Aminoglycoside cross-resistance patterns of gentamicin-resistant bacteria

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SUMMARY Ten strains each of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were habituated to gentamicin by serial passage in antibiotic containing medium. Complete cross-resistance to streptomycin, neomycin, kanamycin, and tobramycin in a linear proportional fashion was demonstrated at all stages of habituation. Most strains of *Staph. aureus* and *Ps. aeruginosa* showed a greater increase in resistance to gentamicin than to the other three aminoglycosides. *E. coli* required more transfers to reach the same degree of resistance than did the other two species. Reversion to greater susceptibility to gentamicin took place after serial passage on antibiotic-free media.

'Wild' gentamicin-resistant strains showed no such proportionality of resistance to kanamycin, neomycin, or streptomycin. But many of these strains showed a proportional increase in resistance to tobramycin.

Acquired resistance of Gram-negative bacilli to gentamicin associated with its clinical usage has been reported from various centres (Greene *et al.*, 1973; Eykyn and Phillips, 1975; Seal and Strange-ways, 1975; Shafi and Datta, 1975). When stated, the sensitivity of these organisms to tobramycin, an aminoglycoside antibiotic which has a very similar spectrum of activity, has been variable. The present study was undertaken to investigate further the cross-resistance between these two and other aminoglycosides. Strains habituated to gentamicin in the laboratory and 'wild' gentamicin-resistant strains were examined for cross-resistance to other aminoglycosides and to some unrelated agents.

Material and methods

BACTERIAL STRAINS

Ten strains each of *E. coli*, *Staph. aureus*, and *Ps. aeruginosa*, all sensitive to 4 µg or less gentamicin per ml, were isolated from clinical material in the Medical Microbiology Department of St Bartholomew's Hospital.

Sixty-eight strains of Gram-negative bacteria
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resistant to 8 µg or more gentamicin per ml were collected from various hospitals in the London area. They were identified by API systems as *Pseudomonas* spp (47), *E. coli* (2), *Klebsiella* spp (4), *Proteus* spp (3), *Providencia* spp (6), *Flavobacter* spp (2), *Alcaligenes* spp (2), and *Acinetobacter* spp (2).

INDUCED RESISTANCE

Gradient plates were prepared using Oxoid DST agar in which the antibiotic-containing wedge included gentamicin at concentrations of 2 µg, 8 µg, 32 µg, and 128 µg. Overnight broth cultures (37°C) of each strain were first flooded onto whole plates containing 2 µg gentamicin per ml and surplus fluid was removed aseptically. After 18 hours' incubation the edge of growth was swept with a loop and applied to a fresh gradient plate containing the same gentamicin concentration, spread with a dry cotton-wool swab, and incubated overnight. The process was repeated until growth was obtained from the whole plate. A sample of this culture was removed for storage on a slope of Oxoid blood-agar base (BAB) containing the concentration of gentamicin to which it had been habituated, and serial passage was continued on gradient plates containing the next higher concentration of gentamicin. In this way 10 strains each of *Staph. aureus*, *E. coli*, and *Ps. aeruginosa* were passaged until growth was obtained

on gradient plates containing 128 µg of gentamicin per ml, so that from each parent strain resistant variants growing in the presence of 2 µg, 8 µg, 32 µg, and 128 µg gentamicin per ml were derived.

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS

Serial doubling dilutions of gentamicin, tobramycin, streptomycin, neomycin, and kanamycin were made in DST agar (Oxoid) and the plates were seeded by means of a multiple inoculation device (Denly-Tech Ltd, Billingshurst, Sussex) with an 18-hour nutrient broth (Oxoid) culture diluted to contain 10^4 colony-forming units/ml. The number of colony-forming units on the control plates was approximately 20. The minimal inhibitory concentration (MIC) was taken to be the lowest concentration of antibiotic that allowed the growth of less than three colonies after 18 hours' incubation. Each parent strain and the resistant progenies were tested together on the same series of plates.

GROWTH CURVES

The growth curves of four parent strains and their derived resistant (128 µg/ml) variants were compared by continuous turbidimetric monitoring before and after 20 serial subcultures on antibiotic-free agar slopes. These growth studies were performed in 'complete' broth (Greenwood and O'Grady, 1973) using the multichannel opacity device described by Mackintosh *et al.* (1973).

Results

HABITUATION STUDIES

Comparison with other aminoglycosides

The number of transfers required to achieve various degrees of resistance to gentamicin is shown in Table 1. *E. coli* required more transfers to reach the same degree of resistance than did *Ps. aeruginosa* or *Staph. aureus*.

After training to various degrees of resistance to gentamicin, the relationship between MICs of

gentamicin and tobramycin is shown in Fig. 1 and the relationship between MICs of gentamicin and kanamycin in Fig. 2. A generally linear relationship was obtained but tobramycin was found to be two to four times more active than gentamicin against *Ps. aeruginosa* and slightly less active against *Staph. aureus*, *E. coli*, and *Klebsiella* spp. The MICs of gentamicin and neomycin, and gentamicin and streptomycin, showed a similar linear relationship although kanamycin, neomycin, and streptomycin were less active against *Ps. aeruginosa* than against the other organisms tested.

Extent of increase in induced resistance

The extent of the corresponding increase in resistance to tobramycin, neomycin, kanamycin, and streptomycin (resistance index) was calculated from the ratio of the MICs of the antibiotic for the parent strain and derived resistant variant multiplied by the reciprocal of the corresponding ratio for gentamicin. For example, if a strain was originally sensitive to 1 µg gentamicin per ml and 2 µg neomycin per ml and the habituated variant was inhibited by 128 µg gentamicin per ml and 256 µg neomycin per ml, the resistance index for neomycin would be $2/256 \times 128/1 = 1$. If the relative increase in the resistance to gentamicin were greater than that to the other aminoglycoside the index would be more than 1, and if it were less, the index would be less than 1. The numbers of strains of *E. coli*, *Staph. aureus*, and *Ps. aeruginosa* showing various resistance indices calculated in this way are shown in Table 2. The majority of *E. coli* strains showed the same degree of resistance to neomycin, tobramycin, and kanamycin as to gentamicin (resistance index = 1). However, most strains of *Staph. aureus* and *Ps. aeruginosa* showed a greater increase in resistance to gentamicin than to these three aminoglycosides (resistance index between 2 and 16). The relative increase in resistance to streptomycin appeared the smallest among the aminoglycosides tested against all three species.

Stability of induced resistance

Two representative strains of each species habituated to high levels of gentamicin were retested in conventional MIC titrations after 20 serial subcultures on antibiotic-free nutrient agar slopes. Reversion to greater susceptibility was demonstrated in all cases, but complete reversion to the original sensitivity was not observed. The strains of *E. coli* and *Staph. aureus* were also investigated by continuous turbidimetric monitoring; *Ps. aeruginosa* does not lend itself to investigation by this means (Greenwood, 1977). Variants of *E. coli* and *Staph. aureus* that had been habituated to high levels of gentamicin grew more slowly than their parent strains but after 20

Table 1 Average number of transfers required to achieve various degrees of resistance to gentamicin

Bacterial species	Average number of transfers required to achieve MIC gentamicin of:		
	2-8 µg/ml	16-64 µg/ml	≥128 µg/ml
<i>E. coli</i>	3 (2-9)*	9 (2-16)	18 (15-31)
<i>Staph. aureus</i>	3 (2-7)	4 (2-9)	11 (7-15)
<i>Ps. aeruginosa</i>	3 (2-5)	7 (3-9)	13 (11-19)

*Figures in parentheses show the range.

Fig. 1 Comparison of MICs of gentamicin and tobramycin at different stages of development of resistance. Coefficients of correlation. *E. coli*, 0.96 (○); *Staph. aureus*, 0.96 (●); *Pseudomonas aeruginosa*, 0.98 (▲)

Fig. 2 Comparison of MICs of gentamicin and kanamycin at different stages of development of resistance. Coefficients of correlation: *E. coli*, 0.8.89 (○); *Staph. aureus*, 0.96 (●); *Pseudomonas aeruginosa*, 0.91 (▲)

Table 2 Relationship of resistance to other aminoglycosides of strains habituated to gentamicin

Organism	Aminoglycoside	No. of strains having resistance index				
		0.25-0.5	1	2-4	8-16	≥32
<i>E. coli</i>	Neomycin	3	7	3	0	0
	Tobramycin	2	10	3	0	0
	Streptomycin	0	0	4	8	2
	Kanamycin	1	10	2	0	0
<i>Staph. aureus</i>	Neomycin	1	1	4	0	0
	Tobramycin	0	1	7	2	0
	Streptomycin	0	1	1	2	4
	Kanamycin	0	1	5	2	0
<i>Ps. aeruginosa</i>	Neomycin	0	1	6	1	0
	Tobramycin	0	2	5	3	0
	Streptomycin	0	0	1	4	4
	Kanamycin	0	1	2	3	0

serial subcultures in the absence of antibiotic these variants exhibited an intermediate growth rate (Fig. 3). The corresponding MIC levels of these revertants were also intermediate between those of the parent and the resistant variant; in the examples shown in Fig. 3, the MIC of gentamicin for the parent of *E. coli* was 0.5 µg per ml, that of the resistant variant 256 µg per ml, and that of the revertant 8 µg per ml. Similarly, the MIC of gentamicin for the parent *Staph. aureus* was 0.125 µg per ml, that of the variant 256 µg per ml, and that of the revertant 16 µg per ml.

Three strains of *Staph. aureus* were found to have

reverted to partial sensitivity despite storage in antibiotic-containing agar slopes. The fall in MICs of gentamicin was accompanied by a reduction in MICs of the other four aminoglycosides except in one case in which resistance to tobramycin was maintained.

Other antimicrobial agents

The sensitivities to tetracycline, chloramphenicol, and polymyxin of all the variants habituated to resistance to at least 128 µg gentamicin per ml and their respective parent strains were compared by titration. No significant differences were found.

'WILD' STRAINS

The MIC of gentamicin was compared with that of four other aminoglycosides for 47 strains of *Pseudomonas* spp and 21 strains of other aerobic Gram-negative bacteria. A random scatter was obtained with kanamycin (Fig. 4). A similar scatter was found with neomycin and streptomycin but the relationship between gentamicin and tobramycin resistance was different (Fig. 5). When MICs of tobramycin were plotted against those of gentamicin, tobramycin appeared to be two to four times more active against the majority of organisms. Three gentamicin-resistant *Pseudomonas* strains with MIC ≥ 32 µg per ml were encountered which remained fully sensitive to tobramycin.

Fig. 3 Growth curves of *E. coli* and *Staph. aureus* in complete broth obtained by continuous turbidimetric monitoring. — = Parent strain; . . . = resistant variant; - - - = resistant variant after 20 subcultures in antibiotic-free media.

Fig. 4 A comparison of MICs of gentamicin and kanamycin in wild strains.

Fig. 5 A comparison of MICs of tobramycin and gentamicin in wild strains.

Discussion

Complete cross-resistance between neomycin, streptomycin, and kanamycin has been reported by many

workers in 'wild' and laboratory-derived resistant strains of bacteria (Kunin *et al.*, 1958; Barber and Waterworth, 1966; Garrod *et al.*, 1973). However, possible cross-resistance between these early antibiotics and newer ones (gentamicin and tobramycin) has not been reported. Cross-resistance to tobramycin among strains habituated to gentamicin has been demonstrated (Weinstein *et al.*, 1971; Waterworth, 1972), but the patterns of cross-resistance emerging during the training procedure were not reported. The present findings indicate that there is complete cross-resistance between kanamycin, streptomycin, neomycin, and tobramycin at all stages of the habituation procedure when strains of *E. coli*, *Ps. aeruginosa*, and *Staph. aureus* are trained to resistance to gentamicin. The degree of cross-resistance obtained is generally directly proportional to the degree of resistance induced to gentamicin, but the ratio appears to vary with species and the aminoglycoside, the smallest increase in cross-resistance being found in streptomycin.

In contrast, 'wild' gentamicin-resistant strains showed no such proportionality of resistance to kanamycin, neomycin or streptomycin, but many of these strains did show a proportional increase in resistance to tobramycin. Gentamicin is more active than tobramycin against sensitive strains of *E. coli* and *Staph. aureus* but tobramycin is two to four times more active than gentamicin against gentamicin-sensitive strains of *Pseudomonas* spp (Waterworth, 1972; Molavi *et al.*, 1973; Klastersky *et al.*, 1973; Wretling *et al.*, 1974). When 47 gentamicin-resistant strains of *Pseudomonas* spp were examined in the present study, this superiority of tobramycin was found to extend to the majority of resistant strains. Three wild strains were encountered which were resistant to ≥ 32 µg gentamicin per ml but sensitive to 1.0 µg tobramycin per ml and, conversely, one strain resistant to 128 µg tobramycin per ml was inhibited by 8 µg gentamicin per ml. Such organisms which do not exhibit cross-resistance between gentamicin and tobramycin may have different mechanisms of resistance from the many strains which show cross-resistance. Similar patterns, observed by Crowe and Sanders (1972), were apparently related to the presence of different R factor mediated aminoglycoside-inactivating enzymes (Jacoby, 1974; Benveniste and Davies, 1973).

It has been suggested that laboratory-induced resistance to aminoglycosides arises from the selection of mutants (Waterworth, 1972). The instability of such resistance suggests rather that resistant variants have a reduced permeability to aminoglycosides and/or that they possess a phenotypically altered ribosome (Davies, 1971). The permeability hypothesis would seem to be the more

attractive of the possibilities in view of the linear relationship between the MICs of gentamicin and the amount of surface lipid that has been demonstrated in wild and induced gentamicin-resistant *Pseudomonas* strains (Pechey and James, 1973). Whatever the resistance mechanism, it appears to be restricted to aminoglycosides because sensitivity to tetracycline, chloramphenicol, and polymyxin remained unchanged.

In addition, adaptation to resistance appears to be accompanied by a reduction in growth rate in broth and frequently variation in colonial morphology on solid media. Such altered growth characteristics are in keeping with the findings of Weinstein *et al.* (1971) that *Pseudomonas* strains lose their virulence for mice and show nutrient dependence when trained to resistance in the laboratory.

Many instances of resistance to gentamicin emerging during therapy have been reported (Greene *et al.*, 1973; Eykyn and Phillips, 1975; Seal and Strangeways 1975; Shafi and Datta, 1975), and some strains have been observed to become less resistant after cessation of gentamicin therapy (Seal and Strangeways, 1975; Snelling *et al.*, 1971). Gentamicin-resistant *Pseudomonas* strains isolated after oral gentamicin therapy have been reported to be less invasive than the sensitive strains isolated before institution of gentamicin therapy, even in patients with agranulocytosis (Greene *et al.*, 1973).

In vivo, R factor mediated resistance undoubtedly plays a part in the emergence of resistance in some cases (Shafi and Datta, 1975; Richmond *et al.*, 1975), but in circumstances where this type of resistance has no opportunity to occur a mechanism similar to that demonstrated in the laboratory may operate. This presumably is most likely to occur with those aminoglycosides in which levels achieved on conventional therapeutic regimes do not greatly exceed the MIC. However, even if resistance does emerge by this mechanism the epidemiological significance of such strains may not be as great as is feared because of the attenuated nature of these bacteria and the relative instability of the resistance induced.

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