol in the case of [^{3}H]Ala-labeled cells (Fig. 1A) and phenylalanine in the case of the [^{3}H]Phe-labeled cells (Fig. 1B).

Once incorporation of individual amino acids had been confirmed, subsequent cultures of serovar 20 were grown in 1-liter volumes containing both [^{3}H]Ala and [^{3}H]Phe. The first batch of serovar 20 that was double labeled was cultivated in 7H9-OADC containing 0.2 μCi of each amino acid per ml. Native lipid acquired 5.2 × 10^4 cpm/mg, and GPL antigens purified from that batch acquired 4.9 × 10^4 cpm/mg. To improve incorporation, subsequent cultures were grown in the presence of 0.3 μCi of each amino acid per ml in 7H9 without the OADC supplement. Under these conditions, incorporation, as determined from three separate batches (each batch consisting of four to nine 1-liter cultures), increased to 1.8 × 10^5 cpm/mg for native lipid and 2.8 × 10^5 cpm/mg (standard deviation, ±0.2) for purified GPL antigens.

Analysis of the double-radiolabeled lipid by TLC in solvent A (Fig. 2) revealed that counts were distributed in two major areas, one corresponding to GPL antigens I through IV (27 ± 1.5%, as determined from three separate batches of lipid) and the other corresponding to their apolar counterparts (44 ± 5%), as described by Brennan and Goren (4). Hydrolysis of purified, double-labeled GPL antigens, either individually or as a mixture, with 6 M Killiani reagent and examination by cellulose TLC demonstrated that radioactivity corresponded to the expected amino acids (Fig. 1C). These findings verify that use of [^{3}H]Ala and [^{3}H]Phe results in the internal labeling of the GPL antigens at the phenylalanine, alanine, and alaninol moieties of the invariant fatty acyl peptide core which is shared by all members of the *M. avium-M. intracellulare* complex (Fig. 3) (4).

Uptake of radiolabeled GPL antigens by mouse peritoneal macrophages. For the uptake studies that follow, a mixture containing only double-radiolabeled GPL antigens I, II, and III was used. As GPL antigen IV contains the least number of acetyl groups, it was eliminated from the antigen fractions used in the following macrophage uptake studies because it was felt that if degradation of the antigens were to take place, part of that process might involve deacetylation. If that were the case, then deacetylation could easily be observed simply by detecting a difference in the R_f values of the other three antigens after they were expected from macrophages and located on TLC.

Unelicited peritoneal cell counts averaged 2.0 × 10^6 cells per ml (standard deviation, ±0.1 × 10^6) for C57BL/6 mice and 3.6 × 10^6 cells per ml (standard deviation, ±0.1) for CBA/J mice. The area of each tissue culture well was 2.0 cm^2, and the addition of 2.0 × 10^6 peritoneal cells to each well resulted in a monolayer of 3.4 × 10^5 adherent cells (standard deviation, ±0.5).

To determine the concentration necessary for maximum uptake of the GPL antigens, macrophage monolayers were pulsed with a concentration of GPL antigens that ranged from 10 to 250 μg/3.4 × 10^5 cells. Macrophages were assayed for radioactivity, and data were expressed as ^{3}H counts per minute per 10^5 cells. Although labeled antigen used in

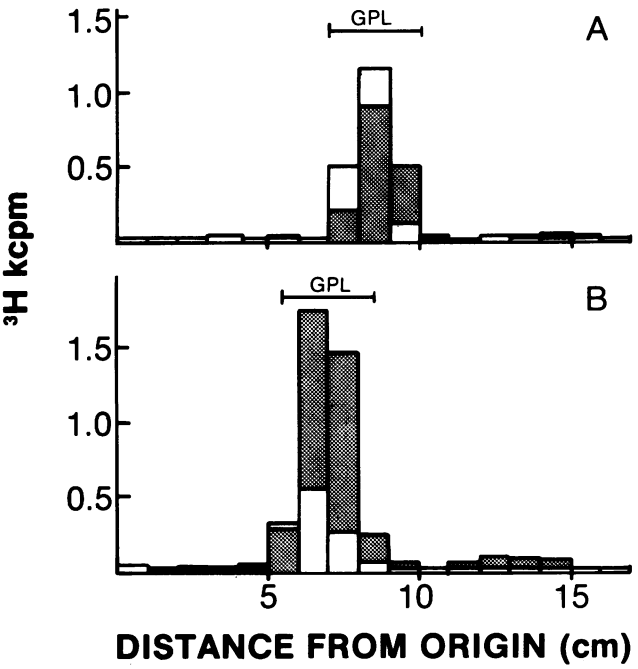
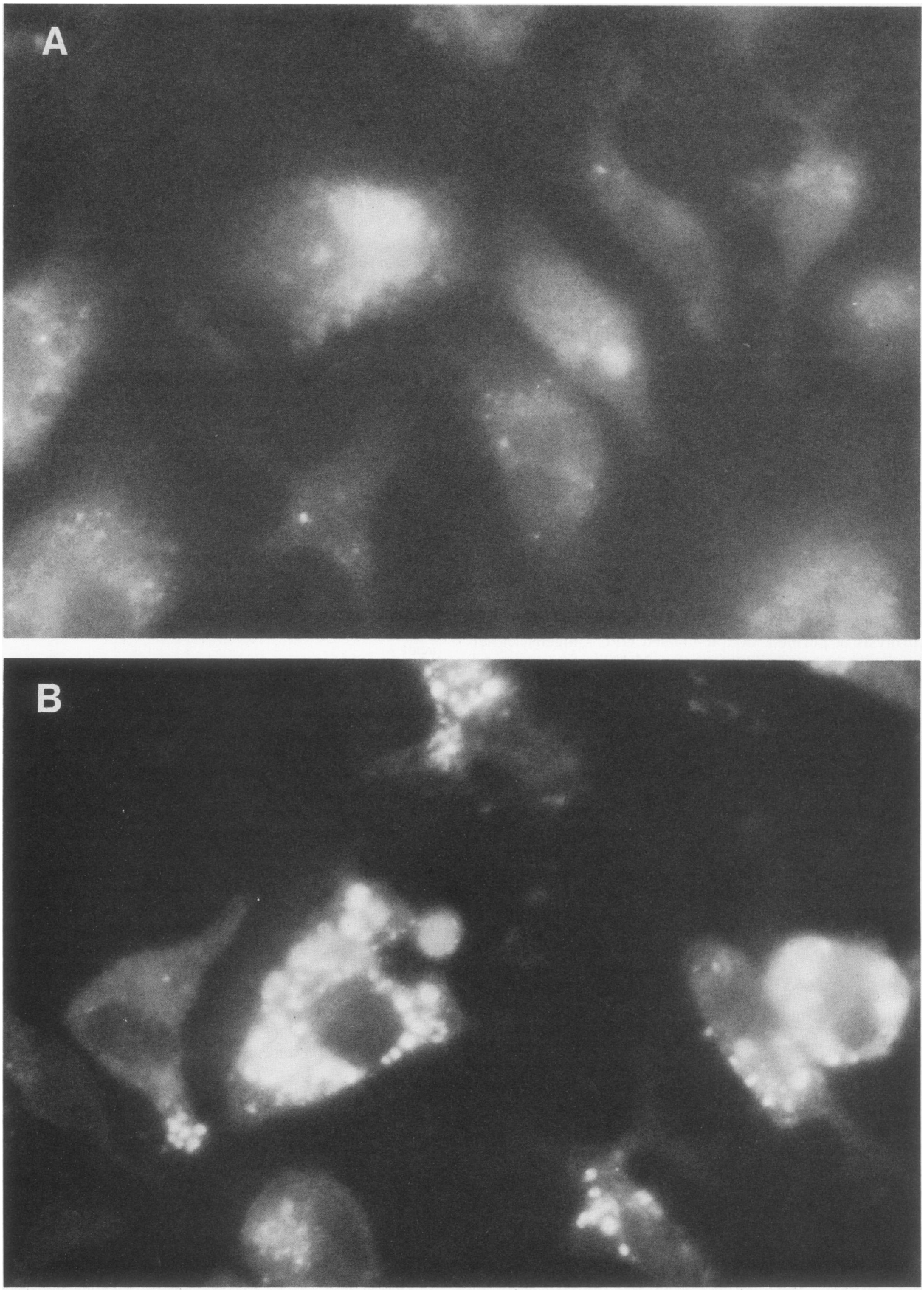


FIG. 7. TLC distribution of radioactive chloroform-extractable components from spent media (▩) and macrophages (□) obtained from C57BL/6 peritoneal macrophage cultures at 24 h (A) and CBA/J peritoneal macrophage cultures at 96 h (B) after the antigen pulse. TLC plates were developed in solvent A, and radioactivity was located by counting sections in Beta Gel. The locations of GPL antigens were confirmed by their migration with respect to verified GPL standards and characteristic color reaction to orcinol-sulfuric acid.

C57BL/6 experiments had lower counts (4.9 × 10^4 cpm/mg) than that used in the CBA/J experiments (2.8 × 10^5 cpm/mg), maximum antigen uptake by both strains occurred at a concentration between 200 and 250 μg of GPL per ml, and the pattern of uptake was similar for both types of macrophages (Fig. 4). The mean percent uptake (plus or minus the standard deviation) of labeled antigen in C57/BL6- and CBA/J-derived macrophages was found to be 63 ± 5.3, 76 ± 5.7, 70 ± 2.1, 67 ± 3.8, 67 ± 1.5, and 60 ± 0.6 for GPL concentrations of 10, 20, 50, 100, 200, and 250 μg/ml, respectively.

A separate series of wells without macrophage monolayers were pulsed with radiolabeled GPL suspended in 1.0 ml of medium 109 to determine how much of the antigens adhered to the plate. It was determined that when 200 μg of radiolabeled GPL antigens (4.4 × 10^4 cpm; standard deviation, ±0.1 × 10^4) was added to tissue culture wells in 1.0-ml volumes, the amount remaining after wash was only 627 cpm (standard deviation, ±251), or 1.4% (standard deviation, ±0.5) of the total.

Retention of radiolabeled GPL antigens by mouse peritoneal macrophages. Examination of GPL antigen retention by mouse peritoneal macrophages was conducted by using the maximum antigen uptake concentration of 200 μg of GPL per ml, as determined in the above experiments. Peritoneal macrophages obtained from C57BL/6 mice had a tendency to disassociate the antigens more rapidly (Fig. 5A) than those obtained from CBA/J mice (Fig. 5B); however, at the end of day 4, the percent total radioactivity remaining in the C57BL/6- and CBA/J-derived peritoneal macrophages was


A
B

23 (standard deviation, ±7) and 19% (standard deviation, ±10), respectively.

Distribution of radioactivity in chloroform-extractable material. To determine how radioactivity was distributed in the macrophage cultures, two separate sets of adherent cells from both C57BL/6 and CBA/J mice were pulsed with 200 μg of GPL per 3.4×10^5 cells after which spent medium and macrophages were extracted with chloroform-methanol-water (8:4:3) at 0, 6, 24, and 96 h after the antigen pulse. It was observed that 98 (standard deviation, ±2) and 99% (standard deviation, ±0.7) of the radioactivity was extractable with chloroform at 0, 6, 24, and 96 h for C57BL/6- and CBA/J-derived macrophages, respectively.

Examination of chloroform-extractable material by TLC revealed that all three GPL antigens (I through III) were present in spent medium and macrophage extracts throughout the 4 days after the pulse (Fig. 6, lanes B and D, respectively). This was determined by the characteristic color reaction that the antigens produced when sprayed with orcinol-sulfuric acid and also their location on the TLC plate with respect to purified GPL standards (Fig. 6). There was no evidence of any component migrating in the region where antigen IV would be present (antigen IV migrates just under GPL antigen III [3]); therefore, it can be assumed that no apparent deacetylation was taking place in the macrophage cultures.

Examination of chloroform-extractable material by TLC revealed that radioactivity was concentrated in an area of the plate that corresponded to GPL antigens I through III, both for the C57BL/6-derived macrophages (Fig. 7A) and the CBA/J-derived macrophages (Fig. 7B). This pattern was observed at 0, 6, 24, and 96 h after the pulse. Because the distribution of radioactivity was the same throughout the 4-day time period for both groups of macrophages, only the results from the 24-h C57BL/6 cultures and the 96-h CBA/J cultures are presented here.

Examination of the spent medium derived from the CBA/J macrophage cultures at 96 h revealed that about 2 to 3% of the radioactivity was located in an area of the TLC plate that corresponded to the apolar GPL components (Fig. 7B). This was not observed in the C57BL/6 macrophage cultures. Because of the limitations with these procedures, quantities of these possible degradation products were not sufficient for further examination.

Immunofluorescent detection of GPL antigens after phagocytosis. After a GPL antigen pulse of 200 μg of GPL per 3.4×10^5 cells, distribution of antigens were observed by means of an indirect immunofluorescence procedure with GPL-specific rabbit antiserum (29). The GPL antigens could be visualized within the macrophages and appeared to be compartmentalized in numerous vacuoles (Fig. 8B). Proper controls (Fig. 8A) included (i) antigen-pulsed cells labeled with preimmune serum followed by FITC conjugate, (ii) nontreated cells labeled with preimmune or immune serum followed by FITC conjugate, and (iii) antigen-pulsed cells labeled with FITC conjugate.

These findings indicate that the GPL antigen suspension used in the macrophage pulsing experiments was prepared in such a way as to allow for proper orientation of antigenic determinants, thus simulating conditions found on the intact mycobacterial cell (29). It can be assumed, therefore, that this model may be useful in studying GPL antigen processing by host macrophages.

DISCUSSION

Our knowledge of factors contributing to the pathogenesis of nontuberculous mycobacteria in the *M. avium-M. intracellulare* group is very limited. Although it is known that members of the *M. avium-M. intracellulare* serogroup are facultative intracellular parasites (25), the reason for their ability to survive phagolysosomal digestion by host macrophages has never been substantiated, only suggested. Members of the *M. avium-M. intracellulare* group can synthesize a superficial network of fibrillar lipid components (1, 9), and for that reason, Draper and Rees originally suggested that these components might act as a protective capsule because of a possible inertness to lysosomal degradation (10). Subsequent reports have referred to this capsule as the L_1 layer (1, 17), and others have revealed that GPL antigens are the major component of this superficial layer (3, 29). In addition, Barrow and Brennan have determined that the GPL antigens are immunogenic when complexed to a carrier such as methylated bovine serum albumin; they have used specific anti-GPL rabbit serum to further demonstrate the superficial location of these potentially important mycobacterial constituents (2).

In previous studies, we have attempted to monitor postphagocytic events that take place between the GPL antigens and mouse peritoneal macrophages by using immunocytochemical techniques (29). Those attempts have been limited to events which immediately follow phagocytosis because of the difficulty in objectively monitoring the exact location of the antigens due to the multiple layer of antigen-antibody-conjugate inherent in indirect immunocytochemical procedures (29). So that a more accurate assessment of postphagocytic events might be made, the radiolabeled procedures reported in this paper were developed.

Glycopeptidolipids of *M. avium* have previously been radiolabeled with L-^{14}C-amino acids, but those components were apparently the apolar C-mycoside counterpart of the GPL antigens (22). As reported by Brennan and Goren, the apolar C-mycosides are similar in structure to the GPL antigens, but they lack the oligosaccharide which is the basis for serologic specificity among members of the *M. avium-M. intracellulare* serocomplex (4). It was therefore necessary to first establish that similar internal labeling experiments could be used to radiolabel the GPL antigens.

In the present study, it was determined that internal labeling procedures can be used to adequately label the GPL antigens from serovar 20 so that they may be used in macrophage uptake studies. Furthermore, it was confirmed that all four of the GPL antigens of serovar 20 (I through IV) can be labeled, thus making it possible to use any mixture of the purified antigens with the confidence that the results would reflect macrophage processing of the entire group. The fact that the antigens were labeled in the fatty acyl peptide core, which is shared by all members of the *M. avium-M. intracellulare* complex (4), implies that these procedures are also applicable for radiolabeling the GPL antigens of other members in that serocomplex.

Results of macrophage pulsing experiments with radiolabeled GPL antigens indicated that the level of maximum

FIG. 8. Photomicrographs of mouse peritoneal macrophages 48 h after a pulse with 200 μg of GPL antigens. Macrophages were labeled by an indirect immunofluorescent antibody procedure, using either preimmune rabbit serum and goat anti-rabbit immunoglobulin G FITC conjugate (A; control groups represented here) or anti-GPL rabbit serum and goat anti-rabbit immunoglobulin G FITC conjugate (B).

antigen association occurred between 200 and 250 μg/3.4 × 10^5 cells for both C57BL/6- and CBA/J-derived peritoneal macrophages. When macrophages were pulsed with the maximum load of 200 μg of GPL per 3.4 × 10^5 cells, there was a gradual release of antigens throughout a 4-day incubation period. Although both C57BL/6 and CBA/J macrophages still retained about 20% of the total antigen load by the end of the 4 days, the C57BL/6 macrophages appeared to disassociate the antigens more rapidly during that time period. It has been reported that C57BL/6 (15, 32) and CBA/J (14) mice differ in their susceptibility to infections with intracellular parasites; therefore, the apparent dissimilarity in antigen handling may be due to differences in mouse strains.

Examination of spent medium and macrophages throughout the 4 days after the antigen pulse revealed that almost all of the total radioactivity was associated with intact GPL antigens I through III, indicating that very little degradation had taken place, not even deacetylation. It is still possible that minor modifications of the antigens may have occurred, but because of the limitations in this procedure, those modifications may not have been detected. Approximately 2 to 3% of the radioactivity in the chloroform-extractable spent medium at day 4 corresponded to components that migrated in the upper portion of the TLC plate. This suggests that removal of a portion of the oligosaccharide might have taken place, thus leaving a degradation product similar to the apolar GPL components which migrate in that area. Attempts are being made to radiolabel the oligosaccharide moiety of the GPL structure so that a more sensitive assay can be used to investigate this latter possibility.

Immunofluorescent examination of the macrophage-associated antigens with GPL-specific antiserum indicated that substantial quantities of the antigens had been internalized. In addition, it was demonstrated by successful immunofluorescent labeling that the GPL suspension used in the pulsing experiments was prepared in such a way that the antigenic determinants were still accessible to antibody attachment, indicating that the preparation simulated the way that the GPL antigens are oriented on the intact mycobacterial cell (29). This latter finding implies that, although the use of a sonicated GPL dispersion may not permit an accurate simulation of in vivo conditions, it at least offers a reasonable model which can be used to study macrophage processing of these potentially important mycobacterial antigens. Furthermore, the fact that immunofluorescent labeling was successful throughout the entire macrophage retention process implies that the GPL components still retained their antigenicity and further substantiates the notion that little degradation had taken place.

From this study, it can be assumed that the GPL antigens, which constitute a major portion of the superficial L_1 layer of *M. avium-M. intracellulare* organisms, are relatively inert to macrophage degradation. These results represent the first evidence that supports previous suggestions regarding the inertness of the superficial L_1 layer (10) and therefore indicate that the GPL antigens may be important in pathogenesis. These results not only imply that the L_1 layer may be a protective device for mycobacteria but also suggest that in an infected host, continued growth of *M. avium-M. intracellulare* organisms would result in the accumulation and persistence of GPL antigens in host tissue. This assumption is supported by previous studies which have demonstrated the accumulation of the L_1 layer in infected animals and in laboratory cultures involving members of the *M. avium-M. intracellulare* group (3, 9, 17) and one other nontuberculous mycobacterium, *M. lepraemurium* (11). In addition, in at least one recent investigation involving *M. avium-M. intracellulare* infections in two patients with AIDS, histologic findings similar to those found with lepromatous leprosy were reported, that is, the appearance of macrophages with foamy-appearing cytoplasm (28). Similar findings have also been reported for other patients suffering from *M. avium-M. intracellulare* infections (5, 6, 21, 23, 24), and it is entirely possible that these histologic observations (i.e., foamy macrophages) might be due to the accumulation of GPL antigens in the host tissue. This would be similar to the deposition of lipids that takes place in lepromatous leprosy, which results in foamy macrophages or lepra cells of Virchow (16).

Although it is not possible to say at this point what effects the accumulation of GPL components might have on an infected host, it is reasonable to speculate that some consequences may occur. Mycobacterial glycolipids have been reported to have a range of activity in host systems, including the immunopotentiating effect of mycobacterial trehalose diesters (19) and the immunosuppressive effects of mycobacterial phosphatidylethanolamine and phosphatidylinositol (31). The ability of the GPL antigens to produce cellular responses characteristic of nonspecific inflammatory reactions has already been demonstrated in this laboratory (submitted for publication); it is therefore entirely possible that accumulating amounts of GPL antigens may affect some parameter of the host response to *M. avium-M. intracellulare* organisms. Further studies involving GPL antigens are currently in progress to answer these questions.

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