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Soluble Blue as a Counterstain in the Ziehl-Neelsen Procedure— A Brief Communication[1]

Paul R. Wheeler and Philip Draper[2]

Suspensions of *Mycobacterium leprae* are commonly stained by the Ziehl-Neelsen method and counterstained with methylene blue. With crude tissue homogenates or partially purified suspensions, the relatively pale staining of the contaminating tissue debris by methylene blue is an advantage since it allows the mycobacteria to be seen easily. In our experiments to prepare highly purified *M. leprae* from animal and human tissues, it has become clear that the conventional counterstain is rather ineffective for demonstrating minor amounts of contamination. We here describe a simple counterstaining procedure using soluble blue which stains tissue particles strongly and allows them to be picked out from among a preponderance of acid-fast bacteria.

MATERIALS AND METHODS

The bacterial suspensions used in this work were obtained at various stages of purification of *M. leprae* from tissues of experimentally infected nine-banded armadillos either by our own (unpublished) method involving homogenization at high pH and digestion with DNAase or by the method of Prabhakaran, *et al.* ([2]).

Suspensions were applied to slides as smears and heat-fixed. They were stained by the version of the Ziehl-Neelsen method used routinely in our laboratory: the slides are flooded with stain, heated until visible vapor is formed and allowed to stand for 5 min, then rinsed in tap water and decolorized in 25% sulfuric acid, followed by rinsing in tap water. The slides were counterstained either with 1% aqueous methylene blue for 5 min or with 1% aqueous soluble blue (C.I.42755) for 5 min or with 0.5% soluble blue for 10 min and rinsed with tap water. The stained smears were examined and photographed under oil-immersion, using panchromatic film (Ilford FP4) and a magenta filter (Wratten 32) to reduce the photographic contrast of the acid-fast bacteria relative to the blue-stained debris.

For electron microscopy, suspensions were negatively stained with 1% uranyl acetate or 1% potassium phosphotungstate (pH 6) and examined in a Philips EM 300 electron microscope operated at 60 kV.

RESULTS

Identical smears of partly purified *M. leprae* counterstained with methylene blue and with soluble blue are shown in Fig. 1. It is clear that the suspension still contained a considerable amount of non-bacterial material derived from the host tissues and staining strongly with soluble blue. The material did not take up methylene blue or did not retain it, and if the smear had been counterstained in the conventional way, it would have been judged to consist only of bacteria. Fibrillar and amorphous debris were present. Electron microscopy (Fig. 2) showed that the fibrillar material had the characteristic band pattern of collagen while the amorphous contaminant, which was the main host-derived component present, consisted partly of amorphous and partly of membranous components. The extent of contamination observed when stained with soluble blue and when seen by electron microscopy seemed similar.

DISCUSSION

Our use of soluble blue arose from the mistaken idea that the major contaminant of our bacterial suspensions was collagen. Soluble blue forms part of the Mallory trichrome method in which collagen is stained green or blue. The stain mixture also in-

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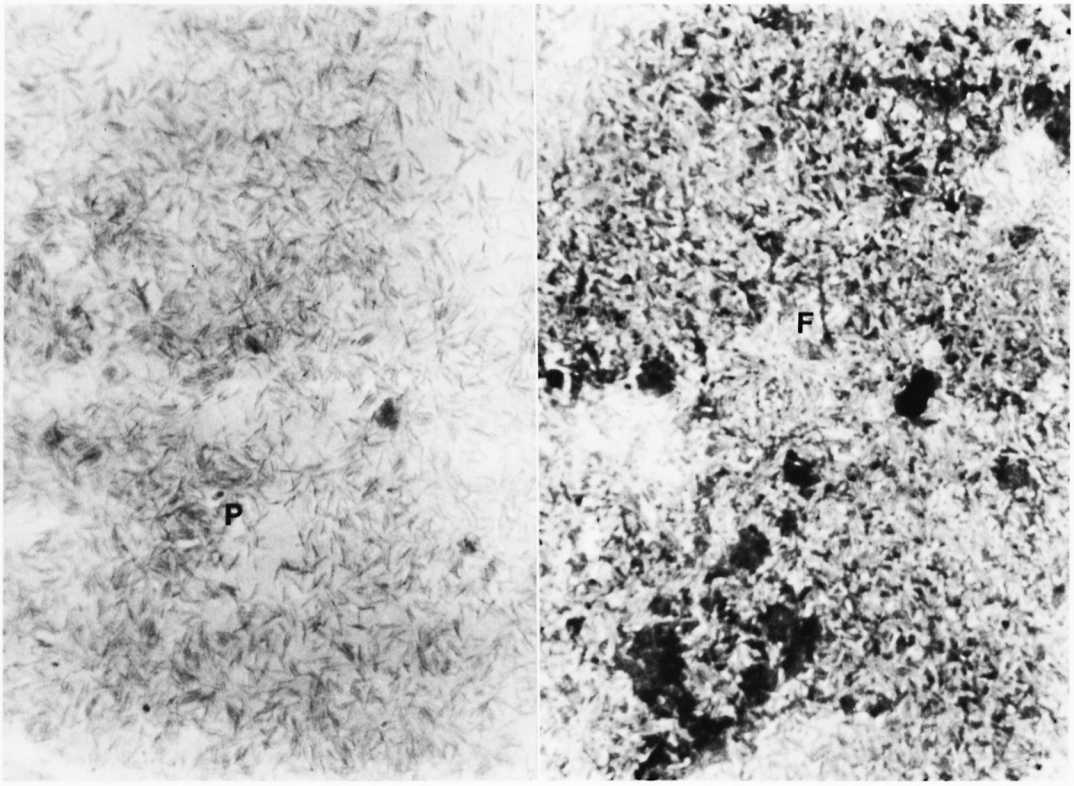
[2] P. R. Wheeler, Ph.D., Member of Scientific Staff; P. Draper, D.Phil., Member of Scientific Staff; National Institute for Medical Research, Mill Hill, London, NW7, 1AA, England.



1a 1b

FIG. 1. Partly purified suspension of *M. leprae* stained by the Ziehl-Neelsen process and counterstained with a) methylene blue and b) soluble blue. p—blue-stained particles; f—fibrillar debris. Photographed through a magenta filter ×1550.

cludes phosphotungstic acid, which apparently increases the specificity of the coloring, as discussed by Lendrum, *et al.* (1), which proves to be unnecessary in the present application. Equivalent results are given by staining for 5 min in 1% dye or for 10 min in 0.5% soluble blue. Soluble blue is a strongly acidic dye (3 sulfonate groups) whereas methylene blue is a weakly basic one, but since the nature of the contaminating tissue material is unknown, its different behavior with the two stains cannot be explained. Observation of the slides while they are being rinsed in tap water after staining suggests that the main advantage of soluble blue is that it is very resistant to being washed out of the stained material whereas methylene blue is easily removed. The powerful coloration given by our technique is unsuitable for very crude bacterial suspensions (e.g., tissue homogenates) since the bacteria are difficult to see. It is also unsuitable for suspensions prepared for counting by dilution with 0.1% bovine serum albumin, which takes up the stain.

The staining method is equally effective when a different acid-fast staining technique is used involving decolorization with acid alcohol (Dr. J. Convit and Mrs. M. Pinardi, personal communication). Its simplicity and sensitivity seem to make it ideal for monitoring the progress of attempts to free *M. leprae* from the animal tissues in which it is grown.

SUMMARY

A technique is described using soluble blue instead of the conventional methylene blue as a counterstain for the Ziehl-Neelsen procedure. This increases the intensity of

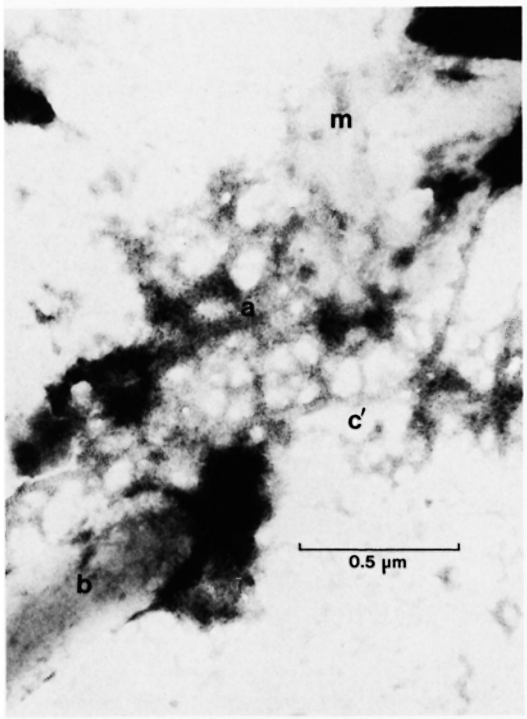


FIG. 2. Partly purified suspension of *M. leprae*, negatively stained with uranyl acetate. a—amorphous debris; b—bacterium; c—collagen fibril; m—membraneous debris. Electron micrograph. Bar represents 0.5 μm.

blue staining of non-bacterial contaminants and is useful in monitoring the progress of purification of *M. leprae* from host tissue.

RESÚMEN

Se describe una técnica en la cual se emplea azul soluble en lugar del azul de metileno convencional como colorante de contraste en el procedimiento de Ziehl-Neelsen. Esto aumenta la intensidad de la coloración azul de los contaminantes no bacterianos y por ésto, la técnica resulta de gran utilidad para seguir el progreso de la purificación del *M. leprae* a partir de tejido infectado.

RÉSUMÉ

On décrit ici les techniques basées sur l'emploi du bleu solide, en lieu et place du bleu de methylène conventionnel comme colorant de contraste dans le procédé de Ziehl-Neelsen. Cette procédure augmente l'intensité de la coloration bleue des contaminants non-bactériens. Elle est utile pour suivre l'évolution de la purification de *M. leprae* récolté à partir des tissus.

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