Technical methods

Staining of mycobacterium leprae in epoxy resin sections

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The continuing investigations into the staining characteristics of acid fast bacilli in epoxy resin sections produced a method for the effective staining of Mycobacterium leprae. This method entails the removal of resin by careful treatment with bromine vapour, as described in a previous communication concerning M tuberculosis.1 In this present procedure the above step is followed by treatment with oil, oxidation with acidified potassium permanganate, immersion for 24 hours in ammoniacal alcohol, and then a modified Ziehl-Neelsen technique, which resulted in M leprae and M tuberculosis being stained acid fast.

Material and methods

TISSUE FIXATION AND EMBEDDING

M leprae and M tuberculosis infected human tissue were fixed in 10% neutral buffered formalin, processed through acetone, and embedded in epoxy resin. Sections were cut at 4 μm.

BROMINE SOLVENT

Sections were placed face downwards on a small Ehlenmeyer flask containing 1 ml of bromine, which was kept rapidly vapourising by means of a magnetic stirrer. The sections were exposed to the bromine vapour for 30–60 seconds followed by two rapid washes in acetone, and a wash in water. This step was done under a fumehood.

REAGENTS

Acidified permanganate: equal parts of 0.5% aqueous potassium permanganate and 0.5% aqueous sulphuric acid were mixed immediately before use. Ammoniacal alcohol comprised: ethyl alcohol 70 ml; distilled water 10 ml; 28% ammonia (20 ml).

STAINING PROCEDURE

Sections were:
1. Exposed to bromine vapour as described above and brought to water.
2. Blotted dry and peanut oil applied at 22°C for 15 minutes.
3. Drained, blotted well, and washed in water.
4. Oxidised for five minutes in acidified permanganate.
5. Washed in water then treated with 1% aqueous oxalic acid for two to five minutes or until the section was bleached.
6. Washed in water for five minutes.
7. Placed in the ammoniacal alcohol reagent and incubated at 37°C for 24 hours.
8. Washed in water for five minutes.
9. Stained with Ziehl-Neelsen’s carbol fuchsin at 22°C for 30 minutes.
10. Rinsed in water and decolourised with 5% aqueous sulphuric acid, usually five minutes.
11. Washed in water and counterstained with 0.1% malachite green for 30 seconds; rinsed with water and air dried.
12. Cleared in xylene and coverslipped using DPX.

Results

M leprae and M tuberculosis stained red. M leprae required all three pretreatments to obtain a positive result. The order of the pretreatment was important. If oil followed the ammoniacal alcohol solution or the oxidation step a negative result ensued. If oxidation came last a negative result was again obtained. M tuberculosis stained well with prior treatment with oil, the oxidation step, and only six hours of pretreatment with ammoniacal alcohol. Excellent positive staining was obtained if 3% hydrogen peroxide was substituted for the acidified permanganate. M leprae did not stain if the hydrogen peroxide was used as the oxidant. When Ziehl-Neelsen’s carbol fuchsin was heated to steaming for 10 minutes and then differentiated with acid alcohol, both M leprae and M tuberculosis stained almost as strongly as with the above described technique.

Discussion

M leprae can be difficult to stain in paraffin sections. In 1938 Faraco suggested the use of warm oils to “refat” the bacillus and restore their acid fast staining. In 1952 Wade avoided alcohol differentiation to enhance the Ziehl-Neelsen stain. Alkali solutions improve the intensity of the stain, as does oxidation of the sec-
tion. These factors were used successfully to restore the acid fastness of *M. leprae* and *M. tuberculosis* in epoxy resin sections.

It must be emphasized that the method described must be followed accurately. If this is done the results are both reliable and consistent.

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References


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Detection of *Campylobacter pyloridis* in gastric mucosa by phase contrast microscopy

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Curved bacteria were recently detected in gastric biopsy specimens using histological stains.† ‡ ³ Marshall and Warren established methods for the isolation of these bacteria and described their Campylobacter pyloridis. They proposed the name *Campylobacter pyloridis* and postulated an aetiological role for these bacteria in gastritis and peptic ulceration. Subsequently, some workers found culture more sensitive than histological stains for detecting *C pyloridis*.† ² ³ whereas others achieved a higher detection rate with the Warthin-Starry silver stain.† ⁴ ⁵ ⁶ We used phase contrast microscopy for the direct examination of gastric antral biopsy specimens for these curved bacteria and compared it with the above mentioned methods.

Material and methods

Gastric biopsy specimens were obtained from consecutive patients undergoing routine upper gastrointestinal endoscopy. Informed consent was obtained from all subjects. Three endoscopic biopsy samples were obtained from the antral mucosa within a 5 cm radius of the pylorus.

**Microscopy and Culture**

Two biopsy specimens were collected in 0.25 ml sterile saline (0–85%) for transport to the laboratory and were processed within three hours. They were minced finely in two drops of saline and about a quarter of this homogenate was pressed firmly between two glass microscope slides. One slide was stained with Gram stain using 0–2% carbol fuchsin as counterstain. A coverslip was placed over the other slide, which was then examined under phase contrast (×400 magnification).

The rest of the sample was cultured on chocolate (horse) blood agar (Columbia agar, Oxoid CM331) and on brain heart infusion agar (Oxoid CM375) supplemented with 7% horse blood, 3 µg/ml vancomycin, 50 µg/ml nalidixic acid, and 5 µg/ml trimethoprim. The plates were incubated at 37°C for up to six days in a humid atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen.

Isolates were considered to be *C pyloridis* if they grew as 1–2 mm translucent greyish colonies after three days on chocolate agar, were Gram negative, curved or S shaped rods which became coccoid in older cultures, and were positive for oxidase and catalase, as described by Marshall and Warren. Further tests were done using brucella broth (BBL 11088) containing 0.16% agar and 5% normal horse serum as basal medium. Tests were incubated as above for 10 days.

**Histoogy**

The third biopsy specimen was fixed in 10% phosphate buffered neutral formalin and 4–5 µm sections stained by the Warthin-Starry method. The three microscopic methods were assessed blindly by independent observers.

Results

Under phase contrast microscopy *C pyloridis* appeared as characteristic black, well defined, curved or S shaped rods, 2–3 µm long, lying in close association with the mucus and epithelial cell layer (Figure). This morphology was retained for at least five hours in preparations kept in a moist chamber at room temperature.
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