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Intramacrophagic *Mycobacterium avium* Bacilli Are Coated by a Multiple Lamellar Structure: Freeze Fracture Analysis of Infected Mouse Liver

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We used freeze fracture electron microscopy to study the fine structure of *Mycobacterium avium* inside phagosomes of murine macrophages. *M. avium*-susceptible C57BL/6 mice were infected with *M. avium* by intraperitoneal inoculation of 10^8 viable bacilli. We studied the microanatomy of the mycobacteria in 3-month infections of mice, a situation in which bacillary multiplication is extensive. In these samples, freeze fracture revealed that intraphagosomal bacilli were surrounded by a multilamellar coat that was apposed to the cell wall. In thin sections, in contrast, the area corresponding to the coat showed no substructure and was electron transparent (the so-called electron-transparent zone that has been previously reported by others). The multiple lamellae resembled an onionlike assembly that was inserted in between the mycobacterial wall outer surface and the phagosomal membrane. Each lamella of the *M. avium* coat was made up of parallel straight fibrils with a width of 5 nm. A variable number of lamellae, sometimes up to 10 or more elements, coated individual bacilli. The multilamellar coat was absent around both extracellular *M. avium* and intramacrophagic *M. avium* after short-term (45-min) inoculation of mice. The supramolecular organization of the *M. avium* lamellar coat as viewed here by freeze fracture is similar to that of purified mycoside C (P. Draper, J. Gen. Microbiol. 83:431–433, 1974; K.-S. Kim, M. R. J. Salton, and L. Barksdale, J. Bacteriol. 125:739–743, 1976), a mycobacterial component currently known as glycopeptidolipid (W. W. Barrow and P. J. Brennan, J. Bacteriol. 150:381–384, 1982). We conclude that *M. avium* bacilli growing in macrophages are surrounded by multilamellar capsulelike structures that contain glycopeptidolipid molecules.

Mycobacterium avium causes life-threatening opportunistic infections in AIDS patients (7, 8, 15, 17, 19, 21) and also in older individuals without apparent predisposing conditions (22). It is an intracellular parasite with the ability to proliferate inside macrophages, where it can live in the adverse environment of the phagosome and survive the assault of the microbicidal armory of the phagocyte (29, 31). A postulated defensive mechanism of mycobacteria is the formation of a capsulelike barrier inside phagocytes (11, 13, 14, 23). The capsulelike layer has been commonly named the electron-transparent zone (ETZ) because of its lack of electron density and substructure in thin-section preparations (11–13, 23). In fact, several electron microscopy studies report that some mycobacterial species (*M. lepraemurium* and *M. avium*) are surrounded by a capsulelike structure after being engulfed by macrophages (11–13, 23). This has been well documented for *M. lepraemurium*, the agent of murine leprosy, by both thin-section and freeze fracture electron microscopy (11, 12).

We studied here the ultrastructure of *M. avium* present in macrophages after short- and long-term infections of an *M. avium*-susceptible mouse strain (C57BL/6). We found that intramacrophagic *M. avium* in samples from mice with 3-month infections were coated by a multilayered structure that we defined with high resolution by freeze fracture electron microscopy. The geometric organization of these lamellae was similar to that previously described by Draper (9) and Kim et al. (16) for mycoside C, identified later by Barrow and Brennan (3) as glycopeptidolipid (GPL). This multilamellar coat was absent from both *M. avium* of the inoculum and *M. avium* present in peritoneal macrophages of mice 45 min after mycobacterial inoculation. Our data suggest that a progressive accumulation of GPL occurs around intramacrophagic *M. avium* in long-term experimental infections of mice.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from a local breeder (Instituto Gulbenkian de Ciência, Oeiras, Portugal) and fed commercial chow and acidified water. The C57BL/6 mouse strain is susceptible to *M. avium* infection (1, 20). Athymic nude mice (C57BL/6 *nu/nu*) were purchased from BomMice (Ry, Denmark) and kept in sterilized housing and fed sterilized chow and water.

Mycobacteria. *M. avium* (ATCC 25291; serotype 2) was grown in liquid medium as described before (27), washed and suspended in saline with 0.04% Tween 80, and briefly sonicated to disrupt bacterial clumps before inoculation in mice. *M. lepraemurium* (Douglas strain, kindly supplied by F. Portaels) was isolated from organ homogenates of infected mice that were used to maintain this mycobacterial species in the laboratory. The bacilli were purified by differential centrifugation, washed, and resuspended in saline with 0.04% Tween 80, the same vehicle used in inocula.

Murine infections. Euthymic (C57BL/6) mice were infected by intraperitoneal inoculation with 10^8 CFU of *M.*

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avium in 0.5 ml of saline. The animals were divided into two groups. One group was sacrificed after a short infection (45 min) and the other one was sacrificed after long-standing infection (3 months). Macrophages were collected from the peritoneal cavity of mice after 45-min infections, and liver samples were obtained from mice after 3-month infections. *M. lepraemurium* was injected intraperitoneally (10^8 bacilli in 0.5 ml of saline) in athymic nude C57BL/6 mice, the animals were sacrificed 6 months later, and liver tissue was collected. The samples were processed for thin-section and freeze fracture electron microscopy.

Freeze fracture electron microscopy. *M. avium* bacilli (used as inocula) or mycobacterium-infected peritoneal exudates or liver fragments were fixed in 4% formaldehyde–1.25% glutaraldehyde–10 mM $CaCl_2$, washed in phosphate-buffered saline (PBS), glycerinated (30% glycerol in PBS), and frozen in a slush of partially solidified liquid nitrogen. In liver samples, the content of *M. avium*-infected macrophages can be ascertained by the freeze fracture method without requiring prior isolation of the Kupffer cells. The samples were freeze fractured at −130°C and shadowed by platinum and carbon evaporation. The metal replicas of the fractured samples were cleaned in normal bleach (Clorox, sodium hypochloride) and distilled water, mounted on Formvar-coated grids, and observed by transmission electron microscopy (Philips EM 410).

Thin-section electron microscopy. The samples were fixed in 4% formaldehyde–1.25% glutaraldehyde–10 mM $CaCl_2$ and postfixed in 1% OsO_4–10 mM $CaCl_2$ and then 1% uranyl acetate as described before (25, 26). The specimens were dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a electron microscope (Zeiss EM 10C).

RESULTS

We found large numbers of *M. avium* bacilli inside liver macrophages after a 3-month infection induced by intraperitoneal inoculation of C57BL/6 mice with 10^8 CFU of *M. avium*. This was an expected finding because the C57BL/6 mice are highly susceptible to the strain of *M. avium* that we used here (1, 27). The mycobacteria were observed inside membrane-bound spaces (phagosomes) of the murine macrophages as shown in thin sections (Fig. 1) and freeze fracture preparations (Fig. 2).

Freeze fracture has the advantage of allowing the study of tissue macrophages in situ, as it does not require previous isolation of the macrophages from the tissues, a procedure that has a good chance of introducing artifactual alterations in the native structure of the macrophages. We therefore studied Kupffer cells in situ in liver tissue fragments. The Kupffer cells could be readily distinguished from circulating blood monocytes because they are much larger and they are positioned on the wall of the liver sinusoids, whereas blood monocytes are seen freely in the liver sinusoid lumen with no attachments to the capillary wall.

The *M. avium*-containing phagosomes could be dissected into different planes by freeze fracture. One such plane split the phagosomal membrane into protoplasmic (Pp) and exoplasmic (Pe) fracture faces (Fig. 2). As observed with most biological membranes, the intramembrane particles of the phagosomal membrane showed a preferential partition with the Pp fracture face. The *M. avium* envelope was fractured at multiple levels, revealing two morphologically distinct types of layers. The deeper fracture plane showed a wavy, ropelike surface (Fig. 3). External fracture planes of the

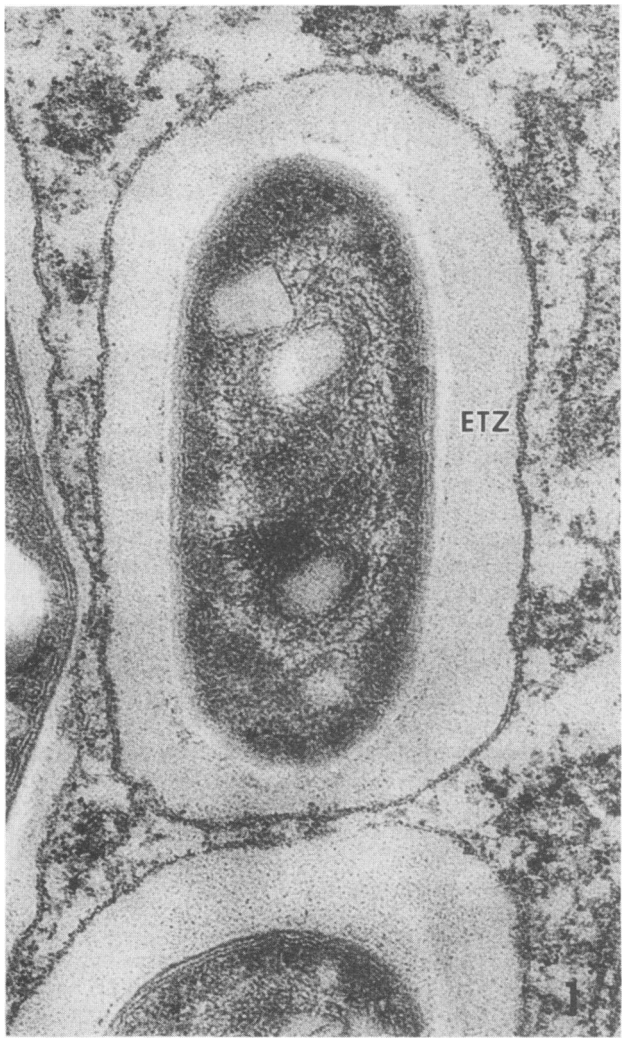


FIG. 1. Thin-section electron micrograph of *M. avium* bacillus inside a phagosome of a murine liver macrophage. An ETZ is seen in between the outer limit of the mycobacterial cell wall and the membrane of the phagosomal vesicle. ×80,640.

envelope revealed several additional planes. Each plane showed a similar structure: a lamella made up of parallel straight fibrils that were each 5 nm wide and closely packed into crystalline assemblies (Fig. 4d and e). The lamellae encircled the intraphagosomal bacilli (Fig. 4a and c). Comparison of contiguous lamellae revealed that the orientation of the parallel fibrils varied from lamella to lamella in the same bacillar coat layer (Fig. 4b and c). The total thickness of the multilamellar layer of the *M. avium* envelope was variable; in some bacilli, a large number (more than 10) of coating lamellae were seen. The inner surface of the phagosomal membrane was often apposed to the outer surface of the multilamellar coat of the ingested *M. avium* bacillus (Fig. 4a and d).

Only seldom was the cell membrane of *M. avium* cleaved by the freeze fracture operation. Lipidic droplets were recognized in the cytoplasm of cross-fractured mycobacteria (data not shown). Ultrastructural comparison of freeze-fractured and thin-sectioned *M. avium* present in liver macrophages indicated that the multilamellar coat identified by

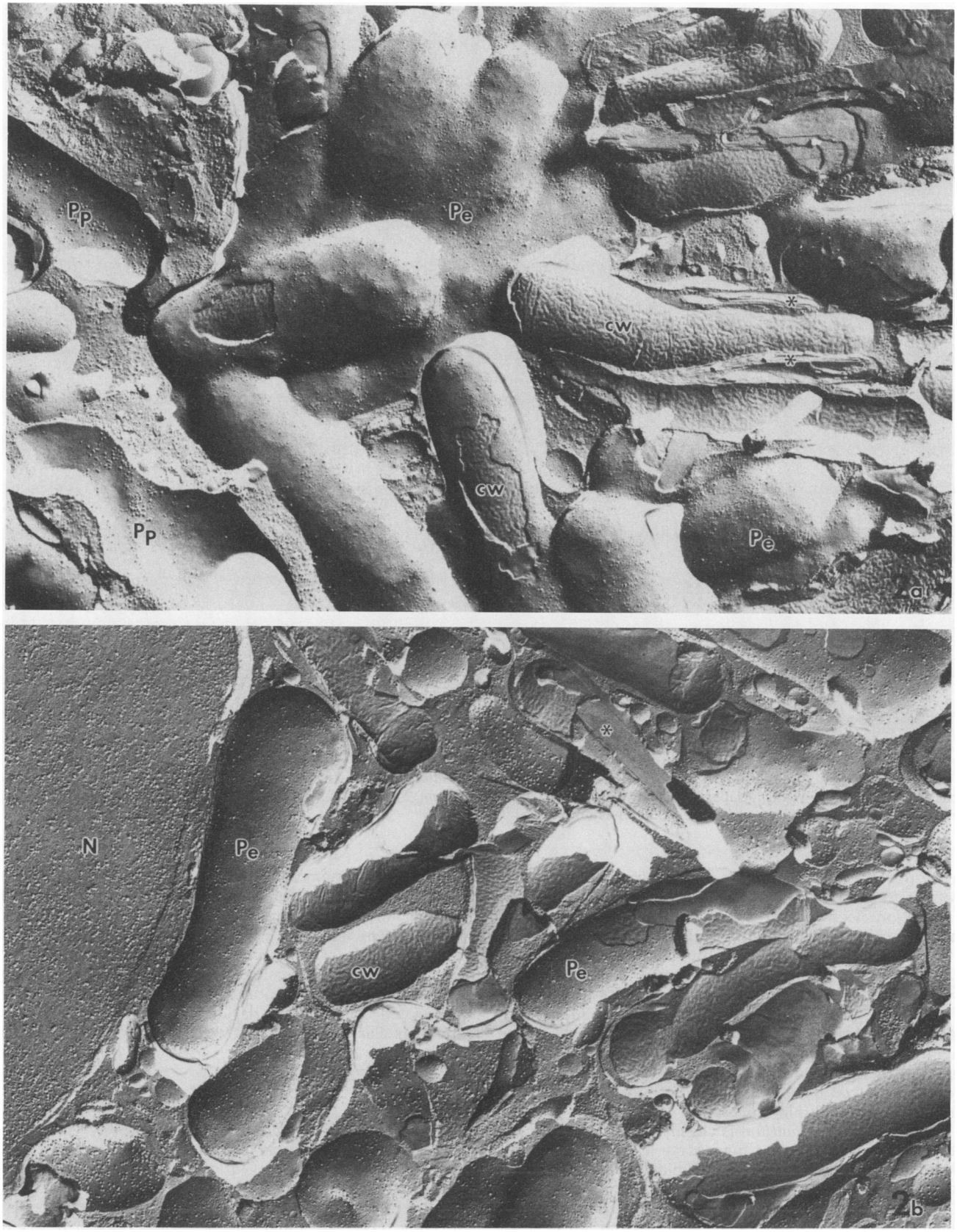


FIG. 2. Freeze fracture electron micrographs of mouse liver macrophages after a 3-month infection with *M. avium*. Numerous *M. avium*-containing phagosomes (P) are present in the cytoplasm of the macrophages. The fracture plane reveals different topographical layers of the *M. avium*-bearing phagosome: protoplasmic (Pp) and exoplasmic (Pe) fracture faces of the vesicle membrane, bacillar cell wall (cw), and a multilamellar layer (asterisk) in between the mycobacterial cell wall and the phagosomal membrane. N, nucleus of the liver macrophage. ×38,000.

FIG. 3. Freeze fracture electron micrograph of mouse liver macrophage after a 3-month infection with *M. avium* showing the multilamellar structure (asterisk) encircling intraphagosomal bacilli. cw, cell wall of *M. avium*; Pp, protoplasmic fracture face of the phagosomal membrane; Pe, exoplasmic fracture face of the phagosomal membrane. ×53,000.

freeze fracture is located in the space corresponding in thin sections to the ETZ (Fig. 1). ETZs were also observed by thin-section electron microscopy in *M. avium* in peritoneal macrophages after a 3-month infection (data not shown).

The multilamellar layer seen around the intramacrophagic *M. avium* after 3-month infections was absent from *M. avium* cells of the inoculum (Fig. 5). The outer fracture plane of the inoculum *M. avium* bacilli showed the amorphous, wavy morphology found in the deeper layer of the *M. avium* bacilli observed inside murine liver macrophages (compare Fig. 5 with Fig. 2). Multilamellar coats were also absent around *M. avium* bacilli present in peritoneal macrophages after short-term (45-min) infections.

Because *M. lepraemurium* is closely related to *M. avium* (18, 28) and it is the other mycobacterial species that was seen encircled by an extracellular lipidic layer when inside macrophages (11, 12), we decided to compare the structure of the *M. lepraemurium* capsular material with the multilamellar assembly herein documented around intramacrophagic *M. avium*. Liver tissue from *M. lepraemurium*-infected nude mice was processed for freeze fracture electron microscopy in the same manner as the *M. avium*-infected liver fragments. We found that the layer surrounding *M. lepraemurium* bacilli was made of long cylinders with a wider diameter (20 nm) than that of the fibrils making up the *M. avium* lamellae (Fig. 6). The cylinders were wrapped around the *M. lepraemurium* bacilli and were not organized into the lamellar assemblies observed around *M. avium*. These morphological features make the *M. lepraemurium*-associated capsular material distinct in structure from the multilamellar investment that we observed coating intramacrophagic *M. avium* bacilli.

DISCUSSION

We used freeze fracture electron microscopy to study the structure of the envelope of *M. avium* cells growing inside mouse liver macrophages after a long-term infection (3 months) and to compare it with that of *M. avium* after a short-term infection of macrophages and with extracellular mycobacteria. We found that *M. avium* present in macrophages of mice after long-term infections were surrounded by multilamellar coats. These were not observed surrounding isolated *M. avium* bacilli or around intramacrophagic *M.*

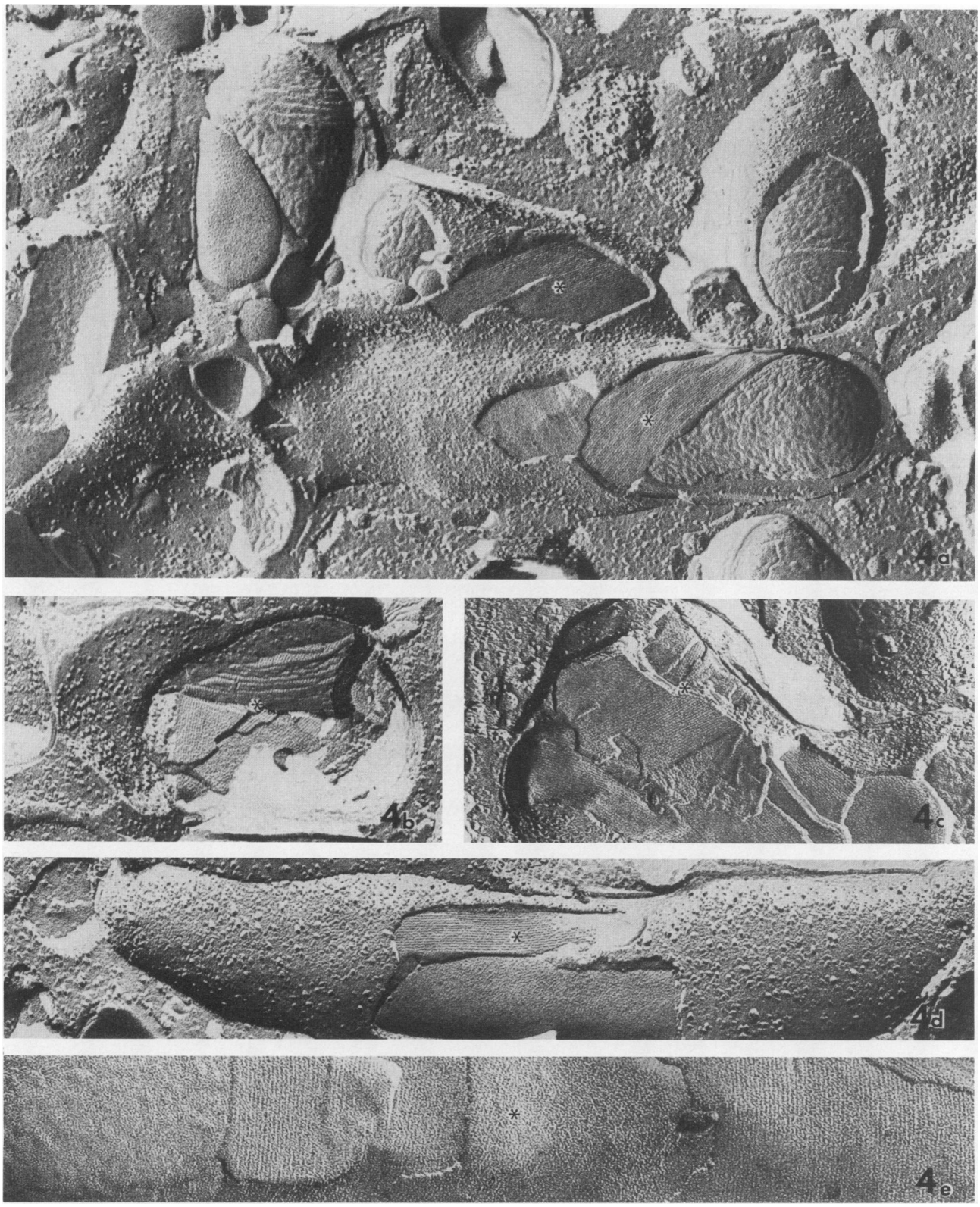
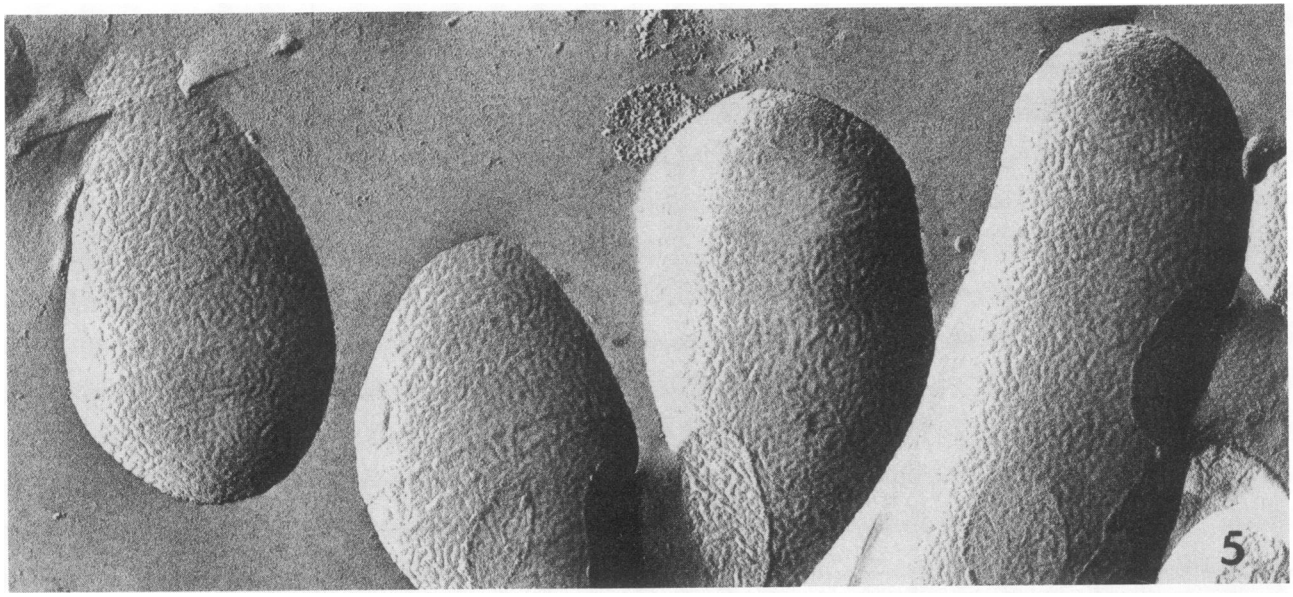


FIG. 4. Different structural aspects of the multilamellar coat (asterisk) that invests intraphagosomal *M. avium* bacilli in mouse liver cells after 3-month infections as revealed by freeze fracture electron microscopy. Face views are shown in panels a, d, and e and document that each lamella is made up of an assembly of straight fibrils that are 5 nm wide. Cross-fractures of the multilamellar coat are illustrated in panels b and c. The lamellae are positioned immediately underneath the exoplasmic fracture face of the phagosomal membrane (Pe) as shown in panels a and d. Each intraphagosomal phagosomal mycobacterial coat may contain numerous apposed lamellae as illustrated in panels b and c. a, ×62,000; b, ×72,000; c, ×71,000; d, ×69,600; e, ×87,000.



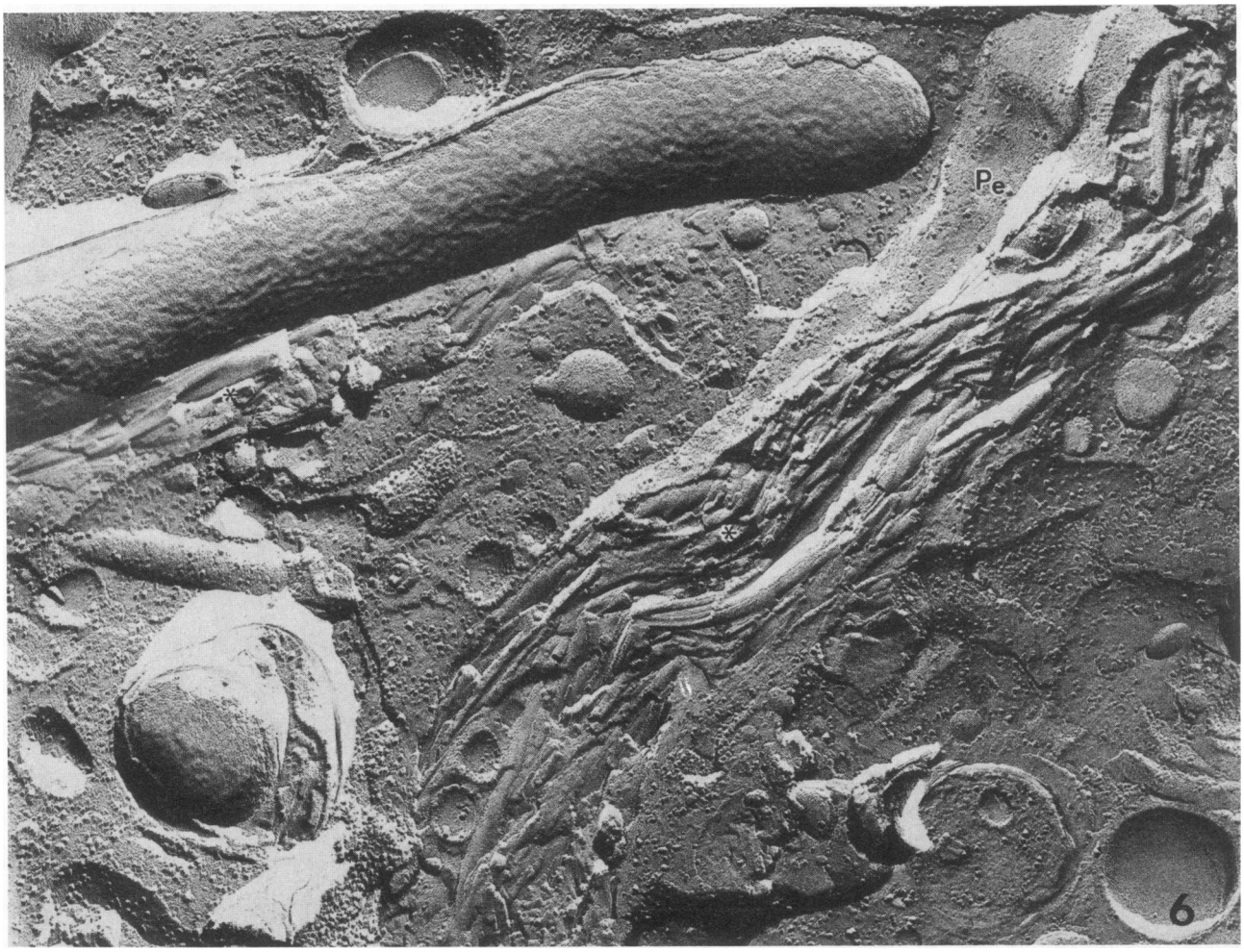


FIG. 5. Electron micrograph of *M. avium* bacilli of the inoculum (used to infect the liver macrophages shown in the previous pictures) processed for freeze fracture directly from liquid culture. These bacilli do not show a lamellar coat; their outer surface is the outer surface of the *M. avium* cell wall showing the same morphology as the cell walls of mycobacteria depicted in Fig. 2 and 3. ×54,500.

FIG. 6. Freeze fracture electron micrograph of mouse liver macrophages after 6-month infection with *M. lepraemurium*. The intraphagosomal coat of *M. lepraemurium* bacilli is made up of narrow cylinders (asterisk) that average 20 nm in diameter and irregularly wrap the mycobacterium. These cylindrical structures are clearly distinct from the multilamellar investments seen around intracellular *M. avium*, as illustrated in the previous figures. ×63,500.

avium bacilli from mice inoculated 45 min before. We also showed that this coat corresponds to the so-called ETZ detected in thin sections.

This is the first study documenting the supramolecular organization of the ETZ layer surrounding intramacrophagic *M. avium* bacilli. The ultrastructure of the *M. avium* multilamellar coat observed by freeze fracture suggests that it is formed by lipidic molecules arranged according to the so-called lamellar phase (5). The microanatomy of the lamellae is similar to that of purified mycoside C as reported before by Kim et al. (16) and Barksdale and Kim (2), also using the freeze fracture method. Mycoside C is currently known as GPL (3, 6). We propose, therefore, that the *M. avium* investment seen around intramacrophagic bacilli is made up of GPL.

Our identification of the capsulelike material as GPL is not based on immunochemistry but on molecular pattern recognition that is based on previous structural data of purified GPL (2, 9, 16). We believe, nonetheless, that the validity of identifying a molecular assembly based on its characteristic structure comes close to the validity of molecular identification by ultracytochemistry. In fact, peculiar arrangements of macromolecules can be identified with specificity by freeze fracture electron microscopy on the basis of pattern recognition. For instance, the characteristic structural patterns of gap and tight junctions allow unambiguous identification of these molecular assemblies by freeze fracture. We propose that GPL, because of the peculiar supramolecular organization of the aggregates seen in purified preparations of the macromolecule, constitutes an elective example of a macromolecule assembly that can be identified by freeze fracture electron microscopy.

There is some controversy whether GPL is the main component of the capsulelike (ETZ) layer surrounding intramacrophagic *M. avium* bacilli. In fact, strains of *M. avium* that synthesize GPL (called Myc^+) or do not synthesize GPL (called Myc^-) were both reported to produce ETZ layers, although the authors added that the Myc^- strain took longer to form its ETZ (23). We used a new approach to investigate this question based on the previous observation that GPL (known before as mycoside C) can be unambiguously identified by freeze fracture electron microscopy because of its characteristic crystalline structure (2, 16). We found that the ETZ layer was formed by the apposition of multiple lamellae each showing the distinct structural features of GPL. Our data sides, therefore, with previous evidence suggesting that GPL is the major component that makes up the capsulelike ETZ layer of intramacrophagic *M. avium* bacilli (9, 11, 30).

We also investigated the freeze fracture morphology of the capsular layer that invests another mycobacterial species, *M. lepraemurium*, also known to produce an ETZ inside macrophages (10–12). We compared this structure with the organization of the multilamellar coat of *M. avium*. We found that the structure of the two ETZ was clearly distinct. This may reflect a different molecular arrangement or chemical composition of the ETZ in the two mycobacterial species.

We view the lamellar coat as the result of accretion of *M. avium*-produced GPL molecules that, because the bacilli are contained inside phagosomes, became sequestrated inside the vesicles. According to this interpretation, the presence of the multilamellar coat is not the result of change of *M. avium* metabolism in response to the adverse environment of the phagosome, but rather the consequence of the GPL molecules being secreted into a closed space, i.e., that of the phagosomal vesicle. Our interpretation is in agreement with the evidence of Kim et al. (16), who have shown that the mycoside C present in the outer mycobacterial integuments can be abundantly secreted by several mycobacterial species in liquid culture.

Because the capsular layer (ETZ) is generally absent around nonpathogenic mycobacteria such as *M. smegmatis* (13), *M. aurum* (24), and *M. gastri* (23) and is present around pathogenic species such as *M. avium* and *M. lepraemurium*, it has been postulated that this layer protects mycobacteria inside macrophages from the antimicrobial actions of the phagocyte (11, 14, 23). Whether this constitutes an important virulence factor of mycobacteria is still a matter of debate. In fact, Belisle and Brennan (4) have recently reported that GPL-devoid *M. avium* causes more severe infections than GPL-containing *M. avium*.

In conclusion, we report here that intramacrophagic *M. avium* bacilli are invested by a multilamellar coat, and we document here for the first time its crystalline arrangement as revealed by freeze fracture electron microscopy. We also show that the ultrastructure of the *M. avium* investment is similar to that of GPL molecules, and we show that the structure of the *M. avium* multilamellar coat is distinct from that of the capsulelike material surrounding intramacrophagic *M. lepraemurium* bacilli. Finally, we suggest that the multilamellar coat is the result of accretion of *M. avium* GPL molecules that are secreted into the closed space of the phagosomal vesicle.

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