INHIBITION OF CLOSTRIDIUM DIFFICILE BY
FAECAL STREPTOCOCCI

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SUMMARY. The inhibitory activity of seven strains of faecal streptococci against
34 strains of Clostridium difficile was examined in vitro after growth of the
streptococci for 24 and 48 h. All strains of C. difficile were inhibited at 48 h but at
24 h the inhibition was variable. Streptococcus faecium, a group D streptococcus and an ungroupable streptococcus exhibited the most striking inhibitory activity. Lowering of pH of the medium occurred at the site of inhibition, but the pH change alone did not explain the inhibition of C. difficile. This antagonism may be related in vivo to the resistance to intestinal colonisation by C. difficile exhibited by the normal bowel flora, and in vitro to failures to isolate C. difficile from faecal specimens.

INTRODUCTION

There have been many studies of bacterial antagonism since this phenomenon was reported originally by Pasteur and Joubert (1877). Although many investigators have studied the antagonistic interactions between streptococci and clostridia (Guelin, Kreguer and Le Bris, 1947; Stark, 1960; Brock, Peacher and Pierson, 1963), the inhibition of Clostridium difficile by streptococci was not described until recently (Rolfe, Helebian and Finegold, 1981). In our laboratory, the inhibitory activity of Streptococcus faecalis against C. difficile was suspected when quantitative culture of a specimen of faeces (from the “index patient”) yielded only S. faecalis (3 × 10⁷ cfu/g faeces), but after the specimen had been treated with ethanol for 1 h C. difficile was isolated (8 × 10⁶ cfu/g faeces). We therefore examined the ability of several strains of faecal streptococci to inhibit the growth of C. difficile because this phenomenon may explain the resistance of the normal intestinal tract to colonisation by C. difficile and failures in vitro to isolate C. difficile from human faeces.

MATERIALS AND METHODS

Bacterial strains. The 34 strains of C. difficile examined were isolated from human faeces on cycloserine-cefoxitin fructose agar (George et al., 1979). They were identified according to George et al. (1979) and Sutter, Citron and Finegold (1980). The strains of streptococci examined were S. faecalis untypable; S. faecalis serotypes 1, 4 and 9; S. faecium, a group-D streptococcus (non-enterococcal), and an ungroupable streptococcus. The untypable S. faecalis was isolated from the faeces of the index patient and serotyped by the Streptococcus Reference Laboratory, Colindale, which also kindly provided the remaining strains.

Antagonism procedure. A modification of the method described by Gillies and Govan (1966) was used. Paper strips impregnated with 24 h Todd-Hewitt broth cultures of each strain of streptococcus were used to inoculate two plates of Viande-Levure Medium (VLM) containing 10⁴% horse blood (Beerens and Fievez, 1971) and modified by the addition of glucose 10 g/L, sodium formaldehyde sulfoxylate 0-3 g/L and L-cysteine HCl 0-5 g/L, pH 7-4. The strips were then removed after 1 min and one plate was incubated for 24 h and the other for 48 h at 37 C. After exposure to chloroform for 30 min, the plates were exposed to air for 1 h and then

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FIG. 1.—Patterns of inhibition of *Clostridium difficile* (lines S3, S2, S1, R) produced by streptococci grown on the middle of a VLM plate. S3 = clearcut zone of inhibition; S2 = zone of inhibition with resistant colonies; S1 = clearcut zone of inhibition limited to the original line of streptococci; R = no inhibition. The pH was measured at points a, b, and c after growth of the streptococci for 24 and 48 h.

transferred to an anaerobic cabinet with a gaseous atmosphere of N₂ 85%, H₂ 10%, CO₂ 5% (Tabaqchali, Fiddian and Atkinson, 1979). Strains of *C. difficile* grown in Robertson's cooked meat broth (RCM) were diluted tenfold in fresh RCM; 1 μl of the diluted broth was inoculated on each of the VLM plates, which were then incubated at 37°C for 48 h in the anaerobic chamber. The resultant zones of inhibition were measured with vernier callipers.

Measurement of the pH of the VLM medium. *S. faecalis* untypable and *S. faecalis* serotype 9 were separately inoculated on VLM plates, as described above, and incubated aerobically at 37°C. At 24 and 48 h the pH of the medium was measured with a Phillips PW 9409 digital pH meter, at three points a, b, c (fig. 1).

**RESULTS**

The inhibition of growth of *C. difficile* by faecal streptococci is illustrated in fig. 2. All seven strains of streptococci, after growth for 48 h, inhibited the 34 strains of *C. difficile*. However, the same strains of streptococci, after 24 hours of growth, did not all inhibit the clostridia. As shown in table I, 31 strains of *C. difficile* were inhibited, in clearcut zones, by *S. faecalis* untypable. One strain was partially inhibited and two strains were resistant. *S. faecalis* serotype 4 inhibited 12 strains of *C. difficile* with clearcut zones; 12 strains were partially inhibited and 10 strains were resistant. *S. faecalis* serotypes 1 and 9 were less inhibitory; each of them inhibited only six strains of *C. difficile*, while 14 and 15 strains were partially inhibited and 14 and 13 strains were resistant to them, respectively. The remaining three strains of streptococci, *S. faecium*, the group-D streptococcus and the ungroupable streptococcus, inhibited all the 34 strains of *C. difficile* after growth for 24 and 48 h. The untypable strain of *S. faecalis*, isolated from the index specimen, inhibited the growth of the *C. difficile* strain isolated from the same specimen after growth for 24 and 48 h.

The zones of inhibition of *C. difficile* after 48-h growth of all the examined streptococci were clearcut and larger than the zones produced after growth for 24 h (table II).
TABLE I
Patterns of inhibition produced by the 24-h growth of streptococci on 34 strains of C. difficile

<table>
<thead>
<tr>
<th>Strain of streptococcus</th>
<th>Number of strains of C. difficile that showed pattern of inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S3</td>
</tr>
<tr>
<td>S. faecalis untypable</td>
<td>31</td>
</tr>
<tr>
<td>S. faecalis serotype 4</td>
<td>12</td>
</tr>
<tr>
<td>S. faecalis serotype 1</td>
<td>6</td>
</tr>
<tr>
<td>S. faecalis serotype 9</td>
<td>6</td>
</tr>
<tr>
<td>S. faecium</td>
<td>34</td>
</tr>
<tr>
<td>Group D</td>
<td>27</td>
</tr>
<tr>
<td>Ungroupable</td>
<td>34</td>
</tr>
</tbody>
</table>

* For patterns of inhibition see fig. 1. S3 and S1 = inhibition; S2 = partial inhibition; R = resistant (no inhibition).

Fig. 2. —Inhibition of six strains of C. difficile by an ungroupable streptococcus grown for 48 h on VLM medium.

The pH at the surface of the VLM medium was lowered by growth of the two strains of streptococci tested over an area extending from point a to point c (figs. 1 and 2), point c representing the largest zone of inhibition of growth of C. difficile produced by the streptococci (table III).

DISCUSSION

The results presented in this study demonstrate that in-vitro growth of C. difficile is inhibited
by a variety of faecal streptococci. Although streptococci are considered to be among the most inhibitory bacteria, exhibiting bacteriocin-like antagonism against other bacteria (Tagg, Dajani and Wannamaker, 1976), this phenomenon has not been reported to occur between streptococci and C. difficile until recently. Rolfe et al. (1981) described the inhibitory activity of group-D enterococci against 20 strains of C. difficile using simultaneous and deferred antagonism procedures. We used a modification of the procedure applied by Gillies and Govan (1966) because the degree of inhibition was easy to detect and the results were reproducible.

After incubation for 48 h all the strains of streptococci inhibited the growth of C. difficile strains, but the degree and the rapidity of their inhibitory activity appeared to vary with the different strains of streptococci. Thus, S. faecium, a group D streptococcus and an ungroupable streptococcus exhibited the most prominent inhibitory activity amongst the seven strains of streptococci. The nature of this inhibitory activity has not yet been clarified. Lowering of the pH of the media during the growth of enterococci may have inhibited the growth of C. difficile, but these pH changes alone do not explain the whole phenomenon. Several species of clostridia can grow on media with pH values of 4.8 and above (Kafel and Ayres, 1969) which is much lower than those observed in our experiments. Thus, apart from the low pH, some other factors might be implicated in this bacterial antagonism such as bacteriocins or bacteriocin-like substances, the production of which is favoured by low pH and by a prolonged period of incubation of the producer strain (Tagg et al., 1976). This may also explain the increased inhibitory activity after 48 h.

Whatever the nature of this antagonism, the phenomenon may have great significance in vivo. Larson et al. (1978) and Fekety et al. (1980) suggested that the normal intestinal flora is capable of suppressing the growth of C. difficile but did not specify the inhibiting organisms. Therefore faecal streptococci, which are one of the major components of the normal flora and important producers of lactic acid (Mitsuoka and Ono, 1977), may play an important role in resistance to colonisation of the intestinal tract by C. difficile.
This phenomenon has recently been exploited in the prevention or treatment of antibiotic-associated colitis (Gotz et al., 1979) by preparations of lactobacilli which inhibit the growth of C. difficile in vitro (Rolfe et al., 1981). An alternative could be the administration of an inhibitory strain of streptococcus which would suppress the growth of C. difficile and enhance the resistance of the intestinal flora. Furthermore, in-vitro antagonism may be implicated in failures to isolate C. difficile from mixed cultures containing faecal streptococci. Such inhibition, which can occur after 24-h as well as at 48-h incubation of faecal specimens may lead to false negative cultures and explain the failure to grow C. difficile from specimens positive for C. difficile cytotoxin (Bartlett et al., 1978; Willey and Bartlett, 1979; Borriello and Larson, 1981). We therefore advocate the treatment of faecal specimens with ethanol before culture in order to eliminate inhibitory bacteria, as was done in the specimen from our index patient.

The different sensitivity patterns observed after 24-h growth of streptococci raise the possibility of utilising and extending this system into a typing scheme for C. difficile and applying it in epidemiological studies. This is under investigation.

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REFERENCES


