Stability of aminoglycoside resistance *in vitro* in gentamicin-resistant *Staphylococcus aureus*

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(Received 31 August 1983; accepted 6 March 1984)

SUMMARY

Stability of aminoglycoside resistance has been investigated in 20 strains of *Staphylococcus aureus* resistant to gentamicin (16 strains were also resistant to methicillin). In view of previous reports that incubation at elevated temperatures can hasten the loss of unstable antibiotic resistance, we passaged strains daily in a liquid medium for 24 days at 43 °C. The nine strains which were resistant to neomycin kept their aminoglycoside resistance virtually intact, whereas most of the other 11 strains (sensitive to neomycin) lost almost all their resistance to gentamicin and kanamycin after 5 days. It thus appears that the stability of aminoglycoside resistances in *Staph. aureus* is closely linked to the resistance of the strains to neomycin. This finding has important possible consequences in terms of the advisability of the clinical usage of preparations containing neomycin or framycetin for topical application and bowel sterilization.

INTRODUCTION

The topical use of neomycin and gentamicin has been held responsible for the emergence of aminoglycoside-resistant strains of *Staphylococcus aureus* (Rountree & Beard, 1965; Alder & Gillespie, 1967; Bint *et al.* 1977; Wyatt *et al.* 1977). However, it was not until the mid 1970's that resistance to both methicillin and gentamicin was observed. The first outbreak of infection by a gentamicin- and methicillin-resistant *Staph. aureus* was reported in London in 1976 (Shanson, Kensit & Duke, 1976). Since then, this type of organism has caused problems in many centres from around the world: Austria, U.S.A., England, Ireland, Denmark, Greece, Belgium and Australia. (Crossley *et al.* 1979; Spitzzy & Rotter, 1979; Peacock, Marsik & Wenzal, 1980; Price, Brain & Dickson, 1980; Giamarellou, Papapetropoulou & Daikos, 1981; Hone *et al.* 1981; King, Brady & Harkness, 1981; Leading Article, 1981; Rosendal, Bang & Rosdahl, 1981; Yourassowsky *et al.* 1981; Linnemann *et al.* 1982; Pavillard *et al.* 1982).

In another outbreak of hospital infection that occurred before the introduction of gentamicin, topical neomycin was implicated (Alder & Gillespie, 1967). It was noted that the antibiotic resistance was stable, and disappeared only when the use of neomycin in therapy was stopped. Later, Ayliffe (1970) found two types of

Table 1. *Characteristics of gentamicin-resistant Staphylococcus aureus strains tested*

Strain no	Phage type	Biotype*	Resistance to		
			Methicillin	Neomycin	Chloramphenicol
1	85 (100 × RTD)	Bx	R	S	R
3	83A/85 (100 × RTD)	B	S	R	S
5	84/85 (RTD)	D	R	R	S
6	Not typable	A	R	R	S
7	Not typable	A	S	R	S
8	85 (100 × RTD)	B	S	S	S
14	84 (100 × RTD)	Ax	R	R	S
17	84/85 (RTD)	D	R	R	S
18	47/83A/84/85 (RTD)	Ax	S	S	S
21	29/84/85 (100 × RTD)	A	R	S	R
27	29/84/85 (100 × RTD)	Dx	R	R	S
29	85 (RTD)	A	R	S	R
31	83A (100 × RTD)	A	R	S	R
35	85 (100 × RTD)	A	R	S	R
36	29/84/85 (100 × RTD)	Bx	R	S	R
37	85 (100 × RTD)	A	R	S	S
40	29/85 (100 × RTD)	A	R	S	R
73	94/96 (RTD)	D	R	R	S
75	84 (100 × RTD)	A	R	S	S
76	85 (100 × RTD)	A	R	R	S

* See Table 2 for interpretation of biotype.

neomycin resistance, one of which was stable and the other unstable. Similarly, resistance to gentamicin has been reported to be unstable by some authors, being lost when the organisms were stored (Porthouse *et al.* 1976; Rosendal *et al.* 1981), but of a stable nature by others (Naidoo & Noble, 1978).

In view of these previous findings, it was decided to investigate the stability of resistance to aminoglycosides *in vitro* in strains of *Staph. aureus* isolated from an outbreak of infection caused by multiresistant organisms in an Australian hospital.

MATERIALS AND METHODS

Bacterial strains

Staph. aureus was identified by a positive tube coagulase test using human plasma and production of DNase. Twenty strains, cultured from patients at St Vincent's Hospital, Melbourne (Stratford & Dixon, 1980), were single isolates from different sites from patients in several wards. Their individual identities were established by means of phage typing, biotyping and other characteristics (Table 1). Results of these tests indicated that no two strains had identical properties, and therefore the strains listed in Table 1 can be considered to be different. Full details of the strains are available on request from the authors. All 20 strains were resistant to benzylpenicillin, minocycline, doxycycline, erythromycin, clindamycin, streptomycin, kanamycin, gentamicin, tobramycin, trimethoprim and sulphonamides. As can be seen from Table 1, all but four of the

Table 2. *Biotyping of Staphylococcus aureus (method of Andrew & Symons, 1982)*

Biotype	Pigments	Lipolysis	Lactose	Haemolysis	Proteolysis
A	Gold	+	+	+	—
Ax	Buff-yellow	+	+	+	—
B	Cream	—	+	—	—
Bx	Cream	—	+	+	—
D	Cream/White	+	—	+	+
Dx	Pink	+	—	+	+
Du	Cream/White	+	—	—	+

strains were resistant to methicillin, nine were neomycin-resistant and seven chloramphenicol resistant.

Phage typing was carried out by Dr R. R. Marples (Central Public Health Laboratory, Colindale) and biotyping by the method of Andrew & Symons (1982). The latter method involves observation of pigment production, lipolysis, haemolysis, proteolysis and the fermentation of lactose. Results are scored by means of an alphabetic code (see Table 2).

Strains were stored in liquid nitrogen after a maximum of four sub-cultures following their initial isolation. They were tested for their resistance to aminoglycosides and the stability of this resistance.

Antibiotics

Streptomycin, neomycin, gentamicin and kanamycin were all of laboratory reference standard, in the form of sulphates with a stated potency and were supplied by the manufacturers. Methicillin, minocycline and erythromycin were obtained from Beecham Research Laboratories, Lederle Laboratories and Abbott Laboratories respectively.

A plate dilution technique was used, doubling dilutions of the antibiotics being incorporated into Iso-Sensitest agar (Oxoid, Basingstoke): 10^4 colony-forming units of each strain were tested by using a multi-point inoculating device (Denley, Sussex). Plates containing methicillin were incubated at 30 °C, others at 37 °C; plates were read at 24 and 48 h.

Resistance mechanisms

The resistance mechanisms to the aminoglycosides were inferred from scrutiny of the results of the MIC values (Shannon & Phillips, 1982). Plasmid analysis was carried out on representative strains by K. G. H. Dyke, Oxford University.

Stability of antibiotic resistance

Staphylococci were grown in Nutrient Broth no. 2 (Oxoid) at 37 °C and sampled at monthly intervals for stability of resistance. The strains were also cultured on slopes of Columbia Agar Base (Oxoid) and incubated at 37 °C. Subcultures were made every seven days on to fresh slopes, which were maintained at 37 °C. Sensitivity testing was carried out on the original slope at the end of each month.

A second set of broth cultures was incubated at 43 °C (Fairbrother, Parker & Eaton, 1954; May, Houghton & Perret, 1964). These broth cultures were inoculated

Table 3. *Activity of six aminoglycosides against 20 strains of gentamicin-resistant strains of Staphylococcus aureus*

Antibiotic	MIC values ($\mu\text{g/ml}$)			
	Range	MIC ₅₀	MIC ₉₀	Geometric mean
Streptomycin	64 to > 512	58	128	90.5
Neomycin	0.25 to 256	1	32	2.3
Kanamycin	32 to > 512	61	256	93.7
Amikacin	1 to 16	0.8	4	1.5
Gentamicin	2 to 128	3	16	5.5
Netilmicin	0.5 to 16	0.8	6.9	1.6

from confluent growth of the organism on an agar plate. They were grown in a divided 10 cm square Petri dish (Sterilin) in a humid atmosphere, and sampled daily for 24 days onto agar plates containing varying concentrations of neomycin and gentamicin, to obtain daily MIC values for these two antibiotics. The inoculum used was c. 7×10^4 c.f.u. on the plate. On day 24 MIC values were also measured for minocycline, doxycycline, erythromycin, streptomycin and kanamycin.

Definition of resistance

Cut-off points for resistance (MIC) were as follows: streptomycin and kanamycin > 4 $\mu\text{g/ml}$, neomycin and gentamicin > 2 $\mu\text{g/ml}$, methicillin > 8 $\mu\text{g/ml}$, all other antibiotics > 1 $\mu\text{g/ml}$.

RESULTS

Phage types

As can be seen from Table 1, there was no correlation between phage type and neomycin resistance.

MIC's

Results are shown in Table 3.

The 20 strains showed wide variation in sensitivity to gentamicin, the two most resistant strains (14 and 76) having MICs of 128 $\mu\text{g/ml}$. The same two strains were also highly resistant to neomycin (MIC 256 $\mu\text{g/ml}$) and to kanamycin (MIC > 512 $\mu\text{g/ml}$), and were also more resistant to amikacin (MIC 16 and 8 $\mu\text{g/ml}$), than were the other 18 strains. However, they were not exceptionally resistant to streptomycin.

The majority of strains were sensitive to netilmicin and amikacin. Thus, resistance to these antibiotics does not seem to follow the patterns shown to the other aminoglycosides.

Mechanisms of resistance

From the MIC results it was inferred that in 19 strains resistance was due to the enzymes *O*-phosphotransferase-APH (2'') -and *N*-acetyltransferase-AAC (6'). In the remaining strain, 73, resistance was judged to be due to impermeability, because of the very high value of the ratio MIC amikacin/MIC gentamicin (Shannon & Phillips, 1982).

Plasmid analysis

Strains 1, 3, 6, 8 and 27 were examined for plasmids. None was found in strain 3, while the others each contained a small plasmid (1.72 Kb, 1.18 MDa), to which no function can be ascribed. Strain 1 also possessed a 4.4 Kb (2.86 MDa) plasmid; as this was the only chloramphenicol-resistant strain tested it is reasonable to suppose that this plasmid codes for such resistance. A plasmid of similar but slightly smaller size was reported by Townsend, Grubb & Ashdown (1983) to be associated with transferable chloramphenicol resistance in *Staph. aureus* strains isolated in Sydney and Melbourne.

No large plasmids of the type described by Townsend *et al.* (1983) and Lyon, May & Skurray (1983) (> 11.2 Kb, > 7.2 MDa) were found in any of the strains. It was therefore considered that gentamicin resistance in these strains involved a transposon (Dyke, personal communication).

There was no connection between plasmid content and resistance to neomycin.

Stability of resistance

The organisms incubated at 43 °C in liquid culture either lost their resistance to aminoglycosides within 3 days or kept it for the duration of the experiment (24 days). Resistance to methicillin and to minocycline was not lost by any strain after the 24 days incubation period, and only two of the 20 strains (3 and 5) lost resistance to erythromycin.

Strains serially sub-cultured on a solid medium at 37 °C usually either lost resistance to aminoglycosides after 1–3 weeks (3–9 sub-cultures) or maintained their resistance for the whole 3 months of the experiment. One strain, however, lost resistance to aminoglycosides only after 18 sub-cultures. No loss of resistance to other antibiotics was observed during the 3 months.

Two strains which lost resistance at 43 °C did not do so when maintained at 37 °C for 3 months.

From these results we conclude that stability of resistance to aminoglycosides can adequately be investigated by testing for sensitivity after strains have been incubated at 43 °C for 5 days.

Patterns of resistance stability

All 20 strains were originally resistant to gentamicin, streptomycin and kanamycin, but only nine were resistant to neomycin (see Table 1). When the results were analysed separately as to whether the strains were originally sensitive or resistant to neomycin, a distinct pattern emerged.

Of the strains initially resistant to neomycin, only one (27) lost neomycin resistance, only two (17 and 27) lost gentamicin resistance, and all retained their resistance to kanamycin and streptomycin. On the other hand, of the 11 strains which were originally sensitive to neomycin, all but two (18 and 35) lost their resistance to gentamicin and all but three (8, 18 and 35) to kanamycin. Resistance to streptomycin in these strains was more stable, however: only two strains (21 and 29) lost their resistance to this antibiotic as a result of passage at 43 °C.

DISCUSSION

From our results it is clear that there are two types of aminoglycoside resistance in *Staph. aureus* – one which is stable and associated with neomycin resistance, and the other which is unstable and is associated with sensitivity to neomycin.

This observation has a possible important implication with regard to use of aminoglycosides in hospital. Neomycin and related compounds (e.g. framycetin) used as bowel preparations or topically as in nasal creams and tulle gras, may well encourage the development of a stable type of aminoglycoside resistance, including that to gentamicin, even though gentamicin is not used. In the past, neomycin resistance of this type has disappeared only when neomycin was withdrawn as a therapeutic agent in the hospital situation. Patients who had become colonized with neomycin resistant strains while in hospital continued to carry them for a long time after their discharge from hospital (Lowbury *et al.* 1964).

Therefore, strains which are resistant to both neomycin and gentamicin present a far greater threat than those which are gentamicin-resistant but neomycin-sensitive, since where there is dual resistance this is stable. Further, they are also more highly resistant to other aminoglycosides.

While it has been proven that neomycin resistance develops as a particular consequence of the topical use of this antibiotic, the evidence associating the emergence of gentamicin-resistant strains with the use of either topical or systemic gentamicin is not so strong. A connection seems to exist between the topical use of neomycin and gentamicin resistance in the hospital environment. However, topical gentamicin was implicated in two outbreaks of gentamicin resistant *Staph. aureus* infections in dermatology units (Wyatt *et al.* 1977; Naidoo *et al.* 1983). This does not seem to occur so often when systemic gentamicin is used.

It is concluded that topical neomycin and related preparations may predispose towards perpetuating a stable type of aminoglycoside resistance, and thus their continued use in hospitals should be discouraged since they may create a risk of producing stable (irreversible) resistance to a number of aminoglycosides, including gentamicin.

We are very grateful to Dr K. G. H. Dyke for plasmid analysis, and to Miss A. Gooding for technical assistance.

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