Freeze-fracture Observations of the Cell Walls and Peribacillary Substances of Various Mycobacteria

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Ultrastructure of the cell wall and peribacillary substances of various mycobacteria (32 strains of 18 species) grown *in vitro* was studied by a freeze-fracture technique. Peribacillary substances differed in shape among species and even among strains of the same species, and were classified into five types: (1) amorphous substances; (2) multi-layered sheaths with no filamentous units; (3) structures composed of filaments of 2-4 nm diameter, which were further classified into three subtypes according to the arrangement of the filaments; (4) helical fibres; and (5) single fibres, or networks of fibrous structures, with no visible substructures. No strains revealed peribacillary structures resembling those of uncultivable *Mycobacterium leprae*. These results have implications for the mechanism of freeze-fracturing in mycobacterial walls.

INTRODUCTION

The ultrastructure of *Mycobacterium* has been studied extensively by thin sectioning, negative staining and scanning electron microscopy (for reviews, see Barksdale & Kim, 1977; Draper, 1982). A few mycobacterial species, including the pathogenic ones *M. avium*, *M. leprae* and *M. lepraemurium*, have also been studied by the freeze-fracture technique. This technique has shown three-dimensional ultrastructure, especially the difference in shape between the peribacillary substances of *M. leprae*, which are composed of amorphous droplets, and those of *M. lepraemurium* and *M. avium*, which are composed of fine crystalline structures (Nishiura *et al.*, 1972; Draper & Rees, 1973; Draper, 1974; Nishiura *et al.*, 1977).

We have studied several species of mycobacteria, grown under various conditions, by means of the freeze-fracture technique, seeking possible new peribacillary structures, since mycobacterial cell walls are known to have a high content of various lipids (for reviews, see Goren, 1972; Barksdale & Kim, 1977; Minnikin, 1982), and fracture is expected to occur between hydrophobic bilayers of lipids (Branton, 1966). We also wished to know whether the peribacillary structures of *M. leprae* exist in cultivable species of mycobacteria.

METHODS

Organisms. Some of the *Mycobacterium* strains were stock cultures in our laboratories, others were obtained from the Institute for Fermentation, Osaka (IFO), Japan or were kindly supplied by Dr M. Tsukamura, Chubu National Sanatorium, Obu, Japan. The species and strain numbers according to the designation of IFO and Dr Tsukamura (T) are shown in Table 1, together with the strain number of the American Type Culture Collection, where possible, in parentheses.

Growth conditions. The organisms were usually grown on Ogawa-egg medium at 30 °C or 37 °C for 3 d to 4 weeks; *M. xenopi* was grown at 45 °C for 4-5 weeks. Some strains were additionally grown by standing in Kirchner medium, by shaking in Dubois-0.4% (v/v) Tween 80 liquid medium, or by standing or shaking in nutrient broth

enriched with 4% (v/v) glycerol, and/or on Bennet's agar, to see whether nutritional differences affected the peribacillary structures.

Preparation of samples and freeze-fracturing. Cultures were usually transferred directly to the specimen holder of the freeze-etching apparatus without any pretreatment. Immediately before freezing, 30% (v/v) glycerol solution was added as a cryoprotectant and to produce a cell paste. Some cultures, especially those of the pathogenic species, were fixed with 2.5% (v/v) glutaraldehyde in 0.07 M-phosphate buffer, pH 7.2, for a few days at room temperature or at 4 °C. They were immersed before use in 30% (v/v) glycerol containing the same buffer overnight at 4 °C, and usually cooled from the room temperature of 10–20 °C but sometimes from 0 °C. Electron micrographs were printed in reverse contrast to facilitate interpretation.

RESULTS

Localized peribacillary substances

Freeze-fracture of the mycobacteria usually occurred near the wall surface (Figs 1–9) and revealed peribacillary structures. Regarding the location of the structures relating to the cell body, three kinds of structures were observed, depending on the species and strains, as follows: (i) localized structures; (ii) structures of rare occurrence or which may adhere to more than two cells; and (iii) layered structures which appear to cover the whole cell. Structures (i) and (ii) represent peribacillary substances and are described below. Structures of type (iii) may represent peribacillary or wall structures and will be described in the next section.

Peribacillary substances observed by freeze-fracturing or freeze-etching showed structural varieties among the various mycobacteria as follows. (1) Amorphous substances, clearly observed in the contour where two cells had been attached before fracturing (Fig. 1). (2) Multilayered sheaths with no filamentous units. This type was observed only in a few cells of *M. fortuitum* IFO 13159 and *Mycobacterium* sp. Nojima 1–3 (Fig. 2). Some of the sheaths encompassed two adjacent cells. (3) Structures composed of filaments 2–4 nm in diameter. These were further classified into three subtypes according to the arrangement of the filaments: (a) a few of the filaments ran parallel with each other to form a narrow sheet- or rod-like structure (Fig. 3); (b) parallel filaments appeared to pile up three-dimensionally (Fig. 4); they were abundant in *M. marinum* T 8010 grown in prolonged culture; (c) sheets, composed of the filamentous units, stacked into ribbon- or tape-like structures, mostly 50–100 nm in width but sometimes wider (Fig. 5); These were found in several mycobacteria, though they were thin and never covered the whole cell (Table 1). The filaments observed in (a) and (b) were thicker and further from each other than those in (c). (4) Rodlets composed of helical fibres, which were found in *M. avium* IFO 3082 and *M. marinum* T 8010 (Fig. 3). (5) Single fibres, or networks of fibrous structures, with no visible substructure. Some of these were easily detached from the wall and were probably peribacillary substances, although they may have arisen from the wall layers. Therefore they will be described in detail in the next section. The occurrence of these features in various strains is summarized in Table 1.

The peribacillary substances differed in shape and amount among strains of the same species, and even among cells grown in the same slant. This fact obscured the interpretation of findings in different nutritional conditions. The peribacillary substances appeared to increase in amount with prolonged cultivation (compare Figs 3 and 4). Some of them appeared to be easily detachable from the wall (Kim *et al.*, 1976; Takeo *et al.*, 1978) because they were also observed apart from the cells (Fig. 2), and because they decreased in amount when the cells were freeze-fractured after treatment with glutaraldehyde and glycerol. Cooling of some of the mycobacteria from room temperature and from 0 °C did not cause appreciable differences in peribacillary structures. This may be partly because room temperature (10–20 °C) is already below the culture temperature of 37 or 30 °C.

Wall and peribacillary layers

All the mycobacteria studied fractured near the wall surface, which is unusual in Gram-positive bacteria, and revealed layered structures of the wall or peribacillary substances. Ultrastructural features of these outer layers differed among species and even among strains of the same species. Some had thick layers, as revealed by the depth of the ridges, while others had

Table 1. *Shapes of peribacillary substances of various mycobacteria observed in the present study*

All strains showed freeze-fracture near the wall surface, which is unusual in Gram-positive bacteria. All but *M. abscessus* showed a band structure (e.g. see Fig. 2).

Species	Strain*	Peribacillary substances†	Species	Strain*	Peribacillary substances†
<i>M. abscessus</i>	T 19977	1, 5B	<i>M. parafortuitum</i>	T 16001 (19687)	1
<i>M. agri</i>	T 90012	1, 5A		T 16003 (19688)	1
<i>M. aurum</i>	T 15001	1	<i>M. phlei</i>	IFO 13160	1, 5A
<i>M. avium</i>	IFO 3082	1, 4, 5B	<i>M. scrofulaceum</i>	(19981)	1, 5A
	Kirchberg	1	<i>M. smegmatis</i>	T 17002	1, 3c
<i>M. bovis</i>	Ravenel	1		T 17027 (14468)	1, 5A
<i>M. chelonae</i>	T 19009 (19235)	1, 5B		IFO 3083	1, 5D
subsp. <i>chelonae</i>			<i>M. thermoresistible</i>	T 1039 (19527)	1, 3c, 5A
<i>M. fortuitum</i>	T 18112 (6841)	1, 3c	<i>M. tuberculosis</i>	H37Ra	1, 5B
	T 18109 (6842)	1, 3c		Aoyama B	1, 5A, B
	IFO 13159	1, 2		Kurono	1, 5B
<i>M. gordonae</i>	T 12109 (14470)	1	<i>M. xenopi</i>	T 28001 (19156)	1
<i>M. kansasii</i>	(12478)	1, 5A		T 28003 (19250)	1, 5A
<i>M. marinum</i>	T 8010 (927)	1, 3a,b, 4, 5A	<i>Mycobacterium</i> spp.	Jucho	1, 5A
	T 8002	1, 3b, 4		Nojima 1-3	1, 2, 5C
<i>M. nonchromogenicum</i>	T 9003 (19530)	1, 3c, 5A		Nojima 4-2	1
	T 9033 (19533)	1, 3c			

* IFO, Institute for Fermentation, Osaka; T, Dr M. Tsukamura, Chubu National Sanatorium. ATCC numbers are shown in parentheses.

† The number signifies observation of the following structures (see also Figures): 1, amorphous substances; 2, multi-layered sheaths with no filamentous units; 3, structures composed of filamentous units (*a*, a few filaments running straight and parallel to each other to form a narrow sheet- or rod-like structure; *b*, filaments gathering three-dimensionally; and *c*, ribbon- or tape-like structures); 4, helical fibres; 5, single fibres or networks of fibrous structures with no visible substructures (A, wrinkled structures; B, short, straight fibres; C, parallel arrays of fibrils with gaps between each fibril; D, fibrils observed in *M. smegmatis* after repeated pipetting).

thin layers. The number of fracture planes was ascertained by the number of layers bounded by ridges in single walls. There were three fracture planes in *M. phlei* IFO 13160 (Fig. 6), *M. smegmatis* T 17027 and *M. gordonae* T 12109, indicating the existence of at least four layers in these organisms. There were one or two in the other strains, though this may be an underestimation of the number of fracturable planes. The exposed faces generally looked smooth.

In some walls, fibrous structures were observed. They showed structural variation but may be grouped into the following four types. (A) Wrinkled structures of 10–20 nm diameter (Fig. 7). They may be paired fibres or ropelike structures (Takeya *et al.*, 1961; Barksdale & Kim, 1977), or thick ramified fibres (Imaeda *et al.*, 1968; Hawley *et al.*, 1977). (B) Short, straight fibres of about 10 nm diameter (Fig. 8). (C) An array of parallel fibrils of about 4 nm diameter and separated by about 4 nm, which appeared to cover the whole wall (Fig. 9). This array was observed only in *Mycobacterium* sp. Nojima 1-3. The fibrils ran parallel to the longer axis of the cell in about one third of the cells, but obliquely in the others. These features were more clearly observed in the E-face than the P-face. (D) *M. smegmatis* IFO 3083 showed several fibrils of about 5 nm diameter when grown in Dubois-0.4% Tween medium with shaking, but not when grown on Ogawa-egg medium. Abundant fibrils were observed if *M. smegmatis* cells, grown on the Ogawa-egg medium, were suspended in the fixation solution by repeated use of a pipette (Fig. 10). It should be noted that areas with abundant fibrils gradually converted to areas having no fibrils and without ridges (Fig. 10). It remains to be resolved whether all these fibrous structures are different from each other, and whether they exist in the wall or the peribacillary layers. Some of them, e.g. those in Fig. 8, appear to show a structural resemblance to bacteriophages (Bradley, 1967).

Some polar regions in the wall showed a concentric-circular appearance (Fig. 11), which may correspond to that reported in *Staphylococcus aureus* as the newly exposed cell wall at division

In all figures (1-11) cells were grown on Ogawa-egg medium and freeze-fractured without any pretreatment unless otherwise described. Bar markers represent 0.5 μ m.

Fig. 1. Freeze-fractured *M. fortuitum* T 18112. Amorphous peribacillary substances (type 1) are thick at the boundary of the contact of the two cells (arrows).

Fig. 2. Freeze-etched *M. fortuitum* IFO 13159 after fixation and cryoprotection. Multilayered sheaths (type 2) are observed (arrows) both on the wall and apart from the cells. The latter were probably detached from the cells during sample preparation. See also band structures (B).

Fig. 3. Freeze-fractured *M. marinum* T 8010 after incubation for 12 d showing narrow sheet- or rod-like structures (type 3a) which are composed of filaments (arrows), and helical fibres (H) (type 4).

Fig. 4. Freeze-fractured *M. marinum* T 8010 from an old culture (about 2 months). Parallel filaments appear to pile up (type 3b) on some parts of the wall (arrows).

Fig. 5. Freeze-fractured *M. nonchromogenicum* T 9033 showing tape-like structures (type 3c) on the wall (T).

Fig. 6. Freeze-etched *M. phlei* IFO 13160 after fixation and cryoprotection showing three fracture planes on the same cell (arrows). See also the band structure (B).

Fig. 7. Freeze-fractured *M. thermoresistibile* T 1039 showing ropelike wrinkles (type 5A) (arrows).

Fig. 8. Freeze-fractured *M. abscessus* T 19977 showing short, straight fibres (type 5B) in the E-face (arrows).

Fig. 9. Freeze-fractured *Mycobacterium* sp. Nojima 1-3 showing long parallel fibrils with gaps between them (type 5C). Two arrays of the fibrils which run parallel to the long axis of the cell are shown in the E-face (arrows).

Fig. 10. Freeze-fractured *M. smegmatis* IFO 3083 showing fibrils (arrows) which are revealed when cells were freeze-fractured after repeated pipetting (type 5D). See also the P-face of the plasma membrane (M).

Fig. 11. Freeze-etched *M. scrofulaceum* ATCC 19981 showing a concentric-circular appearance of the wall in the polar area of the cell (arrows), which is surrounded by the band structure (B).

(Giesbrecht & Wecke, 1971; Amako *et al.*, 1982). Surrounding the concentric-circular area of the wall, a band-like swelling of the wall about 20 nm wide and 20 nm high could usually be seen (Fig. 11). It has been suggested that this forms at the new cell pole at binary fission, because in cell pairs just after fission it appears at the new cell pole (Izumi *et al.*, 1970). Our results for various mycobacteria confirmed this earlier observation, though only a few cells of *M. thermoresistible*, *M. nonchromogenicum*, *M. marinum* and *M. xenopi* showed the bands and none could be seen in *M. abscessus*. This fact indicates deterioration of bands or deposition of substances on the walls of these species. In contrast, the bands were usually seen in some strains of *M. phlei*, *M. scrofulaceum*, *M. fortuitum* and *Mycobacterium* sp. Nojima 1-3, showing that the bands are conserved for a few generations. In these organisms the bands serve as good natural markers to reveal the mode of growth, especially the localized wall growth of mycobacteria (K. Takeo, unpublished), since they mark on the wall the places where cell divisions have occurred.

DISCUSSION

The crystalline form of a substance reflects its physicochemical properties. Some lipids are known naturally to form helices (see Tachibana & Kambara, 1965). Mycobacterial peribacillary substances which take a helical shape (type 4) may be composed of such lipids. Multilayered sheaths (type 2) are reasoned to be composed of polar lipids in which polar and hydrophobic bilayers are stacked alternately (Moor & Mühlethaler, 1963). C-mycosides are known to exist in *M. avium*, *M. marinum* (= *M. scrofulaceum*), *M. butyricum* and *M. kansasii* (for a review see Goren, 1972). They are serotype-specific substances of the *M. avium*-*M. intracellulare*-*M. scrofulaceum* complex (Brennan & Goren, 1979) to which *M. lepraemurium* is closely related (Draper, 1974). Ribbon-like structures composed of C-mycosides have been reported in *M. lepraemurium*, *M. avium* and *Mycobacterium* sp. (Draper & Rees, 1973; Draper, 1974; Kim *et al.*, 1976). We found similar structures in some strains of *M. fortuitum*, *M. nonchromogenicum*, *M. smegmatis* and *M. thermoresistible*, though it is not certain whether all of them contain C-mycosides. In no strains were the sheets as thick as in *M. lepraemurium* (Draper & Rees, 1973; Nishiura *et al.*, 1977) nor did they cover the whole cell. The stacking of hydrophobic bilayers (where freeze-fracture occurs) in capsular form would hinder the exchange of hydrophilic metabolites, except for organisms growing very slowly, such as *M. lepraemurium*, though on the other hand, it appears to be a good protection against host defence mechanisms (Draper & Rees, 1970).

Cultivation of *M. leprae* *in vivo* has been successful in armadillos and nude mice (Storrs *et al.*, 1974; Kohsaka *et al.*, 1976) though it has been complicated sometimes by naturally occurring or contaminating mycobacteria. Among the 32 strains of 18 species of cultivable mycobacteria that we studied, no strains revealed peribacillary structures resembling those of *M. leprae*, which may offer one basis to distinguish *M. leprae* ultrastructurally from natural or contaminating mycobacteria (Fukunishi *et al.*, 1982).

Imaeda *et al.* (1968) proposed, on the basis of negative staining, that mycobacterial walls consisted of three layers. Freeze-fracturing and negative staining led Barksdale & Kim (1977) to propose a four-layered model as follows: (smooth) sheetlike surface glycolipid or peptidoglycolipid (L_1); wrinkled and superficial (L_2); the underlying fibrous ropelike structure (L_3); and (smooth) innermost peptidoglycan. Draper (1982) has regarded the outer two layers in both models as capsule, not covalently bound to the wall. Our results support his view: outer layers of mycobacterial walls differed, even among strains of the same species, in the number of the fracture planes bounded by distinct ridges, in the ridge thickness of these layers and in the shape of structures found in these layers. We use the term 'peribacillary substances' because some structures exist only locally.

The freeze-fracture technique is useful for visualizing layered structure. There seems to be an implicit supposition that exposed faces showing an essential difference in appearance represent different layers or, in other words, that a certain face exposed by the fracture between certain layers always looks essentially the same. As suggested by the following four lines of reasoning, this may not be valid in mycobacterial walls, including peribacillary layers, unless they are split

into improbably large numbers of layers. First, the high content of various kinds of lipids in mycobacterial walls (for reviews, see Goren, 1972; Barksdale & Kim, 1977; Minnikin, 1982), which may reach 60% of the dry weight (Kotani *et al.*, 1959), probably forms plenty of hydrophobic bonds. A group of hydrophobic substances which nearly cover a plane possibly offers one fracture plane. Thus, different walls may have different fracturable planes or even one wall may have different fracturable planes in a certain layer. In other words, certain double layers in mycobacterial walls may be split along visible structures in some cells but only along amorphous substances in others. Secondly, different layers in the model of Barksdale & Kim are wrinkled to different extents as described above. However, it is unlikely that the two exposed faces (the E-face of the upper layer and the P-face of the underlying layer) were in full contact by hydrophobic bonds before fracturing. This conflict can be resolved if some layers which have wrinkled structures also have amorphous substances, and if they fracture in some cells along the wrinkled structures, revealing wrinkled faces, both in the E- and P-faces, but fracture in others only along amorphous substances, revealing smooth faces, as discussed above. Thirdly, single walls of *M. phlei* IFO 13160 revealed three fracture planes separated by ridges (Fig. 6), all of which were usually smooth, although sometimes ropelike wrinkles were observed. These facts suggest changes of the fracture path in wrinkled layers, though they may simply imply the existence of four smooth layers and in addition some wrinkled layers in *M. phlei*. Lastly, *M. smegmatis* IFO 3083 revealed plenty of fibrils in the wall (Fig. 10) when grown on Ogawa-egg medium and freeze-fractured after repeated pipetting, while it revealed few fibrils when this procedure was not used. Furthermore, areas with plenty of fibrils gradually changed to areas having no fibrils, without showing any ridges between them (Fig. 10). If these two different areas belong to two different layers of the wall, there should be a ridge in the transition areas. These facts appear to show that the fibrils may or may not be revealed due to subtle changes in the arrangement of the wall matrix and in the fracture pathway. Thus, it is suggested that exposed faces showing differences in appearance do not necessarily represent different layers in organisms whose envelopes are rich in hydrophobic substances.

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