Freeze-Etching Study of Human and Murine Leprosy Bacilli[1]

M. Nishiura, S. Izumi, T. Mori, K. Takeo and T. Nonaka[2]

In order to examine the morphologic details of the electron-transparent zones (ETZ) around human and murine leprosy bacilli, we have carried out freeze-etching studies of this problem since 1969, and the results so far obtained have been published in several preliminary reports ([11-13, 15]). In this paper we present the details of freeze-etched findings of both human and murine leprosy lesions. A culture of murine leprosy bacilli on cell-free culture medium (Ogawa's 1% egg yolk medium) was also studied by the same technic.

Draper and Rees in 1973 ([5]) reported on the freeze-etching finding of the ETZ of murine leprosy bacilli, and by their chemical studies the ETZ material was identified as mycocide of type C. As a result of our present study, it became clear that ETZ material of human leprosy bacilli is very much different from that of murine leprosy bacilli. The ETZ of human leprosy bacilli is composed of hydrophobic droplets having a liquid nature, whereas the ETZ material of murine leprosy bacilli is of crystalline solid nature even at the body temperature of mice.

MATERIALS AND METHODS

Lepromas biopsied from eight cases of nodular and infiltrative lepromatous cases have been used as specimens for freeze etching. All murine lepromas were of Hawaiian strain of *M. lepraemurium* and were biopsied three to six months after inoculation of mice.

Culture of murine leprosy bacilli. In 1969, murine leprosy bacilli were successfully cultured by Ogawa using Ogawa's 1% yolk medium ([16, 17]). Bacilli used in the present study were subcultures of the bacilli originally cultured by Ogawa. This strain has been maintained in successive subcultures by T. Mori of the Research Institute for Microbial Diseases, Osaka University. The pathogenicity of this strain was confirmed by Mori by inoculation to mice ([9]). Colonies of this subculture of Mori were processed for freeze etching at Kyoto University.

Freeze-etching technic. All biopsy specimens were fixed in 2.5% glutaraldehyde (with phosphate buffer) for three hours at pH 7.2. After that, specimens were immersed in 30% glycerol-in-saline for 12 to 24 hours.

Colonies of murine leprosy bacilli grown on culture media were collected and put directly into 30% glycerol-in-saline without fixation just before freezing. Because of the comparatively low water content of bacillary colonies as compared to lepromas, long immersion in glycerol-in-saline was not necessary in the specimen preparation of colonies of murine leprosy bacilli. Glycerol-in-saline was used only as a supporting media for the colonies of murine leprosy bacilli for the freeze fracturing and etching.

The principles of the freeze-etching technic were the same as the usual technics of freeze etching ([2, 8, 18]). However, we have developed our own model of freeze-etching apparatus for this study ([11]). This model is a modification of Bullivant's metal block method ([2]). In our model, the fracturing is done in a vacuum and this model is especially suitable for freeze-fracture replication at a low temperature of −150°C. This model has been further developed as the Hitachi Freeze Replica Unit.

Freezing of the specimen is done first in liquid Freon 12 and later the frozen specimen is transferred to liquid nitrogen. Fracturing of the specimen is done at 5×10^{-6} Torr vacuum. For freeze-fracture replication, platinum-carbon shadowing and carbon

[1] Received for publication 8 January 1977.

[2] M. Nishiura, M.D., Professor, Leprosy Research Laboratory, Kyoto University School of Medicine, Sakyo-Ku, Kyoto, Japan, and S. Izumi, M.D., Seisho-En National Leprosarium, Aji-Cho, Kida-Gun, Kagawa and Leprosy Research Laboratory, Kyoto University School of Medicine; T. Mori, M.D., M.D.Sci., Associate Professor, Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; K. Takeo, Ph.D., Instructor, Chest Disease Research Institute, Kyoto University; and T. Nonaka, B.S., EM Technologist, Leprosy Research Laboratory, Kyoto University School of Medicine.

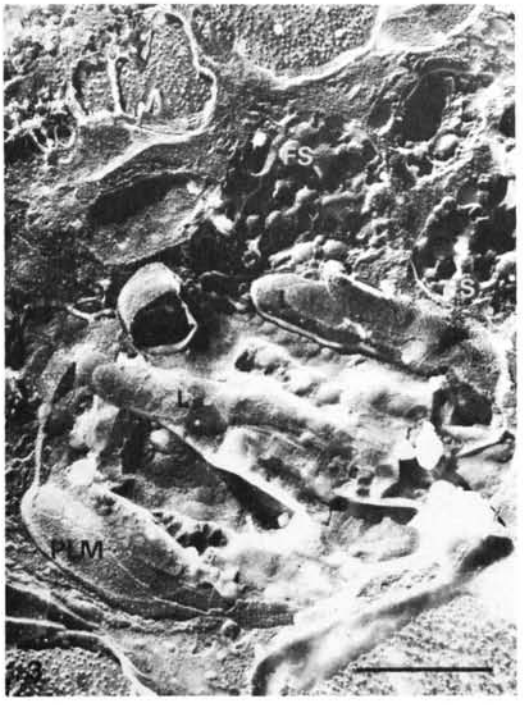

FIG. 1. Typical human lepra cell with fairly well developed foamy structures in the cytoplasm. Human leprosy bacilli are seen wrapped in a single phagolysosomal membrane. Small spherical droplets are present around human leprosy bacilli in the phagolysosomes. ×36,000. Symbols: FS, intracytoplasmic foamy structure; L, human leprosy bacillus; PLM, phagolysosomal membrane. Scale: 1 μ.

FIG. 2. Mature intracytoplasmic foamy structure of human lepra cell. Various sized droplets are wrapped within phagolysosomal membrane. The spherical droplets have no lamellar structures and they are composed of homogeneous hydrophobic material. In ultrathin sections, these spherical droplets look like electron-transparent foamy structures and they form ETZ by coalescing with each other. ×16,500. Symbols: FS, intracytoplasmic foamy structure; PLM, phagolysosomal membrane. Scale: 1 μ.

evaporation are done immediately after the fracturing at −150°C. For freeze etching, the specimen is heated to −100°C and after one minute of etching, the cleavage surface of the specimen is replicated with platinum-carbon and carbon evaporation. Replica film thus formed is removed by immersing the tissue in a commercial bleach solution of sodium hypochlorite. Replicas detached from the tissue surface are washed twice in distilled water and mounted on the copper grids for electron microscopy.

RESULTS

Findings of human lepra cells and M. leprae. The electron-transparent zone around human leprosy bacilli was originally defined in 1958 ([22]), based on the ultrathin section pictures of lepromatous lesions. It is the electron-transparent area around leprosy bacilli inside phagolysosomes of lepra cells which increases distinctly when leprosy bacilli start

FIG. 3. Group of human leprosy bacilli are seen in phagolysosomes in the cytoplasm of human lepra cell. In this picture most of the bacilli are solid and seem to be actively growing. The content of phagolysosomes at this stage is hydrophilic. ×16,500. Symbols: CY, cytoplasm of lepra cell; L, human leprosy bacillus; PL, phagolysosome; PLM, phagolysosomal membrane. Scale: 1 μ.

to degenerate. Actually there are two kinds of materials other than bacilli inside the phagolysosomes in the cytoplasm of lepra cells. One is the homogeneous moderately electron-dense material which we termed opaque droplets ([22]) and most probably it is the lysosome material. The other consists of small spherical droplets accumulated around leprosy bacilli in the phagolysosome. Spherical droplets in the phagolysosome are electron-transparent in ultrathin section and never show lamellar structure in freeze replication as is observed in the case of neutral fat droplets. These small droplets are produced around leprosy bacilli (Fig. 1) and after increasing in the phagolysosomes, they finally occupy the whole space in these structures (Fig. 2). Spherical droplets are hydrophobic, but the homogeneous material inside the phagolysosome is hydrophilic. This hydrophilic nature was confirmed by the artifact of water crystal formation in the homogeneous part of the phagolysosome. Usually we can observe a single membrane of phagolysosome very clearly by the freeze-etching technic (Figs. 1, 3). Even when leprosy bacilli are few in the cytoplasm of the lepra cell, isolated bacilli are also covered with a single membrane of phagolysosome.

The freeze-etching study of human leprosy bacilli in lepra cells has shown clearly that the band structures around the cell wall of the human leprosy bacillus are composed of two thin strings (Fig. 4). Band structures of human leprosy bacilli ([6, 14]) (Fig. 4) are usually observed on the surface of the cell wall, whereas those of murine leprosy bacilli are embedded in the bacillary cell wall (Fig. 5).

Findings of murine lepra cells and murine leprosy bacilli. Crystalline material is seen around murine leprosy bacilli and has a ribbon-like or membranous form. Usually on the surface of the cell wall of the murine leprosy bacillus, this crystalline material has a multi-layered membranous appearance, and its crystalline nature is evident because of the presence of parallel straight lines on the membrane and the tendency of being split along these parallel straight lines. Draper and Rees showed a ribbon-like structure by negative staining of murine leprosy bacilli ([4]). Regarding the crystalline structure around murine leprosy bacilli, we have reported the findings of freeze etching in 1972 ([11]), and Draper and Rees also studied this

FIG. 4. Freeze fracture of a human lepra cell. Solid leprosy bacilli are seen in the lepra cell. There are band structures on the surface of leprosy bacilli and each band is made of two thin strings. ×72,000. Symbols: BS, band structure which is composed of two thin strings; L, human leprosy bacillus. Scale: 1 μ.

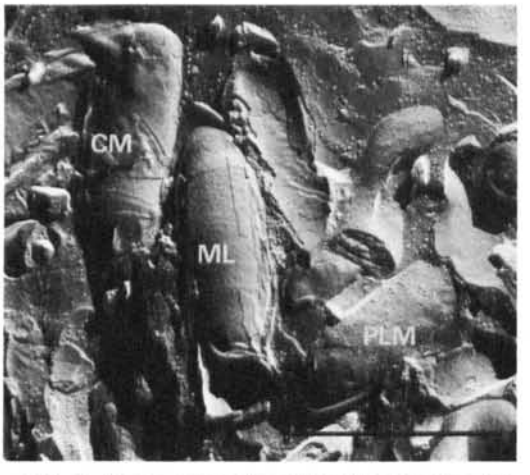

FIG. 5. Just outside the cell wall of murine leprosy bacilli, crystalline material shows membranous appearance. However, this membrane also has a crystalline nature and it is easily split along the straight lines of the crystal. Band structures of murine leprosy bacilli are usually embedded in the cell wall. ×55,000. Symbols: BS, band structure; CM, crystalline material; ML, murine leprosy bacillus; PLM, phagolysosomal membrane. Scale: 1 μ.

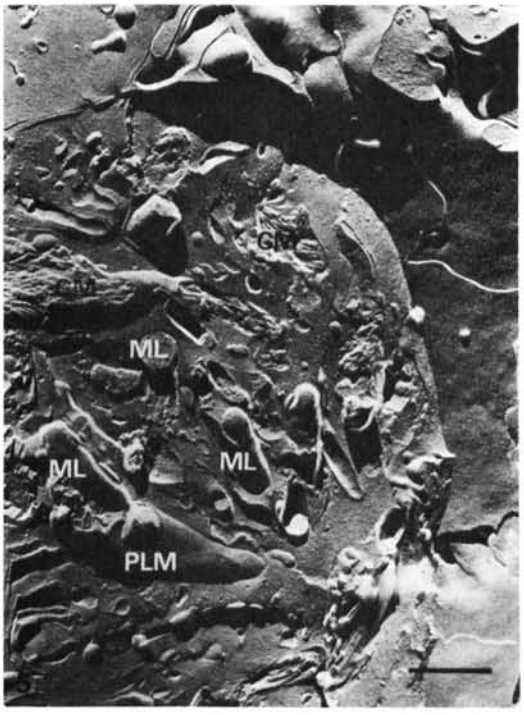

FIG. 6. Murine lepra cell in mouse liver. There are many phagolysosomes containing leprosy bacilli and crystalline material. This crystalline material is the cause of the random arrangement of murine leprosy bacilli inside murine lepra cells. Separated by solid crystalline material around each murine leprosy bacillus the bacilli cannot assume a side-by-side arrangement. X21,000. Symbols: CM, crystalline material; ML, murine leprosy bacillus; PLM, phagolysosomal membrane. Scale: 1 μ.

FIG. 7. Dividing murine leprosy bacillus wrapped in a phagolysosome in the cytoplasm of a murine lepra cell. Crystals are also seen wrapped inside the same phagolysosome together with a murine leprosy bacillus. X27,000. Symbols: ML, dividing murine leprosy bacillus; PLM, phagolysosomal membrane. Scale: 1 μ.

structure by the same freeze-etching technic ([5]). Draper's detailed study on the chemical nature of this material showed that it is made of type C mycocide ([5]). The crystalline material is usually wrapped with phagolysosomal membrane together with murine leprosy bacilli (Figs. 6, 7), but in old murine lepra cells this crystal is also found in the cytoplasm of murine lepra cells separated from murine leprosy bacilli.

Freeze-etching observation of the cell-free culture of M. lepraemurium. Colonies of murine leprosy bacilli grown on the surface of culture media were collected and processed in the same way as in the freeze-etching experiments on human and murine leprosy bacilli *in vivo*.

Electron microscopic observation of the colonies of murine leprosy bacilli on the culture media revealed large amounts of crystalline material around each bacillus (Fig. 8). The presence of large amounts of crystalline material (mycocide C according to Draper) around bacilli grown on cell-free culture media suggests that this material is produced by murine leprosy bacilli and not by host cells.

DISCUSSION

In ultrathin sections, electron-transparent zones around both human and murine leprosy bacilli appear electron-transparent and homogeneous and no further morphologic details can be observed in them except for occasional fine electron-dense granules in human lepra cell ETZ and fibrous structures in murine lepra cell ETZ. This is due to the drastic change of the content of ETZ by dehydration in alcohol or acetone during the process of embedding for ultrathin sectioning. In the freeze fracture and etching technics, these lipid materials are not lost in the processes of fixation in glutaraldehyde and freezing in liquid Freon 12 and liquid nitro-

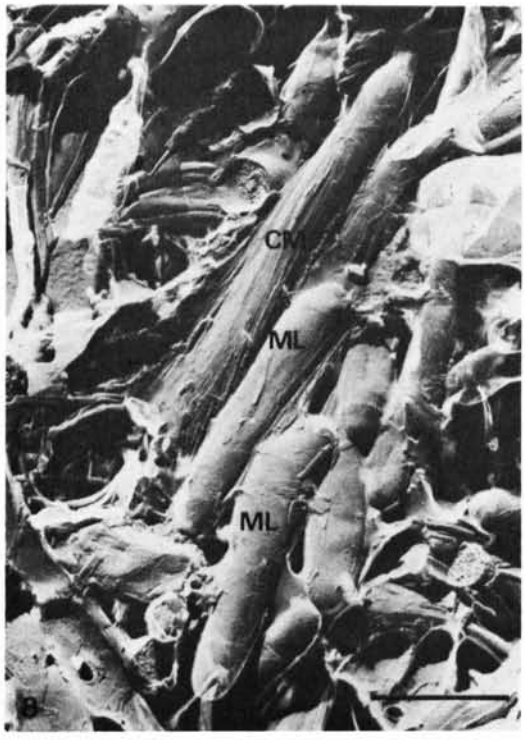

FIG. 8. Murine leprosy bacilli grown on Ogawa's 1% egg yolk medium. Large amounts of crystalline material are present around the cell wall of the bacilli. Such quantity of crystalline material was not seen in cultures of other mycobacteria. Probably this is the same substance which is found around murine leprosy bacilli *in vivo*. × 36,000. Symbols: CM, crystalline material around murine leprosy bacillus; ML, murine leprosy bacillus. Scale: 1 μ.

gen. Thus the morphologic feature of the ETZ is preserved in a more natural condition.

Spherical droplets of foamy structures of human lepra cells can be differentiated from neutral fat droplets of subcutaneous tissue. In the case of neutral fat droplets, the freezing usually causes a change in molecular arrangement and all fat droplets show lamellar structures after freezing in liquid nitrogen. Spherical droplets of foamy structures in human lepra cells never show lamellar structures even at the temperature of liquid nitrogen. They are always of liquid nature at body temperature, and because of this human leprosy bacilli can be packed together closely in phagolysosomes and show characteristic side-by-side arrangement.

The crystals around murine leprosy bacilli are, however, in solid crystalline state even at the body temperature of mice since these crystals can be demonstrated by negative staining of murine leprosy bacilli ([4]). This is also clear from the fact that neighboring murine leprosy bacilli can never come very close to each other as they are separated by solid crystalline material around each murine leprosy bacillus. Toda and Nishiura ([20]) studied the difference between human and murine lepra cells by ultrathin sectioning in 1963 and found fibrous structures around murine leprosy bacilli in old murine lepra cells. This fibrous structure was actually osmium tetroxide infiltrating into the spaces between each lamella of crystalline material. At that time we did not realize the crystalline nature of this fibrous structure in the murine lepra cell.

Draper *et al* identified this crystalline material around the murine leprosy bacillus as mycocide C. Mycocide C has also been reported in avian tubercle bacilli ([21]). Draper, in 1974, reported ([3]) fibrils of 6.8 nm in diameter arranged in parallel bands as related to *M. avium* by negative staining of chloroform soluble material removed from homogenized bacilli. Crystalline mycocide was reported recently in relation to Mycobacterium sp. NQ by Kim *et al* ([7]).

Takeo *et al* made freeze-etching studies of various mycobacteria in our laboratory ([16]). The mycobacteria examined thus far are *M. tuberculosis* (Aoyama B, Kurono and H37Ra), *M. avium* (Kirchberg, Jucho and I 3082), *M. bovis* (Ravenel), *M. fortuitum* (18112, 18001, 18009, and I 13159), *M. phlei* (I 13160), *M. smegmatis* (17020, 17002, and I 3083), *M. marinum* (08010 and 08002), *M. gordonae* (T-12109), *M. agri* (90012), *M. rhodochrous* (I 13161), *M. aurum* (15001), *M. chelonei* (19009), and *M. parafortui* (16001 and 16003). Although some of these mycobacteria *(M. avium, M. fortuitum, M. smegmatis, M. marinum, M. aurum, M. chelonei* and *M. phlei)* showed small amounts of crystalline material around their cell wall, none of them showed such large amounts of crystalline material as in the case of the cultured murine leprosy bacilli.

Mori *et al* ([10]) found that murine leprosy bacilli are rich in palmitic acid and 10-methyl stearic acid by gas chromatography. The peculiar straight shape of the crystals found by freeze etching around murine leprosy bacilli might be due to the rich content of these saturated fatty acids.

SUMMARY

Morphologic features of the electron-transparent zone (ETZ) material around human and murine leprosy bacilli were examined by a freeze-etching technic. The ETZ around human leprosy bacilli is composed of spherical droplets of hydrophobic material. These are always liquid at body temperature and they never show crystalline lamellar structure even at the temperature of liquid nitrogen. The ETZ around murine leprosy bacilli is composed of ribbon-like or membranous crystalline structures. This material is solid and crystalline at the body temperature of mice, and this solid material is the chief cause of the random arrangement of murine leprosy bacilli inside the cytoplasm of murine lepra cells. This crystalline structure has also been observed around murine leprosy bacilli grown on cell-free culture media.

RESUMEN

Se examinaron las caracteristicas morfológicas del material de la zona transparente a los electrones (ZTE) que rodea a los bacilos de la lepra humana y murina usando la técnica de "impresión por congelación" o freeze-etching. La ZTE que rodea al bacilo de la lepra humana está compuesta por gotitas esféricas de material hidrofóbico que siempre están líquidas a la temperatura del cuerpo y que nunca presentan una estructura cristalina laminar aún a la temperatura del nitrógeno líquido. La ZTE que rodea al bacilo de la lepra murina está compuesta por estructuras membranosas cristalinas de apariencia acintada. Este material es sólido y cristalino a la temperatura corporal del ratón y es la causa principal del arreglo desordenado que presentan los bacilos de la lepra murina dentro del citoplasma de las células parasitadas. Esta estructura cristalina también se ha observado alrededor de los bacilos de la lepra murina crecidos en medios de cultivo libres de células.

RÉSUMÉ

On a utilisé une technique de microtomie de congélation pour étudier les caractéristiques morphologiques du matériel provenant de la zone transparente aux électrons (ETZ) entourant des bacilles de lèpre humaine ou murine. Cette zone, autour de bacilles de lèpre humaine, est composée de gouttelettes sphériques de matériel hydrophobe. Ce matériel est toujours liquide à la température du corps; il ne montre jamais de structures lamellaires cristallines, même à la température de l'azote liquide. Cette zone transparente autour des bacilles de lèpre murine est composée de structures cristallines en rubans ou membraneuses. Ce matériel est solide et cristallin à la température corporelle de la souris. Ce matériel solide explique la répartition au hasard des bacilles de lèpre murine à l'intérieur du cytoplasme des cellules de lèpre murine. Cette structure cristalline a également été observé autour de bacilles de lèpre murine qui se multipliaient sur des milieux de culture sans cellules.

REFERENCES

1. Akahori, H., Okamura, S., Nishiura M. and Uehira, K. A new model of freeze etching apparatus. Proc. 30th Ann. EMSA Meeting, 1972, pp 294-295.
2. Bullivant, S. and Ames, A. A simple freeze-fracture replication method for electron microscopy. J. Cell. Biol. **29** (1966) 435-447.
3. Draper, P. The mycocide capsule of *Mycobacterium avium 357*. J. Gen. Microbiol. **83** (1974) 431-433.
4. Draper, P. and Rees R. J. W. Electron-transparent zone of mycobacteria may be a defense mechanism. Nature **228** (1970) 860-861.
5. Draper, P. and Rees, R. J. W. The nature of the electron-transparent zone that surrounds *Mycobacterium lepraemurium* inside host cells. J. Gen. Microbiol. **77** (1973) 79-87.
6. Izumi, S., Nishiura, M., Sugimori, M., Okada, S. and Takizawa, H. A method of classifying solid leprosy bacilli based on the number of band structures on them. Int. J. Lepr. **38** (1970) 126-133.
7. Kim, K., Salton, M. R. J. and Barksdale, L. Ultrastructure of superficial mycocidic integuments of Mycobacterium sp. J. Bacteriol. **125** (1976) 739-743.
8. Moor, H., Muhlethaler, K., Waldner, H. and Frey-Wyssling, A. A new freeze ultramicrotome. J. Biophys. Biochem. Cytol. **10** (1961) 1-13.
9. Mori, T. Cultivation of *M. lepraemurium* on the 1% Ogawa's yolk medium and animal inoculation with cultivated *M. lepraemurium*. Lepro **43** (1974) 226-233.
10. Mori, T., Dohmae, K. and Kohsaka, K. Lipids and nucleic acid in *M. lepraemurium*. Lepro **37** (1968) 311-316.
11. Nishiura, M. Designing of a new freeze-etching apparatus. Kagaku **42** (1972) 431-438 (In Japanese)
12. Nishiura, M. Three-dimensional observation of leprosy lesions by freeze-etching technic. Abstract in Int. J. Lepr. **40** (1972) 467.
13. Nishiura M., Izumi S. and Nonaka, T. Observation of human and murine leprosy ba-

cilli. A freeze-etching study. Metabolism Dis. **12** (1975) 1-2. (In Japanese)

14. NISHIURA, M., OKADA, S., IZUMI, S. and TAKIZAWA, H. An electron microscope study of the band structure of the leprosy bacillus and other mycobacteria. Int. J. Lepr. **37** (1969) 225-238.
15. NISHIURA, M., UEHIRA, K., HASEGAWA, T. and TAKEUCHI, M. The morphologic difference between human and murine leprosy bacilli as revealed by freeze-etching technic. Abstract in Int. J. Lepr. **40** (1972) 89-90.
16. OGAWA, T. Studies on the cultivation of *Mycobacterium lepraemurium*. Lepro **43** (1974) 216-225.
17. OGAWA, T. and MOTOMURA, K. Studies on murine leprosy bacillus. I. Attempt to cultivate *in vitro* the Hawaiian strain of *Mycobacterium lepraemurium*. Kitasato Arch. Exp. Med. **43** (1970) 21-36.
18. STEERE, R. L. Electron microscopy of structural detail in frozen biological specimens. J. Biophys. Biochem. Cytol. **3** (1957) 45-63.
19. TAKEO, K., NONAKA, T. and NISHIURA, M. Freeze-etching observation of various mycobacterial walls and extracellular material adhered to them. Unpublished data.
20. TODA, E. and NISHIURA, M. Electron microscopic difference of human and murine lepra cells. Abstract in Lepro **32** (1963) 166-167.
21. VOILAND, A. BRUNETEAU, M. and MICHEL, G. Etude du mycoside C_2 de *Mycobacterium avium*. Eur. J. Biochem. **21** (1971) 285-291.
22. YAMAMOTO, T., NISHIURA M., HARADA, N. and IMAEDA, T. Electron microscopy of ultrathin sections of lepra cells and *Mycobacterium leprae*. Int. J. Lepr. **26** (1958) 1-8.