INFECTION AND IMMUNITY, Sept. 1983, p. 1312–1321
0019-9567/83/091312-10$02.00/0

Vol. 41, No. 3

Postphagocytic Detection of Glycopeptidolipids Associated with the Superficial L_1 Layer of *Mycobacterium intracellulare*

MARK J. TERELETSKY† AND WILLIAM W. BARROW*

Department of Microbiology and Immunology, Texas College of Osteopathic Medicine, Fort Worth, Texas 76107

Received 28 March 1983/Accepted 7 June 1983

The superficial L_1 layer of *Mycobacterium intracellulare* serovar 20 was detected by immunocytochemical procedures based on C-mycoside glycopeptidolipid (GPL) antigens. Specific immune serum was produced by injecting a GPL-methylated bovine serum albumin complex into rabbits with Freund incomplete adjuvant. The resulting antibodies were used to label serovar 20 with goat anti-rabbit immunoglobulin G (IgG) conjugated with either fluorescein or ferritin. The labeled GPL antigens were then detected by fluorescent and electron microscopy. With these procedures, it was possible to observe the superficial distribution of the GPL antigens on serovar 20 and to confirm their intraphagosomal location within the macrophages after phagocytosis. These immunocytochemical procedures now make it possible to monitor these mycobacterial antigens during phagocytosis and may be helpful in determining their role in pathogenesis.

Mycobacteria are perhaps best noted for the role that they play in severe chronic diseases, such as tuberculosis and leprosy; however, in more recent years, the severity of infections caused by the nontuberculous mycobacteria has increased (9, 10, 15, 26). One of the major reasons that mycobacteria are such severe pathogens lies in their ability to survive within host macrophages (19). In the case of certain nontuberculous mycobacteria, the ability to persist within host macrophages has been attributed to the presence of a capsule (11, 12), which has more recently been referred to as the L_1 layer by Barksdale and Kim (1). Although it has been suggested that the L_1 layer acts as a protective sheath, this has not been specifically demonstrated.

Recent investigations have revealed that the L_1 layer of serovar 20 of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* (MAIS) complex comprises predominantly a group of polar glycopeptidolipid (GPL) antigens (3). Because similar GPL antigens have been identified in other MAIS serovars by Brennan and Goren (5) and Brennan et al. (4, 6), it is possible to assume that individual serovars likewise contain L_1 layers comprising their respective GPL antigens.

Further studies by Barrow and Brennan revealed that the GPL antigens from serovar 20 were immunogenic when complexed with a carrier, such as methylated bovine serum albumin, and the specific antibodies thus produced could be used in an indirect fluorescent antibody procedure to label the superficial L_1 layer of serovar 20 (2). The purpose of the previous investigation (2) was to produce specific antibodies that could be used to develop immunocytochemical techniques for observing the intracellular fate of the superficial GPL antigens during phagocytosis. Using the immunocytochemical techniques, we hoped to observe the integrity of the L_1 layer during phagocytosis and thus better determine its effectiveness as a protective capsule.

This paper describes the immunocytochemical techniques that were developed to detect the superficial GPL antigens of serovar 20 after phagocytosis by mouse peritoneal macrophages. By using indirect immunofluorescent and immunoferritin procedures, we further confirmed the superficial location of the GPL antigens and established their intraphagosomal location within infected mouse peritoneal macrophages. With these procedures, it will now be possible to monitor the GPL antigens during phagocytosis and better determine their role in pathogenesis.

MATERIALS AND METHODS

Mycobacterium. The mycobacterial strain used in this investigation was serovar 20 of the MAIS serocomplex (3). The mycobacteria were cultured by shaking at 37°C in 7H11 medium as described previously (3).

Animals. Male and female C57BL/6 mice were purchased from Harlan-Sprague-Dawley (Indianapolis, Ind.) and raised in small groups on a diet of Ralston Purina mouse chow and water. Matings were conduct-

† Present address: Flint Osteopathic Hospital, Flint, MI 48502.

ed randomly, and the mice used in this investigation were 6 to 10 weeks of age.

GPL extraction and purification. The GPL antigens were extracted from lyophilized mycobacteria with $CHCl_3$-CH_3OH (2:1) and purified by a combination of silicic acid-Celite and DEAE-cellulose column chromatography, as described previously (3).

Immune serum. A complex of the GPL antigens and methylated bovine serum albumin (4.7 mg of GPL, 0.32 mg of protein) was emulsified in Freund incomplete adjuvant and injected intramuscularly into New Zealand white rabbits once a week for 3 weeks, as described by Barrow and Brennan (2). The titers of the rabbit antiserum were measured by the agglutination procedure described by Schaefer (21), and the specificity of the antisera was determined by gel diffusion, immunoelectrophoresis, and immunofluorescence as described by Barrow and Brennan (2).

Isolation and cultivation of macrophages. Normal peritoneal macrophages were isolated and cultured by the procedure of Chang (8). The mice were sacrificed by cervical dislocation, and the peritoneal cells were removed by the irrigation of the peritoneal cavity with 2.0 ml of ice-cold NCTC medium 109 (Difco Laboratories, Detroit, Mich.) containing 5 U of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml of solution. The viability of the cells was determined by trypan blue exclusion to be always ≥95%. The cells were pooled and counted in a hemacytometer (AO Spencer).

One-milliliter volumes of the peritoneal cell suspension were added to plastic Leighton tubes (Costar, Cambridge, Mass.), which contained plastic cover slips, and incubated for 2 h at 37°C. After the cover slips were washed with medium 109 to remove nonadherent cells, the cultures were maintained at 37°C in medium 109 containing 40% heat-inactivated horse serum (KC Biologicals, Inc., Lenexa, Kans.) and 10% of a 1:5 dilution of bovine embryo extract (Difco Laboratories) as described by Chang (8). The medium also contained penicillin at 100 U/ml. After 24 h of incubation, the adherent cells were washed once more to ensure the removal of nonadherent cells.

Infection of adherent cell cultures with serovar 20. The adherent cells were infected with serovar 20 which had been cultured in 7H11 as described previously (3). Mycobacteria in exponential phase were washed and suspended in 7H11 to give a reading of 50 on a Klett-Summerson colorimeter with a no. 45 filter. This corresponded to 4.6×10^7 viable U/ml as determined by the plate-count technique. The mycobacterial suspension was further diluted by adding 0.5 to 9.5 ml of medium 109 containing 10% horse serum, and 1.0 ml was added to each Leighton tube to infect the adherent cell cultures. Infection was allowed to take place at 37°C for 2 h, after which, the nonphagocytosed bacteria were removed by washing with medium 109 containing 10% horse serum. Fresh Chang medium was added, and the cultures were maintained at 37°C for 24 h. The numbers of phagocytosed bacteria were determined by staining the adherent cells by the Ziehl-Neelsen acid-fast technique and enumerating the number of acid-fast bacilli within the adherent cells (24).

Preparation of adherent cells for immunofluorescence. Infected and noninfected cells were fixed in phosphate-buffered saline (PBS) containing 1% paraformaldehyde for 24 h and then washed thoroughly in PBS. An indirect fluorescent antibody procedure was used to label the phagocytosed mycobacteria. Either preimmune or immune (anti-GPL, titer = 1,280) rabbit serum was diluted with PBS, applied to the adherent cells, and allowed to incubate at room temperature for 30 min. The cells were washed for 1 h with two changes of PBS and treated with goat anti-rabbit immunoglobulin G (IgG) fluorescein conjugate (Miles Laboratories, Elkhart, Ind.) diluted with PBS. After 30 min of incubation at room temperature, the adherent cells were washed with PBS for 1.5 h (three changes). Cover slips containing fluorescein-labeled cells were attached to glass microscope slides with Duro superglue (Loctite Corp., Cleveland, Ohio) and mounted with buffered glycerol saline. Fluorescence was observed on a Zeiss photomicroscope III fluorescent microscope, using incident-light excitation with exciter-barrier filter and reflection combination for fluorescein isothiocyanate (FITC) fluorescence. Photomicrographs were taken with Kodak Ektachrome 400 film.

Immunoferritin labeling of serovar 20. A modification of a procedure described by Van Driel et al. (25) was used to label serovar 20 with ferritin. Mycobacteria in exponential phase were collected by centrifugation ($5,000 \times g$) and washed with PBS. The cells were suspended in PBS, and the density was adjusted to give a reading of 50 on a Klett-Summerson colorimeter (no. 45 filter). Rabbit antiserum was then added to the bacterial suspension to achieve the desired dilution and allowed to incubate at 37°C for 30 min. The cells were washed and suspended in PBS, after which ferritin-labeled goat anti-rabbit IgG (Miles Laboratories) was added to achieve the desired dilution. The cell suspension was then incubated at 37°C for 1 h. The cells were washed with PBS, allowed to fix overnight at 4°C in cacodylate buffer (0.1 M, pH 7.2) containing 3% glutaraldehyde, embedded in 1.5% agarose as described by Higgins (14), and cut into blocks approximately 1 mm^3. The blocks were allowed to fix overnight at room temperature in cacodylate buffer (0.1 M) containing 1% osmium tetroxide (EMS, Ft. Washington, Pa.). Osmium was removed by thorough washing in cacodylate buffer (0.1 M), and the blocks were prestained in uranyl acetate (0.5% in triple-distilled water). The blocks were dehydrated with a graded series of ethyl alcohol and embedded in Spurr resin mixture (EMS) (22), and the specimens were thin sectioned on a Sorvall ultramicrotome and either poststained with uranyl acetate and lead citrate or examined unstained. The sections were examined on an AEI Corinth 500 transmission electron microscope operating at 60 kV.

Infection of adherent cells with ferritin-labeled serovar 20. Mycobacteria were prelabeled with ferritin as described above, and the ferritin-labeled bacteria were used to infect adherent cells. The ferritin-labeled bacteria were first diluted in medium 109 (10% horse serum) and added to adherent cells as described above. Infection was allowed to take place at 37°C for 2 h, after which the adherent cells were washed free of nonphagocytosed bacteria and fixed in cacodylate buffer (0.05 M, pH 6.2) containing 3% glutaraldehyde overnight at 4°C. The adherent cells were then fixed with 1% osmium tetroxide in cacodylate buffer (0.05 M) overnight at 4°C, washed with cacodylate buffer,

and prestained as before with uranyl acetate. The cells were then dehydrated in a graded series of ethyl alcohol, embedded in Spurr resin mixture (EMS) (22), sectioned, and observed by transmission electron microscopy as described above. Fixation, dehydration, and embedding were carried out with the adherent cells attached to the plastic cover slips.

RESULTS

Infection of adherent cells with mycobacteria. In developing a procedure for the infection of adherent cells with mycobacteria, we initially employed glass Leighton tubes and cover slips. These were replaced with plastic Leighton tubes and cover slips to facilitate the preparation of the adherent cell populations for electron microscopy. Plastic tubes and cover slips were therefore utilized throughout the investigation for the fluorescent and ferritin labeling procedures involving adherent cells.

The area of the plastic cover slips was 495 mm^2. When 1.0 ml of the peritoneal cell suspension (3.8×10^6 cells per ml [standard deviation, $\pm 0.6 \times 10^6$]; 38% monocytes [standard deviation, ±5.5%]) was added, approximately 2×10^5 to 4×10^5 cells adhered to the cover slip. The addition of 2.3×10^6 mycobacteria to this adherent cell population resulted in a ratio of about 6 to 12 bacteria per adherent cell, and this ratio resulted in an infection of about 48% (standard deviation, ±13%).

The ratio of infected cells to noninfected cells could be adjusted either by increasing or decreasing the dilution factor when the bacteria were mixed with medium 109 (10% horse serum) or by increasing or decreasing the infection time. However, for this investigation, the infection schedule described above was utilized. When lighter infections were used, electron microscopy was difficult to perform because of the scarcity of infected cells. When heavier infections were used, the bacterial load caused a faster depletion of macrophages because of lysing.

Fixation of adherent cells for immunofluorescence. Three fixation procedures were evaluated for this investigation—one using Formalin-acetone (20), one using 3% glutaraldehyde, and one using 1% paraformaldehyde. Fixation with Formalin-acetone, using a modification of a procedure described by Rausch et al. (20), proved to be too destructive to the macrophage membrane. Fixation with 3% glutaraldehyde resulted in autofluorescence that interfered with the interpretation of immunofluorescence studies. We found that the overnight treatment of adherent cells with 1% paraformaldehyde resulted in the best fixation, as judged by intact macrophage morphology, penetrability of the macrophage membrane by antibodies and antibody conjugates, and preservation of GPL antigens.

Postphagocytic detection of GPL antigens by immunofluorescence. We determined the presence of the GPL antigens by an indirect fluorescent antibody technique, using the specific antibodies previously described (2). Various dilutions of immune serum and FITC conjugate were evaluated, but a dilution of 1:20 for each proved to give the best results for nonspecific staining and even distribution of FITC.

Control slides included (i) noninfected macrophages labeled with preimmune or immune serum followed by FITC conjugate, (ii) infected cells labeled with preimmune serum followed by FITC conjugate, and (iii) infected cells labeled only with FITC conjugate. An example of the control sets is shown in Fig. 1A. No fluorescence was observed in any of the controls. By this immunofluorescence procedure, the GPL antigens could be detected throughout the infection period (Fig. 1B). In some experiments, infection was allowed to continue for 5 to 7 days. However, because the intracellular multiplication of the bacteria began to destroy the macrophage population after a few days, the experiments reported here are those limited to 24 h.

Attachment of ferritin to serovar 20. The detection of the GPL antigens, and thus the L_1 layer, was accomplished by attaching goat anti-rabbit ferritin conjugate to serovar 20 which had been previously labeled with rabbit anti-GPL serum. Several dilutions of immune serum and ferritin conjugate were tested, but the best proved to be a 1:20 dilution for both. The pattern of ferritin granules revealed the superficial distribution of the GPL antigens and accentuated the L_1 layer described by Barksdale and Kim (1) and Kim et al. (16) (Fig. 2A). The distribution of the ferritin granules indicates that the GPL antigens are evenly dispersed throughout the superficial L_1 layer of serovar 20. Figure 2B represents the results of the controls that were employed in this study. In one set, serovar 20 was labeled with preimmune serum followed by ferritin conjugate. In another control, serovar 20 was treated with neither serum nor ferritin conjugate, and in a third control, only ferritin conjugate was used. No ferritin label was detected on the mycobacteria in the control sets.

Postphagocytic detection of L_1 layer by indirect immunoferritin technique. Postphagocytic detection of the L_1 layer was accomplished by infecting peritoneal macrophages with ferritin-labeled serovar 20 and observing the infected cells by transmission electron microscopy. Figure 3A to C represents peritoneal macrophages immediately after infection with ferritin-labeled serovar 20. The L_1 layer is clearly outlined by ferritin granules and appears to be continuous and uniform in nature. In most sections, the phagosomal (or phagolysosomal) membrane appears to run adjacent to the L_1 layer. This was the case whether the phagosomes contained a single my-

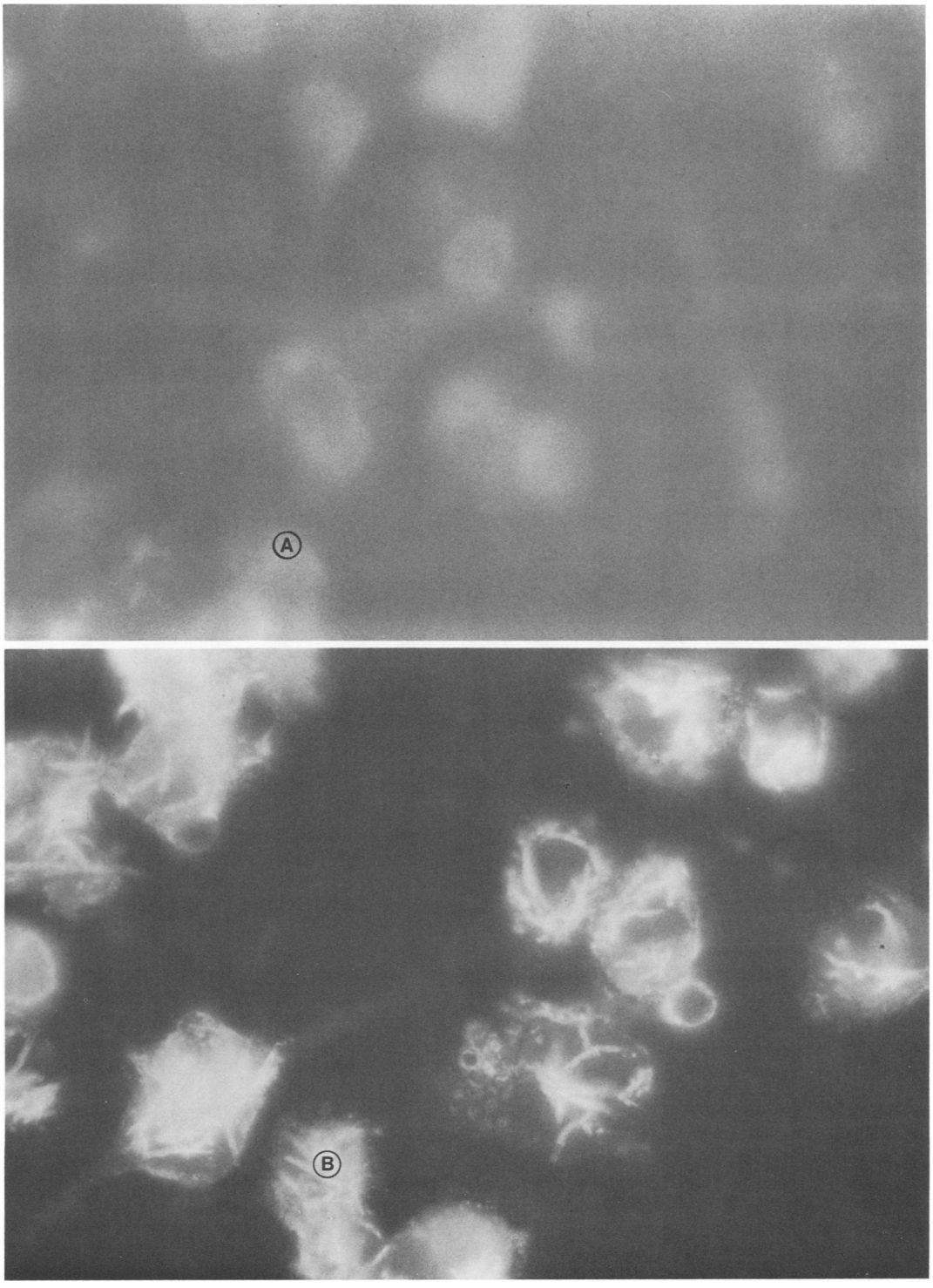

FIG. 1. Photomicrographs of mouse peritoneal macrophages infected with serovar 20. The cells were labeled by an indirect immunofluorescent antibody procedure, using either (A) preimmune rabbit serum and goat anti-rabbit IgG FITC conjugate (control groups, in which no fluorescence was observed, are shown here) or (B) anti-GPL rabbit serum and goat anti-rabbit IgG FITC conjugate.

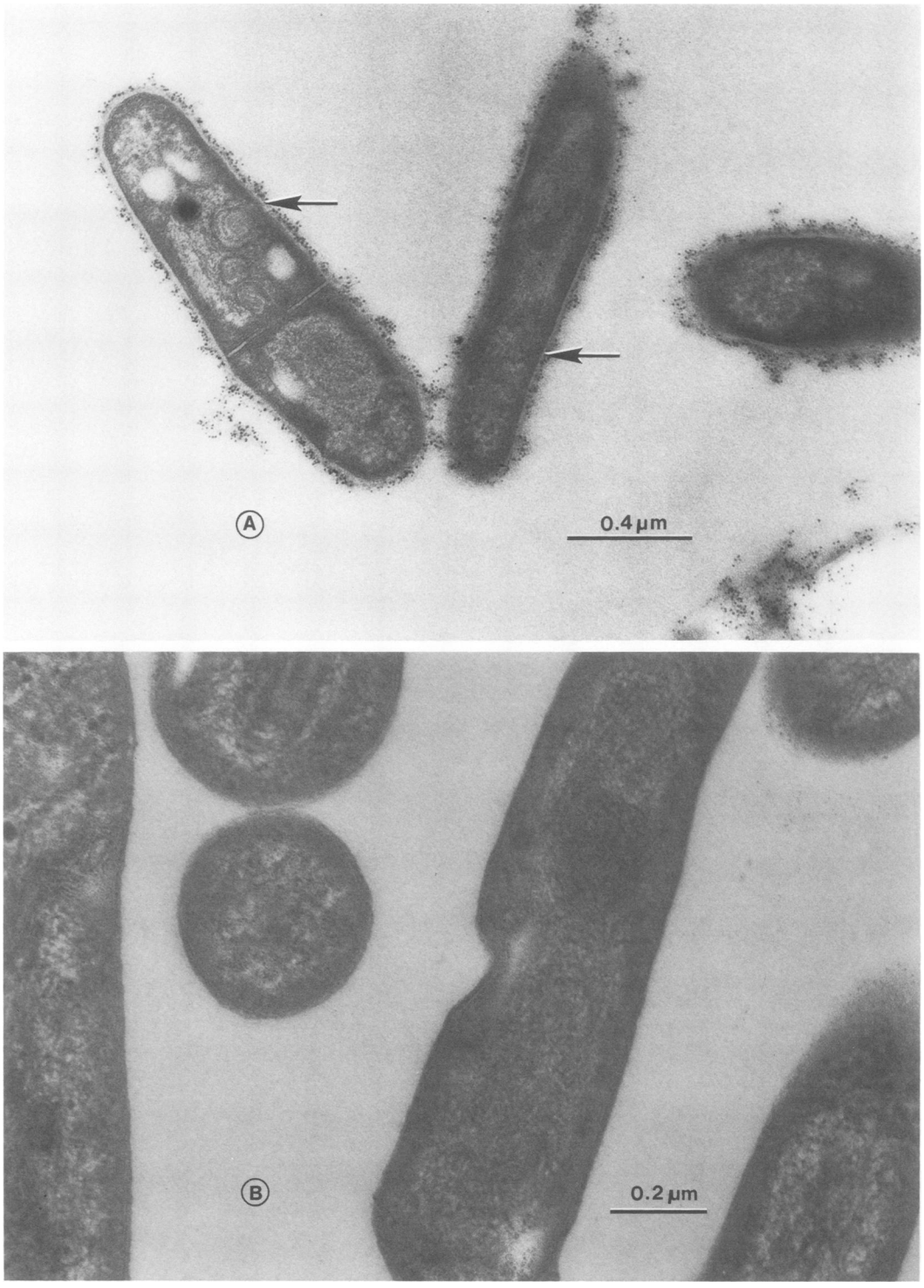

FIG. 2. Ultrathin sections of serovar 20 treated with either (A) anti-GPL rabbit serum followed by goat anti-rabbit IgG ferritin conjugate, or (B) preimmune rabbit serum and goat anti-rabbit IgG ferritin conjugate (control sets, in which no ferritin granules were present, are shown here). The cells were poststained with uranyl acetate and lead citrate. Ferritin granules outline the L_1 layer (arrows).

cobacterium (Fig. 3B and C) or numerous mycobacteria (Fig. 3A). Immediately inside the ferritin layer was an electron-transparent zone which varied in thickness but was also continuous and uniform in nature. This variation in thickness can be seen by comparing Fig. 3A and B with C. The L_1 layers observed in Fig. 3A and B are noticeably thinner than that seen in Fig. 3C. The difference in the thickness of the L_1 layers was most likely due to the differing amounts of GPL antigens present on individual mycobacteria. It should be pointed out that in this investigation serovar 20 was cultured by a shaking method. As discussed previously, serovar 20 derived from shaken cultures does not possess as much of the filamentous L_1 layer as cells cultured in a static mode (3, 16). Therefore, the ferritin markers in this investigation probably represent the more rigidly attached L_1 layer.

Also detected in some sections were what appeared to be vacuoles located within the cytoplasm of the macrophages; some of these contained ferritin granules, whereas others did not (Fig. 3C). Although the exact nature of these ferritin-containing vacuoles is not known at this time, they may be important in antigen processing. This point will be discussed later.

The results of experiments in which macrophages were infected with unlabeled serovar 20 are represented in Fig. 3D. No ferritin granules were observed in the cells infected with unlabeled serovar 20; however, in this group, we detected an electron-transparent zone identical to the one shown in Fig. 3A to C. Because of its presence in phagosomes containing either ferritin-labeled or unlabeled cells, it is possible to assume that this is the electron-transparent zone previously associated with the protective capsule (11, 12) and subsequently referred to as the L_1 layer (1). However, it is likely that this electron-transparent zone also contains the L_2 and L_3 layers described by Barksdale and Kim (1). This point will be commented upon below.

DISCUSSION

Lipid antigens have not been considered of much importance until rather recently; however, they constitute a unique class of antigens which have been significant in mammalian, as well as microbial, immunology (18). An important aspect of lipid research has been the use of certain immunological techniques to identify the cellular location of lipid antigens. These localization techniques have been employed in studies of mammalian tissues (17), as well as bacterial systems (25), where lipid antigens were detected by fluorescein- or ferritin-conjugated globulins specific for antibodies to the antigens.

In the present study, immunocytochemical techniques were developed to detect a group of lipid antigens that comprise the superficial L_1 layer of serovar 20 (3). These lipid antigens, which are referred to as polar GPL antigens, represent a unique group of mycobacterial antigens that may play an important role in mycobacterial pathogenicity (11). The detection of these GPL antigens was accomplished by indirect immunological methods, using fluorescein- and ferritin-conjugated antibodies.

After phagocytosis by mouse peritoneal macrophages, serovar 20 was detected by an indirect fluorescent antibody procedure, using antibodies specific for the GPL antigens. The penetration of antibodies and antibody conjugates was made possible by prior fixation of the infected macrophages with 1% buffered paraformaldehyde. With this method, the detection of mycobacteria was possible throughout an infection period of several days; however, because of the eventual loss of macrophages due to the bacterial infection, the experiments in this investigation were limited to 24 h. If this procedure is to be used to monitor the GPL antigens during long-term infections, this problem will have to be overcome. A method already being developed in this laboratory is to infect the macrophages with mycobacteria that have been killed by a method which does not affect the antigenicity of the GPL components. Thus, the GPL antigens could be monitored throughout an infection period without the loss of macrophages due to bacterial multiplication.

A more detailed analysis of the superficial distribution of the GPL antigens was achieved by an indirect immunoferritin technique. It was found that the L_1 layer of serovar 20 is evenly distributed around the entire surface of the cell, further substantiating the results of other investigations concerning the L_1 layer of nontuberculous mycobacteria (1, 16). The L_1 layer was also detected in macrophages which had been infected with ferritin-labeled serovar 20. Again, the ferritin granules revealed the superficial distribution of the GPL antigens and clearly demonstrated their intraphagosomal location within the macrophage. The fact that the L_1 layer completely surrounds the phagocytosed mycobacteria, as revealed by the ferritin label, strongly suggests that it might protect the pathogen from digestion by lysosomal enzymes. Current studies, using the immunocytochemical techniques, are attempting to determine whether the L_1 layer is inert and can resist the phagolysosomal environment.

Although ferritin granules were observed on the surface and not within the electron-transparent zone, this does not indicate that the GPL antigens are absent from that zone. Apparently, homogeneous materials (e.g., pneumococcal polysaccharide capsules) are not very accessible

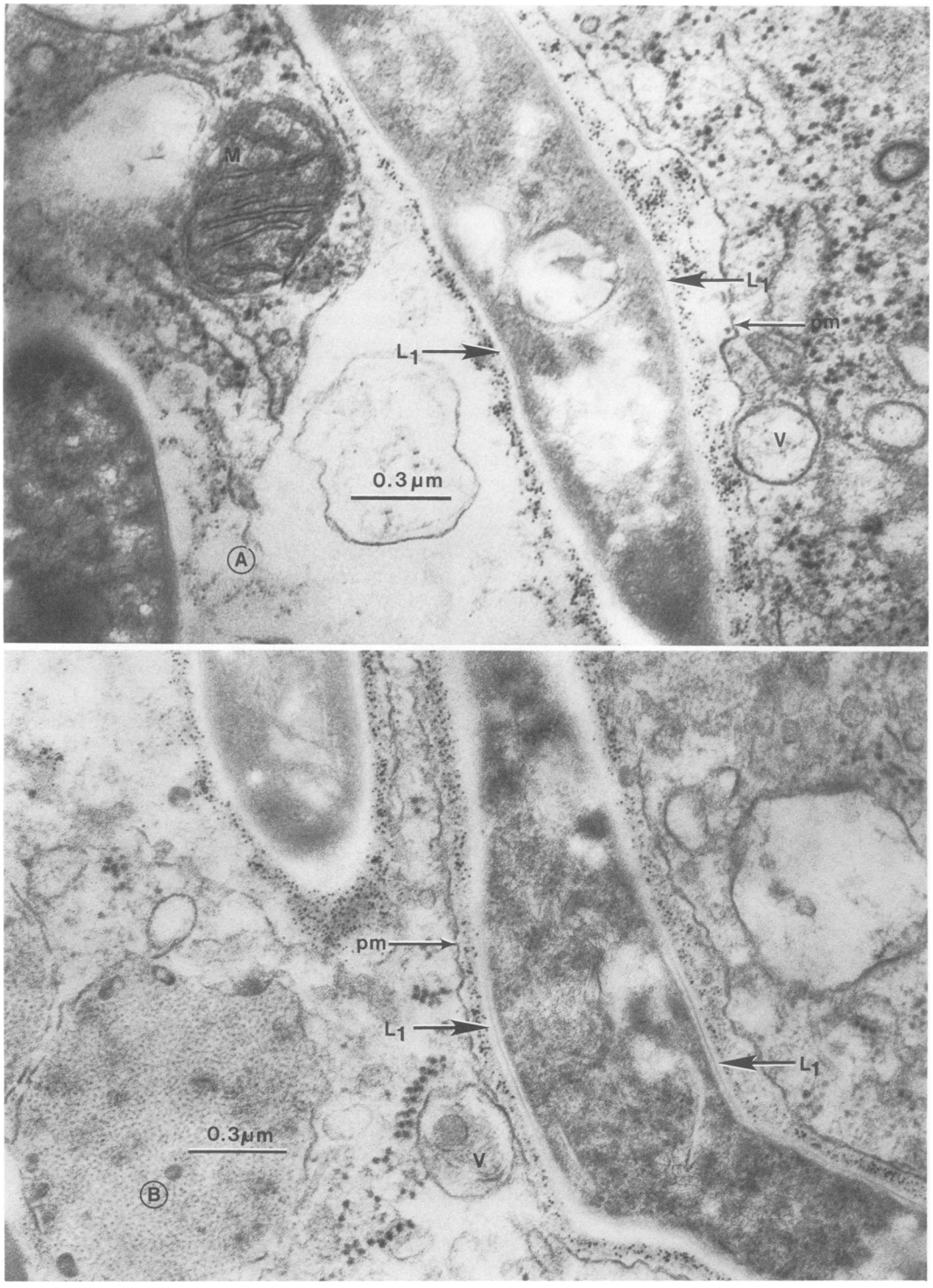

FIG. 3. Ultrathin sections of mouse peritoneal macrophages infected with either (A to C) serovar 20 previously treated with anti-GPL rabbit serum and goat anti-rabbit IgG ferritin conjugate or (D) unlabeled serovar 20. Ferritin granules outline the L_1 layer (L_1) within the phagosomal membrane (pm), and the electron-transparent zone (et) can be observed within the phagosomal membrane. M, Mitochondrion; V, vacuoles. The cells were poststained with uranyl acetate and lead citrate.

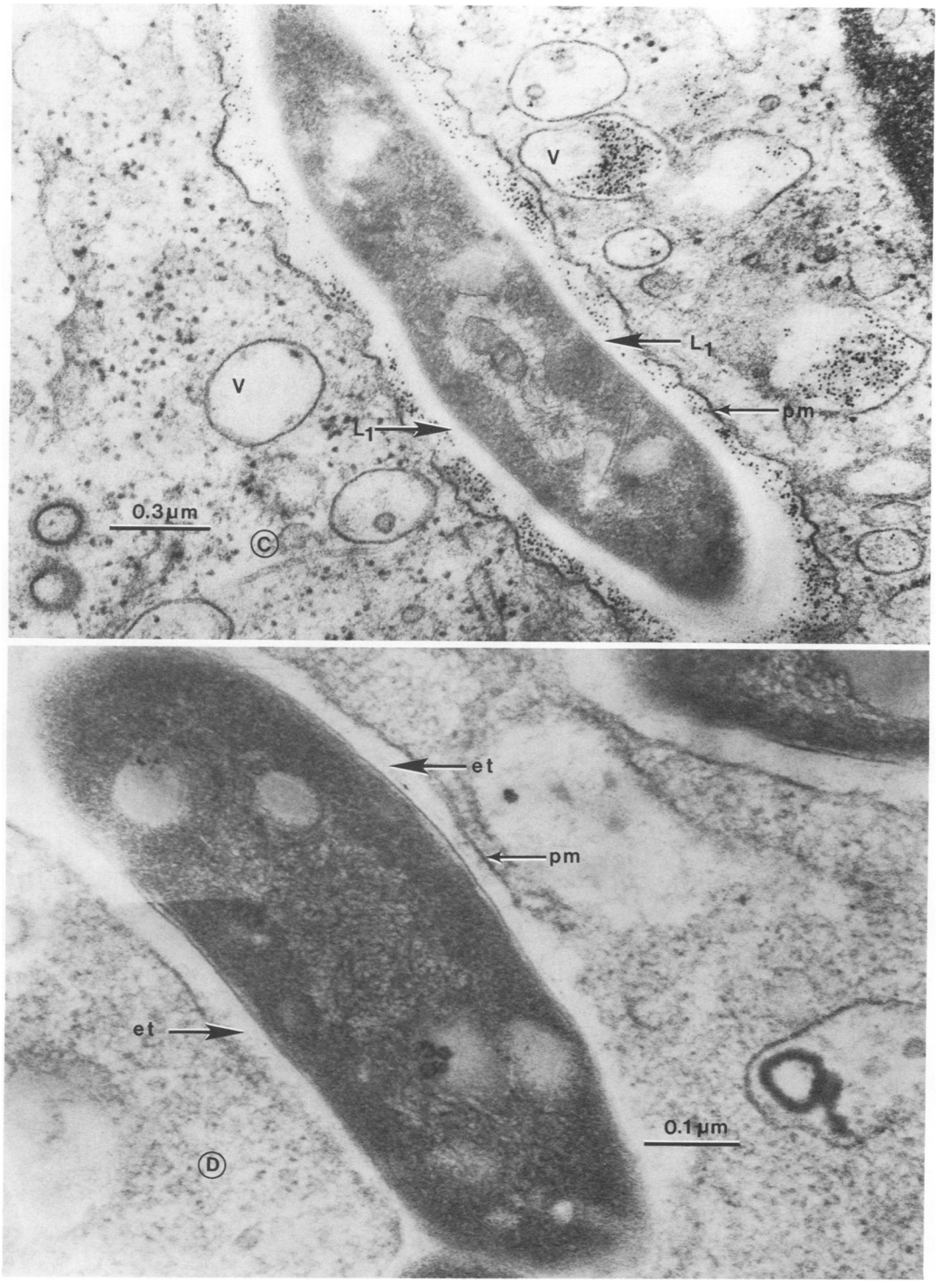

FIG. 3—*Continued*

to ferritin conjugates (23). The presence of the GPL antigens throughout most of the L_1 layer can be assumed, however, because of the varying thickness observed in Fig. 3A to C. This is not at variance with the fact that the L_2 and L_3 layers are also present in the electron-transparent zone and occupy an area immediately external to the murein layer. Thus, by comparing electron micrographs of macrophages infected with ferritin-labeled serovar 20 and those infected with unlabeled serovar 20, we were able to confirm the relationship of the L_1 layer with the electron-transparent zone previously described (11–13, 27).

Also noted in sections of macrophages infected with ferritin-labeled serovar 20 were cytoplasmic vacuoles which contained ferritin granules. Similar vacuoles were observed by Catanzaro et al. (7) when guinea pig peritoneal macrophages were allowed to pinocytose horseradish peroxidase. These investigators associated the cytoplasmic vacuoles with antigen localization and suggested that they may be important in antigen processing (7). In the present report, the ferritin-containing vacuoles may also be associated with antigen processing; however, it is not possible to say for certain that they are associated with the processing of the GPL antigens. The degradation of the antigen-antibody complex may have taken place at the antibody-conjugate level instead of the L_1 layer on the bacterial cell. Thus, the ferritin granules may not represent GPL antigens but only the ferritin conjugate. Another possibility is that the ferritin-containing vacuoles represent portions of the L_1 layer which have been displaced from the surface of the mycobacteria. This would not be unlikely because of the nature of the L_1 layer. It appears from previous studies that the L_1 layer, at least in part, is loosely attached and can be easily removed by agitation (3, 16).

Attempts were made to label serovar 20 with ferritin after phagocytosis. This would have made it possible to detect the exact location of the GPL antigens at the time of fixation, thus giving a truer picture of the phagocytic events. Unfortunately, the attempts were not successful, probably because the penetration of the ferritin conjugate was poor. The penetration of ferritin conjugate is a problem when whole tissue is stained before embedding but is sometimes successful with cells in monolayers (23). Other studies are in progress to overcome this problem so that postphagocytic detection of the GPL antigens can be more accurately accomplished with electron microscopy.

Since it has been established that other serovars of the MAIS complex contain similar GPL antigens, the results of this investigation can be extended to include them. Although the observations described in this paper do not confirm the protective nature of the L_1 layer, they do increase the likelihood of that assumption. Further immunocytochemical studies are being conducted in this laboratory in an effort to establish the protective nature of the L_1 layer and to determine what role the GPL antigens play in pathogenesis.

ACKNOWLEDGMENTS

This research was supported by funds from the Heiser Program for Research in Leprosy and Texas College of Osteopathic Medicine Faculty Research Grant no. 34820.

We thank James E. Carnes and Jay Pearce, Department of Anatomy, Texas College of Osteopathic Medicine, for their assistance in the preparation and sectioning of specimens for electron microscopy.

LITERATURE CITED

1. **Barksdale, L., and K. S. Kim.** 1977. *Mycobacterium*. Bacteriol. Rev. **41**:217–372.
2. **Barrow, W. W., and P. J. Brennan.** 1982. Immunogenicity of type-specific C-mycoside glycopeptidolipids of mycobacteria. Infect. Immun. **36**:678–684.
3. **Barrow, W. W., B. P. Ullom, and P. J. Brennan.** 1980. Peptidoglycolipid nature of the superficial cell wall sheath of smooth-colony-forming mycobacteria. J. Bacteriol. **144**:814–822.
4. **Brennan, P. J., G. O. Aspinall, and J. E. Nam Shin.** 1981. Structure of the specific oligosaccharides from the glycopeptidolipid antigens of serovars in the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex. J. Biol. Chem. **256**:6817–6822.
5. **Brennan, P. J., and M. B. Goren.** 1979. Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* serocomplex. J. Biol. Chem. **254**:4205–4211.
6. **Brennan, P. J., H. Mayer, G. O. Aspinall, and J. E. Nam Shin.** 1981. Structures of the glycopeptidolipid antigens from serovars in the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* serocomplex. Eur. J. Biochem. **115**:7–15.
7. **Catanzaro, P. J., R. C. Graham, Jr., and H. J. Schwartz.** 1969. Ultrastructural identification of possible sites of antigen processing in macrophages. J. Immunol. **103**:618–621.
8. **Chang, Y. T.** 1964. Long-term cultivation of mouse peritoneal macrophages. J. Natl. Cancer Inst. **32**:19–35.
9. **Christensen, E. E., G. W. Dietz, C. H. Ahn, and G. A. Hurst.** 1979. Pulmonary manifestations of *Mycobacterium intracellulare*. Am. J. Roentgenol. **133**:59–66.
10. **Damsker, B., and E. J. Bottone.** 1980. Nontuberculous mycobacteria as unsuspected agents of dermatological infections: diagnosis through microbiological parameters. J. Clin. Microbiol. **11**:569–572.
11. **Draper, P., and R. J. W. Rees.** 1970. Electron-transparent zone of mycobacteria may be a defense mechanism. Nature (London) **228**:860–861.
12. **Draper, P., and R. J. W. Rees.** 1973. The nature of the electron-transparent zone that surrounds *Mycobacterium lepraemurium* inside host cells. J. Gen. Microbiol. **77**:79–87.
13. **Edwards, R. P.** 1970. Electron-microscope illustrations of division in *Mycobacterium leprae*. J. Med. Microbiol. **3**:493–499.
14. **Higgins, M. L.** 1973. A fixation and embedding procedure for thin-sectioning bacteria. CRC Handbook Microbiol. **1**:686–689.
15. **Kelsey, D. S., R. T. Chambers, and A. S. Hudspeth.** 1981. Nontuberculous mycobacterial infection presenting as a mediastinal mass. J. Pediatr. **98**:431–432.

16. **Kim, K. S., M. R. J. Salton, and L. Barksdale.** 1976. Ultrastructure of superficial mycosidic integuments of *Mycobacterium* species. J. Bacteriol. **125**:739–743.
17. **Marcus, D. M., and R. Janis.** 1970. Localization of glycosphingolipids in human tissues by immunofluorescence. J. Immunol. **104**:1530–1539.
18. **Marcus, D. M., and G. A. Schwarting.** 1976. Immunochemical properties of glycolipids and phospholipids. Adv. Immunol. **23**:203–240.
19. **Ratledge, C.** 1976. The physiology of the mycobacteria. Adv. Microb. Physiol. **13**:116–245.
20. **Rausch, P. G., K. B. Pryzwansky, and J. K. Spitznagel.** 1978. Immunocytochemical identification of azurophilic and specific granule markers in the giant granules of Chediak-Higashi neutrophils. N. Engl. J. Med. **298**:693–698.
21. **Schaefer, W. B.** 1975. Serological identification and classification of atypical mycobacteria by their agglutination. Am. Rev. Respir. Dis. **92**(Suppl.):85–93.
22. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
23. **Sternberger, L. A.** 1979. Immunocytochemistry, p. 68–73. John Wiley & Sons, Inc., New York.
24. **Suter, E.** 1952. The multiplication of tubercle bacilli within normal phagocytes in tissue culture. J. Exp. Med. **96**:137–150.
25. **Van Driel, D., and A. J. Wicken.** 1973. Cellular location of the lipoteichoic acid of *Lactobacillus fermenti* NCTC 6991 and *Lactobacillus casei* NCTC 635. J. Ultrastruct. Res. **43**:483–497.
26. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119**:107–159.
27. **Yamamoto, T., M. Nishiura, N. Harada, and T. Imaeda.** 1958. Electron microscopy of ultra-thin sections of lepra cells and *Mycobacterium leprae*. Int. J. Lepr. **26**:1–8.