Action and interaction of penicillin and gentamicin on enterococci

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SUMMARY The action and interaction of benzylpenicillin and gentamicin on *Streptococcus faecalis* was studied using mainly turbidimetric methods. The minimum antibacterial concentration (MAC) of each antibiotic lay considerably below the conventionally determined minimum inhibitory concentration, and levels of the two agents exceeding the MAC were necessary in order to obtain a synergic interaction.

Evidence was obtained that gentamicin interfered with bacterial lysis induced by penicillin, and this suggests that the aminoglycoside is responsible for the bactericidal activity of the combination, the role of the penicillin being solely to facilitate access of the aminoglycoside to its target site. Our findings do not, however, fully support the generally held view that the increased permeability of enterococci to aminoglycosides is due to penicillin-induced cell wall damage. 'Persisters'—cells surviving prolonged exposure to the optimum lethal concentration of penicillin—were not killed by subsequent exposure to gentamicin if the penicillin was removed but were killed if the penicillin remained present.

For many years the combination of benzylpenicillin and streptomycin has been the treatment of choice for enterococcal endocarditis because the marked bactericidal synergy that is readily shown against many enterococcal strains *in vitro* is matched by clinical success. However, strains of enterococci that are highly resistant to streptomycin are not killed by the combination (Standiford *et al.*, 1970), and gentamicin, which acts synergically with penicillin against almost all enterococci (Watanakunakorn, 1971; Calderwood *et al.*, 1977), should probably replace streptomycin as the aminoglycoside of choice (Watt, 1978).

Synergy between penicillins and aminoglycosides is thought to be due to penicillin-mediated wall damage, facilitating entry of the aminoglycoside into the cell (Moellering and Weinberg, 1971). A great deal of work has been done on the interaction of these compounds against enterococci (see Jawetz (1975) for review), but relatively little has been done to study systematically the synergic interaction at different concentrations, particularly those lying below the minimum inhibitory concentration (MIC) where

a variety of antibacterial effects are known to occur (Greenwood, 1975; Lorian, 1975), or those at which penicillin exerts its optimal lethal effect (Eagle and Musselman, 1948).

In this investigation of the action and interaction of benzylpenicillin and gentamicin we have paid special attention to effects observed at sub-MIC and optimum bactericidal levels and have examined the susceptibility of penicillin-induced 'persisters' to the action of gentamicin.

Material and methods

The strain of *Streptococcus faecalis* used, designated *Hook*, was originally isolated from a case of sub-acute bacterial endocarditis. The strain belonged to Lancefield's group D, was bile tolerant, hydrolysed aesculin and arginine, and fermented mannitol but not arabinose.

Growth medium was the TRIS-buffered 'complete' medium (pH 7.2) described previously (Mawer and Greenwood, 1978).

Benzylpenicillin sodium (Glaxo Laboratories Ltd) and gentamicin sulphate (Nicholas Laboratories Ltd) were standard pharmaceutical preparations. Appropriate concentrations were freshly prepared, as required, in sterile distilled water.

TURBIDIMETRIC STUDIES

Twenty-millilitre volumes of broth were inoculated

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with bacteria from an overnight broth culture to achieve an inoculum of $ca 5 \times 10^5$ bacteria per ml and incubated in the multichannel continuous opacity monitoring device described by Mackintosh *et al.* (1973). Antibiotic was either included in the broth at the beginning of the experiment or, in dense inoculum experiments, added when bacterial growth had reached a standard level of 30% of maximum (viable count $ca 5 \times 10^7$ bacteria per ml). The action of penicillin was halted, when required, by the addition of 0.1 ml penicillinase (broad-spectrum mixture of β -lactamases I and II; Whatman Biochemicals Ltd).

VIABLE COUNTS

Surface viable counts were performed by standard methods. In certain experiments, small numbers of survivors were detected by filtering cultures exposed to antibiotic through membrane filters (average pore size 0.45μ) and incubating the washed filters on the surface of blood agar plates.

Results

A continuous opacity record of *Str. faecalis* Hook, in the presence of various concentrations of benzylpenicillin, is shown in Figure 1. Concentrations below $1 \mu\text{g}$ per ml had little effect on bacterial growth, and $1 \mu\text{g}$ per ml was regarded as the minimum antibacterial concentration (MAC), defined in the turbidimetric system as that concentration producing a reproducible deviation from the normal growth curve. The MIC, the concentration suppressing growth for the whole of the 20-hour incubation period, was $4 \mu\text{g}$ per ml. Cultures growing during the overnight incubation period in the presence of 1 and $2 \mu\text{g}$ benzylpenicillin per ml were shown, on retesting in the turbidimetric system, to have acquired a small increment of resistance to the antibiotic in that they were able to grow without a prolonged lag phase in that concentration to which they had been previously exposed.

The response of the *Hook* strain to gentamicin is shown in Figure 2. Again, concentrations below the

Fig. 1 Continuous opacity records of *Str. faecalis* (Hook strain). Penicillin added at time 0 to achieve concentrations (μg per ml) shown.

Fig. 2 Continuous opacity records of *Str. faecalis* (Hook strain). Gentamicin added at time 0 to achieve concentrations (μg per ml) shown.

MIC ($16 \mu\text{g}$ per ml) had a partial antibacterial effect, and cultures growing during overnight incubation after initial suppression of growth (ie, cultures exposed to 4 and $8 \mu\text{g}$ gentamicin per ml in Fig. 2) exhibited a small increase in resistance to gentamicin. The MAC of gentamicin was found to be $2 \mu\text{g}$ per ml.

Viable counts of cultures exposed to benzylpenicillin overnight (Fig. 3) showed that the optimal bactericidal concentration of benzylpenicillin was $4 \mu\text{g}$ per ml; concentrations above that level were less lethal, the so-called 'Eagle effect' (Eagle and Musselman, 1948). This effect was also seen in high inoculum experiments in the turbidimetric system in which high concentrations of penicillin ($>16 \mu\text{g}$ per ml) induced less lysis than lower levels (Fig. 4); in such experiments no inoculum effect was observed in that the MAC remained at $1 \mu\text{g}$ per ml and the MIC at $4 \mu\text{g}$ per ml.

An inoculum effect was, however, observed with gentamicin. When tested against an inoculum of

Fig. 3 Optimum dosage effect of penicillin against *Str. faecalis* (Hook strain). Number of colony-forming units (CFU) recovered after exposure of an inoculum of 5×10^8 CFU to various concentrations of penicillin overnight.

Fig. 4 Continuous opacity records of *Str. faecalis* (Hook strain). Penicillin added at arrow to achieve concentrations ($\mu\text{g per ml}$) shown.

ca 5×10^7 bacteria per ml, the MAC rose to $8 \mu\text{g per ml}$ (Fig. 5).

COMBINATION STUDIES

Viable counts of cultures of the *Hook* strain of *Str. faecalis* exposed to penicillin and gentamicin, alone and in combination, demonstrated the classical bactericidal synergy between these compounds (Fig. 6). Concentrations at or above the MAC of the two components were necessary to achieve a combined bactericidal effect. Filtration experiments on cultures exposed to penicillin and gentamicin for 24 hours showed that complete sterilisation of the culture could be achieved with $\frac{1}{2}$ MIC gentamicin in the presence of concentrations of penicillin at or above the MIC (Table).

The interaction of sub-MIC concentrations of the two agents was also examined in the turbidimetric system. When *Str. faecalis Hook* was exposed to increasing concentrations of gentamicin (spanning the concentrations 1MAC to $\frac{1}{2}$ MIC) in the presence of $1 \mu\text{g}$ benzylpenicillin per ml, it was found that a concentration above the MAC of gentamicin was necessary to influence significantly the recovery of organisms during the overnight incubation period

Fig. 5 Continuous opacity records of *Str. faecalis* (Hook strain). Gentamicin added at arrow to achieve concentrations ($\mu\text{g per ml}$) shown.

Fig. 6 Killing curves of *Str. faecalis* (Hook strain) exposed to various concentrations ($\mu\text{g per ml}$) of penicillin (PN) and/or gentamicin (GM).

Table Recovery of bacteria by membrane filtration after exposure to benzylpenicillin and/or gentamicin

Conc. of penicillin ($\mu\text{g per ml}$)	Number of colony-forming units recovered in presence of:	
	No gentamicin	Gentamicin $8 \mu\text{g per ml}$
0	C	C
1	C	25
2	C	8
4	590	0
8	N	0

C = confluent ($> 10^6$); N = numerous (> 3000)

(Fig. 7). In dense inoculum experiments, inhibitory concentrations of gentamicin appeared to interfere with the lytic activity of penicillin (Fig. 8).

FATE OF 'PERSISTERS'

The fate of penicillin-induced 'persisters' was examined by removing penicillin enzymically from cultures exposed to the antibiotic in the presence and absence of gentamicin. When cultures were exposed to levels of penicillin at, or just below, the MIC for 7 hours, regrowth of persisting bacteria was affected only by the simultaneous presence of concentrations of gentamicin exceeding the MAC. Overnight suppression of persisting bacteria was achieved with a level of $4 \mu\text{g}$ gentamicin per ml (2MAC; $1/4$ MIC) (Fig. 9).

When gentamicin was withheld for 7 hours and added together with penicillinase, $4 \mu\text{g}$ gentamicin

Fig. 7 Continuous opacity records of *Str. faecalis* (Hook strain). A: no antibiotic; B-F: antibiotic added at time 0 to achieve concentrations ($\mu\text{g per ml}$) of B: gentamicin 8; C: penicillin 1; D: penicillin 1 plus gentamicin 2; E: penicillin 1 plus gentamicin 4; F: penicillin 1 plus gentamicin 8.

Fig. 8 Continuous opacity records of *Str. faecalis* (Hook strain). Penicillin (PN) and/or gentamicin (GM) added at arrow to achieve concentrations ($\mu\text{g per ml}$) shown.

Fig. 9 Continuous opacity records of *Str. faecalis* (Hook strain). A: no antibiotic; B-H: antibiotic added at time 0 to achieve concentrations ($\mu\text{g per ml}$) of B: gentamicin 2; C: gentamicin 4; D: penicillin 2; E: penicillin 4; F: penicillin 2 or 4 plus gentamicin 1; G: penicillin 2 or 4 plus gentamicin 2; H: penicillin 2 or 4 plus gentamicin 4. Penicillinase added to cultures D to H after 7 hours (arrow).

per ml was insufficient completely to suppress growth of persisters. An experiment was therefore designed to establish how long penicillin and gentamicin must be present together for complete suppression of persisters to occur. A series of cultures was exposed to $2 \mu\text{g}$ penicillin per ml, and sufficient gentamicin was added at hourly intervals to achieve a concentration of $4 \mu\text{g}$ per ml. The action of penicillin was halted after 7 hours in each case. The

experiment is depicted diagrammatically in Figure 10. It was found that gentamicin had to be present together with the penicillin for at least 5 hours for persisters to be completely suppressed.

Fig. 10 Diagrammatic representation of experiment in which cultures of *Str. faecalis* (Hook) were exposed to penicillin ($2 \mu\text{g per ml}$; solid line) and gentamicin ($4 \mu\text{g per ml}$; dotted line) for various periods of time. Penicillin activity was halted enzymically after 7 hours. Arrows indicate time at which regrowth occurred.

Further evidence that gentamicin was unable to kill persisters in the absence of penicillin was obtained by exposing cultures to the optimal bactericidal concentration of penicillin ($4 \mu\text{g per ml}$) overnight. Penicillin action was halted with penicillinase, and sufficient gentamicin was immediately added to achieve concentrations of 4 or $8 \mu\text{g per ml}$. Growth of persisters occurred after 13 hours' exposure to $4 \mu\text{g}$ gentamicin per ml and after 16 hours' exposure to $8 \mu\text{g}$ gentamicin per ml.

Each of the main findings of this study was confirmed using another strain of *Str. faecalis* freshly isolated from a case of subacute bacterial endocarditis.

Discussion

Benzylpenicillin and gentamicin both exhibit considerable inhibitory activity at levels below those detected by the conventional MIC, and the end-point of MIC titrations appears to be influenced in both cases by the ability of part of the population to break through inhibitory levels to re-establish a slightly more resistant culture during the incubation period. This raises the question whether bacteriostatic synergy between these compounds, when detected by chessboard titrations, may be due to the mutual suppression of such adaptive variants.

Bactericidal synergy between penicillin and gentamicin is manifested by a marked enhancement of the early lethal activity of the compounds. The present results reveal two interesting aspects of this interaction.

Firstly, levels of the two antibiotics exceeding the MAC were required for bactericidal synergy to occur. Thus, if it is true that the basis of synergy is that penicillin weakens the cell wall and the aminoglycoside is then admitted more readily (Moellering and Weinberg, 1971), the increase in accessibility must be fairly small (and perhaps embraces a larger fraction of the population), because the concentration of gentamicin required to obtain a synergic response was not less than that which alone could cause some inhibition of growth.

Secondly, gentamicin appears to antagonise the lytic activity of penicillin (Fig. 8). This suggests that once the gentamicin is admitted, it is responsible for most of the subsequent bactericidal effect.

One of the weaknesses of the permeabilisation theory of penicillin/aminoglycoside interaction is that it does not satisfactorily explain how persisters are killed by aminoglycosides. Persisters, first described by Bigger (1944), are those cells that survive exposure to lethal concentrations of penicillins while displaying no increase in intrinsic resistance to the drug. One theory of persistence depends on the fact that when penicillins cause lysis of Gram-positive bacteria, wall components, including lipoteichoic acid (LTA), may be released into the medium. LTA is an inhibitor of cell wall autolytic activity in Gram-positive cocci (Höltje and Tomasz, 1975), including *Str. faecalis* (Cleveland *et al.*, 1976), so that, if the lethal activity of penicillins is a consequence of continued autolytic activity, as has been suggested (Rogers, 1967), persisters may be bacteria protected by LTA released by those cells that lyse first. It is possible that such bacteria incur sufficient structural damage to admit aminoglycosides even though they are not killed by the penicillin.

An alternative theory of persistence is that such cells are caught by the penicillin in a particular metabolic state—when they happen to have no cell wall growth points. Such cells need to employ autolytic enzymes—mucopolysaccharide hydrolases—to provide gaps for the insertion of fresh cell wall material, so that if these enzymes are inhibited by penicillins, as has been shown for *Escherichia coli* (Hartmann *et al.*, 1972), the cell is unable to grow until the penicillin is removed (Greenwood, 1972). If this hypothesis is correct, there is no reason why persisters should be hypersusceptible to aminoglycosides, because they do not have damaged walls. The present work confirms that persisters are not susceptible to gentamicin: even those cells

conforming to a very strict definition of persisters—those surviving exposure to the optimal bactericidal level of penicillin for 24 hours—were not completely suppressed by subsequent exposure to gentamicin. Furthermore, as shown by Miles *et al.* (1951) using streptomycin and confirmed in the present study for gentamicin, the simultaneous presence of penicillin and aminoglycoside is required for synergy to occur. This suggests that increased permeability to aminoglycosides due to cell wall damage caused by the penicillin is not the whole explanation, because such damage takes time to repair and the aminoglycoside should be admitted for some time after the penicillin has been removed. In the present study, benzylpenicillin and gentamicin had to be present together for at least 5 hours for complete sterilisation of the culture to occur.

If gentamicin alone cannot kill persisters (even after prolonged exposure to penicillin) and yet can do so together with penicillin after several hours' simultaneous exposure, the conclusion seems inescapable that binding of penicillin to the cell facilitates entry of the aminoglycoside in some way, even in the absence of cell wall damage.

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