A MODIFIED ZIEHL-NEELSEN STAINING METHOD FOR IDENTIFYING DEAD *MYCOBACTERIUM LEPRAE MURIUM*

R. J. W. Rees, B.Sc., M.B., B.S.
and R. C. Valentine, M.A., Ph.D.

National Institute for Medical Research
Mill Hill, London, N.W.7, England

Our experimental studies ([1]) have shown that degenerate changes in the morphology of the rat leprosy bacillus, *Mycobacterium leprae murium*, as seen with the electron microscope, permit the identification of dead forms, and we believe that the similar degenerate forms of the human leprosy bacillus, *M. leprae*, are likewise dead organisms. Furthermore, there is sufficiently good agreement between the proportion of degenerate forms of *M. leprae* identified with the electron microscope and the proportion of bacilli irregularly stained by the Ziehl-Neelsen method as seen with the light microscope to suggest that the bacilli which stain irregularly are dead ([1]). Further evidence for this view has come from comparing the appearances of individually identified and stained human leprosy bacilli in the light and electron microscopes ([2]).

Although this correlation between the observations with the light and electron microscopes have held for *M. leprae* under all conditions so far investigated, the correlation has not extended to *M. leprae murium* under certain *in vitro* conditions. For example, when the rat leprosy bacilli were incubated in phosphate buffer at 37°C, the proportion of degenerate forms seen with the electron microscope increased within a few weeks, but irregular staining only became apparent after several months. Rat leprosy bacilli from tissue cultures also showed degenerate forms in the electron microscope, but did not show obvious irregular staining with carbol-fuchsin. We report here briefly a modification of the standard Ziehl-Neelsen staining method which does allow degenerate forms of the rat leprosy bacilli to be identified with the light microscope. The method may also be useful in making more certain the identification of degenerate human leprosy bacilli.

MATERIALS AND METHODS

The methods for preparing suspensions of *M. leprae murium* and examining these bacilli with the light and electron microscopes have been previously described ([1, 2]). Suspensions of bacilli containing high proportions of degenerate (dead) organisms were produced by incubating in phosphate buffer at 37°C for several months.

Ziehl-Neelsen method and modifications.—The carbol-fuchsin stain was prepared as follows: Basic fuchsin, 5 gm.; phenol crystals, 25 gm.; absolute alcohol, 50 cc.; and distilled water to make 500 cc.

In order to determine the effect of the suspending fluid used for carbol-fuchsin on the morphology of the bacilli seen with the electron microscope, a control "solvent" was prepared as follows: phenol crystals, 25 gm., absolute alcohol, 50 cc., and distilled water to make 500 cc.

Dried films of bacilli were stained on the electron-microscope support by exposure to steaming carbol-fuchsin or its solvent for 2 minutes, decolorizing in 2.5 per cent sulfuric acid, and counterstaining with 1 per cent aqueous methylene blue for 1 minute.

The routine method for staining bacilli on slides was to expose to steaming carbol-fuchsin for 5 minutes, decolorize in 25 per cent sulfuric acid for 10 minutes, and counterstain in 1 per cent aqueous methylene blue for 1 minute. Modifications of the method applied to the temperature and the time of exposure to carbol-fuchsin. Comparisons were made at 18°, 32°, 37°, 42° and 48°C with exposure times of 1, 3, 6, 12, 15 and 24 hours. For these latter tests the slides were immersed in Coplin jars containing carbol-fuchsin held at the appropriate temperature in the incubator.

RESULTS

EXAMINATION OF *M. LEPRAE MURIUM* WITH THE ELECTRON MICROSCOPE AFTER VARIOUS TREATMENTS

(1) *Standard carbol-fuchsin staining.*—When dead bacilli were examined with the electron microscope after being stained by the standard Ziehl-Neelsen technique, it was no longer possible to determine with certainty the degenerate organisms. The staining process had redistributed their remaining cytoplasm to more nearly resemble the uniform appearance of normal organisms, which in fact correlated with their more or less regular staining seen with the light microscope. By mapping out the area of the bacilli occupied by electron-dense material it could be seen that this filled 60 per cent of the stained bacilli on the average, compared with 45 per cent before staining. A series of studies was undertaken in order to determine which stage of the Ziehl-Neelsen method leads to changes in the distribution of electron-dense material in the degenerate bacilli.

(2) *Effect of the fuchsin solvent.*—When the bacilli after exposure to steaming fuchsin solvent were examined with the electron microscope, it was found that this procedure alone resulted in a spread of the electron-dense material in the cytoplasm. As a further control the bacilli were exposed to steaming water, but this caused no change in the distribution of the electron-dense material, and the bacilli could still be identified as degenerate organisms with the electron microscope.

These observations led us to ascertain whether staining of degenerate *M. leprae murium* at lower temperatures would avoid the redistribution of electron-dense material.

(3) *Staining with carbol-fuchsin at lower temperatures.*—A series of smears was stained at different temperatures varying from 18° to 48°C, and varying in staining time from 1 to 24 hours. The results of these studies showed that at temperatures higher than 37° C, dead bacilli generally showed uniform staining, whereas at 37°C irregular staining was retained. However, staining at 37°C for less than 6

hours left a significant proportion of the organisms unstained or poorly stained; below 37°C, even prolonged staining left many bacilli too poorly stained to be identified. This suggested, therefore, that for routine procedures the smears should be stained at 37°C for not less than 6 hours and for convenience usually 6 to 15 hours.

COMPARISON OF THE PROPORTION OF DEGENERATE FORMS OF *M. LEPRAE MURIUM* SEEN WITH THE ELECTRON MICROSCOPE AND IRREGULARLY-STAINING BACILLI SEEN WITH THE LIGHT MICROSCOPE, USING THE NEW PROCEDURE

A series of 21 samples of *M. leprae murium* from a variety of *in vitro* experiments carried out either in tissue culture or in cell-free media were examined independently with the light microscope, after staining at 37°C for 6 to 12 hours, and the proportion of irregularly stained bacilli in each was recorded. The same suspensions were independently examined be electron microscopy and the proportion of degenerate forms counted. The results of these studies are given in Table 1, which shows the very close agreement between the two methods.

TABLE 1.—*Comparison of the proportion of degenerate forms of* Mycobacterium leprae murium *seen with the electron microscope, and irregularly-stained bacilli seen with the light microscope in the same sample, after the modified Ziehl-Neelsen method.*

Degenerate in electron microsepe (per cent)	Irregularly stained by carbol-fuchsin (per cent)
100 (48/48)	100 (40/40)
100 (20/20)	100 (30/30)
100 (40/40)	96 (96/100)
100 (25/25)	86 (38/44)
100 (25/25)	77 (36/47)
98 (98/100)	95 (95/100)
97 (133/137)	99 (106/107)
90 (63/70)	76 (64/84)
86 (63/73)	85 (85/100)
73 (66/91)	66 (66/100)
45 (5/11)	50 (20/40)
20 (5/25)	5 (5/108)
9 (5/53)	13 (9/69)
6 (3/50)	6 (7/110)
6 (3/50)	5 (3/65)
6 (2/33)	5 (5/100)
4 (2/50)	6 (12/191)
4 (2/50)	4 (4/107)
3 (3/100)	8 (4/49)
3 (4/126)	6 (3/50)
2 (2/102)	8 (4/50)

CONCLUSIONS AND SUMMARY

On examination of degenerate forms of *M. leprae murium* with the electron microscope after staining with hot carbol-fuchsin, the typical

features of degenerate organisms can no longer be identified. Electron microscopy revealed that the staining process led to spreading of the cytoplasmic content, so that the organisms had the appearance of more or less uniformly-filled bacilli. By examining degenerate forms of these microorganisms with the electron microscope after different stages of the Ziehl-Neelsen method, it was found that the redistribution of the cytoplasmic content of these bacilli occurs when the organisms are heated in the presence of the Ziehl-Neelsen solvent, even without the dye. However, the characteristic features of degenerate *M. leprae murium* can be retained by staining at lower temperatures. The best results were obtained by staining with carbol-fuchsin at 37°C for 6 to 15 hours. Using this modified Ziehl-Neelsen method, the viability of these bacilli can be assessed with the light microscope.

CONCLUSIONES Y RESUMEN

Al examinar formas degeneradas del *M. leprae murium* con el microscopio electrónico después de la coloración con carbol-fuchsina en caliente, no pueden identificarse ya más las características típicas de los microbios generados. La microscopía electrónica reveló que el procedimiento de coloración fomentaba la dispersión del contenido citoplásmico, de modo que los microorganismos revestían el aspecto de bacilos henchidos más o menos uniformemente. Examinando formas degeneradas de estos microorganismos con el microscopio electrónico después de aplicar diversas etapas del método de Ziehl-Neelsen, se observó que la redistribución del contenido citoplásmico de estos bacilos tiene lugar cuando se calientan los microorganismos en presencia del solvente de Ziehl-Neelsen, aun sin el colorante. Sin embargo, pueden retenerse las características típicas del *M. leprae murium* degenerado coloreando a temperaturas más bajas. Se obtuvieron los mejores resultados tiñendo con carbol-fuchsina a 37° C. por espacio de 6 a 15 horas. Usando este método de Ziehl-Neelsen modificado, puede justipreciarse la viabilidad de estos bacilos con el microscopio luminoso.

RESUMÉ

Lorsqu'on examine au microscope électronique les formes dégénérées de *M. leprae murium* après coloration par la fuchsine phéniquée á chaud, on ne peut plus identifier les caractéristiques typiques des organismes dégénérés. La microscopie électronique révèle que la méthode de coloration entraîne une diffusion du contenu cytoplasmique, telle que les organismes revêtent l'apparence de bacilles plus ou moins uniformément remplis. En examinant les formes dégénérées de ces microorganismes au microscope électronique après différentes étapes de la technique de coloration de Ziehl-Neelsen, il a été constaté que la redistribution de contenu cytoplasmique de ces bacilles a lieu lorsque les organismes sont chauffés en présence de la solution de Ziehl-Neelsen, même si le colorant est absent. Néanmoins, les caractéristiques typiques de *M. leprae murium* dégénéré peuvent être retenues par la coloration à plus basse température. Les résultats les meilleurs ont été obtenue par la coloration avec la carbol-fuchsine à 37°C durant 6 à 15 heures. En employant cette méthode de Ziehl-Neelsen modifiée, la viabilité de ces bacilles peut être estimée au microscope ordinaire.

REFERENCES

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