OBSERVATIONS ON THE MORPHOLOGY OF *MYCOBACTERIUM LEPRAE* BY ORDINARY OPTICS, PHASE MICROSCOPY, AND ELECTRON MICROSCOPY

K. R. CHATTERJEE
Leprosy Enquiry, Indian Council of Medical Research
School of Tropical Medicine
N. N. DAS GUPTA AND M. L. DE
Biophysics Division, Institute of Nuclear Physics
Calcutta, India

Although in recent years *Mycobacterium leprae* has been examined to a certain extent with the aid of the phase contrast microscope (7) and by electron microscopy (1, 3, 5, 8), the bulk of our knowledge about its morphology rests on observations made with preparations stained by the Ziehl-Neelsen method and examined with classical optics. The interpertations regarding the biological significance of the morphological characters observed during such studies are often confusing, since the observed differences in shape and form have been explained differently by various workers. For example, the segmented and granular forms have been variously described as due to degeneration and disintegration (4), as manifestations of growth and proliferation corresponding to bacterial activity (6), or due—at least in part—to effects of the staining procedure (9).

In the work reported here, attempts have been made to establish with certainty whether the variants observed are merely due to effects of technique in the preparation, or whether they actually represent different morphological characteristics of the leprosy bacillus. For this purpose, photomicrographs of ordinary stained preparations and of suspensions examined by phase microscopy, and electron micrographs of properly fixed specimens, have been carefully compared.

MATERIAL AND METHODS

Specimens for examination were collected from three male Indian patients suffering from the lepromatous type of the disease, moderately advanced, who had had no antileprosy treatment. The site selected for collection of the material was thoroughly cleansed and secured with a pair of sterile forceps. A small piece of affected skin was removed aseptically with a sharp scalpel, avoiding inclusion of fat, which interferes in the process of separation of bacilli. The piece of tissue was lightly pressed between two pieces of filter paper to remove excess of fluid, and it was then finely minced with a pair of sclera scissors.

Preparation of the specimens.—The minced tissue was divided into four parts. One part was triturated in physiological saline, and the bacilli separated by centrifugation were utilized for ordinary and phase-contrast microscope studies. The remaining three portions were processed for electron microscopy, as follows:

Osmic acid fixation: A second part of the minced tissue was exposed to the vapor of 2 per cent osmic acid solution for 10 minutes, to fix the cells. After fixation, the tissue was triturated in distilled water and the mixture was centrifuged

at 1,000 rpm for 10 minutes. The supernatant fluid containing the bacilli was separated, and drops of it were placed on collodion-coated grids and dried in a desiccator for electron microscope examination.

Water treatment without fixation: A third part of the minced tissue was ground up in distilled water. This suspension was treated like the previous one: after centrifuging, drops of the bacillus-containing supernatant fluid were dried on collodion-coated grids.

Chloroform extraction: The remaining portion of the minced tissue was ground in chloroform; the chloroform extract containing bacilli—and some tissue lipids—was removed to another tube, and a drop of this mixture was evaporated on a grid.

Of these three methods of preparing the specimen for electron microscopy, the osmic acid-fixation method was found to give the best results. Specimens of unfixed material prepared in distilled water give micrographs lacking in details, and cytoplasmic artefacts are sometimes formed due to the sequence of swelling and then sudden contraction of the bacillus content during the processing. Specimens prepared by the chloroform extraction method show no bacillus details except the cell wall and some cytoplasmic matrix.

Technique of microscopy.—1. Light microscopy: A Leitz research microscope with a 12 × eyepiece and 90 × achromatic oil immersion objective was used. Photomicrographs were made from air-dried and heat-fixed thin smears, stained with cold carbol-fuchsin for one-half hour at room temperature, rinsed in water, decolorized with 2.5 per cent hydrochloric acid in 70 per cent alcohol and counterstained with dilute methylene blue. The photomicrographs were made at a magnification of 1200 × on the photographic film and then subsequently enlarged.

2. Phase contrast microscopy: For this study a Zeiss microscope with phase attachments was used. Saline suspensions of the bacilli were examined with a 12 × eyepiece and 90 × phase objective, for both bright-contrast and dark-contrast details. Photomicrographs were of necessity confined to cells which lay in contact with the slide or coverglass and did not suffer Brownian movement.

3. Electron microscopy: A 50 kilovolt electron microscopy recently installed in the Institute of Nuclear Physics (2) was used in this work. Some of the specimens were first shadowed with 20A° chromium at an angle of tan −1/5. The micrographs were made at a magnification of about 7000 × and subsequently enlarged optically. The total magnifications have been indicated in each micrograph with a micron mark.

FINDINGS

Light microscopy.—Figures 1 to 7 are photomicrographs of stained bacilli, processed as described. Several characteristic forms are apparent. A number of them (Figs. 1 and 3, *a* and *b*) are oval-shaped bodies slightly more tapered at one end than at the other. Some of the oval bodies have each a single dark polar region (Fig. 1, *a*), but usually they have two dark polar ends separated by a central light region (Figs. 1 and 3, *b*). Bacilli of this oval type are often found in clusters (Fig. 4). Bacilli marked *c* in Figures 1 and 3 consist of two cells about to separate. In such cases the daughter cells are again found with well-developed light and dark regions, as noticed in the already-separated cells (Figs. 1 and 3, *a* and *b*).

Besides the oval-shaped structures, bacilli with elongate bodies are also frequently observed. These appear to be of two distinct types. In

the first type a number of well-differentiated light and dark regions are apparent (Figs. 1 and 2, *d*). This type looks like the forms often observed in other bacteria before cell division. A further stage in the differentiation within the body of the cell is represented by the bacilli marked *d* in Figure 5, where the dark regions seem to be very prominent compared to the rest of the cells. In the extreme condition of this stage there are seen only a number of dark bodies, well separated from each other and situated in a line (Fig. 5, *e*).

The second type of elongate bacilli consists of homogeneously-stained rod-like structures of uniform diameter throughout (Figs. 3 and 6, *f*). Figure 7 shows a homogeneously-stained bacillus in the process of division. A constriction has formed at the place of separation and the daughter cells, also of the solid type, are formed with narrow and pointed ends.

Phase microscopy.—Figures 8 and 9 show bright-phase photomicrographs of living bacilli. The short oval bodies seen in the pictures of stained bacilli are found here as white bodies with small dark dots. The phase photographs show the oval-shaped type with a single white region at one pole (Fig. 8, *a*), as well as oval bodies each with two prominent, bright polar regions separated by a central dark dot (Figs. 8 and 9, *b*). These correspond exactly with the stained cells *a* and *b* in Figures 1 and 3. The two poles of the longer bacilli (Fig. 8, *h* and *j*) appear to be of longer optical path, and hence brighter, in the bright-contrast photographs. The central longitudinal dark region is of shorter optical path. A white region (S) divides transversely the bacillus marked *h*. Possibly a septum will form at this point.

Electron microscopy.—The observations with the stained and phase preparations have been strikingly confirmed by the electron micrographs of osmic-fixed specimens. Figures 10 to 14 are micrographs taken from the same field, showing bacilli with single (Figs. 10 and 11) and double (Figs. 12-14) polar condensations. Bacilli with single polar condensations may be compared with those marked *a* in the ordinary and phase pictures (Figs. 1 and 8). These masses seem to taper off toward the lighter zone. Bacilli with double polar condensations are more frequent; they correspond to the bacilli marked *b* in Figures 1, 3, 8 and 9. The double polar condensations are usually found at the two ends (Figs. 13 and 14), but sometimes the condensed regions may be slightly displaced, the lighter zone extending to the pole (Fig. 12, P). Figure 14 is an example of a bacillus with double polar condensations showing evidence of branching.

Sometimes very elongate forms of bacilli with alternate light and dark areas are observed (Figs. 15 and 16) which may be compared with Figure 1, *e*. A lighter print (not included in the plate) of Figure 15 shows that division has already taken place through the condensed portion of S (cf the septum S in Fig. 8). Budding seems to have taken place at one end of the long bacillus in Figure 16.

Figures 17 to 19 are negative prints of electron micrographs of shad-

owed bacilli. Here, in contrast to the positive prints, the bright zones are the denser ones and the dark zones the thinner. In all cases the polar regions seem to be more dense or thick than the rest of the cell. In Figure 17 a longitudinal light region, interrupted by small scattered condensations, is observed along the length of the bacillus (cf Fig. 8, *h* and *j*). In Figure 18, a constriction in the cell wall has occurred in the intermediate region p, where there is also evidence of condensation. Possibly a division will take place at this region along the line PQ, after which one of the daughter bacilli (*a*) would be shaped like those marked *a* in Figures 10 and 11 with a single polar condensation and a tapering clear zone. The other daughter cell (*b*) would have two polar condensations, one of them (P) will be displaced away from the pole with a tapering transparent region extending beyond the condensation (cf Fig. 12). Figure 19 shows a bacillus with budding, having condensed areas at the poles and lighter areas scattered throughout the body of the cell.

The electron micrographs also reveal the existence of the solid type of bacillus with more or less uniform diameter and rounded ends (Fig. 20), as already shown in a photomicrograph (Fig. 6, *f*). There is evidence of slight variations in density within the bacterial cell, but these are not arranged in alternate light and dark zones as is characteristic of the other type marked *d*. The dividing stage of a solid bacillus (Fig. 21) is strikingly similar to that already seen in Figure 7. A constriction has formed at the place of division, and the ends of the daughter cells are very narrow and pointed. That the regions of condensation within the bacillus are of greater height compared with the central transparent zone is evident from corresponding shadow lengths of Figure 22.

Bacilli enclosed within different structures are shown in three electron micrographs, Figures 23-25. Figure 23 shows bacilli lying probably within a nerve terminal generally found inside a tactile corpuscle; the fibrillar structure represents an axon without its myelin sheath. In Figure 25 bacilli are seen inside what is possibly a cell of Schwann. Figure 24 indicates the presence of a fine membranous sheath around the bacillus with a definite outline.

A bacillus in the process of division into a number of oval-type daughter cells is shown in Figure 26. The daughter cells are still intact, but constrictions have already formed at the places of future separation.

Electron micrographs of water-treated and chloroform-extracted specimens are shown in Figures 27 and 28, respectively. It is evident that in both cases only the cell-wall outline is preserved, and that intracellular dense matter was removed in the process.

DISCUSSION

There have been conflicting opinions regarding the biological significance of the morphological variants presented by *M. leprae*. Inability to culture the organism in any artificial medium makes it necessary to

study bacilli taken directly from the human host. This puts the investigator in a disadvantageous position, because the isolation of the bacilli from tissue without injuring them or producing artefacts is difficult and needs great care. Because of this complicating factor, doubts often arise as to whether some of the forms observed may have been due to artefacts caused by the processing techniques. In the present work the phase photographs of living bacilli without any previous treatment, and the electron micrographs of properly-fixed specimens, have been carefully compared with the photomicrographs of stained bacilli, in order to make sure that the observed forms are really characteristic of the mycobacterium itself.

With regard to the suitability of different methods of preparing specimens for electron microscopy, it was found that the specimens fixed with osmic acid gave the best results. Preparations of unfixed material made in distilled water lack in details because of the cytoplasmic artefacts sometimes formed by first swelling, and then sudden contraction, of the bacillary content during the processing. Specimens prepared by the chloroform extraction method show no details except the cell wall, and probably some cytoplasmic matrix. In our opinion, therefore, fixation of material in the vapor of 2 per cent osmic acid solution gives the best results for electron microscope study. The chloroform-extraction method may, however, possibly be of use for study of the bacillus cell wall.

Among the characteristic forms confirmed by this study are: (*a*) oval-shaped bodies with one or two polar condensations, of lengths varying from 0.8 to 1.6 microns; (*b*) an elongate type approximately 2.2 microns long, with double polar condensations, very frequently observed (1, 5); (*c*) an elongate type, 3.0 microns or more in length, with alternate light and dark regions (3), and (*d*) homogeneously dense types, either of uniform diameter throughout (5) or wider in the middle with tapering ends (7). In addition to these forms, we have encountered bacilli in the process of budding, branching and dividing. Lateral budding has been observed in both the phase and electron microscopy, and budding from one end of the bacillus has also been noticed.

The fact that comparable forms have been seen with all three methods of examination, including phase microscopy of living bacilli, indicates very strongly that the various forms observed are not the results of preparation technique, but that they represent the various characteristic features of the leprosy bacillus itself. Further, the fact that all these forms have been observed in the same individual suffering from the untreated, active disease rules out the possibility that some of them could have been the result of treatment. It is, however, possible and likely that some of the observed forms may be encountered more frequently in patients under treatment than in others; this matter is under study.

It is likely that the observed variants represent different stages of the development of the leprosy bacillus under different conditions. Morphological characters observed in our study may be broadly divided into

two types: (1) solid, uniformly dense bacilli, and (2) bacilli containing alternate light and dark zones. Both of these types may show the process of division. From the study of these different variants it seems to us that there possibly exist two phases of the growth cycle—a slow phase of multiplication resulting in solid, homogeneously dense forms, and a rapid phase resulting in forms possessing alternate light and dark regions. Investigations are in progress to gather further definite information on this point.

SUMMARY

Mycobacterium leprae has been studied with the aid of the ordinary and phase microscopes, and the electron microscope.

It has been found that treatment of *M. leprae* with distilled water or chloroform prior to examination produces artefacts. Fixation of material is the vapor of 2 per cent osmic acid solution gives the best results for the electron microscope study of the bacillus.

The following forms representing the morphological variants of this mycobacterium have been established with certainty from this study: (*a*) a short, oval type of cells with one or two polar condensations; (*b*) elongate types with double polar condensations; (*c*) very long types with alternate light and dark zones; and (*d*) homogeneously dark, elongate types.

The possible significance and relationships of these variants have been discussed.

RESUMEN

El *Mycobacterium leprae* fué estudiado con la ayuda de los microscopios ordinario y de fase y del microscopio electrónico.

Se descubrió que el tratamiento del *M. leprae* con agua destilada o cloroformo antes del examen produce artefactos. La fijación del ejemplar en los vapores de una solución de ácido ósmico al 2 por ciento da los mejores resultados en el estudio del bacilo leproso con el microscopio electrónico.

Por este estudio se han establecido con certeza las siguientes formas que representan las variantes morfológic as de esta micobacteria: (*a*) una forma corta y oval de células con una o dos condensaciones polares; (*b*) formas alargadas de dobles condensaciones polares; (*c*) formas muy largas con zonas claras y obscuras alternadas; y (*d*) formas alargadas uniformemente obscuras.

Se discuten las posibles importancia y relaciones de estas variantes.

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DESCRIPTION OF PLATES

PLATE (12)

Figures 1 to 7 are photomicrographs of leprosy bacilli stained by carbol-fuchsin, 4,800 × (enlargements from the original negatives).

FIGS. 1-3. (*a*) Oval-shaped bacilli with single polar condensations. (*b*) Oval-shaped bacilli with double polar condensations. (*c*) Bacilli dividing into daughter cells. (*d*) Elongate bacilli with dark and light regions. (*e*) Very long bacilli similar to the (*d*) type. (*f*) Solid, homogeneously dense bacilli.

FIG. 4. Oval forms, as in Figs. 1 and 3, but in clusters.

FIG. 5. (*d*) Bacilli with dark regions more prominent than the rest of the cells. (*e*) The dark bodies are well separated and arranged in a line.

FIG. 6. (*f*) Homogeneously dense bacilli.

FIG. 7. (*g*) Homogeneously dense bacillus in the process of division.

Figures 8 and 9 are bright-contrast phase photomicrographs of living leprosy bacilli, 4,800 ×.

FIGS. 8 & 9. (*a*) Oval-shaped bacilli with single polar condensations. (*b*) Oval-shaped forms with double polar condensations. (*h*) Bacilli of elongate type, with denser regions at both poles and central longitudinal lighter regions, interrupted by denser zones; S indicates the site of possible septum formation. (*j*) As in (*h*) but without septum formation.

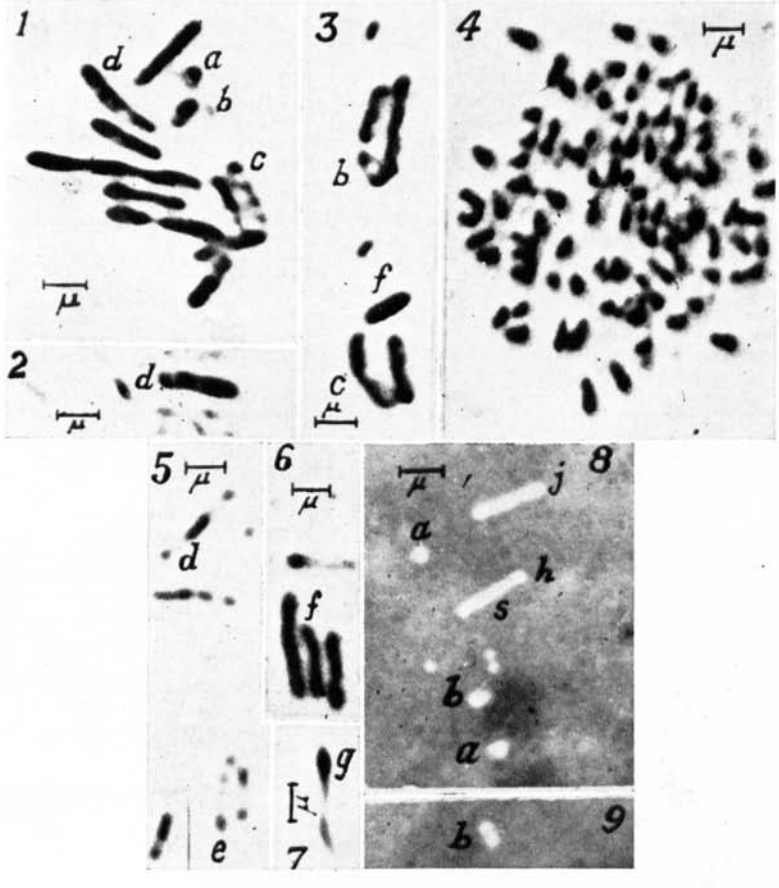

PLATE 12

PLATE (13)

Figures 10 to 25 are electron micrographs of osmic acid-fixed bacilli. Figures 26 to 28 are from material prepared by other methods.

FIGS. 10 & 11. Bacilli with single polar condensations. 11,000 ×.

FIGS. 12 & 13. Bacilli showing double polar condensations. 12,000 ×.

FIG. 14. Bacillus showing lateral budding, 12,000 ×.

FIG. 15. Very elongate form of bacillus, with alternate light and dense areas, showing division through a dense region at S (see text). 12,000×.

FIG. 16. Very elongate bacillus showing budding at one end. 18,000 ×.

FIG. 17. Negative print of a bacillus with polar condensations and intermediate lighter zones. Chromium shadowed, 16,000 ×.

FIG. 18. Negative print of a bacillus in the dividing stage, with three condensed zones. Chromium shadowed, 16,000 ×.

FIG. 19. Negative print of a bacillus with budding from the side and a pointed end. Chromium shadowed, 16,000 ×.

FIG. 20. Bacillus of the solid type, with rounded ends. 22,000 ×.

FIG. 21. Bacillus of the solid type but with pointed ends, in the process of division. 10,000 ×.

FIG. 22. Bacillus with two long condensations which appear, from the shadow lengths, to be of greater height than the transparent region. 13,000 ×.

FIG. 23. Bacilli inside a pacinian body, with attached nerve ending. 7,000 ×.

FIG. 24. Bacillus with a fine membranous sheath with definite outline. 7,000 ×.

FIG. 25. Bacilli inside a tissue cell. 7,000 ×.

FIG. 26. Bacillus from a distilled-water preparation, showing three round forms in the process of separation. 25,000 ×.

FIG. 27. Elongate-type bacillus from a distilled-water preparation, showing only the cell membrane. 15,000 ×.

FIG. 28. Bacillus from a chloroform-extraction preparation, showing only the cell wall and the outline of the cytoplasmic matrix. 15,000 ×.

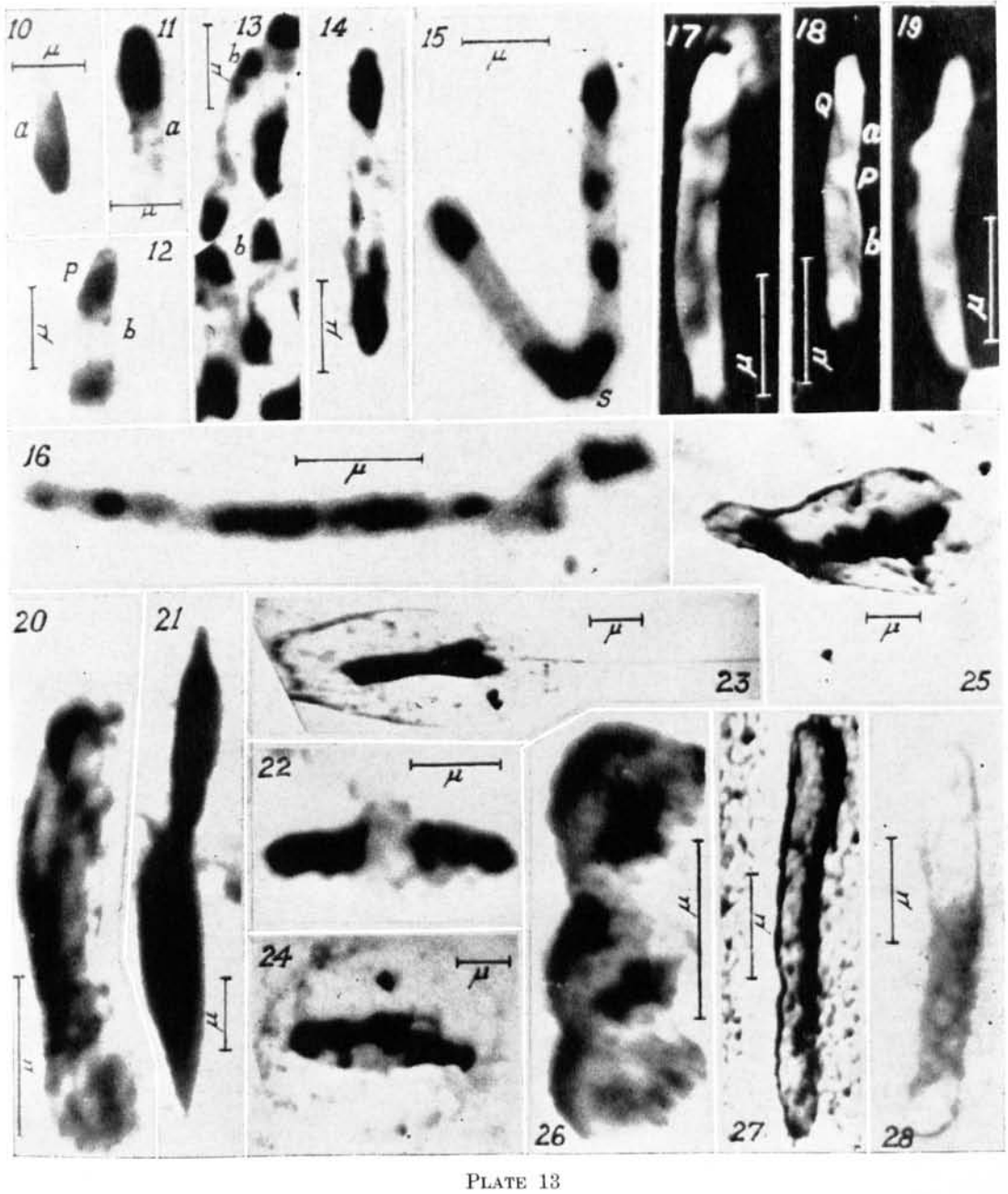

PLATE 13