An aid to the preliminary identification of non-sporing anaerobes

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One of the problems associated with the isolation of anaerobic bacteria from clinical material is the presence of aerobic bacteria which, by virtue of their more rapid growth rate, are inclined to obscure the anaerobes. *Proteus* species pose a particular problem because of their tendency to swarm over the surface of the medium. Such mixed infections are frequently encountered in otogenic cerebral abscesses (McFarlan, 1943; Heineman and Braude, 1963; Ingham *et al.*, 1977) and intra-abdominal sepsis (Altemeier, 1942; Gillespie and Guy, 1956; Leigh, 1974). In this laboratory a nalidixic acid strip placed on the surface of the medium is used to inhibit the growth of proteus in anaerobic cultures. Preliminary studies showed that this allowed the growth of bacteroides, fusobacterium, and anaerobic streptococci, while inhibiting most Gram-negative aerobic bacilli, *Haemophilus* spp, and clostridium. It was therefore decided to incorporate nalidixic acid in the medium to aid the more rapid isolation of anaerobic organisms and, additionally, by the use of appropriate antibiotic sensitivity discs (Sutter and Finegold, 1971) it was hoped a presumptive identification of the anaerobes could be made. The manner in which this technique is used is presented in this paper.

Material and methods

The basic culture medium used consisted of nutrient agar supplemented with 5% defibrinated horse blood. Anaerobic culture was carried out in a Mackintosh and Fildes jar fitted with a cold catalyst using an atmosphere of 90% hydrogen and 10% carbon dioxide, incubated at 37°C for a minimum of 48 hours and in some instances up to five days.

All specimens were inoculated onto two blood agar plates containing 50 µg/ml nalidixic acid (Nal 50 plates), one of which was spread to obtain single colonies. The other (the Nal 50 sensitivity plate) was uniformly inoculated, and discs containing penicillin IU, metronidazole, 5 µg, and clindamycin, 2 µg, were placed at equidistant points on the surface. The two Nal 50 plates were incubated anaerobically,

together with a blood agar plate inoculated to obtain single colonies on which a strip of paper 5.5 × 0.5 cm containing nalidixic acid had been placed, at right angles to the well of inoculum, so that it extended into the area where single colonies would be expected.

In addition, specimens were inoculated onto a blood agar plate and an Oxoid MacConkey agar plate, which were incubated aerobically at 37°C and onto a chocolate agar plate incubated in a candle jar at 37°C.

Results and discussion

The technique of using the Nal 50 sensitivity plate is as follows. The variety of aerobes present is determined by examination of the blood agar and MacConkey plates incubated aerobically. Inhibition of growth of Gram-negative aerobes by nalidixic acid is indicated by their absence around the clindamycin disc on the Nal 50 sensitivity plate. Examination of a Gram film prepared from the centre of the Nal 50 plate, that is, beyond the zones of inhibition produced by the discs, enables an assessment to be made of the types of bacteria present. Complete inhibition of growth around the metronidazole disc shows that obligate anaerobes only are present. Growth of Gram-negative bacilli up to the penicillin disc can be assumed to be *Bacteroides fragilis*. Similarly, any Gram-positive cocci on the plate which are inhibited by metronidazole may be identified as obligate anaerobes, which may or may not grow up to the penicillin disc, according to their sensitivity. A zone of inhibition around the metronidazole disc with intrazonal growth consisting of Gram-positive cocci is indicative of the presence of obligate anaerobes together with a facultative anaerobe, for example, *Streptococcus faecalis* or *Staphylococcus aureus*, the presence of which would already have been noted on the aerobic plates. If, however, Gram-positive cocci have not grown on the aerobic plate this is presumptive evidence for the presence of carbon dioxide dependent streptococci, for example, *Streptococcus mutans* or some strains of *Streptococcus milleri*.

The presence of carbon dioxide dependent streptococci and anaerobic streptococci in mixed culture may be suspected if there are differences in size and morphology in films prepared from the centre of the plate. In contrast, films from within the zone of inhibition of the metronidazole disc show marked uniformity in this respect due to the inhibition of growth of the anaerobic streptococci.

Further incubation of the Nal 50 sensitivity plate allows the isolation of slow-growing organisms such as *Bacteroides melaninogenicus*, *Actinomyces*, and

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other anaerobes. The presence of a multiplicity of anaerobes is indicated by the development of concentric zones of inhibition of growth around the metronidazole disc. Gram films of smears prepared from within these zones provide useful preliminary indications of the identity of these organisms, which can be obtained in pure culture from the Nal 50 plate spread for single colonies.

This technique has been in regular use in this laboratory for the last two years. It has allowed the demonstration of the presence of obligate anaerobes, often after overnight incubation, their preliminary identification, and sensitivity to antibiotics. A quantitative evaluation of the technique was not carried out but it was used in our recent investigations of the bacteriology of otogenic cerebral abscesses (Ingham *et al.*, 1977). In this study 37 obligate anaerobes were isolated from nine patients, a frequency of isolation which, to our knowledge, has not previously been achieved in such patients.

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Letters to the Editor

Cell counting in gut mucosa

Counts of cells in the lamina propria have almost all been reported with the area of the lamina propria as the reference unit. This seems illogical. To take an obvious example, the lamina propria in coeliac disease is both densely cellular and increased in area in proportion to the length of gut wall examined; there is no evidence that the surface area of the muscular wall of the gut is altered in coeliac disease, and the muscularis mucosae should therefore provide a constant reference unit whereas the volume of lamina propria is not constant. An incidental advantage of using the muscularis mucosae for reference is the relative toughness of the muscle, compared with the loose connective tissue of the lamina propria, which is much more readily disrupted and theoretically more liable to artefactual volume changes because of its high proportion of extracellular fluid.

In diagnostic histopathology, the usual abnormality is both a large increase in cellular density and a lesser increase in area of lamina propria (Meinhard *et al.*, 1975), and in this situation the choice of area of lamina propria as unit produces few anomalies. However, if the area of lamina propria is reduced then the same absolute number of cells will appear to be an increased number, and this effect was well shown by a recent biopsy which on subjective assessment was clearly normal by all criteria; the mean mucosal thickness ($\pm$ standard deviation) excluding villous height was $91 \pm 19 \mu$ (6 controls: $108 \pm 33 \mu$), area of lamina propria was 0.086 ± 0.014 sq mm per 1 mm length of muscularis mucosae (controls 0.126 ± 0.026), and total plasma cells (methyl green pyronin) were 260 ± 57 per 1 mm length of muscularis mucosae (controls 223 ± 89). Estimation of total plasma cells per sq mm lamina propria gives

3023 ± 663 (controls 1770 ± 706); it is difficult to accept this apparent statistical difference as a valid biological difference.

I suggest that the length of muscularis mucosae is a rational choice of reference unit for cell counts in gut mucosa, and that where possible the area of lamina propria per unit length of muscularis mucosae should also be given.

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