INTERNATIONAL JOURNAL OF LEPROSY Volume 55, Number 1
Printed in the U.S.A.

Orientation Staining for the Demonstration of *Mycobacterium leprae* in Semithin Sections[1]

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For ultrastructural examination of leprosy tissue, we usually use biopsies embedded in a low-viscosity, epoxy-resin medium according to Spurr ([9]). However, significant staining of *Mycobacterium leprae* in the semithin sections could not be achieved using the staining method of Richardson ([8]), since the *M. leprae* hardly differed from the surrounding tissues and could be confused with mast cell granules. We therefore tried to apply the usual histological staining methods according to Ziehl-Neelsen and Fite ([3]) to stain the semithin sections. However, before and even after separation of the embedding plastic medium from the semithin sections we did not achieve positive results with the use of these techniques. The reasons could be, on the one hand, that these staining materials do not enter into epoxy-embedded material or, on the other hand, that the corresponding protein structures were destroyed after the aggressive deepoximation. The demonstration of *M. leprae* in semithin sections is necessary for the correct determination of the area for ultrastructural analysis. Therefore, we used for staining of *M. leprae* the methylene blue-borax and basic fuchsin technique which, in a similar manner, has been described for labeling purposes with plastic-embedded materials ([2, 4–6]).

MATERIALS AND METHODS

Patients. Biopsies were taken from 11 patients with Hansen's disease. The tissue of all 11 patients was studied by routine histopathological examination, and the diagnosis was confirmed using the Fite stain.

Electron microscopy. For electron microscopic (EM) examination, the tissue was prepared in two different steps:

a) First, the tissue of two patients was fixed in 10% Formalin and embedded in paraffin. After a histopathological examination, using Fite staining for the determination and localization of *M. leprae* in the tissue, the remaining material was deparaffined and prepared for consecutive semithin and ultrathin sections. For EM purposes, the material was cut into sections of 1–2 mm lengths and fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4) for 1 hr at 4°C. The post-fixation was developed in 1% osmium tetroxide in 0.15% phosphate buffer. Dehydration was performed in 30% and 50% of ethanol, and contrasting was done in 1% phosphotungstic acid and 0.5% uranyl acetate in 70% ethanol. After further dehydration, the material was embedded according to Spurr ([9]) and, after polymerization, cut into semithin sections (0.7 μm) using a Reichert and Jung microtome. The sections were put onto carriers, dried at 30°C, and heat-fixed at 80°C for 1 hr. The bacilli found in the semithin sections were compared to the Fite-stained *M. leprae* of identical specimens which were fixed in Formalin and embedded in paraffin.

b) Second, a part of the fresh biopsies of the remaining nine patients was immediately fixed in a 0.15 M phosphate-buffered (pH 7.4) glutaraldehyde solution and prepared in the same way as described in a) above. The ultrathin sections were cut with a Reichert and Jung ultramicrotome and examined by a Zeiss EM 9.

Staining

Solutions. Methylene blue (1 g) was dissolved in 100 ml of 1% water borax. This solution can be stored for an indefinite period but should be filtered prior to use in order to avoid deposits of the color. The original solution of basic fuchsin was prepared as follows: 1 mg basic fuchsin was dissolved in 10 ml of 50% ethanol and 3 ml

[1] Received for publication on 20 March 1986; accepted for publication in revised form on 10 October 1986.

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of this original solution was made up to 50 ml with distilled water. The solution does not need filtering and can be stored up to 4 months.

Procedure. The staining technique for the semithin specimens is summarized in Table 1. A decoloration step using acid alcohol for the demonstration of the acid resistance of the bacilli was performed, but the stain of the plastic-embedded material was not acid-fast.

TABLE 1. *Procedure for the staining technique.*

Methylene blue-borax	5 min at 70°C
Warm running water	2–5 min
Alkaline-fuchsin	5 min at room temperature
Cold running water	1 min
Air dry and cover with Eucitt®	

RESULTS

Histological examination. In all 11 patients, the bacilli were bright violet using the staining method of Fite.

Semithin sections. Using the methylene blue-borax and basic fuchsin technique, all biopsies were characterized by the same staining picture (Table 2). The stratum corneum stained red to dark violet; the connective tissue was pink and red. Endothelial cells, fibroblasts, leukocytes, and histiocytes stained blue. Included material in foamy degenerated cells (bacilli) stained an intense violet (Fig. 1), while the mast cell granules stained dark blue (Fig. 2). The Fite-stained bacilli in the paraffin sections corresponded completely with the structures which stained violet with the methylene blue-borax basic fuchsin technique.

Electron microscopy. In all cases, the stained structures were corroborated as *M. leprae* by EM analysis. In the ultrathin sections, typical *M. leprae* ([7]) were found in

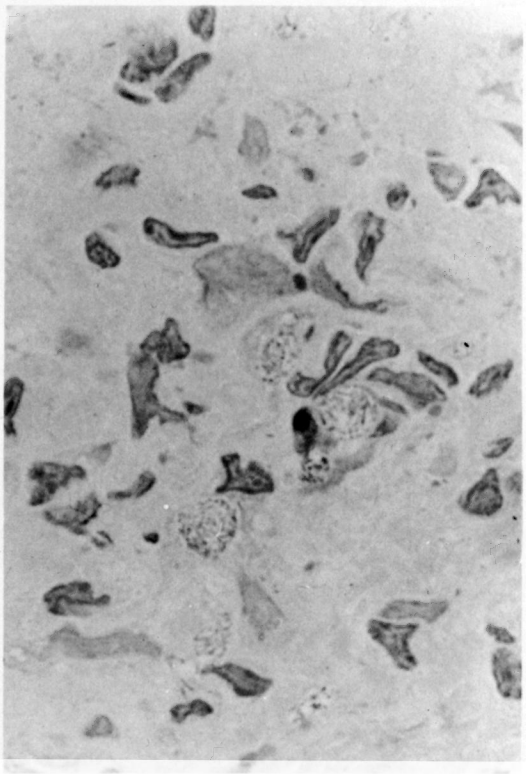

FIG. 1. Semithin section of multibacillary leprosy using the methylene blue-borax/basic fuchsin technique. In the original color, *M. leprae* stain an intense violet (×400).

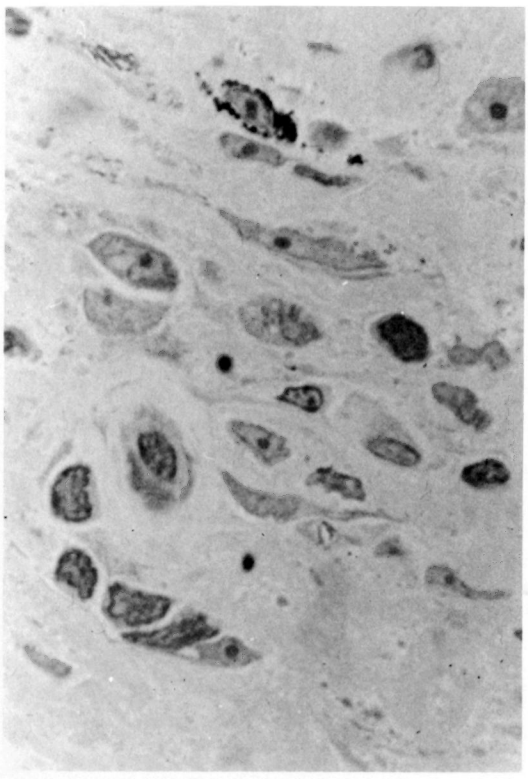

FIG. 2. Semithin section of leprosy. There is clear differentiation in the original color between the intense violet-stained *M. leprae* and the dark-blue-labeled mast cell granules (methylene blue-borax/basic fuchsin ×400).

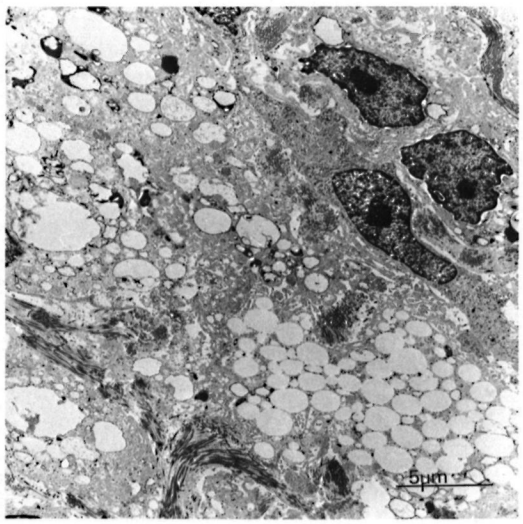

FIG. 3. Electron microscopic examination of leprosy lesion after precise demarcation of ultrathin section area using methylene blue-borax/basic fuchsin technique (×4750).

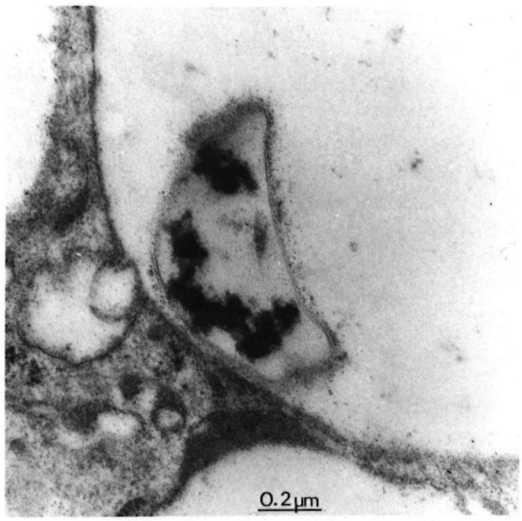

FIG. 4. *M. leprae* as seen with high magnification (×75,000).

histiocytes (Figs. 3 and 4), endothelial cells, and extracellular tissue. Even in the paucibacillary stages of Hansen's disease, *M. leprae* could be detected by EM analysis. When the microorganisms in semithin sections were marked by the methylene blue-borax basic fuchsin orientation stain, an exact demarcation of the ultrathin area could be carried out.

DISCUSSION

With the methylene blue-borax basic fuchsin staining technique, healthy skin presents a considerably more differentiated picture (Figs. 1 and 2) than with other comparable staining methods, e.g., that of Richardson ([8]). With regard to the demonstration of *M. leprae,* we succeeded in demarcating the microorganisms in the semithin section technique with the staining described above. This method does not represent a diagnostic tool, since the stain of *M. leprae* is not acid-fast in plastic-embedded material using acid alcohol. However, this fact is not important, since the diagnosis of Hansen's disease was already confirmed using Fite staining of the previous histological examination. Therefore, the methylene blue-borax basic fuchsin technique should only be used for orientation purposes in semithin sections. Nevertheless, the cell inclusions corresponded totally in regard to their localization to the *M. leprae* in the paraffin sections stained according to Fite, so that we can assume that it also constitutes *M. leprae* here. These findings were corroborated in subsequent EM examinations in which the fine structure of the *M. leprae* could be shown (Figs. 3 and 4).

The semithin sections thus prepared allow, by the clear demonstration of the bacilli, a precise demarcation of the ultrathin section area, which is not only oriented by histological accompanying factors (foamy cells, cellular infiltrate). Compared with

TABLE 2. *Characteristics of the methylene blue-borax/alkaline-fuchsin stain in semithin sections.*

Structure	Color
Epidermis	
Str. corneum	Red to dark violet
Str. Malpighi	Dark blue to light blue
Melanin	Brown
Corium	
Connective tissue	Pink to red
Elastic fibers	Blue-violet
Inflammatory cells	Dark blue (nuclei) Light blue (cytoplasm)
Mast cell granules	Dark blue
Material included in foamy degenerated cells	Intense violet

other methods ([2]), the staining solutions presented here are stable for a long time, the method allows easy handling and presents a precise descrimination between *M. leprae* and mast cell granules. In this context it must be stressed that the laboratory expenses, as compared with the Richardson staining, are only inconsequentially increased. In addition, this staining can be applied not only to individual sections but also as a cuvette method in the incubator.

SUMMARY

In Spurr-embedded biopsies for ultrastructural examinations, *Mycobacterium leprae* hardly differed from the surrounding tissues using the staining technique of Richardson. Also, the usual histological staining methods of Ziehl-Neelsen and Fite did not achieve positive results for the determination of *M. leprae*. Therefore, we applied the methylene blue-borax and basic fuchsin technique for the demonstration of the bacilli in plastic-embedded tissue of 11 patients suffering from Hansen's disease. In every patient the diagnosis was confirmed by histological examination of skin biopsies. Portions of the biopsies of nine patients were then fixed in glutaraldehyde and osmium tetroxide and embedded according to Spurr. In the other two cases, the material was first fixed in 10% Formalin and embedded in paraffin. After cutting 3-μm sections for routine histological examination, the remaining material was prepared adequately for ultrastructural examination.

Using the methylene blue-borax and basic fuchsin technique, the semithin sections of plastic-embedded material presented a considerably more differentiated picture than other comparable methods. *M. leprae* located in foamy cells or in the tissue stained violet. These findings were corroborated in subsequent electron-microscopic examinations. The semithin sections thus prepared allow, through the clear demonstration of the microorganisms, a precise demarcation of the ultrathin area.

RESUMEN

En las biopsias embebidas en plástico para exámenes ultraestructurales, el *Mycobacterium leprae* dificilmente se distingue del tejido circundante cuando se usa la técnica de tinción de Richardson. Los métodos usuales de tinción de Ziehl-Neelsen y de Fite, tampoco dan resultados satisfactorios. Debido a ésto, nosotros empleamos la técnica de azul de metileno-bórax y fuchsina básica para demostrar la presencia de bacilos en el tejido incluído en parafina proveniente de 11 pacientes afectados por la enfermedad de Hansen. En cada paciente el diagnóstico se confirmó por examen histológico de las biopsias de piel. Una porción de las biopsias de 9 pacientes se fijaron además con glutaraldehído y tetróxido de osmio y se embebieron en formalina al 10% en parafina. Después de cortar secciones de 3 μm para el examen histológico de rutina, el resto del material se procesó para su examen ultraestructural.

Usando la técnica del azul de metileno-bórax y fuchsina básica, las secciones semidelgadas del material embebido en plástico presentaron una imagen considerablemente más diferenciada que la obtenida con otros métodos comparables. El *M. leprae* localizado en las células espumosas o en el tejido, se tiñó de color violeta. Estos hallazgos fueron corroborados subsecuentemente en exámenes por microscopía electrónica. Así, las secciones semidelgadas permiten una clara demostración de los microorganismos y una precisa demarcación del tejido.

RÉSUMÉ

En utilisant la technique de coloration de Richardson en vue d'une examination ultra-structurelle des biopsies enrobées par la méthode de Spurr, on a montré que *Mycobacterium leprae* ne se différenciait que très mal des tissus environnants. De même, les méthodes de coloration histologiques habituelles de Ziehl-Neelson et de Fite n'entraînent pas de résultats positifs pour la détermination de *M. leprae*. Dès lors, les auteurs ont appliqué une technique à base de bleu de méthylène-borax et de fuchsine basique, pour démontrer la présence des bacilles dans du tissu enrobé dans du plastique chez 11 malades souffrant de lèpre. Chez chaque malade, le diagnostic a été confirmé par l'examen histologique des biopsies cutanées. Des fragments de biopsies obtenus chez 9 malades ont été fixés par la glutaraldéhyde et par le tétroxyde d'osmium, et enrobés suivant la méthode de Spurr. Chez deux autres cas, le matériel a d'abord été fixé dans du formol à 10% et enrobé dans de la paraffine. Après avoir été coupé en sections de 3-μm pour l'examen histologique de routine, le matériel restant a été préparé de manière adéquate pour l'examen des ultrastructures.

Avec la technique à base de bleu de méthylène-borax et de fuchsine basique, les sections d'épaisseur moyenne du matériel enrobé dans le plastique ont présenté une image beaucoup plus différenciée qu'avec les autres méthodes utilisées aux fins de comparaison. *M. leprae* était situé dans les cellules spumeuses ou dans le tissu coloré en violet. Ces observations corroborent les résultats des examens menés ensuite au microscope électronique. Les coupes d'épaisseur moyenne préparées de cette manière permettent de procéder à une dé-

marcation très précise de zones extrêmement fines, grâce à la mise en évidence de manière très claire, des microorganismes.

Acknowledgment. The excellent technical assistance of Ms. A. Henn is kindly appreciated.

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