A COMPARATIVE STUDY BY ELECTRON MICROSCOPY OF THE MORPHOLOGY OF MYCOBACTERIUM LEPRAE AND CULTIVABLE SPECIES OF MYCOBACTERIA¹⁻²

FRANCIS W. BISHOP AND LEIF G. SUHRLAND University of Rochester School of Medicine and Dentistry Rochester, New York AND CHARLES M. CARPENTER School of Medicine, University of California Los Angeles, California

The usefulness of the electron microscope in the study of bacteria is well established. It has provided a new technique for studying the internal structure of the cell, not revealed by the present methods of light microscopy. It should yield clues which will lead to a better understanding of the reactions between infectious and bactericidal agents, as well as those between host and parasite in disease.

The differentiation of a bacterial cell wall from a fluid or potentially fluid protoplasm has been clearly observed in electron micrographs of several species of the genus bacillus (1, 2). Other observers have shown capsules, vacuoles, cytoplasmic membranes, the distribution of granules of various types, and nuclear structures in a number of micro-organisms (3, 4, 5, 6). The present studies were undertaken to determine whether similar findings could be demonstrated in Mycobacterium leprae. That organism is difficult to study because cultures are not available and the cells must be obtained from suspensions of lepromatous tissue. The tenacity with which the human strain adheres to tissue cells makes their separation particularly arduous. The bacillus of murine leprosy, on the other hand, is more readily freed from tissue elements, and thus a source of material can be obtained more readily. Although most of the observations were made on M. leprae murium (Puerto Rican strain) and on M. leprae, electron micrographs of several other species of mycobacteria were studied for purposes of comparison.

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MATERIALS AND METHODS

The technique for obtaining acid-fast bacilli from suspensions of tissue for electron micrography was as follows:

A leproma was removed aseptically, minced with scissors, and ground in a sterile mortar with from 3.0 to 5.0 ml. of an 0.85 per cent saline solution. After thorough grinding, sufficient saline solution was added to make approximately a 5.0 per cent suspension of the tissue. It was then freed of coarser tissue particles by filtration through several layers of sterile gauze. The resulting suspension was allowed to stand for 30 minutes. The supernatant was removed, centrifuged, resuspended in saline, and recentrifuged, after which the supernatant was removed and discarded. The top layer of the sediment, which contained masses of lepra bacilli, was then carefully removed with a small loop and resuspended in saline. This suspension was centrifuged and the sediment was again washed, after which a final suspension was made in distilled water.

Suspensions of the other acid-fast organisms studied were prepared from cultures grown on Dubos' at 37° C. When growth was evident microscopically the cultures were centrifuged and the cells were washed three times in saline solution; the final suspension was made in distilled water. The incubation period varied with the organism concerned. *M. tuberculosis* and the vole bacillus were harvested after incubation for 14 days. *M. phlei* and *M. smegmatis* were incubated only 72 hours. A loopful of a suspension of bacteria in distilled water was placed on the specimen screen, which had been covered with a thin collodion film. The excess water was removed with filter paper and the specimen permitted to dry. It was then placed in the electron microscope and a suitable field located and photographed according to standard procedures. An RCA Universal Model electron microscope, Type EMU, in which the voltage was fixed at 50 k. v. was employed. The magnification was usually set at 6,100 X directly on the plate.

OBSERVATIONS

A comparative study of the electron micrographs of the different species of mycobacteria, especially M. leprae murium, has shown interesting differences which are presented in the Description of Plates.

DISCUSSION

The findings presented in this report are preliminary studies of more extensive comparisons now in progress. Inasmuch as M. leprae has not been cultivated in vitro, electron microscope studies are especially valuable in furthering our knowledge of its morphology and physiology. The electron microscope reveals internal structures in this micro-organism as well as in other acid-fast species, which cannot be seen with the ordinary light microscope. Conspicuous variations of internal structure are revealed in different cells from the same source in the electron micrographs. The significance of these variations in terms of viability, cell division, and autolytic changes can be determined only after a long-continued study. Photographs of a large number of acid-fast bacilli under a variety of conditions and environments are necessary before definite conclusions on their morphology can be drawn.

The polar bodies of increased density seen in all the species of mycobacteria examined are similar to those observed in *B. mycoides* by Knaysi and Baker (7). They observed at least two distinct types of internal bodies, designated as types A, and B, which were differentiated on the basis of their capacity, size, form, and behavior. Type A is large and opaque and shows no definite signs of internal arrangement; these bodies they identified as nuclei of the cells. This type is well illustrated in the electron micrographs of the mycobacteria, particularly *M. tuberculosis*, and to a lesser degree, in *M. leprae* and in *M. leprae murium*. From one to several bodies are found in a single cell, although bipolar orientation is most common. Bodies of the B type are small, very thin, and generally more numerous than the other kind. Type B bodies were not prominent in the mycobacteria examined.

There is a growing suspicion, among investigators working in the field of electron microscopy, that artifacts may be introduced by the technique now used in suspending the bacteria for final deposition on the specimen screen. During the evaporation of an 0.85 per cent saline solution, for example, concentration of salt increases, and by osmosis it may produce alterations in the normal architecture of the cell. Distilled water may likewise produce swelling. Effects of this nature have already been observed in certain species of bacteria in this laboratory. The mycobacteria studied, however, seem to be fairly resistant to these forces, but their effects should be considered and further work carried out. Artifacts may also be produced as a result of excessive heating of the specimen by the electron beam. This is particularly true in microscopes which use the biased electron gun. The differentiation of living and dead organisms in lepromatous tissues was not definitely established. Although the observations with the saponified fraction of moogrol³ and with sodium oleate yielded little information, a new approach to the study of the in vitro effect of drugs on M. leprae is presented. Such studies are now in progress.

³Moogrol was supplied by Burroughs Wellcome and Company, Inc., New York City.

SUMMARY

The morphological features of M. leprae, M. leprae murium, M. tuberculosis, M. smegmatis, M. phlei, and of the vole bacillus were compared by electron microscopy. A cell wall and nuclear structures were observed in the different species, and their characteristics were defined. Changes in the morphology of M. leprae murium as a result of exposure in vitro to saponified fractions of moogrol, to oleic acid, as well as to boiling in 0.85 per cent saline solution, to strong bases and to formaldehyde, are described.⁴

The morphologic pattern of M. leprae and of M. leprae murium, as revealed by electron micrographs, is in general respects different from that of the cultivable mycobacteria. On the other hand, these two leprosy strains closely resemble each other.

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*See Description of Plates .- EDITOR.

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DESCRIPTION OF PLATES

(Note: All photographs, except Fig. 9, were 30,500 X before reduction. As reproduced, the magnification is around 20,000 X or slightly more.)

PLATE 14

Fig. 1. A relatively thick suspension of M. tuberculosis (hominis). Large dense granules are present, and are particularly prominent at the poles of the cells. A peripheral zone of increased transparency visible around most of the cells may represent the cell wall, and possibly a capsule.

Fig. 2. Another tubercle bacillus in which polar bodiés similar to those of the previous figure are particularly well illustrated. A slight retraction of the protoplasm from the cell wall at one end is apparent.

Fig. 3. *M. tuberculosis* (bovis), BCG Vaccine. Polar bodies are smaller than those in Figs. 1 and 2, and the bacilli are somewhat longer. The apparent halo about the cells is presumably due to a collection of medium which has retracted. Small, dense granules are distributed irregularly throughout the cells; these may represent particulate matter in the medium.

Fig. 4. *M. phlei.* Polar granules are present, but they are considerably smaller than those of *M. tuberculosis.* BISHOP, ET AL]

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PLATE 14

Fig. 5. Vole bacillus. The individual bacillus pictured is considered to be an early stage of a filamentous or long form prior to division. The polar granules appear to be intermediate in size between those of M. phlei and M. tuberculosis.

Fig. 6. *M. smegmatis.* A few large, as well as numerous small, dense granules are present in the cell. Moderate shrinkage of the protoplasm from the delicate cell wall is evident.

Fig. 7. "*M. leprae*," strain No. 67, National Type Culture Collection, isolated from a case of human leprosy. Dense granules are concentrated at the poles, with a moderate quantity scattered throughout the cell. Some of the shadows within the cell are due to underlying debris.

Fig. 8. *M. leprae murium*, after fixation 1.5 hours in 10 per cent osmic acid. Dense polar bodies more elongated than those of other species of *Mycobacteria* are located at the extreme ends of the organism and many occupy from 30.0 to 50.0 per cent of each cell. In general, the nuclear material is more-widely distributed throughout the cell than occurs in the other species of *Mycobacteria*.



PLATE 15

Fig. 9. M. leprae murium. From same specimen as Fig. 8, higher magnification.

Fig. 10. *M. leprae murium.* This cell shows an irregular distribution of nuclear material, which is in contrast with that of other mycobacteria where it has a tendency toward polar localization. This distribution of granular material may be an indication of lytic processes, or possibly represents a step preparatory to division.

Fig. 11. M. leprae murium. Five cells from the same field. Two of them, A and B, have a rather irregular distribution of dense granules. Cell C, however, has a fairly marked polar concentration. Two others D and E, are of more uniform density, though some polar concentration is evident. A distinct cell wall is visible. These marked differences in morphology may represent different stages in the life cycle of the organism.

Fig. 12. *M. leprae murium*, from lepromatous tissue which had been boiled in 0.85 per cent sodium chloride for 45 minutes in the preparation of lepromin. The cell outline is still quite distinct, and dense granules appear throughout. The cells do not appear markedly different from unheated cells.



Fig. 13. *M. leprae murium*, after boiling in 0.05 normal sodium hydroxide for 15 minutes in an effort to break down the cell into smaller morphological components. Partial destruction of the cell body reveals small, dense polar bodies surrounded by a less dense concentration of other material, while the cytoplasm remains quite transparent.

Fig. 14. *M. leprae murium*, after boiling in 0.05 normal sodium hydroxide for 30 minutes. Many of the cells have disintegrated.

Fig. 15. *M. leprae murium*, boiled 15 minutes in formaldehyde. The cell has shrunk and exhibits an irregular outline and diffuse opacity.

Fig. 16. *M. leprae murium*, exposed for 24 hours to a 1 per cent saponified fraction of moogrol. The cells are swollen and show some disintegration, the granules are less distinct and appear more diffuse.



PLATE 17

Fig. 17. M. leprae murium, after exposure to saponified moogrol (same as Fig. 16).

Fig. 18. *M. leprae murium*, exposed for 24 hours to 1 per cent sodium oleate. There is complete lack of orientation of dense granules at the poles, and their pattern of distribution is irregular.

Fig. 19 and 20. *M. leprae*, from a human leproma which had first been noted by patient about one month previously. Large dense polar bodies, similar to those observed in *M. leprae murium*, are located at the ends of the cells. Much debris is present, because of difficulty encountered in freeing the cells from the tissue elements.



PLATE 18

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