INTERNATIONAL JOURNAL OF LEPROSY

Volume 50, Number 1
Printed in the U.S.A.

Ultrastructural Features of the Multiplication of Human and Murine Leprosy Bacilli in Macrophages of Nude Mice[1]

Yukiko Fukunishi, Seitaro Okada, Mitsugu Nishiura, and Kenji Kohsaka[2]

The main purpose of the present experiment was to study the ultrastructural features of the growth of *M. leprae* and *M. lepraemurium* in macrophages of nude mice. When the lesions of human and murine leprosy are examined by electron microscopy using ultrathin sections ([6,8,9]), it becomes clear that the two species of mycobacteria produce slightly different electron-transparent zones around the bacillary bodies in their respective host cells. In the case of *M. leprae* ([8]), the electron-transparent zones have a tendency to coalesce with each other and to form distinct intracytoplasmic foamy structures when the lesion becomes old. On the contrary, in the case of *M. lepraemurium*, an electron-transparent zone is formed around each bacillus, and the typical foamy structure like that of human lepra cells is never formed.

In 1970 Draper and Rees ([1]) reported ribbon-like structures in murine leprosy bacilli observed by negative staining. In 1972 ([4]) and 1977 ([5]) Nishiura, *et al.* reported the differences in peribacillary substances in human and murine leprosy bacilli observed by the freeze-etching technique. Many small spherical droplets accumulated around *M. leprae* in phagolysosomes in human lepra cells. They corresponded to the electron-transparent zone and foamy structures around *M. leprae*. In the case of *M. lepraemurium* growing in murine lepra cells, a peculiar membranous or crystalline substance was found around their bacillary bodies. In 1973 Draper, *et al.* ([2]) reported freeze-etching findings of the peribacillary substance of *M. lepraemurium* in the C3H strain mouse and identified this substance as mycoside C by detailed biochemical analysis. From the above studies, it is clear that the peribacillary substances around *M. leprae* and those around *M. lepraemurium* differ very much from each other.

In 1960 Shepard ([7]) described the multiplication of *M. leprae* in mouse foot pads, and made a breakthrough in animal experiments on leprosy. In 1976, Kohsaka, *et al.* ([3]) reported the formation of lepromatoid lesions in the foot pads of nude mice inoculated with *M. leprae* and bred under specific pathogen free (SPF) conditions in a vinyl isolator. They inoculated 10^4 *M. leprae* into each foot pad and confirmed the growth of lepromata after a year and a few months.

In 1977, when we compared the ultrastructures of human lepra cells containing *M. leprae* and murine lepra cells of C3H strain mice caused by *M. lepraemurium*, it was difficult to decide whether the ultrastructural differences observed were due to the difference of mycobacteria or due to the difference of macrophages.

When it became possible to grow *M. leprae* in nude mouse foot pads, we decided to compare the ultrastructural morphology of the peribacillary substances of these two species of mycobacteria in the same kind of host cell, namely the macrophage of the nude mouse.

MATERIALS AND METHODS

Inoculation of *M. leprae*. A suspension of *M. leprae* was prepared from a leproma from a patient with lepromatous leprosy provided by the Leprosy Research Labo-

[1] Received for publication on 5 January 1981; accepted for publication in revised form on 27 August 1981.

[2] Y. Fukunishi, M.D., Ph.D., Medical Officer, National Sanatorium Oshima Seisho-en, Aji-cho, Kida-gun, Kagawa Pref. 761-02, Japan; S. Okada, M.D., Ph.D., Associate Professor; M. Nishiura, M.D., Ph.D., Professor, Leprosy Research Laboratory, Kyoto University School of Medicine, Sakyo-Ku, Kyoto 606, Japan; K. Kohsaka, Ph.D., Assistant Professor, Department of Leprology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita City, Osaka, Japan.

ratory, Kyoto University School of Medicine. After mincing the leproma with scissors, a bacillary suspension was made with Hanks' solution by grinding in a mortar, and 10^7 *M. leprae* in a volume of 0.03 ml were inoculated into the right hind foot pads of 30 each of nude mice (nu/nu) bred off a BALB/c background and bred under SPF conditions using a vinyl isolator. Food and water were given after complete sterilizing by autoclaving.

Inoculation of *M. lepraemurium*. A bacillary suspension was prepared from a C3H strain mouse inoculated with *M. lepraemurium* by the same method as for that of *M. leprae*. *M. lepraemurium*, 10^7 in a volume of 0.03 ml, were inoculated into right hind foot pads of each of 10 nude mice bred under conventional conditions.

Electron microscopy. The tissues of sacrificed nude mice inoculated with *M. leprae* or *M. lepraemurium* were fixed with 3% glutaraldehyde in 0.06 M phosphate buffer (pH 7.4) for 24 to 48 hr for ultrathin section study, washed three times with the buffer, and post-fixed with 2% OsO_4 in distilled water for 14 to 24 hr at 4°C. After dehydration with a graded ethanol series, the tissues were embedded in methacrylate, cut by an ultra-microtome with glass knives, and then stained with uranyl acetate and lead citrate.

The tissues for study by freeze-etching techniques were fixed with 3% glutaraldehyde under the same conditions as those for ultrathin sectioning, and then immersed in 20 to 40% glycerol for 1 to 2 days at 4°C. Other procedures were the same as described previously ([5]).

Other examinations. *M. leprae* obtained from human lepra cells and from experimental nude mouse lepromata and *M. lepraemurium* from the C3H murine leprosy lesions and from the experimental nude mouse lesions caused by *M. lepraemurium* inoculation were examined by the dopa-oxidase test ([13, 14]) and the pyridine extraction test ([15]).

RESULTS

Macroscopic findings.

a) Lesions in nude mice caused by *M. leprae* inoculation. Foot pads, spleen, liver and skin were examined. Specimens were taken at 24 hr and at 1, 2, 3, 5, 6, 10, 14 and 18 months after inoculation respectively.

FIG. 1. Arrow indicates a swollen right hind foot pad of a nude mouse inoculated with *M. leprae*.

FIG. 2. Arrows indicate lepromata in the spleen of a nude mouse inoculated with *M. leprae*.

FIG. 3. Arrows indicate two lepromata originating from the lymph nodes of a nude mouse inoculated with *M. leprae*.

FIG. 4. Arrows indicate lepromata of a nude mouse inoculated with *M. leprae*.

FIG. 5. Arrow indicates a leproma of a nude mouse inoculated with *M. leprae*.

No lesions were found up to 8 months after inoculation, but almost all inoculated right hind food pads became swollen about 10 months after inoculation. Twelve months after inoculation, the inoculated right hind foot pads, the spleen and some lymph nodes showed distinct lepromata (Figs. 1, 2, 3, 4, and 5). When the lesions of this stage were examined by electron microscopy, a large number of bacilli inside phagolysosomes of macrophages were observed.

Lesions in nude mice caused by *M. lepraemurium* inoculation. Foot pads, spleen, liver and skin were studied at 24 hr, 25, 30, and 35 days and 2, 3, 5, and 6 months after inoculation.

Macroscopic signs appeared by 1 month

after inoculation, when the inoculated right hind foot pads became swollen. When the lesions were examined by electron microscopy, a large number of bacilli were seen inside macrophages. Two months after inoculation, the tails and backs of some of these nude mice also showed lepromata and they soon became ulcerated. The growth of murine leprosy lesions in nude mice was much faster than that seen in C3H strain mice.

Ultrastructural findings.

a) Ultrastructural features of the multiplication of *M. leprae* inside nude mouse macrophages. *M. leprae* multiply in phagolysosomes of macrophages of nude mice in essentially the same fashion as they do in human lepra cells. In ultrathin sections, electron-transparent zones or intracytoplasmic foamy structures were seen around the growing leprosy bacilli (Fig. 6), but opaque droplets (lysosomes), which can be seen in human lepra cells, were less frequently encountered in nude mouse macrophages. The findings with freeze-etching of the nude mouse lesions were strikingly similar to those in human lepra cells. Almost all of the bacilli in these nude mice lesions were long and slender, and had band structures on smooth cell wall surfaces like those in human lepra cells (Fig. 7). Distinct accumulations of small spherical droplets were observed inside phagolysosomes (Fig. 8).

b) Ultrastructural features of the multiplication of *M. lepraemurium* inside nude mouse macrophages. The fundamental ultrastructural features of the multiplication of *M. lepraemurium* inside the macrophages of nude mice (Fig. 9 and 10) were identical to those seen in the macrophages of C3H strain mice (Fig. 11). In ultrathin sections, distinct electron-transparent zones were observed around each bacillus (Fig. 9), but intracytoplasmic foamy structures were not seen. In freeze-etching pictures, crystalline material was seen around each bacillus growing in the phagosomes of nude mice (Fig. 10). Contrary to the findings in human lepra cells and in nude mouse macrophages infected with *M. leprae*, no spherical droplets were observed in the lesions of nude mice caused by *M. lepraemurium*. *M. lepraemurium* in nude mouse macrophages are surrounded by membranous or crystalline material just as they are in murine lepra cells in C3H strain mice. Almost all of the bacilli in these nude mice inoculated with *M. lepraemurium* were long and slender, and band structures were seen on them.

→

FIG. 6. Electron-microscopic finding of intracytoplasmic foamy structures inside the macrophages of nude mouse inoculated with *M. leprae* by ultrathin sectioning. N = nucleus of macrophages. (Scale 1 μm, Magnification ×8500.)

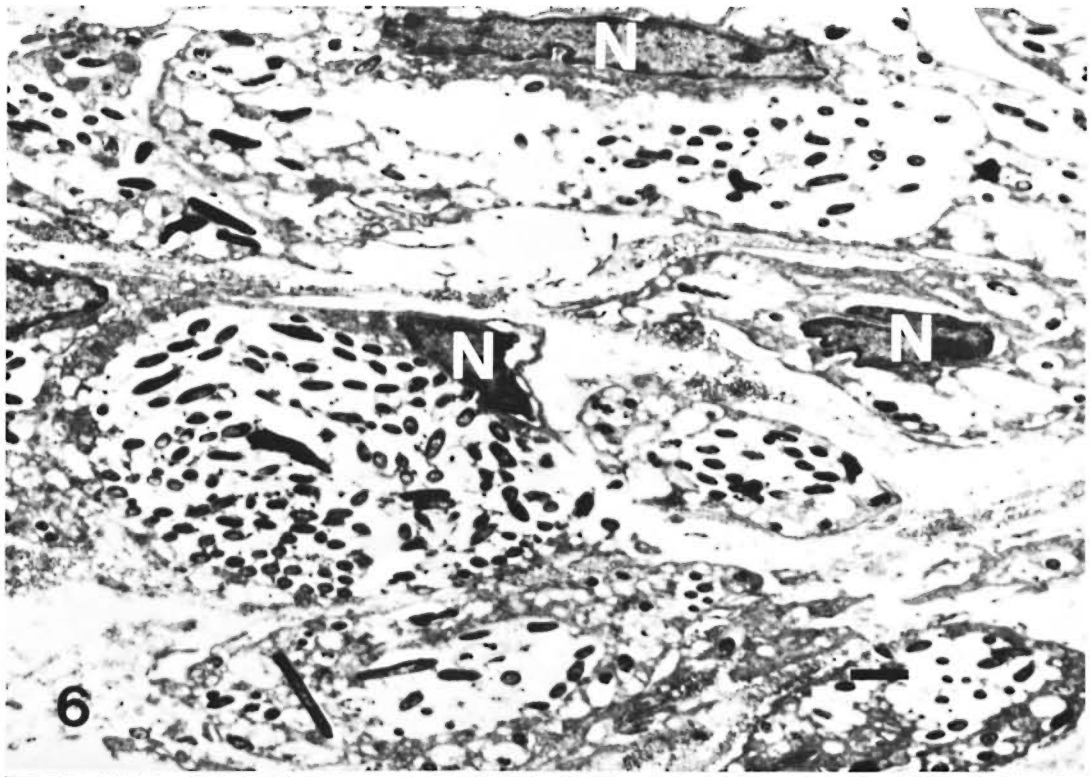
FIG. 7. Electron microscopic finding of many spherical droplets (**S**) around leprosy bacilli (**B**) inside a macrophage of a nude mouse inoculated with *M. leprae* by freeze-etching. Arrows indicate band structures on the cell wall of leprosy bacilli. (Scale 1 μm, Magnification ×39,000.)

FIG. 8. Electron microscopic finding of intracytoplasmic foamy structures inside the phagolysosome of a macrophage of a nude mouse inoculated with *M. leprae* by freeze-etching. A large amount of spherical droplets (**S**) are observed around the leprosy bacilli (**B**) inside the phagolysosome. (Scale 1 μm, Magnification ×15,500.)

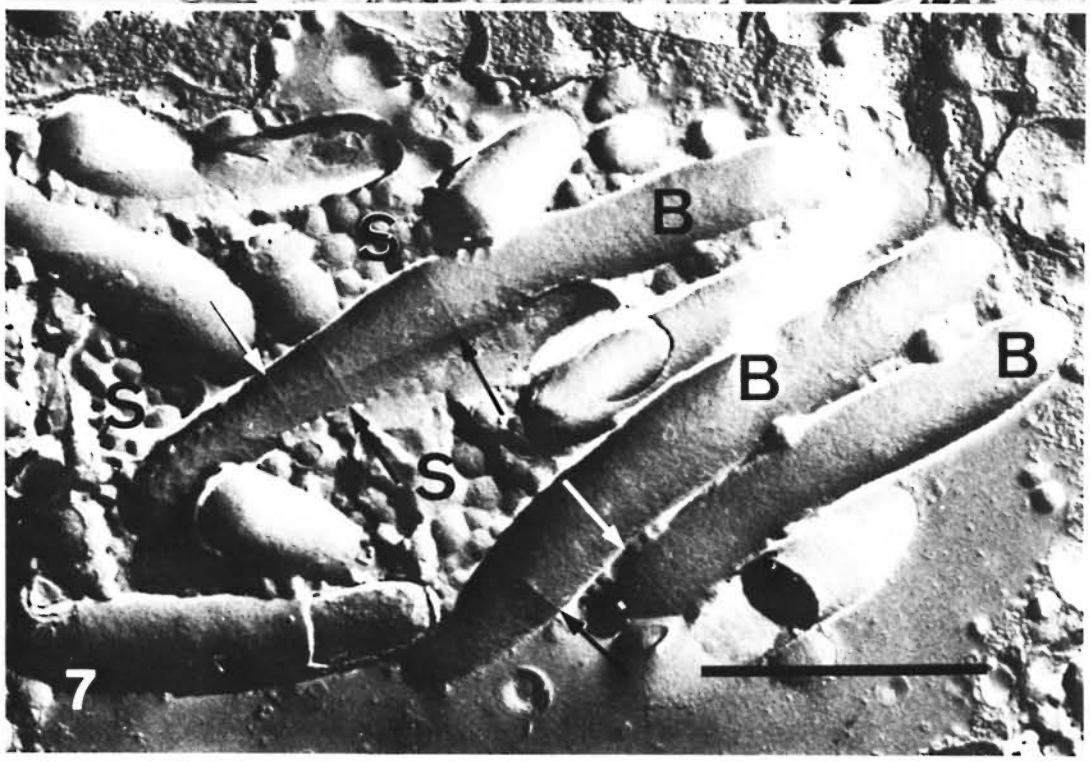
FIG. 9. Electron microscopic findings of murine leprosy bacilli inside the macrophage of a nude mouse inoculated with *M. lepraemurium* by ultrathin sectioning. Arrows indicate electron-transparent zones (**E**) around murine leprosy bacilli (**B**). (Scale 1 μm, Magnification ×22,000.)

FIG. 10. Electron microscopic finding of murine leprosy bacilli inside the macrophage of a C3H strain mouse inoculated with *M. lepraemurium* by freeze-etching. Thick arrows indicate crystalline materials (**C**) around the murine leprosy bacilli (**B**). Thin arrows indicate band structures on the cell wall of the murine leprosy bacilli. (Scale 1 μm, Magnification ×36,500.)

FIG. 11. Electron microscopic finding of murine leprosy bacilli inside the macrophage of a nude mouse inoculated with *M. lepraemurium* by freeze-etching. Thick arrows indicate crystalline materials (**C**) around the murine leprosy bacilli (**B**). Thin arrows indicate band structures on the cell wall of the murine leprosy bacilli. (Scale 1 μm, Magnification ×51,000.)

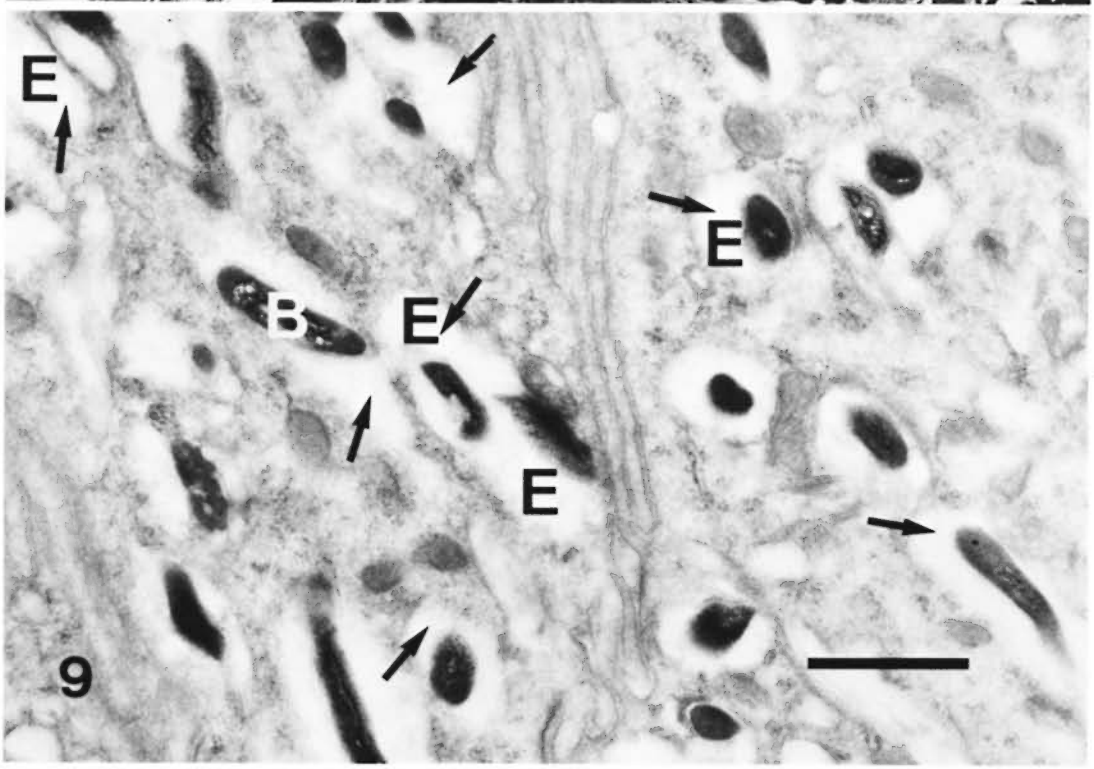


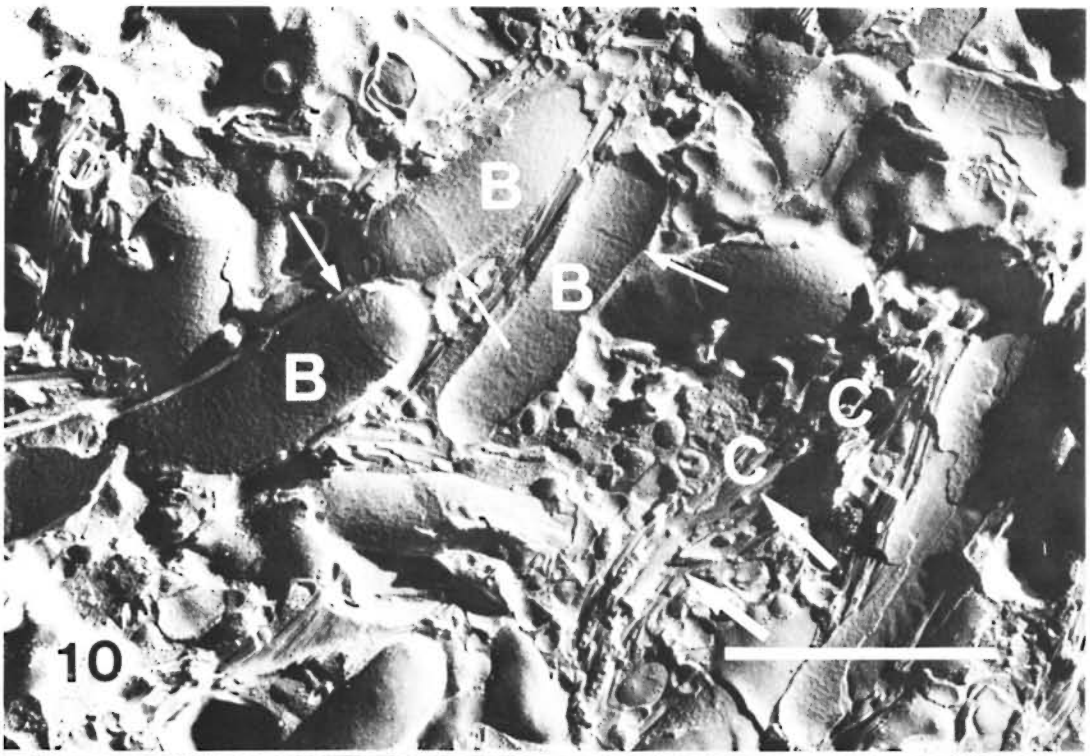
6

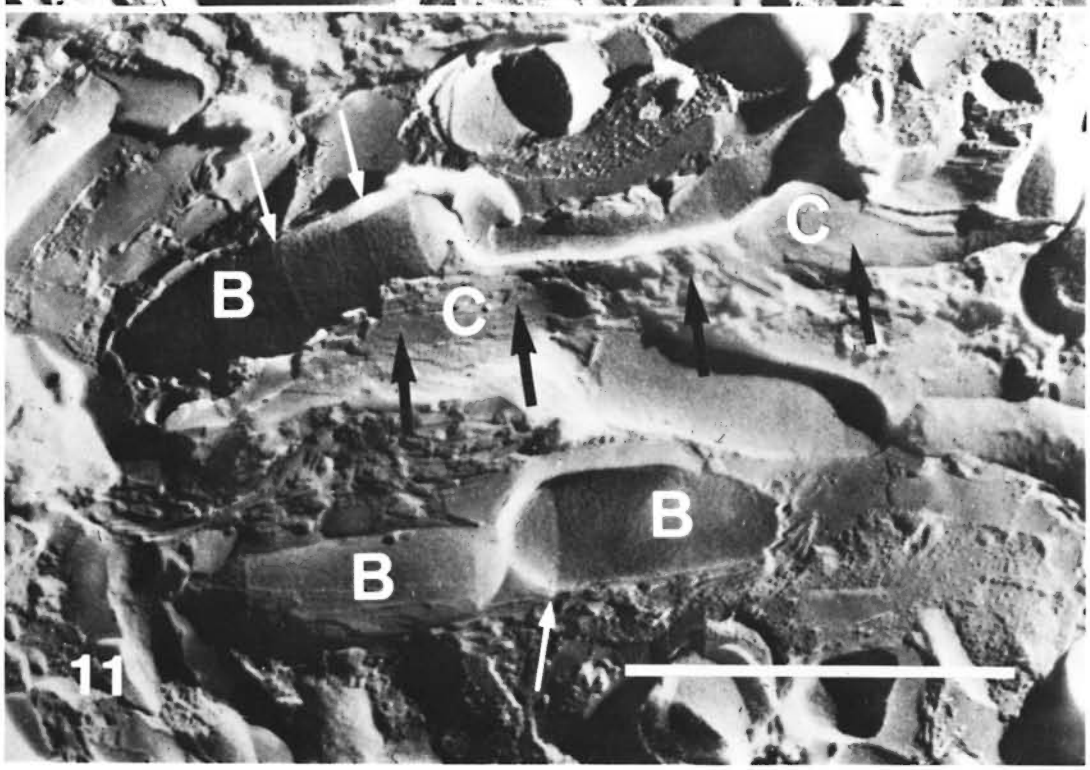


7


B
B
B
S
S
S
8


E
E
B
E
E
9


B
B
B
C
C
10


B
C
C
B
B
11

THE TABLE. *A summary of ultrastructural findings with the multiplication of* M. leprae *and* M. lepraemuriam *in different host cells.*

	Human lepra cell	Nude mice inoculated with *M. leprae*	C3H Mice inoculated with *M. lepraemuriam*	Nude mice inoculated with *M. lepraemuriam*
Spherical droplets	Positive	Positive	Negative	Negative
Crystalline material	Negative	Negative	Positive	Positive
Band structure	Positive	Positive	Positive	Positive
Form of bacilli	Long and slender	Long and slender	Long and slender	Long and slender
Dopa-oxidase test	Positive	Positive	Negative	Negative
Pyridine extraction test	Positive	Positive	Negative	Negative

These findings and the results of the dopa-oxidase tests and the pyridine extraction tests are shown on The Table.

DISCUSSION

The present findings demonstrate by electron microscopy that the ultrastructural features around *M. leprae* and *M. lepraemurium* inside phagolysosomes or phagosomes of nude mouse macrophages show striking differences from each other. *M. leprae* growing inside nude mouse macrophages produce the same ultrastructural changes as those in human lepra cells. On the other hand, *M. lepraemurium* produce crystalline material around the bacillary bodies inside the phagosomes of the nude mouse macrophages. This finding is the same as that in murine lepra cells of the C3H strain mouse. In the present experiment, the host cells for both *M. leprae* and *M. lepraemurium* were the macrophages of nude mice. Thus, differences produced in these macrophages by inoculations with *M. leprae* and *M. lepraemurium* respectively seem clearly to be due to the differences between the metabolism of *M. leprae* and that of *M. lepraemurium*.

In 1977 Nishiura, *et al.* ([5]) and Takeo, *et al.* ([10]) examined the peribacillary substance of various cultivable mycobacteria by freeze-etching. Of the 14 species of cultivable mycobacteria which were examined, none showed the spherical droplet as observed around *M. leprae* in both human and nude mouse macrophages.

On the other hand, peribacillary spherical droplets similar to those found around *M. leprae* have been seen in experimental armadillo (*Dasypus novemcinctus*) leprosy lesions ([12]) and, in recent studies, in some of the armadillos with naturally acquired leprosy-like disease ([11]).

The nature of the mycobacteria in naturally acquired leprosy-like disease of armadillos is not fully clarified as yet. However, when *M. leprae* multiply in various host cells (human, nude mouse, and armadillo macrophages), spherical droplets always appear around the organisms growing in macrophages. Thus, the spherical droplets around *M. leprae* are most probably made up of specific substances produced by the multiplication of *M. leprae* in suitable host cells.

SUMMARY

Ultrastructural features of the growth of *M. leprae* and *M. lepraemurium* in nude mouse macrophages were studied by ultrathin sectioning and freeze-etching. In nude mouse macrophages, *M. leprae* produced spherical droplets (foamy structures) similar to those in human lepra cells. On the other hand, *M. lepraemurium* produced typical crystalline material in nude mouse macrophages, which is quite the same as that observed in the C3H strain mouse. Spherical droplets in the form of foamy structures seem to be made up of a specific substance produced by the multiplication of *M. leprae* in suitable host cells (human, nude mouse, and armadillo macrophages).

RESUMEN

Se estudiaron las características ultraestructurales del crecimiento del *M. leprae* y del *M. lepraemurium* en los macrófagos de los ratones desnudos por las técnicas de microscopía en cortes ultradelgados y de impresión por congelación (freeze-etching). En los

macrófagos de los ratones desnudos, el *M. leprae* produjo gotitas esféricas (estructuras espumosas) similares a las observadas en las células de la lepra humana. Por otro lado, el *M. lepraemurium* produjo, en los macrófagos de los animales desnudos, un típico material cristalino muy similar al observado en la cepa de ratones C3H. Las gotitas esféricas en forma de estructuras espumosas parecen estar hechas de una substancia específica producida por la multiplicación del *M. leprae* en células huésped susceptibles (macrófagos humanos, de ratón desnudo, y de armadillos).

RÉSUMÉ

On a étudié les caractéristiques de l'ultrastructure de *M. leprae* et de *M. lepraemurium* en cours de croissance, chez des macrophages de souris glabres, au moyen de sections ultra-minces et de cryofractures. Dans les macrophages de la souris glabre, *M. leprae* produit des goutelettes sphériques (structures spumeuses) semblables à celles que l'on observe dans les cellules de la lèpre humaine. D'autre part, *M. lepraemurium* donne naissance, dans les macrophages de souris glabres, à du matériel cristallin typique, tout à fait semblable à celui que l'on observe chez les souris de la souche C3H. Les goutelettes sphériques, à l'aspect de structures spumeuses, semblent constituer une substance spécifique qui serait produite par la multiplication de *M. leprae* dans des cellules-hôtes appropriées (macrophages humains, de souris glabres, et de tatous).

REFERENCES

1. Draper, P. and Rees, R. J. W. Electron-transparent zone may be a defense mechanism. Nature **228** (1970) 860–861.
2. 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.
3. Kohsaka, K., Mori, T. and Ito, T. Lepromatoid lesion developed in nude mouse inoculated with *Mycobacterium leprae*—Animal transmission of leprosy. Lepro **45** (1976) 177–187.
4. Nishiura, M. Designing of a new freeze-etching apparatus. Kagaku **42** (1972) 431–438.
5. Nishiura, M., Izumi, S., Mori, T. and Nonaka, T. Freeze-etching study of human and murine leprosy bacilli. Int. J. Lepr. **45** (1977) 248–254.
6. Okada, S. I. Electron microscopic observation of murine and human leprosy bacilli. II. On the granules in the bacterial cells of murine leprosy bacilli. III. Optical and electron microscopic observation of murine leprosy bacilli. IV. Electron microscopic observation of leprosy bacilli by ultra-thin preparation. V. Electron microscopic observation of murine leprosy bacilli by ultra-thin preparation. Research Activities of the National Institute for Leprosy Research, Special Issue of "Leprosy in Japan" (1958) 97–106.
7. Shepard, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J. Exp. Med. **112** (1960) 445–454.
8. Yamamoto, T., Nishiura, M., Harada, N. and Imaeda, T. Electron microscopy of ultra-thin sections of lepra cells and *Mycobacterium leprae*. Int. J. Lepr. **26** (1958) 1–8.
9. Yamamoto, T., Nishiura, M., Harada, N. and Imaeda, T. Electron microscopy of *Mycobacterium lepraemurium* in ultra-thin sections of murine leprosy lesions. Int. J. Lepr. **26** (1958) 111–114.
10. Takeo, K., Nonaka, T. and Nishiura, M. Freeze-etching observation of various mycobacterial walls and extracellular material adhered to them. Unpublished data.
11. Nishiura, M., Fukunishi, Y., Okada, S., Abe, M., Kohsaka, K., Yoneda, K., Mori, T., Ito, T., Bretana, A., Convit, J., Walsh, G. P., Meyers, W. M. and Binford, C. H. A joint study on the naturally acquired leprosy-like disease of armadillos. Int. J. Lepr. **48** (1980) 493–494.
12. Fukunishi, Y., Meyers, W. M., Walsh, G. P., Johnson, F. B., Binford, C. H., Okada, S. and Nishiura, M. Ultrastructural features of the multiplication of leprosy bacilli in macrophages of armadillos inoculated with *M. leprae*. Int. J. Lepr. **48** (1980) 494.
13. Prabhakaran, K. Oxidation of 3,4-dihydroxyphenylalanine (DOPA) by *Mycobacterium leprae*. Int. J. Lepr. **35** (1967) 42–51.
14. Prabhakaran, K. A rapid identification test for *Mycobacterium leprae*. Int. J. Lepr. **41** (1973) 121.
15. Convit, J. and Pinardi, M. E. A simple method for the differentiation of *Mycobacterium leprae* from other mycobacteria through routine staining technics. Int. J. Lepr. **40** (1972) 130–132.