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ACTIVITY OF AMOXYCILLIN AGAINST ENTEROCOCCI AND SYNERGISM WITH AMINOGLYCOSIDE ANTIBIOTICS

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ENHANCEMENT of the bactericidal activity of benzylpenicillin against enterococci by streptomycin is one of the earliest and best known examples of antibiotic synergism (Jawetz, Gunnison and Coleman, 1950). Enterococci are moderately sensitive *in vitro* to benzylpenicillin, which may also exert marked bactericidal activity though it generally fails to bring about complete sterilisation of cultures of these organisms. However, when benzylpenicillin is combined with streptomycin, which by itself is relatively ineffective, a marked synergistic action occurs with many strains of *Streptococcus faecalis*, resulting in a striking increase in the bactericidal activity of the penicillin. Treatment of enterococcal endocarditis with benzylpenicillin alone has often failed, whereas treatment with combinations of penicillin and streptomycin has achieved a large number of cures (Robbins and Tompsett, 1951; Geraci and Martin, 1954; Mandell *et al.*, 1970). The use of penicillin+streptomycin combinations for this purpose is probably the most widely accepted example of clinical synergism (Jawetz, 1968).

However, a number of strains of *S. faecalis* show a high level of resistance to streptomycin and in such cases synergism is not observed *in vitro* with combinations of penicillin and streptomycin (Harvard, Garrod and Waterworth, 1959; Standiford, de Maine and Kirby, 1970; Moellering *et al.*, 1971a). Other aminoglycoside antibiotics, namely kanamycin and gentamicin, are generally more active than streptomycin against enterococci (Toala *et al.*, 1970) and streptomycin-resistant strains are often sensitive to kanamycin and are almost always sensitive to gentamicin (Moellering, Wennersten and Weinberg, 1971b; Watanakunakorn, 1971). Combinations of benzylpenicillin with kanamycin or gentamicin are usually synergistic against *S. faecalis* (Watanakunakorn, 1971; Wilkowske *et al.*, 1971) provided that the strain is sensitive to the aminoglycoside (Standiford *et al.*, 1970; Moellering *et al.*, 1971b). Ampicillin has been shown to be more active than benzylpenicillin against *S. faecalis* (Sonne and Jawetz, 1968) and synergism has been demonstrated with ampicillin in combination with streptomycin or kanamycin (Fekety and Weiss, 1967; Sonne and

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Jawetz, 1968; Wilkowske *et al.*, 1971). Combinations of ampicillin and streptomycin have been used successfully in the treatment of enterococcal endocarditis (Mandell *et al.*, 1970).

Amoxycillin (D- α -amino-*p*-hydroxybenzylpenicillin) is a new semi-synthetic penicillin with an antibacterial spectrum and level of activity generally similar to that of ampicillin (Neu and Winshell, 1971*a*; Sutherland and Rolinson, 1971); it is particularly well absorbed after oral administration to human subjects (Croydon and Sutherland, 1971; Neu and Winshell, 1971*b*; Kosmidis *et al.*, 1972; Sutherland, Croydon and Rolinson, 1972). This new penicillin has been shown to be slightly more active than ampicillin against *S. faecalis* (Sutherland *et al.*, 1972) and this report is concerned with a study of the extent of in-vitro bactericidal activity of amoxycillin against the enterococcus, alone and in combination with aminoglycoside antibiotics.

MATERIALS AND METHODS

Enterococcal strains. The 55 strains of enterococci used in this study had been isolated from a variety of clinical sources (blood, urine and wounds). They were identified according to the criteria of Wilson and Miles (1964) and comprised *S. faecalis* (25 strains), *S. faecalis* subsp. *zymogenes* (15 strains), *S. faecalis* subsp. *liquefaciens* (13 strains) and *S. faecium* (2 strains).

Minimum inhibitory concentrations. Serial dilutions of the test antibiotics were added to 18-ml volumes of molten 5% blood agar (Blood Agar Base, Oxoid CM55, and Horse Blood, defibrinated, Wellcome) at 50°C and poured into petri dishes. The plates were dried at 37°C and inoculated with a drop (0.001 ml) of an undiluted overnight broth culture (Nutrient Broth no. 2, Oxoid CM67) of each strain of enterococcus by means of a replicating device delivering 20 cultures to each plate. Minimum inhibitory concentrations (MIC) were determined after incubation for 18 h at 37°C, the MIC being taken as the lowest concentration of antibiotic completely preventing visible growth.

Minimum bactericidal concentrations. Minimum concentrations of the antibiotics or of combinations of them inhibitory to large inocula were determined. Serial dilutions of drugs were made in tubes containing 5-ml volumes of nutrient broth. The tubes were inoculated with 0.03 ml of an overnight culture of the test organism, giving an inoculum of about 10^6 cells per ml, and the MICs were read after overnight incubation at 37°C. A loopful of culture was taken (loop diameter 5 mm) from each tube showing no visible growth after overnight incubation and was streaked on antibiotic-free blood agar containing a 1 in 100

TABLE I

Distribution of minimum inhibitory concentrations (MICs) of penicillins and aminoglycosides against 55 strains of enterococci

Antibiotic	Number of strains with an MIC (μ g per ml) of											
	0.25	0.5	1.25	2.5	5.0	12.5	25	50	125	250	500	>500
Amoxycillin	5	49	1	0	0	0	0	0	0	0	0	0
Ampicillin	0	25	29	1	0	0	0	0	0	0	0	0
Benzylpenicillin	0	2	49	4	0	0	0	0	0	0	0	0
Gentamicin	0	0	1	19	26	7	2	0	0	0	0	0
Kanamycin	0	0	0	0	5	19	22	5	2	0	0	2*
Streptomycin	0	0	0	0	0	7	9	21	2	0	1	15*

* MIC > 5000 μ g per ml.

dilution of Wellcome penicillinase (Burroughs Wellcome Limited). The plates were incubated for 18 h at 37°C and the minimum bactericidal concentration (MBC) was determined as the lowest concentration of antibiotic from which subculture failed. Absence of growth corresponded to a viable count of 10^2 cells per ml or less. In a number of tests the inoculum was reduced to 0.03 ml of a 1 in 100 dilution of an overnight culture to determine the effect of a reduced inoculum size on the MBC of the compounds.

Bactericidal activity. The bactericidal activities of the penicillins and aminoglycosides, alone and in combination, were determined by measurement of the reduction in the viable count of an organism after exposure to the antibiotics. Tubes of nutrient broth (20-ml volumes) containing known concentrations of the antibiotics were inoculated with a drop (0.03 ml) of an overnight broth culture and incubated at 37°C. Samples taken after incubation for 3, 6, 24 and 48 h were suitably diluted in sterile distilled water and 0.02-ml volumes were pipetted on to the surface of blood-agar plates containing penicillinase. In tests with combinations of penicillins and aminoglycosides or where it was thought that the viable counts had fallen to very low levels, subcultures were made by spreading 1.0-ml volumes of the undiluted cultures over the plates. Colonies were counted after overnight incubation at 37°C and the viable counts were calculated.

RESULTS

Minimum inhibitory concentrations

The MICs of the penicillins and aminoglycosides for 55 strains of enterococci are shown in table I. All three penicillins were inhibitory at relatively low concentrations; amoxycillin was the most active, being about twice as active as benzylpenicillin which was itself rather less active than ampicillin. The aminoglycosides were less active than the penicillins against the enterococci, and gentamicin, the most active aminoglycoside, was two to four times less active than benzylpenicillin. Kanamycin was four times less active than gentamicin and was at least twice as active as streptomycin. All the strains of enterococci were sensitive to the penicillins, but 15 showed a high level of resistance to streptomycin ($MIC > 5000 \mu\text{g per ml}$) and two of these were also insensitive to kanamycin ($MIC > 5000 \mu\text{g per ml}$). None of the enterococci tested was resistant to gentamicin. There were no marked differences in the

TABLE II
Effect of inoculum size on the minimum bactericidal concentrations (MBCs) of amoxycillin, ampicillin and benzylpenicillin for 10 strains of enterococci

Inoculum	Penicillin	Number of strains with an MBC ($\mu\text{g per ml}$) of									
		0.25	0.5	1.25	2.5	5.0	12.5	25	50	100	>100
Large (c. 10^8 cells per ml)	Amoxycillin	0	0	0	0	0	0	0	0	0	10
	Ampicillin	0	0	0	0	0	0	0	0	0	10
	Benzylpenicillin	0	0	0	0	0	0	0	0	0	10
Small (c. 10^4 cells per ml)	Amoxycillin	1	0	5	4	0	0	0	0	0	0
	Ampicillin	0	0	1	8	0	1	0	0	0	0
	Benzylpenicillin	0	0	1	1	6	1	1	0	0	0

sensitivities of the various sub-species of *S. faecalis* to the penicillins or aminoglycosides, but the two strains of *S. faecium* were rather more resistant to the aminoglycosides than were the rest. The streptomycin-resistant enterococci included strains of *S. faecalis*, *S. faecalis* subsp. *liquefaciens* and *S. faecalis* subsp. *zymogenes*.

Minimum bactericidal concentrations

In tests in which a large inoculum (10^6 cells per ml) was used the MBCs of the penicillins exceeded 100 μg per ml for all 10 strains tested (table II) but with a smaller inoculum (10^4 cells per ml) MBCs were much lower and were quite close to the MICs of the compounds. In tests on 12 strains (10^6 cells per ml) the aminoglycosides showed some evidence of bactericidal activity against aminoglycoside-sensitive enterococci, but not against resistant strains (table III). When sub-lethal concentrations of the aminoglycosides were combined with amoxycillin, ampicillin or benzylpenicillin, the bactericidal activities of all combinations were very much greater than those of the compounds alone against aminoglycoside-sensitive strains of enterococci. However, bactericidal activity was not detected when the strain was resistant to the aminoglycoside in the combination under test. Thus the streptomycin-resistant

TABLE III

Minimum bactericidal concentrations (MBCs) of penicillins and aminoglycosides alone, and in combination, for 12 strains of enterococci

Penicillin	Aminoglycoside and its concentration (μg per ml)	Number of strains with an MBC (μg per ml)* of									
		0.25	0.5	1.0	2.5	5.0	10	25	50	100	>100
Amoxycillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	0	2	2	0	0	0	0	0	7†
	Kanamycin (20)	0	5	4	1	0	0	0	0	0	2‡
	Gentamicin (10)	6	3	3	0	0	0	0	0	0	0
Ampicillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	1	2	1	0	0	0	0	0	7†
	Kanamycin (20)	0	5	4	1	0	0	0	0	0	2‡
	Gentamicin (10)	1	3	7	0	1	0	0	0	0	0
Benzylpenicillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	0	0	0	4	0	0	0	0	7†
	Kanamycin (20)	1	0	0	3	5	1	0	0	0	2‡
	Gentamicin (10)	3	2	2	4	1	0	0	0	0	0
None	Streptomycin	0	0	0	0	0	0	0	1	3	8
None	Kanamycin	0	0	0	0	0	0	0	2	5	5
None	Gentamicin	0	0	0	0	0	1	1	4	4	2

* For an inoculum size *c.* 10^6 cells per ml of medium.

† Streptomycin MIC > 5000 μg per ml.

‡ Kanamycin MIC > 5000 μg per ml.

strains were not killed by penicillin+streptomycin but were susceptible to the combinations that included kanamycin or gentamicin, except for two strains that were also resistant to kanamycin and these were not killed by the penicillin+kanamycin combinations.

Bactericidal activity

The bactericidal effects of the penicillins, aminoglycosides and penicillin+aminoglycoside combinations are shown in greater detail in figs. 1-4. Thus, in a comparison of the bactericidal activities of amoxycillin and benzylpenicillin, amoxycillin was more effective than the latter in killing *S. faecalis* subsp. *zymogenes* strain no 816 (fig. 1). At a concentration of 1.0 µg per ml, benzylpenicillin was largely ineffective whereas amoxycillin reduced the viable count by greater than 99% after 6 h although this was followed by resumption of growth to a visible level at 48 h. At a higher concentration (10 µg per ml) benzylpenicillin showed greater bactericidal activity and brought about a 99% reduction in the viable count after 24 h, but was distinctly less active than amoxycillin and produced a slower rate of killing than did amoxycillin during the first 6 h. Amoxycillin reduced the viable count at 48 h by 99.99% but failed to sterilise the culture.

The results of a viable count experiment with combinations of amoxycillin with streptomycin or with kanamycin against the streptomycin-sensitive

FIG. 1 (left).—The bactericidal activities of amoxycillin and benzylpenicillin against *Streptococcus faecalis* subsp. *zymogenes* strain no. 816; ●—● control; ▲—▲ amoxycillin 1.0 µg per ml; △—△ amoxycillin 10 µg per ml; ■—■ benzylpenicillin 1.0 µg per ml; □—□ benzylpenicillin 10 µg per ml.

FIG. 2 (right).—Bactericidal activities of amoxycillin, streptomycin and kanamycin alone and in combination against the streptomycin-sensitive *S. faecalis* strain no. 14; ●—● control; ○—○ amoxycillin 1.0 µg per ml; △—△ streptomycin 20 µg per ml; □—□ kanamycin 20 µg per ml; ▲—▲ amoxycillin 1.0 µg per ml + streptomycin 20 µg per ml; ■—■ amoxycillin 1.0 µg per ml + kanamycin 20 µg per ml.

S. faecalis strain no. 14 are shown in fig. 2. Streptomycin (20 μg per ml) inhibited growth of the organism for 6 h but by 24 h the viable count was the same as that in the control culture. Kanamycin (20 μg per ml) was bactericidal for 6 h but thereafter growth increased to equal that of the control. Similarly, amoxycillin (1.0 μg per ml) was bactericidal over the first 6 h but this was followed by a resumption of growth to a level greater than the initial count after 24 h. In contrast, a combination of amoxycillin (1 μg per ml) with streptomycin (20 μg per ml) or kanamycin (20 μg per ml) brought about a 99.99% reduction in viable count within 6 h, and the combinations sterilised the culture within 24 h in the case of kanamycin and within 48 h with streptomycin.

On the other hand a combination of amoxycillin and streptomycin failed to show synergistic effects against the streptomycin-resistant *S. faecalis* subsp. *zymogenes* strain no. 816 (MIC > 5000 μg streptomycin per ml), the bactericidal activity of amoxycillin with streptomycin being no greater than that of amoxycillin alone (fig. 3). This strain was sensitive to kanamycin (MIC 25 μg per ml) and to gentamicin (MIC 25 μg per ml) and combinations of amoxycillin with kanamycin (20 μg per ml) or with gentamicin (10 μg per ml) showed significant bactericidal effects, sterilising the culture within 6 h with gentamicin or within 24 h with kanamycin. Similarly, combinations of amoxycillin with streptomycin or kanamycin were no more effective than amoxycillin alone in reducing the viable count of a culture of *S. faecalis* subsp. *zymogenes* strain no. 830 which was resistant to both streptomycin and kanamycin (MIC > 5000 μg per ml) (fig. 4). However, the organism was sensitive to gentamicin and a combination of the latter with amoxycillin was synergistic, reducing the viable count to zero after 48 h (fig. 4).

FIG. 3.—Bactericidal activities of amoxycillin, streptomycin, kanamycin and gentamicin alone and in combination against the streptomycin-resistant *S. faecalis* subsp. *zymogenes* strain no. 816: ●—● control; ○—○ amoxycillin 1.0 μg per ml; △—△ streptomycin 20 μg per ml; ▲—▲ amoxycillin 1.0 μg per ml + streptomycin 20 μg per ml; □—□ kanamycin 20 μg per ml; ■—■ amoxycillin 1.0 μg per ml + kanamycin 20 μg per ml; ◇—◇ gentamicin 10 μg per ml; ◆—◆ amoxycillin 1.0 μg per ml + gentamicin 10 μg per ml.

DISCUSSION

The results reported here confirm that *in vitro* amoxycillin is more active than ampicillin (Sutherland *et al.*, 1972) and that ampicillin is more active than benzylpenicillin (Sonne and Jawetz, 1968) against enterococci, although the differences in the activities of the three penicillins are not pronounced. The enterococci tested were all sensitive to moderately low concentrations of the penicillins, but a number of strains showed a high level of resistance to streptomycin or kanamycin. Toala *et al.* (1970) state that there has been no apparent decrease in recent years in the sensitivity of enterococci to benzylpenicillin but that the contrary is true for other antibiotics including aminoglycosides. A relatively high incidence of resistance to streptomycin and kanamycin among enterococci was reported by Standiford *et al.* (1970) and Moellering *et al.* (1971a and b), but all strains were sensitive to gentamicin. In those studies it was found that streptomycin-resistant strains of enterococci were often sensitive to kanamycin but kanamycin-resistant strains were almost invariably resistant to streptomycin. A similar pattern of cross-resistance to the aminoglycoside antibiotics was observed among the enterococci described here.

The penicillins appeared to show poor bactericidal activity against a large inoculum of enterococci in terms of the minimum bactericidal concentrations (MBC) of the compounds. However, determination of the MBC was a particularly stringent test, requiring a fall in viable count to 10^2 cells per ml or less, which corresponded to a 99.99% kill of a large inoculum (10^6 cells per ml), for a bactericidal effect to be recorded. Under these conditions the MBCs of the penicillins were above 100 μg per ml. With a smaller inoculum (10^4 cells

FIG. 4.—Bactericidal activities of amoxycillin, streptomycin, kanamycin and gentamicin alone and in combination against *S. faecalis* subsp. *zymogenes* strain no. 830, highly resistant to streptomycin and kanamycin; ●—● control; ○—○ amoxycillin 1.0 μg per ml; △—△ streptomycin 20 μg per ml; ▲—▲ amoxycillin 1.0 μg per ml + streptomycin 20 μg per ml; □—□ kanamycin 20 μg per ml; ■—■ amoxycillin 1.0 μg per ml + kanamycin 20 μg per ml; ◇—◇ gentamicin 10 μg per ml; ◆—◆ amoxycillin 1.0 μg per ml + gentamicin 10 μg per ml.

per ml), the penicillins readily reduced the viable count to less than 10^2 cells per ml and the MBCs were quite close to the MICs. In fact, the bactericidal activity experiments showed that moderately low concentrations of the penicillins rapidly produced marked bactericidal effects against a large inoculum of enterococci although the compounds failed to sterilise the cultures within the period of the test. In these tests amoxycillin was more effective than benzylpenicillin.

The synergism against enterococci shown by amoxycillin when combined with streptomycin, kanamycin or gentamicin is homologous with that of combinations of benzylpenicillin or ampicillin with aminoglycoside antibiotics. However, in the studies reported here, not all strains of enterococci showed increased susceptibility to penicillin+aminoglycoside combinations since synergism occurred only when the organism was sensitive to (inhibited by) the aminoglycoside being investigated. This finding is in agreement with data reported by Standiford *et al.* (1970) and Moellering *et al.* (1971a) who found that there was a good correlation between failure of penicillin+aminoglycoside combinations to produce synergism and resistance of the test strain of enterococcus to the bacteriostatic action of aminoglycoside antibiotics. The relevance of the in-vitro synergism between penicillins and aminoglycosides against enterococci to the clinical situation has been questioned (Tompsett and Pizette, 1962) and clinical cures have been obtained with penicillins alone (Hein and Berg, 1949; Geraci and Martin, 1954), but the weight of evidence suggests that more favourable results are obtained with combinations of antibiotics (Garrod and Waterworth, 1962; Jawetz and Sonne, 1966; Mandell *et al.*, 1970). The treatment of choice for enterococcal endocarditis is generally considered to be a combination of benzylpenicillin with streptomycin but the fairly frequent occurrence of highly streptomycin-resistant strains of enterococci throws some doubt upon this. It is evident that there is a relatively high incidence of resistance to streptomycin and kanamycin among enterococci and that appropriate laboratory tests need to be carried out to assist in the choice of therapy. Many hospital laboratories find tests for synergism difficult and time-consuming, and it has been suggested that knowledge of the aminoglycoside sensitivity of the strain of enterococcus can be used to predict bactericidal synergism because of the good correlation already mentioned between these two characteristics (Standiford *et al.*, 1970; Moellering *et al.*, 1971a). The results of this limited study are in accord with this suggestion.

Our results suggest that amoxycillin may be superior to ampicillin or benzylpenicillin in the treatment of enterococcal infections. In addition to its greater antibacterial activity, amoxycillin is particularly well absorbed after oral administration to man and produces penicillin serum-concentrations that are higher than those obtained with oral ampicillin and of about the same order as those produced by intramuscular benzylpenicillin. These considerations seem to justify clinical trial of amoxycillin in the treatment of enterococcal infection.

SUMMARY

Amoxycillin was more active *in vitro* than ampicillin or benzylpenicillin against clinical isolates of enterococci. All 55 strains tested were sensitive to the three penicillins but 15 strains showed a high level of resistance to streptomycin and two of these were also insensitive to kanamycin. All strains were sensitive to gentamicin, which was the most active of the aminoglycoside antibiotics.

The penicillins showed pronounced bactericidal activity against the enterococci but failed to sterilise cultures of these organisms. Combinations of penicillins and aminoglycosides invariably produced synergistic bactericidal effects which resulted in sterilisation of cultures of enterococci provided that the strain was sensitive to the aminoglycoside moiety of the antibiotic combination. Synergism was not observed with a combination of a penicillin and an aminoglycoside when the enterococcus was resistant to the aminoglycoside.

The data reported suggest that amoxycillin may have certain advantages, compared with ampicillin or benzylpenicillin, for the treatment of enterococcal infections.

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