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Ultrastructural Features of Macrophages of Armadillos Infected with Actively Multiplying *Mycobacterium leprae*[1]

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In 1974, Yoshizumi, *et al.* ([13]) reported the electron-microscopic findings of lesions in the nerves of an armadillo (*Dasypus novemcinctus*) inoculated with *Mycobacterium leprae* and, in ultrathin sections, demonstrated intracytoplasmic foamy structures in the macrophages and Schwann cells of the peripheral nerves. This confirmation of foamy structures in the lesions of experimental leprosy in the armadillo is important for the identification of the etiologic agent as *M. leprae*; however, direct observation of the three-dimensional ultrastructure of the bacilli in the experimental lesions is not possible in ultrathin sections. Employing the freeze-etching technique, we have studied the three-dimensional ultrastructure of lesions in armadillos experimentally infected with *M. leprae*. This freeze-etching technique is highly useful because it reveals both the surface structure of the walls of the bacilli and the details of the intracytoplasmic foamy structures. We compared the findings in experimental leprosy in armadillos with those in lepra cells in humans and in macrophages of nude mice infected with *M. leprae* ([3, 9]).

MATERIALS AND METHODS

By ultrathin sectioning and freeze-etching, we examined the ultrastructure of experimental lesions in lepromas, lymph nodes, spleens, livers, and lungs of 7 nine-banded armadillos (*Dasypus novemcinctus*) inoculated with *M. leprae*. One armadillo was inoculated directly with 3.4×10^8 *M. leprae* isolated from a human leproma. All of the other six animals were inoculated with 2×10^{10} organisms isolated from lesions of armadillos infected with *M. leprae* of human origin that had been passaged once in armadillos. Specimens were obtained 18 months after inoculation from the armadillo inoculated with bacilli of human origin and 11–13 months after inoculation in the six animals which received bacilli from armadillo passage.

All tissues were from *M. leprae*-infected armadillos that had been killed with an overdose of ketamine hydrochloride. Specimens for ultrathin sectioning were fixed with 3% glutaraldehyde in 0.06 M phosphate buffer (pH 7.4) for 24–48 hr, and then with 2% OsO_4 in distilled water for 14–24 hr at 4°C. The tissues were embedded in methacrylate, cut by ultramicrotome, and then stained with uranium acetate and lead citrate. Tissues for study by the freeze-etching technique were fixed with 3% glutaraldehyde under the same conditions as for ultrathin sectioning, and then immersed in 20–40% glycerol for 1–2 days at 4°C. Other procedures were the same as described previously by Nishiura ([9]).

RESULTS

In 6 of the 7 armadillos, ultrathin sections demonstrated typical intracytoplasmic foamy structures (Fig. 1). In all of the armadillos the freeze-etched preparations revealed intracytoplasmic foamy structures in phagolysosomes. These structures contained small spherical droplets that appeared to have accumulated around the leprosy bacilli (Figs. 2–4).

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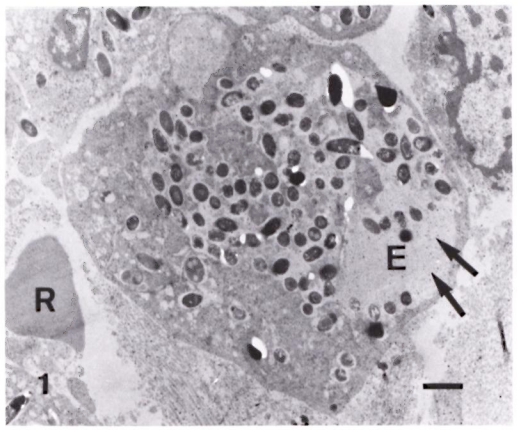

FIG. 1. Ultrathin section of leproma from an armadillo with experimental leprosy. Note the large number of leprosy bacilli in a phagolysosome of a macrophage. Arrows indicate an electron-transparent zone in the intracytoplasmic foamy structure (×10,000).

B = leprosy bacilli; E = electron-transparent zone; R = red blood cell; S = spherical droplet; scale = 1 μm.

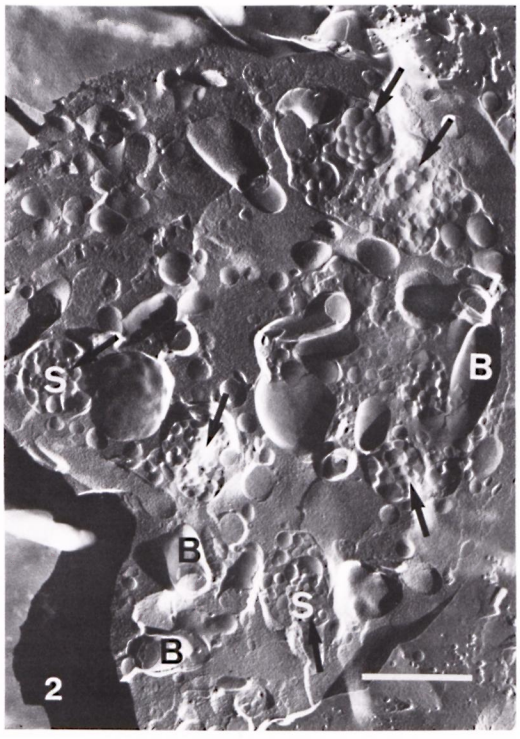

FIG. 2. Freeze etching of leproma from an armadillo with experimental leprosy. Arrows indicate spherical droplets in an intracytoplasmic foamy structure in a macrophage (×26,000). (See Fig. 1 for symbol legend.)

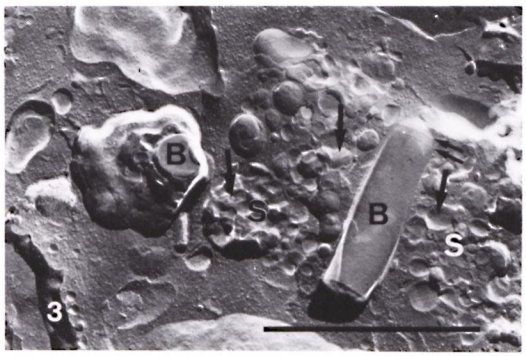

FIG. 3. Freeze etching of a lepra cell from an armadillo with experimental leprosy. A large amount of spherical droplets are observed around leprosy bacilli. Arrows indicate spherical droplets (×59,500). (See Fig. 1 for symbol legend.)

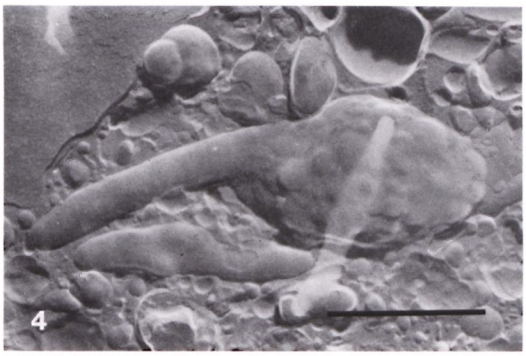

FIG. 4. Freeze etching of lepra cell from an armadillo with experimental leprosy. Observe the leprosy bacilli and spherical droplets surrounded by phagolysosomal membrane (×44,000). (See Fig. 1 for symbol legend.)

The organic solvents employed in tissue processing for ultrathin sections extract lipids from the foamy structures, and this extraction is believed to be responsible for the electron-transparent zones in the intracytoplasmic foamy structures in macrophages in the ultrathin sections. In freeze-etching replication, however, the fine details of the intracytoplasmic foamy structures are preserved as aggregations of small spherical droplets around bacilli in phagolysosomes.

Almost all of the bacilli in these armadillos were long and slender, with band structures visible on the smooth cell wall surfaces (Figs. 5–7). Each band is composed of two circumferential fine lines on the surface of the cell wall (Fig. 7). All of these characteristics of the cell wall and band

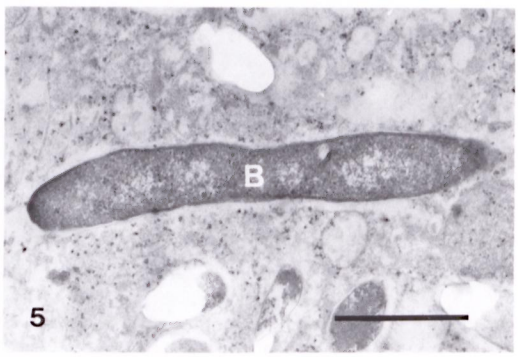

FIG. 5. Ultrathin section of leprosy bacilli in a lepra cell from an armadillo with experimental leprosy (×37,000). (See Fig. 1 for symbol legend.)

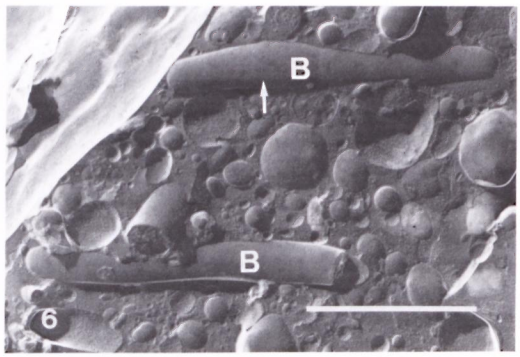

FIG. 6. Freeze etching of leprosy bacilli in a lepra cell from an armadillo with experimental leprosy. Bacilli are long and slender with band structures on the cell wall of the bacillus (arrow) (×47,000). (See Fig. 1 for symbol legend.)

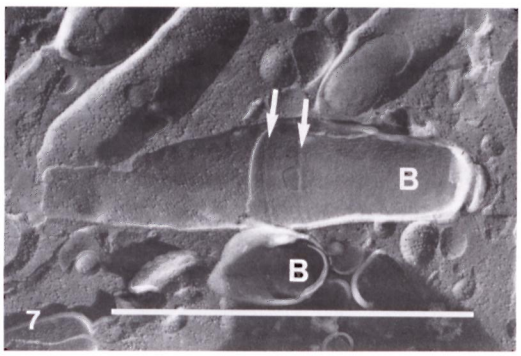

FIG. 7. Freeze etching of leprosy bacilli in a lepra cell from an armadillo with experimental leprosy. Arrows indicate band structures on the cell wall surface. Each band structure is composed of two thin lines (×100,000). (See Fig. 1 for symbol legend.)

structures of the bacilli are typical of those of *M. leprae* as seen in tissues from leprosy patients. The ultrastructural findings in lesions of armadillos infected with *M. leprae* obtained directly from patients and from first passage *M. leprae* were the same.

In one of the armadillos receiving passaged bacilli, there were early lesions only in the spleen. We believe that the *M. leprae* had not multiplied sufficiently in this animal to produce typical intracytoplasmic foamy structures; however, the bacilli showed the characteristic features described above.

DISCUSSION

The intracytoplasmic foamy structures in experimental armadillo leprosy have been confirmed by ultrathin sectioning (Yoshizumi, *et al.*[13]) and freeze etching (present study). Since similar intracytoplasmic foamy structures are consistently observed in human lepra cells and in nude mice lesions caused by inoculation with *M. leprae,* this foamy substance seems to have a close relationship to the multiplication of *M. leprae* in host cells ([3]).

However, similar foamy structures were also found in naturally acquired leprosy-like disease in armadillos by Marchiondo, *et al.* ([7]) and Walsh, *et al.* (personal communication). According to the studies already published, the pathogens of this natural leprosy-like disease in armadillos have biological characteristics very similar to those of human leprosy bacilli ([8, 12]).

Although morphological criteria including ultrastructural features are very useful for the detection of differences among different mycobacteria, they are not the decisive criteria for the identification of bacilli from experimental models. All of the ultrastructural, immunological, and biological data must be taken into consideration.

Kanai, *et al.* ([6]) studied the ultrastructural features of *M. bovis,* Ravenel R-KM strain, and reported that the electron-transparent zone between the phagosomal membrane and the bacillary cell wall is mostly artifact. They suggested that in the artifact-free condition the gap between the phagosomal membrane and the bacillary cell wall is very narrow and it might actually be a tight phagosome in which a bacillus is wrapped tightly by a phagosomal membrane. They did not report the presence of foamy struc-

tures around *M. bovis* in this study. In the case of *M. leprae* infections, leprosy bacilli are observed in tight phagosomes in the early stage of development of leprae cells, but as the leprosy bacilli multiply in the phagosomes of macrophages, lipidic material is accumulated and this always has the appearance of spherical droplets in the phagolysosomes of the lepra cells.

The ultrastructural features of the lipidic material which accumulates around *M. lepraemurium* are very much different from those of the spherical droplets of *M. leprae.* In the case of *M. lepraemurium,* the peribacillary substance is chiefly made up of crystalline material attached to the mycobacterial cell wall when observed by the freeze-etching technique. Chemically, this crystalline material is principally mycoside C according to Draper, *et al.* ([1, 2]). Takeo, *et al.* ([11]) studied 19 species of mycobacteria grown *in vivo* by the freeze-etching technique, and spherical droplet-like structures were never observed in any of the mycobacteria which they examined.

Recent studies by Brennan, *et al.* ([4, 5]) elucidated the biochemical structures of the peribacillary substances of *M. leprae* grown in armadillo tissue. The immunological data using these lipid materials from infected armadillo tissue, which are available now, suggest the identity of the peribacillary substance of bacilli in an experimental armadillo lesion and the peribacillary substance of *M. leprae* in human lepromatous tissue ([10, 14]). From the available data, it seems most probable that the peribacillary substance found in the armadillo lesion by freeze etching in this study corresponds to the lipidic substance which Brennan's group studied.

As to the forms of mycobacteria found in experimental armadillo leprosy, the bacilli were usually slender and smooth surfaced with band structures located on the surface of the cell wall just like human leprosy bacilli.

SUMMARY

Experimental leprosy lesions in the armadillo (*Dasypus novemcinctus*) were studied by freeze etching and ultrathin sectioning. Infected macrophages have distinct intracytoplasmic foamy structures in the form of spherical droplets accumulated around multiplying bacilli. This finding is the same as those observed in human lepra cells and nude mice macrophages infected with *M. leprae.*

RESUMEN

El estudio al microscópio electrónico de las lesiones experimentales en el armadillo (*Dasypus novemcinctus*) por la técnica de impresión por congelamiento y en secciones ultradelgadas, mostró la presencia de estructuras espumosas peculiares en los macrófagos de las lesiones. Las características de tales estructuras espumosas son las mismas que las de las "células de la lepra" en los humanos y que las de los macrófagos de ratones desnudos infectados con *Mycobacterium leprae.*

RÉSUMÉ

Une étude menée par microscopie électronique sur les lésions de lèpre expérimentale chez le tatou (*Dasypus novemcinctus*) par cryodécapage et des coupes ultra minces a révélé des structures intracytoplasmique spumeuses distinctes dans les macrophages des lésions. Les caractéristiques de ces structures spumeuses étaient les mêmes que celles que l'on relèvent dans des cellules de lèpre humaine et dans des macrophages de souris glabres infectées par *Mycobacterium leprae.*

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