Characterisation of and polysaccharide production by amoxycillin-resistant streptococci

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Summary. Small numbers of bacteria capable of growing on agar supplemented with amoxycillin 40 mg/L were isolated from the saliva of 9 out of 20 adult volunteers in a previous study. All the bacteria were identified as *Streptococcus sanguis* although no strains produced dextran in conventional tests. However, using a specific assay, all the antibiotic-resistant strains were found to secrete glucosyltransferases (GTF), the enzymes that synthesise these extracellular polysaccharides; the production of GTF-S, the enzyme that synthesises dextran, was 22–43% less than that of an antibiotic-sensitive control strain. Enzyme production by both antibiotic-resistant and sensitive bacteria was markedly inhibited by dextran primer. The amoxycillin-resistant bacteria were resistant to other penicillins; their resistance to erythromycin was variable but they were uniformly sensitive to cephalothin and clindamycin. As dextran production has been proposed as a key factor in the colonisation of damaged heart valves by bacteria such as *S. sanguis*, these highly resistant bacteria may not pose a threat to the susceptible individual.

Introduction

In a recent study of the effect on the oral flora of adult volunteers of repeated high-dose prophylaxis with amoxycillin, several antibiotic-resistant streptococci were isolated from saliva (Woodman *et al.*, 1985). Of particular significance were the low numbers of streptococci isolated from 9 out of 20 subjects before prophylaxis that were able to grow on media supplemented with amoxycillin 40 mg/L. This concentration corresponds to the peak serum levels of the antibiotic and, consequently, these organisms could pose a threat to patients at risk of infective endocarditis. In a similar study of antibiotic prophylaxis, Southall *et al.* (1983) reported that all 17 of their amoxycillin-resistant streptococci were dextran-negative *Streptococcus sanguis*. Although dextran-negative *S. sanguis* strains have been described (Price *et al.*, 1986), most studies report that an overwhelming majority of strains produce extracellular polysaccharides from sucrose. Some of the highly amoxycillin-resistant streptococci from our recent study (Woodman *et al.*, 1985) have now been identified. These strains were also unusual in that they consistently failed to produce dextran (glucan) during growth in the presence of

sucrose although they were able to secrete low concentrations of glucosyltransferases, the enzymes responsible for polysaccharide production.

Materials and methods

Samples and subjects

Stimulated whole saliva was collected from 20 healthy adult volunteers before their participation in a study of the effect of repeated high-dose prophylaxis with amoxycillin on the normal oral flora (Woodman *et al.*, 1985).

Bacteriology

The bacteria present in saliva were dispersed by vortex mixing, serially diluted in nutrient broth and samples were plated on Mitis-Salivarius (MS) Agar (Oxoid) containing amoxycillin 40 mg/L to enable streptococci resistant to peak serum levels of the antibiotic to be enumerated (Woodman *et al.*, 1985). All plates were incubated anaerobically for 72 h at 37°C in an atmosphere of CO₂ 10% in H₂. Bacteria were confirmed as streptococci on the basis of their colonial morphology, their appearance in gram-stained smears and their negative reaction in a catalase test; they were freeze-dried for subsequent identification by the criteria of Hardie and Bowden (1976) and by the commercially-available API 20 Strep System, (API, Basingstoke). Production of

extracellular polysaccharide from sucrose was assessed by colonial texture on sucrose-containing agar, and by the production of a white precipitate after the addition of 1.2 volumes of ethanol to 5 ml of culture filtrates (Hehre and Neill, 1946).

Glucosyltransferase (GTF; EC 2.4.1.5) and fructosyltransferase (FTF; EC 2.4.1.10) activity

Amoxycillin-resistant strains were grown in batch culture in a complex medium (LHP) containing potassium salts and supplemented with glucose 1% w/v at a constant pH 7.0 by the automatic addition of 2M KOH (Keevil *et al.*, 1984). The bacteria were grown until late log phase and the enzymes that synthesise glucans and fructans from sucrose were assayed in culture supernates in the presence and absence of dextran primers (60 µg/ml, final concentration) by the method of Keevil *et al.* (1984). The enzymes that produce insoluble glucan (mutan) and insoluble fructan are GTF-I and FTF-I, respectively; activities were expressed as the amounts (µg) of insoluble glucan or fructan that were polymerised from sucrose (/ml of culture supernate)/h. Similarly, soluble glucan (dextran) and soluble fructan are produced by the enzymes GTF-S and FTF-S, respectively; activities were also expressed as the amounts (µg) of ethanol-precipitable glucan (dextran) or fructan that were polymerised from sucrose (/ml culture supernate)/h.

Antibiotic sensitivity patterns

The minimum inhibitory concentrations (MIC) of several antibiotics for the strains were determined in multiwell polystyrene plates containing appropriate concentrations of dried, stabilised antibiotics arranged to give two-fold dilutions after addition of nutrient broth (Sensititre®; Gibco Ltd, Paisley, Scotland). After incubation at 37°C for 24–48 h, bacterial growth could be seen as a button of cells in the bottoms of wells.

Results

Low numbers of streptococci able to grow on agar supplemented with amoxycillin 40 mg/L were isolated from the saliva of 9 out of 20 volunteers. The mean viable count of these resistant bacteria was low (116 cfu/ml) but the range between individuals was wide (0–2.4 × 10³ cfu/ml). In all subjects these highly-resistant bacteria represented <0.1% of the total cultivable flora.

Some of these highly-resistant bacteria died when freeze-dried. All of the remaining highly-resistant streptococci, when passed through the two established typing schemes, were identified as *S. sanguis* although none synthesised extracellular polysaccharides from sucrose. In the tests recommended by Hardie and Bowden (1976), all strains failed to ferment mannitol and sorbitol, or to produce acetoin

from glucose, but they all liberated ammonia from arginine, hydrolysed aesculin and produced hydrogen peroxide. Similarly, using the API-20 Strep galleries, the only positive reactions were hydrolysis of aesculin, production of leucine arylamidase and arginine dehydrolase, and fermentation of lactose and starch. Colonies of the antibiotic-resistant strains were soft and non-adherent on sucrose-agar whereas a control culture-collection strain had a hard texture and could be detached from the agar surface only with great difficulty. Unlike the antibiotic-sensitive control strain, our highly-resistant streptococci did not produce ethanol-precipitable polysaccharide when grown in sucrose broth. However, when assays for the enzymes responsible for synthesising these polysaccharides were performed on culture supernates, low activities of GTF-S and GTF-I were detected (table I). The levels of GTF-S produced by the amoxycillin-resistant strains were only 23–43% of that of an antibiotic-sensitive type-culture-collection strain (NCTC 7865) grown in identical conditions; the production of GTF-I was relatively similar and low in all the *S. sanguis* strains tested. The presence of low-molecular-weight dextran primer was markedly inhibitory to GTF-S secretion by all strains, but particularly by the antibiotic-resistant bacteria.

Table I. Extracellular glucosyltransferase (GTF) and fructosyltransferase (FTF) production by amoxycillin-resistant oral streptococci

Strain no.	Primer present or absent	Transferase activity (µg/ml/h; ±SD)* detected as			
		GTF-S	GTF-I	FTF-S	FTF-I
247	–	164 ± 30	22 ± 24	0	0
	+	0	16 ± 27	0	0
254	–	168 ± 41	10 ± 5	5 ± 6	0
	+	36 ± 26	0	0	0
297	–	185 ± 49	26 ± 8	0	0
	+	50 ± 20	3 ± 7	0	0
151	–	228 ± 30	12 ± 5	0	0
	+	155 ± 23	2 ± 2	0	0
195	–	117 ± 25	25 ± 6	0	0
	+	93 ± 8	17 ± 5	0	0
NCTC 7865†	–	530 ± 40	26 ± 7	90 ± 15	5 ± 6
	+	350 ± 23	10 ± 5	40 ± 7	5 ± 6

* Activities are expressed as the mean ± SD of between 3–5 determinations on cultures grown on different days.

† This strain is sensitive to amoxycillin and is included as a control.

Strain 247 produced no soluble glucan in the presence of primer and glucan production by strain 254 was inhibited by nearly 80%. None of the amoxycillin-resistant strains produced FTF-I and only one, strain 254, secreted FTF-S (table I).

The MIC of amoxycillin for the streptococci isolated on agar supplemented with amoxycillin 40 mg/L, fell on sub-culture although the MIC of amoxycillin for several strains exceeded 10 mg/L. By use of a proprietary test system, these strains were found to be moderately resistant to a range of other penicillins and variable in their resistance to erythromycin, but were uniformly sensitive to cephalothin and clindamycin (table II).

Discussion

Transient bacteraemia commonly follows several routine dental procedures. Consequently, patients at risk of infective endocarditis receive antibiotic prophylaxis before such treatment (Cawson, 1981). A common misconception is that streptococci, the bacteria most frequently isolated from patients with infective endocarditis (Lowes *et al.*, 1980; Moulds *et al.*, 1980), are always sensitive to the penicillins. Several studies have reported the widespread carriage of small numbers of penicillin-resistant streptococci in the oral cavity of healthy subjects (Drucker and Jolly, 1971; Sukchotiratana *et al.*, 1975; Phillips *et al.*, 1976; Southall *et al.*, 1983; Woodman *et al.*, 1985). Significantly, the numbers of these resistant bacteria are greater in people exposed more frequently to antibiotics, such as those at risk of infective endocarditis (Phillips *et al.*, 1976; Shanson and Namayak, 1982), or in those who have recently received prophylactic cover or a short course of antibiotic treatment (Southall *et al.*,

1983; Harrison *et al.*, 1985a; Woodman *et al.*, 1985). Erythromycin is recommended as an alternative antibiotic when there is concern that penicillin-resistant bacteria may have been selected. The amoxycillin-resistant bacteria in our studies showed some resistance to erythromycin but were uniformly sensitive to clindamycin, another alternative antibiotic.

These antibiotic-resistant streptococci have not been identified routinely. However, Southall *et al.* (1983) reported that the amoxycillin-resistant streptococci isolated in their study were similar to *S. sanguis* except that all 17 isolates from 11 volunteers were atypical in that they did not produce dextran. Dextran-negative erythromycin-resistant strains of *S. sanguis* have also been described recently (Harrison *et al.*, 1985b). This is surprising because although dextran-negative *S. sanguis* have been reported, they are relatively uncommon and most strains characteristically synthesise polysaccharides (Price *et al.*, 1986). In our previous study, bacteria were isolated that could grow on agar supplemented with amoxycillin 40 mg/L and, as this concentration corresponds to the peak serum levels of the antibiotic, they were considered to pose a potentially significant threat to the patient. These highly-resistant bacteria were identified as dextran-negative *S. sanguis* on the basis of conventional criteria including colonial texture on sucrose agar and precipitation of supernates with ethanol. However, using a specific assay, all strains were found to be capable of secreting glucosyltransferases that synthesise such extracellular glucans, although the activities of GTF-S, the enzyme responsible for dextran production, was lower in culture supernates of resistant strains than in that of an antibiotic-sensitive strain included as a control. This apparent paradox might be explained

Table II. Antibiotic sensitivity profile of amoxycillin-resistant streptococci

Strain no.	Minimum Inhibitory Concentration (mg/L)										
	Pen G	Amp	Oxa	Gent	Amik	Tet	Chlor	Eryth	Ceph	Clind	Co-trim
151	4	> 16	> 16	1	8	2	2	0.5	< 0.25	< 0.12	4
195	2	8	4	1	1	0.5	1	< 0.25	< 0.25	< 0.12	4
247	> 8	> 16	> 16	2	16	2	2	1	0.25	< 0.12	4
254	4	8	8	0.5	4	2	1	0.5	< 0.25	< 0.12	4
297	> 8	> 16	> 16	1	4	8	1	2	< 0.25	< 0.12	> 8
NCTC 7865*	< 0.06	< 0.12	0.5	2	8	0.5	4	0.25	< 0.25	0.25	4

Pen G, penicillin G; Amp, ampicillin; Oxa, oxacillin; Gent, gentamicin; Amik, amikacin; Tet, tetracycline; Chlor, chloramphenicol; Eryth, erythromycin; Ceph, cephalothin; Clin, clindamycin; Co-trim, co-trimoxazole.

* *S. sanguis* NCTC 7865 is an antibiotic sensitive strain included as a control.

by the finding that, unlike *S. mutans* (Fukui *et al.*, 1982), dextran synthesis by *S. sanguis* (Keevil *et al.*, 1984), and particularly by our antibiotic-resistant strains, is markedly inhibited by low-molecular-weight dextran primers. Sucrose can act as both substrate and inducer to some enzymes; therefore, the relatively high concentrations of this sugar in the TYC agar plates and in the broth cultures might have inhibited polysaccharide production.

The adherence of streptococci to damaged heart valves *in vitro* (Ramirez-Ronda, 1978) and to a platelet-fibrin matrix (Scheld *et al.*, 1978) has been shown to be enhanced by the ability of cells to synthesise glucans (Mills *et al.*, 1984). Similarly, pre-exposure of bacteria or valve-leaflets to pre-formed dextrans of low molecular weight can interfere with this attachment (Ramirez-Ronda, 1980) confirming the important role of extracellular polysaccharides in the pathogenicity of certain oral

streptococci, and particularly *S. sanguis*. Thus, although these amoxycillin-resistant bacteria possess glucosyltransferases but appear to be unable to synthesise glucans under certain environmental conditions, they may not be able to attach to a damaged endocardium. If this is confirmed, these highly-resistant bacteria would not be considered a threat to a susceptible patient nor would their selection following repeated antibiotic administration invalidate prophylaxis (Southall *et al.*, 1983; Harrison *et al.*, 1985a; Woodman *et al.*, 1985). We are continuing to investigate these possibilities in in-vitro attachment models and in in-vivo studies of pathogenicity in an animal endocarditis model.

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