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THE APPEARANCE OF DEAD LEPROSY BACILLI BY LIGHT AND ELECTRON MICROSCOPY

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One of the most characteristic features of a smear of human leprosy bacilli stained by the Ziehl-Neelsen method is the presence of a significant proportion of organisms which stain irregularly with the carbol-fuchsin. Although these irregularly-stained bacilli are well recognized, there is no straightforward way to test their infectivity because human leprosy bacilli cannot either be cultured or caused to infect animals. It is not surprising, therefore, that very different interpretations have been given to these irregularly-stained bacilli. For example, Hoffman, in 1933 ([5]), suggested that the "granular" forms resulted from degeneration and disintegration of the bacilli, whereas Manalang, in 1937 ([6]), concluded that they represented an active phase in the life cycle of the bacilli and that irregularly-stained organisms corresponded to a proliferative phase.

More recently, a number of authors have suggested that irregular staining represents degeneration or death of the bacilli. Davey ([2, 3]), from his experience in chemotherapeutic work, concluded that effective therapy was associated with the appearance of increasing proportions of bacilli showing irregular staining with carbol-fuchsin, and that these morphologic changes were associated with loss of viability of the organisms. A similar interpretation has been accepted by Ridley ([10]), who has introduced a "granularity index" to assess the proportion of irregularly stained bacilli.

Our own experimental studies ([9]) have shown that degenerative changes in the morphology of the rat leprosy bacillus, *Mycobacterium leprae murium*, seen in the electron microscope allow dead forms to be identified and can be used as a guide to the viability of these organisms. We believe that the similar degenerate forms of human leprosy

bacilli, which can also be identified in the electron microscope and the proportion of irregularly-stained bacilli seen in the light microscope to suggest that the bacilli which stain irregularly were dead. We report here further studies on individually identified and stained leprosy bacilli as seen by light and electron microscopy which strengthen the evidence for identifying degenerative changes seen with the electron microscope with irregular staining seen by the light microscope.

MATERIALS AND METHODS

Suspensions of leprosy bacilli.—(*a*) Rat leprosy bacillus: Partially purified suspensions of the Douglas strain of *M. leprae murium* were prepared by the method previously described ([9]), from the livers of mice infected intravenously with bacilli 4 to 6 months previously. The bacilli, with a small amount of remaining tissue, were finally suspended in either physiologic saline containing 1 per cent bovine plasma albumin (fraction V) or 0.01M phosphate buffer (pH 7) to give not less than 10^7 acid-fast bacilli per cubic centimeter.

(*b*) Human leprosy bacillus: Biopsy material heavily infected with *M. leprae* was obtained from patients with lepromatous leprosy in East Africa or Malaya who had received no known treatment. The material was despatched by air, on ice, in especially constructed containers ([1]), and was processed within 48 hours of removal from the patient. The method for preparing the suspension of human bacilli was the same as that used for rat bacilli except for the addition of small amounts of silver-sand to assist homogenization. The suspension of *M. leprae* contained a higher proportion of tissue homogenate than the suspension of *M. leprae murim,* and the bacilli were more clumped.

Suspensions of rat or human bacilli containing clumped bacilli were dispersed by exposure to ultrasonic vibration for 30 seconds ([4]). The suspension of bacilli was fixed, immediately after preparation, by adding formaldehyde to a final concentration of 2 per cent.

Preparation of cell-walls from mycobacteria.—Fresh, unfixed suspensions of *M. leprae murium,* or of *M. phlei* (from the surface of egg cultures), were prepared in phosphate buffer. Eight cubic centimeters of the suspension, containing approximately 10^8 bacilli per cc., was placed in the cup of a sonic generator (250W, 25 kc/sec magnetostriction transducer coupled to a stainless steel cup) and vibrated for 8 to 12 minutes. The suspension was kept cool (below 12°C) by surrounding the cup with crushed ice in water. Samples were removed after 8, 10 and 12 minutes' exposures to the sonic vibration for examination with the light and electron microscopes.

Preparation of bacilli for examination with the light and electron microscopes.—The fixed bacilli were suspended in distilled water, and a drop was dried on the film (nitrocellulose covered with carbon) on a platinum electron-microscope specimen support. The dried film was washed briefly in water to remove any remaining traces of soluble material.

The dried film of bacilli on the support was stained by the Ziehl-Neelsen method, modified to avoid breaking or losing the film. The support was held throughout in the vertical position in a platinum loop. (Steel forceps in contact with the platinum support evolve gas bubbles in an acid solution which lift the film.) The film was stained by immersing the support in steaming carbol-fuchsin for 2 minutes. Excess carbol-fuchsin was removed, first by touching the side of the support with filter-paper and then washing in distilled water. The film was decolorized in 2.5 per cent sulfuric acid and again washed in water. Before counterstaining, the support was dried by filter paper and placed, film upwards, on a microscope slide and examined to make sure that excess carbol-fuchsin had been removed. The film was then further decolorized if necessary, and finally counter-

stained in 0.2 per cent methylene blue for 1 minute, washed in water, and finally dried. All washing and staining procedures were carried out in petri dishes.

The support was placed vertically in a drop of immersion oil on a clean microscope slide, further oil added until it was completely immersed, and the support was then turned in the pool of oil to lie horizontally, film upwards. With care it was possible to avoid trapping bubbles of air below the film. The support was centered under the microscope using 2 mm. oil-immersion objective and X6 binocular eyepieces. Each support has 7 holes, one central and 6 peripheral. All the holes were examined and drawn diagrammatically for future identification in the electron microscope (i.e., holes were numbered 1 to 7 and any particular features, such as folded film or debris, were noted). Each numbered hole was then examined in detail, and the position of each clearly-defined bacillus was mapped out. When all the bacilli had been mapped particular ones were selected, and numbered drawings were made detailing the stained features of each individually-identified organism. Drawings were used because photographs failed to record sufficient detail.

When the drawings were completed the oil was removed from the support by immersing it in xylol for 1 minute, after which it was transferred through three lots of absolute alcohol and dried. The preparation was then examined with the electron microscope, and the bacilli that had been drawn were located and electron micrographs taken.

RESULTS

Appearance of cell-walls from mycobacteria stained by carbol-fuchsin.—This part of the investigation was undertaken to determine whether the surface and/or the protoplasm of mycobacteria take up carbol-fuchsin when stained by the Ziehl-Neelsen method. Sonic vibration was used to prepare cell walls, and the quality of the preparation was checked in the electron microscope.

Stained smears of the bacillus suspension before exposure to sonic vibration showed about 100 well-stained organisms per microscope field. Similarly-stained smears prepared from the suspension after 8, 10 or 12 minutes exposure to sonic vibration showed only an occasional well-stained bacillus per 5 to 10 microscope fields, against a background of extremely fine acid-fast granules.

Similar samples examined in the electron microscope showed, after 8 minutes of sonic treatment, large numbers of almost complete cell walls free from attached or intracellular electron-dense material, with only a very occasional normal (uniformly electron dense) intact bacillus. The 10- and 12- minute samples contained much higher proportions of ruptured cell-wall material. It was evident that cell walls prepared by these methods are not acidfast, suggesting, therefore, that in intact bacilli and acid-fast moiety is not the cell wall but is an intracellular component. The findings were similar for *M. phlei* and *M. leprae murium.*

Appearance of stained M. leprae murin *compared by light and electron microscopy.*—In order to have suspensions with a high proportion of nonviable bacilli (showing "degenerate" forms in the electron microscope and irregularly-stained acid-fast forms by the light microscope), as judged by failure to produce leprosy lesion in mice, the sus-

pensions in phosphate buffer were incubated at 37°C for 1 to 3½ months (9). A direct comparison of individual bacilli with the light and electron microscopes was made on many stained organisms.

By comparing drawings with electron micrographs of the stained murine bacilli, a high correspondence was observed between electron density and carbol-fuchsin staining. Bacilli which were uniformly acidfast were also uniformly electron opaque. This type of bacillus (Figs. 1a and 1b) corresponds to the "normal" viable form. Many of the bacilli showed along their length irregular staining ("beaded forms") with carbol-fuchsin. Nevertheless, there was usually no difficulty in deciding that these more intense acid-fast moieties were a part of one intact bacillus, because the rest of the bacillary skeleton was faintly acidfast (Fig. 2a). The electron micrograph of the same organism (Fig. 2b) showed an intact cell wall containing electron-dense aggregates of disorganized protoplasm ("degenerate" type), and the more dense areas exactly corresponded to those staining strongly with carbol-fuchsin (Figs. 2 and 3).

Although there were many variations in the pattern of the irregularly-stained areas in the beaded form of bacilli seen with the light microscope, these patterns could be identified generally with the denser aggregates seen in the electron microscope. The only form of bacillus whose electron microscope appearance did not closely match the drawing of the stained organism is shown in Figs. 4a and 4b. With the light microscope the organism showed uniform staining—denoting a normal healthy type—whereas the electron micrograph revealed a longer organism with only a central electron-dense area and an otherwise empty cell wall—denoting a degenerate type. It is clear that only the electron-dense area of the bacillus became sufficiently stained with carbol-fuchsin to be visualized with the light microscope, and the empty cell wall was invisible. Usually, however, sufficient cytoplasm remains within the cell wall of degenerate organisms (e.g., Fig. 2b) for the complete outline of such bacilli to be clear when the stained preparation is viewed with the light microscope. They can then be correctly identified as degenerate.

Appearance of stained M. leprae *compared by light and electron microscopy.*—Although the human leprosy bacilli examined have come

DESCRIPTION OF PLATE

The pictures in the first column (Figs. 1a to 7a) are drawings of individual stained bacilli seen with the light microscope. The corresponding pictures in the second column (Figs. 1b to 7b) show the appearance of the same individual bacilli with the electron microscope. All magnifications, approximately X30,000.

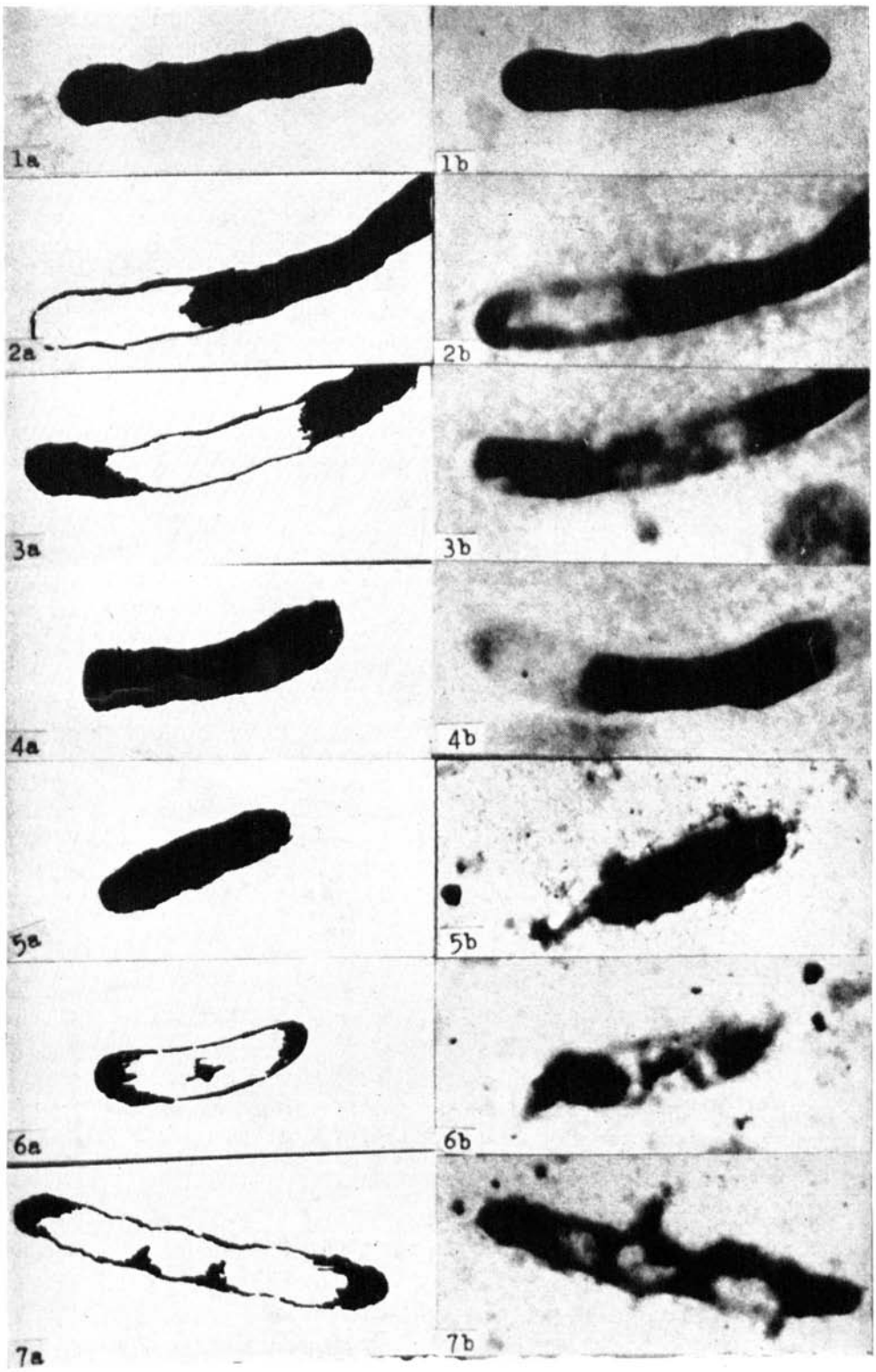
FIG. 1. Normal form of *M. leprae murium.*

FIGS. 2 and 3. Degenerate forms of *M. leprae murium.*

FIG. 4. Degenerate form of *M. leprae murium,* showing that the empty cell wall at the ends of the bacillus is not seen with the light microscope.

FIG. 5. Normal form of *M. leprae.*

FIGS. 6 and 7. Degenerate forms of *M. leprae.*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from untreated patients, more than one-half of the organisms were of the degenerate type. More than a hundred individual bacilli have been examined, after staining, with the light and electron microscopes, and the results have been similar to those found with *M. leprae murium*. In fact, the irregular staining and degenerate changes are more obvious in the human than in the rat bacilli. Again, those bacilli that stained uniformly (Fig. 5a) showed as more or less uniform dense rods, "normal" bacilli (Fig. 5b) by the electron microscope, with the exception of the type already referred to in *M. leprae murium* in which one or both ends of the bacillus were completely transparent (cf Fig. 4b). This latter type, if the proportion of stained protoplasm was small, appeared by the light microscope as short rods (coccobacilli). Two examples of irregularly-stained human leprosy bacilli and their appearance in the electron microscope are shown in Figs. 6a and 6b, and Figs. 7a and 7b. These two bacilli are representative of the degenerate type of organism, and show again the close agreement between areas staining irregularly with carbol-fuchsin and areas of electron density.

DISCUSSION

The earlier studies of McFadzean and Valentine ([7, 8]) suggested that the electron microscope could provide a quantitative guide to the viability of rat leprosy bacilli by allowing dead forms to be identified. These observations were extended using rat leprosy bacilli and *E. coli* ([9]). The murine bacillus was chosen as a model for the human leprosy bacillus, because viability of the organisms could be checked finally in the experimental animal. *E. coli* was chosen because viability could be checked on simple culture medium. In both organisms, loss of viability was associated with similar morphologic changes identified with the electron microscope. These studies provided good evidence that the electron microscope could be used to identify loss of viability in two totally different groups of bacilli. It was reasonable, therefore, to conclude that human leprosy bacilli manifesting similar morphologic changes when examined with the electron microscope were also dead, particularly since rat and human leprosy bacilli are closely related.

The determination of loss of viability of human leprosy bacilli is of the greatest importance, both for our understanding of the development of the infection and also for assessing progress during treatment because, as yet, the organism cannot be cultured in ordinary medium and there is no susceptible experimental animal. Unfortunately, the practical application of the electron microscope to determine the proportion of dead organisms in studies of human leprosy is very restricted, not only because of the limited availability of the microscope but also because high concentrations of relatively pure suspensions of bacilli are required. However, our more recent studies with the electron microscope ([9]) have suggested that the light microscope may be used equally

well to identify nonviable bacilli by their irregular staining with carbol-fuchsin applied by the ordinary Ziehl-Neelsen method. These latter conclusions were made by showing that there was a close agreement between the proportion of degenerate forms of human leprosy bacilli as shown by the electron microscope and irregularly-staining bacilli with the light microscope. Our aim here has been to confirm this by examining the same individually-stained bacilli, first with the light microscope and then in the electron microscope.

Before undertaking these comparative studies on individually-stained intact rat and human leprosy bacilli, the staining properties, using Ziehl-Neelsen method, of rat leprosy bacilli and *M. phlei* were investigated. The results show clearly that suspensions containing very large numbers of cell walls do not stain with carbol-fuchsin. It is concluded, therefore, that the constituent of mycobacteria which binds the carbol-fuchsin (believed to be mycolic acid or a derivative) resides in the cytoplasm and not in the cell wall.

The subsequent studies on rat and human leprosy bacilli first stained with carbol-fuchsin and then individually identified and examined with the electron microscope have shown a high correspondence between the electron-dense material inside the cell wall of the organism and the carbol-fuchsin-staining moiety. Uniformly-staining bacilli had a uniform electron density, whereas irregularly-staining bacilli had an irregular distribution of electron-dense material inside the still distinct and intact cell wall, and the features corresponded exactly.

Since cell walls do not stain with carbol-fuchsin, the irregular staining of the degenerate form of the organism can only be identified by the light microscope when there is sufficient residual cytoplasm to outline the bacillary shape. In fact, the only degenerate form of bacillus seen with the electron microscope which could not be identified as such in the stained preparation (see Fig. 4) was where one or both ends of the organism were completely empty and therefore invisible by the light microscope. Here the bacillus was wrongly identified as a normal but shorter, uniformly-staining bacillus. The correlation between irregular staining seen by the light microscope and degenerate changes seen with electron microscopy has been shown both in rat and human leprosy bacilli, although with the standard staining technique the correlation is most clearly shown in human leprosy bacilli.

The present studies clearly demonstrate for the first time that irregularly-stained forms seen in human leprosy bacilli can be identified exactly with degenerate forms of bacilli seen with the electron microscope, and, from previous experimental studies with rat leprosy bacilli and with *E. coli,* these irregularly-stained organisms are likely to be nonviable. It is reasonable, therefore, to conclude that only the uniformly-staining human leprosy bacilli, the so-called "solid" forms, are

likely to be viable. All forms of irregularly-stained bacilli, whether defined as "fragmented" or "granular" or "beaded" can be considered dead organisms. This finding supports and adds to the significance of the observations of Davey that an increased proportion of irregularly-stained leprosy bacilli found in patients improving under treatment can and should be used in assessing successful chemotherapy, and should be added to the criteria for determining the therapeutic activity of antileprosy drugs. It is of particular interest, therefore, that in our own studies on experimental chemotherapy in rat leprosy (R.J.W.R.) and in the studies of Davey ([3]), deterioration in rat and human infections was associated with the reappearance of uniformly-stained bacilli in smears that hitherto had shown predominantly irregularly-stained bacilli.

SUMMARY

Preparations of leprosy bacilli stained by the Ziehl-Neelsen method were examined first by the light microscope and then with the electron microscope. By comparing the same individual bacilli it is shown that the material which stains red is in the cytoplasm of the bacilli and not in the cell wall, and that every bacillus which appears irregularly stained in the light microscope is shown by the electron microscope to be completely degenerate and dead. The conditions under which the light microscope can be used to assess viability are discussed.

RESUMEN

Se examinaron preparaciones de bacilos leprosos teñidos con la técnica de Ziehl-Neelsen primero con el microscopio luminoso y luego con el electrono-microscopio. Comparando los mismos bacilos dados queda demostrado que el material que toma el color rojo radica en el citoplasma de los bacilos y no en la pared de la célula y que todo bacilo que aparece teñido irregularmente en el microscopio luminoso se halla, según patentiza el microscopio electrónico, absolutamente degenerado y muerto. Se discuten las condiciones en las que cabe usar el microscopio luminoso para valorar la viabilidad.

RESUMÉ

Des préparations de bacilles de la lèpre colorées par la méthode de Ziehl-Neelsen ont êté examineés, d'abord par la microssoci classique, ensuite au microscope electronique. Losque l'on compare les mêmes individus bacillaires, on constate que les éléments colorés en rouge sont localisés dans le cytoplasme, et non dans la paroi cellulaire, et que chaque bacille qui semble irregulièrement coloré à l'examen par le microscope classique apparaît en fait, au microscope electronique, complètement dégeneré et mort. Sont débattues dans cette communication les conditions sous lesquelles la microscopie classique peut être utilisée pour discuter de la viabilité des bacilles.

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REFERENCES

1. Brown, J. A. K. and Rees, R. J. W. A simple apparatus for the transport of leprosy tissue on ice. East African Med. J. **36** (1959) 495-499.
2. Davey, T. F. Progress with new antileprosy drugs. Trans. VIIth Internat. Congr. Leprol., Tokyo, 1958; Tokyo, Tofu Kyokai, 1959, pp. 252-259; *also* Internat. J. Leprosy **26** (1958) 299-304.
3. Davey, T. F. Some recent chemotherapeutic work in leprosy. With a discussion of some of the problems involved in clinical trials. Trans. Roy. Soc. Trop. Med. & Hyg. **54** (1960) 199-206.
4. Garbutt, E. W., Rees, R. J. W. and Barr, Y. M. Multiplication of rat-leprosy bacilli in cultures of rat fibroblasts. Lancet **2** (1958) 127-128.
5. Hoffman, W. M. The granular forms of the leprosy bacillus. Internat. J. Leprosy **1** (1933) 149-158.
6. Manalang, J. The morphology of *M. leprae* during the course of treatment. Month. Bull. Bur. Hlth. (Manila) **17** (1937) 3-10.
7. McFadzean, J. A. and Valentine, R. C. An attempt to determine the morphology of living and dead mycobacteria by electron microscopy. (Preliminary communication.) Trans. VIIth Internat. Congr. Leprol., Tokyo, 1958; Tokyo, Tofu Kyokai, 1959, pp. 89-90.
8. McFadzean, J. A., and Valentine, R. C. The value of acridine orange and of electron microscopy in determining the viability of *Mycobacterium leprae murium*. Trans. Roy. Soc. Trop. Med. & Hyg. **53** (1959) 414-422.
9. Rees, R. J. W., Valentine, R. C. and Wong, P. C. Application of quantitative electron microscopy to the study of *Mycobacterium lepraemurium* and *M. leprae*. J. Gen. Microbiol. **22** (1960) 443-457.
10. Ridley, D. S. A bacteriologic study of erythema nodosum leprosum. Internat. J. Leprosy **28** (1960) 254-266.