# ELECTRON MICROSCOPE STUDIES OF THE MURINE LEPROSY BACILLUS[1]

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The specimens of murine leprosy bacilli employed for observation with the electron microscope were as follows: (1) bacilli from untreated animals; (2) bacilli stained with a dye, or treated with chemicals or ferments; (3) ultra-thin sectioned bacilli; (4) ultra-thin sectioned murine lepra cells, and bacilli in those cells which had not undergone the artificial effects of separation, the relations between the cells and bacilli being studied; (5) fractions obtained from fractional centrifugation of bacilli destroyed by repeated freezing and thawing, the granules isolated from the bacilli being studied microchemically.

For comparison the bacilli, and especially their granules, stained with various double stains were observed with the optical microscope. The electron- and optical-microscopic structures were compared with each other, to study their significance, and in the optical microscope work the results of different staining of the same bacillus were compared.

## RESULTS

Various results were obtained in this study. Those here reported concern only two problems, namely, the various granules of the murine leprosy bacilli, and the relation between lipids and bacilli.

With the electron microscope, in the untreated bacilli there were sometimes demonstrated granules with distinct boundaries, like the so-called A-body of tubercle bacilli. However, most of the large granule-like structures seen were irregularly shaped and indistinctly bordered, and they should be called dense zones rather than granules. Granule-like structures were frequently observed with the optical microscope in the bacilli stained with various stains, but these are not actual granules except nuclei, mitochondria and some kinds of granules, but correspond to the electron-dense zone.

The dense zone is considered to be the area in which various substances and functions are aggregated densely. The following can be cited as examples. The granules stained positively with Altmann's mitochondria stain coincided well with the gram-positive granules. Heidenhain's iron hematoxylin stain produced the same result. Osmophilic granules also appeared to coincide with the gram-positive granules, accordingly also

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the granules positive with mitochondria stains such as Altmann's and Heidenhain's method. But in the observations with the electron microscope the osmophilic granules were much larger than mitochondria-like structures which could be found in the ultra-thin sections of bacilli, and most of them were irregular dense zones rather than granules. The optical-microscope granules are not always real granules under the electron microscope. The gram-positive or osmophilic granules may not be mitochondria. However, it can be concluded at least that some areas including or neighboring mitochondria show marked gram-positiveness or osmophilia.

In the ultra-thin sections, few sharply-defined large granules were seen, except mitochondria-like structures which mostly existed in the dense zone. And the granule-like structures observed with the optical microscope after various stainings also existed in the part corresponding to the dense zone. Therefore, it is concluded that the dense zone is a part in which various substances and functions are aggregated densely, and cannot be explained by only one interpretation.

Coincidence of the existing places of granules positive with the Gram stain, Altmann's stain, Heidenhain's stain or osmium tetroxide treatment is due to this fact. The existence of polysaccharide could not be proved in any granules, visible with the optical microscope, that had been isolated from the destroyed bacilli. Therefore, the granule-like structures that are positive with the polysaccharide stain are not real granules, but merely zones in which polysaccharide is concentrated.

The murine leprosy bacilli employed were destroyed by repeated freezing and thawing. This treatment, repeated 82 times, was followed by centrifuging at 4,000 r.p.m. for 30 minutes, after which the supernatant was centrifuged at 11,000 r.p.m. for 40 minutes and divided into supernatant and sediment. The former was again centrifuged, at 38,000 r.p.m. for 40 minutes. The sediments were washed with distilled water.

In the sediment of the 11,000 r.p.m. rotation there were found large granules 100-200 mμ in diameter. In the sediment of the 38,000 r.p.m. rotation were found rather flat granules, nearly 50 mμ in diameter, which resembled the granules of tubercle bacilli which have been called "particulate partile" by Yamamura; and also numerous minute spherical granules 15-20 mμ in diameter. In the last supernatant, fine rod- or rosary-shaped granules were observed, in addition to these spherical granules.

Some large granules contained DNA, and others contained RNA. Some large granules could be stained positively with mitochondria stains such as Altmann's and Heidenhain's. Some of them were gram-positive, and others gram-negative. At any rate, these large granules are not identical. Some may correspond to a nucleus, and some others may be mitochondria, or the so-called A-body, or other particles.

As regards the minute, 15-20 mμ granules, the orcein-HCl reaction of

both the suspension of the sediment and the supernatant after 38,000 rotation failed to be positive, owing to the scanty samples. From the findings of methyl green-pyronin and Gram stains of the sediment, and the electron microscope pictures of the granules after treatment with RNAase and 1M/NaCl, the minute granules seemed to contain RNA. From the electron microscope appearance after trypsin digestion, they seemed also to contain protein. They were negative for fat.

Judging from the characteristics of the positive Gram stain of the sediment, Gram positiveness of the murine leprosy bacilli seems to be due to that of these granules in the cytoplasm of the bacilli. Considering the existence of RNA, and the size, shape, etc., of the minute granules, they seemed to have some significance like that of the fine granules composing the endoplasmic reticulum of various animal cells, and to be related to protein synthesis and other intracellular metabolism, although the existence of ferments in them remained uncertain because of the difficulty of obtaining a sufficiently large amount of the sample to determine them.

Cell walls distinctly observed in the ultra-thin section preparation of bacilli were 10-20 mμ thick, even, and of high density. Some of the findings frequently seen in the collected bacilli appeared as if the cell wall had been stripped off from the cytoplasm and swelled out. This seemed certainly to be a result of mechanical force, or of osmotic pressure owing to the use of distilled water during the collecting manipulation.

On the other hand, in some bacilli an amorphous substance of low density but different from that of the background filled a space between outward-bulging cell wall and the dense cytoplasm, and in such places, conversely, the ordinary cytoplasm was often made thin. This condition was occasionally observed among the collected bacilli, and also in bacilli in the ultra-thin sections of the murine leproma, and for that reason it is difficult to consider that it was an artifact due to the manipulation in the collection of the bacilli. This figure resembles closely those which can occasionally be observed by the optical microscope among bacilli stained with Sudan III, but the same bacilli stained by the Ziehl-Neelsen method after Sudan III staining showed no such swollen parts at all, and were not distinguishable from usual rod-shaped ones.

The bacilli that had not been destroyed by the repeated freezing and thawing described were stained with Sudan III and observed with the optical microscope. They were thicker in appearance than after the Ziehl-Neelsen stain. In most of them an unstained inner part was bordered by a stained outer part, thus showing an annular figure. Sometimes a stained transverse zone could be seen, in addition to the annular zone. The bacilli stained by Ziehl-Neelsen following Sudan III revealed a contrary result, namely, the inner part not stained with Sudan III was stained by the fuchsin, and the septum-like part positively stained with Sudan

III did not stain with Ziehl-Neelsen to show segmentation of the bacilli. The same results were obtained with Sudan black or osmium tetroxide.

On electron microscope observation of these bacilli unstained, a dense zone about 50-140 mμ in width was found to encircle the bacilli. This density is assumed to be due to lipoid.

Such staining results and osmophilic structure were also found in bacilli which had not been subjected to any treatment such as freezing and thawing. From these findings it seems that the lipoid which stains with Sudan III has no concern with the acid-fast substance. Deformation of bacilli stained with Ziehl-Neelsen, such as the segmented or rosary-like appearance, may represent fatty degeneration of bacilli.

Some of the large granules isolated from destroyed bacilli contained fat. But, as said, the minute (15-20 mμ) granules did not contain fat.

In bacilli kept in some medium for a long period, for the study of cultivation of bacilli, granules of various sizes that stained black with Ziehl-Neelsen could occasionally be observed, especially in the ends of bacilli. These granules contained fat. Sometimes those granules were separated from cell bodies to fuse into larger granules. Those also represented a kind of fatty degeneration.

When murine leprosy bacilli were observed with the electron microscope after Ziehl-Neelsen staining, the inner part with high density which seemed to be stained with fuchsin was distinctly bordered by a peripheral clear zone about 60-150 mμ in width which seemed not to be stained with fuchsin. This width coincided well with the width of the annular dense zone containing fat in some bacilli as described above. Although no zone of this width can be distinguished from the inner part in the electron microscope observation of unstained bacilli, except some bacilli with a dense annular zone, this zone is considered to have some specific significance. At any rate, the amount and distribution of lipoid varies much with individual bacilli.

Slightly elevated folds running across the surface of bacilli were occasionally observed. Short bacilli which looked as if cut transversely, and bacilli of ordinary size in which the transverse line was found in the ultra-thin sections, were sometimes found. In some cases a part with low density, or that looked like a ghost cell, was separated from another, normal, part by the transverse line. All these findings lead me to the supposition that a process of demarcation followed by fragmentation different from ordinary cell division may happen in the murine leprosy bacilli.