Improved staining of leprosy bacilli in tissues

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Summary A technique which reliably demonstrates *Mycobacterium leprae, M. tuberculosis* and fungi in tissues is described. It is based on the oxidation of cell wall lipid substances by chromic or periodic acid, and the subsequent release of aldehydes which are then capable of reducing ammoniacal silver salt solutions to metallic silver. The organisms so demonstrated appear uniformly solid. The sensitivity of the method and the ease of examination and recognition of bacilli and their products are recommendations for the use of the method in diagnosis and research, disregarding morphological appearances.

Introduction

For unknown reasons it is often difficult to demonstrate *Mycobacterium leprae* in tissues. This is especially so with material which has been fixed for long periods in formalin, or when organisms are in an advanced state of decay, or if they are not acid-fast by the conventional Ziehl–Neelsen staining method. Greater sensitivity of staining can be achieved using prior oxidation with periodic acid and subsequent staining in carbolfuchsin,[1,2] or carbol-pararosanilin.[3,4] Silver impregnation, originally used to demonstrate fungi in tissues[5] is another reliable means of staining mycobacteria.[6-8] Harada[9,10] made use of prior oxidation followed by silver impregnation to enhance the demonstration of mycobacteria in tissues.

The method described below is based on the same principle. Leprosy bacilli, tubercle bacilli, other mycobacteria and certain fungi can be clearly visualized by this technique.

Materials and methods

TISSUES

Human skin lesions of various forms of leprosy, pulmonary lesions of tuberculosis and fungal lesions were examined. Tissues were fixed in 10% neutral buffered formalin for 24–48 h, dehydrated, blocked in paraffin wax and sectioned at 5 μm. Egg albumin–glycerin adhesive was used to attach the sections on to clean glass slides.

METHOD

The chromic acid or periodic acid-ammoniacal silver reaction to demonstrate mycobacteria and fungi in tissues is carried out as follows.

1   Deparaffinize sections in xylene and hydrate through graded alcohols to water.
2   (a) Oxidize in 5% chromic acid for 1 h followed by 2% sodium bisulphite briefly, or (b) oxidize in 1% periodic acid overnight.
3   Wash in running tap water, and rinse in 3 changes of distilled water.
4   Place sections in a Coplin jar containing freshly prepared ammoniacal silver solution, and place the jar in an oven at 60°C for about $1-2\frac{1}{2}$ h, until the sections turn brown to black (see below).
5   Rinse in 2 or 3 changes of distilled water at 60°C, and then in several changes of distilled water at room temperature.
6   Tone in 0·1% gold chloride for 5 min.
7   Rinse in distilled water.
8   Fix in 2% sodium thiosulphite for 2 min.
9   Wash in tap water.
10 Stain nuclei with nuclear-fast red solution for 15 min.
11 Rinse in tap water.
12 Counterstain with 0·01% methyl blue (Merck, art. 16316), or with 0·03% methyl blue (Merck, art. 16315) in saturated aqueous picric acid solution for 5 min.
13 Dehydrate directly in absolute alcohol.
14 Clear in terpineol-xylene (1 : 1), followed by xylene. Mount.

AMMONIACAL SILVER SOLUTIONS

Solutions are prepared in one of three ways.
1   Ammoniacal silver nitrate: to 25 ml of 10% aqueous silver nitrate solution add 28% ammonia water until a clear solution is formed. Then add 10% silver nitrate drop by drop till a faint cloudiness appears. Add 25 ml distilled water.
2   Ammoniacal silver carbonate: to 10 ml of 10% aquaeous silver nitrate add 40 ml of 5% anhydrous silver carbonate. When the precipitate has settled, remove the supernatant and wash the deposit several times with distilled water. Decant. Add 28% ammonia water drop by drop till the precipitate is dissolved. Add 10% aquaeous silver nitrate drop by drop till the solution becomes cloudy. Make up to 100 ml with distilled water.
3   Ammoniacal silver hydroxide solution: to 10 ml of 10% silver nitrate solution add 5 ml of 4% NaOH till a black precipitate is formed. Add 28% ammonia drop by drop till the solution is cloudy. Make up to 100 ml with distilled water.

STAINING TIMES	After chromic acid oxidation	After periodic acid oxidation
1 Ammoniacal silver nitrate:	2 h	2h
2 Ammoniacal silver carbonate:	1 hr 40 min	2 h 45 min
3 Ammoniacal silver hydroxide:	50 min	1 h 10 min

NUCLEAR STAIN   1 g nuclear-fast red (CI 60760)
                 5 g aluminium sulphate
            100 ml distilled water.

Results

Mycobacteria and fungi stain black, mucin brown-black, melanin grey, nuclei pink-red, and connective tissue green-grey-black. In pulmonary lesions carbon remains black (see Figures 1, 2 and 3).

Discussion

The method described for mycobacteria, including *M. leprae* and *M. tuberculosis*, and fungi in tissues is based on a chemical reaction which is both sensitive and reliable. The mechanism of the reaction depends on the oxidation of lipid hydroxy-amino groups in the mycobacterial cell wall and the release of free aldehydes. These aldehydes can be revealed either by a modified Schiff procedure using carbol-pararosanilin,[3,4] or by treatment with silver salt solutions which are reduced to metallic silver. The present method utilizes ammoniacal silver salts rather than the methanamine used previously.[9,10] Ammoniacal silver is used to demonstrate melanin. After oxidation melanin is only poorly recognizable but other substances like argentaffin, ascorbic acid, uric acid and polyphenols react with argyrophilic stains in a manner similar to aldehydes, and silver is deposited at the site of interaction. This in no way hinders the recognition of the mycobacteria or fungi, though carbon particles in the lung can sometimes mask bacilli in that tissue.

Chromic acid has the same oxidizing potential as periodic acid, but strict control of the oxidation time is necessary. Prolonged oxidation in chromic acid carries the risk of losing the aldehydes released, upon which the subsequent binding by carbol-pararosanilin,[3,4] or impregnation
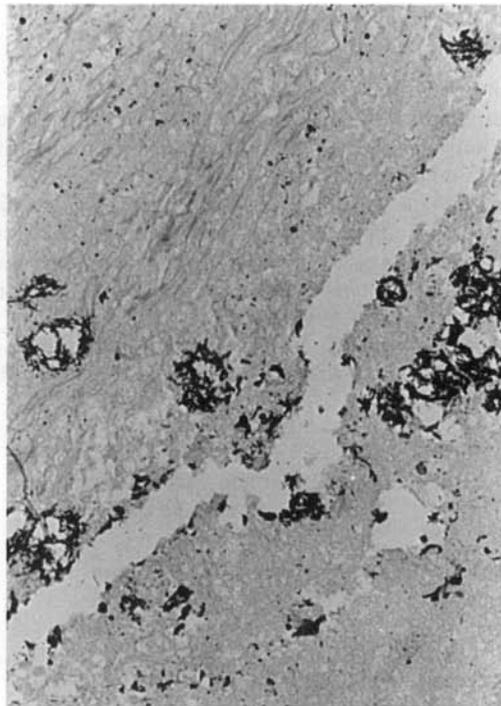

Figure 1. Tubercle bacilli in pulmonary tuberculosis. Periodic acid-ammoniacal silver hydroxide stain.
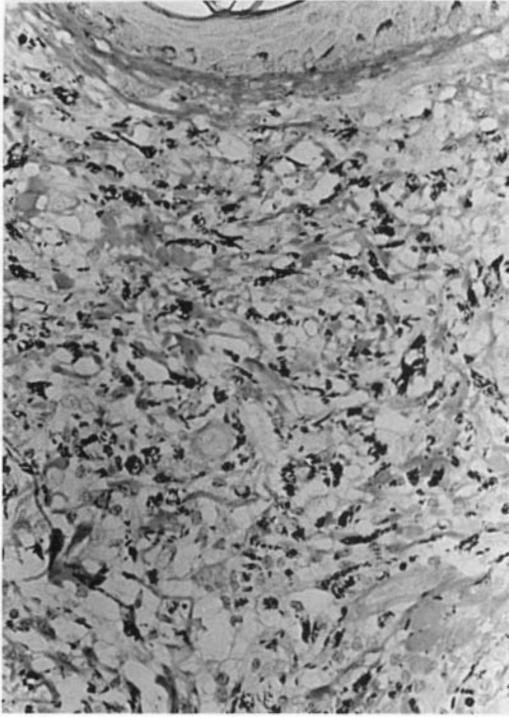
Figure 2. Leprosy bacilli in lepromatous lesion of skin. Periodic acid-ammoniacal silver hydroxide stain.
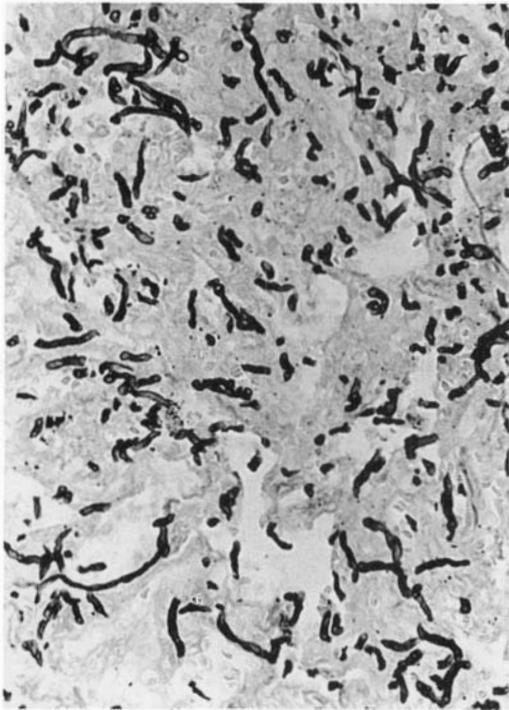

Figure 3. Fungi in pulmonary tuberculosis. Periodic acid-ammoniacal silver hydroxide stain.

with silver depends. For this reason periodic acid is to be preferred in spite of the longer time required for the oxidation.

The majority of the *M. leprae* stained by the chromic or periodic acid-ammoniacal silver technique, stain with a uniform solid appearance. Even nonsolid bacilli appear rod-shaped. Thus the usefulness of the method does not extend to studies on viability or assessment of the bacteriological state of the patient. Nevertheless, its application in diagnosis and research cannot be emphasized too strongly. In research the ease of identifying organisms and degradation products, which are often difficult to detect by acid-fast staining, is comparable in many ways to the sensitivity of immunocytochemical methods using antiBCG antibody. Material stained by the two methods in parallel correlated very well (M J Ridley, personal communication 1988). The fact that granular and degraded bacilli stain with a solid appearance is understandable considering that it is the lipid moiety in the bacterial cell wall itself that is the key substance on which the reaction is effected. The disintegration of cell walls is achieved with difficulty in the case of *M. leprae*, especially so in lepromatous infections in which clearance of bacilli presents further problems.

Acknowledgments

References

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