RESISTANCE OF BACTERIA TO THE NEWER AMINOGLYCOSIDE ANTIBIOTICS: AN EPIDEMIOLOGICAL AND ENZYMATIC STUDY

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Four hundred and thirty-five *Staphylococcus aureus* strains and 395 strains of gram-negative bacteria were tested for susceptibility to gentamicin, sisomicin, tobramycin, netilmicin and amikacin. None of the staphylococcal strains were resistant to the 5 drugs, except two strains that were resistant to amikacin. Eighteen percent of the gram-negative bacteria were resistant to gentamicin, 16% to tobramycin, 14% to sisomicin, 7% to netilmicin and 2% to amikacin. Tests from representative strains showed that these differences were mainly due to the production of aminoglycoside-modifying enzymes. The lower incidence of strains resistant to sisomicin compared to gentamicin was due to a slightly better antimicrobial activity of sisomicin.

Results of experiments on the efficiency of enzymatic phosphorylation, acetylation and adenylylation, and the inactivation of aminoglycoside antibiotics were not always in concurrence with the degree of phenotypic expression of resistance. Thus, the aminoglycoside 3′-phosphotransferase of staphylococci modified amikacin, but the strains were susceptible to the drug. Similarly, the aminoglycoside 2″-nucleotidyltransferase from members of *Enterobacteriaceae* and *Pseudomonas* efficiently adenylylated and inactivated netilmicin, but the strains were susceptible to netilmicin. On the other hand, there can be factors, intrinsically inherent in some strains, contributing to their phenotypic expression of resistance level. It is inferred, therefore, that the contribution of enzymes to the degree of resistance to aminoglycoside antibiotics in a given strain can be evaluated only by comparative examination of resistance in the wild type strain and its corresponding enzyme-negative variant.

In recent years gentamicin, with or without the concomitant administration of beta-lactam antibiotics, has been used with increasing frequency in the treatment of hospital-acquired, gram-negative bacterial infections. This resulted in increasing emergence of gentamicin-resistant strains. The situation is clearly demonstrated in Table 1, which contains the total number of strains examined at our institution from 1974 to 1976 and the percentage of gentamicin-resistant isolates. Since the Institute of Medical Microbiology in Zurich has to perform diagnostic microbiology for the University Hospital and for about 30 smaller hospitals in the area and has to serve also as a Public Health Laboratory for the Kanton of Zurich, these data can be considered to represent the prevailing situation in the eastern part of Switzerland. The frequency of 9% of gentamicin-resistant cultures isolated from hospitalized patients in 1976 is appallingly high.

Because resistance to gentamicin occurred mostly in strains having resistance to many other antibiotics a comparative examination of the newly developed or recently introduced aminoglycoside antibiotics seemed appropriate and necessary.

This paper presents the result of examination of susceptibility of 830 gram-positive and gram-negative bacterial strains of clinical origin to five selected aminoglycoside antibiotics. In addition, data on susceptibility to modification of these antibiotics by enzymes isolated from representative gentamicin-resistant strains, are presented. These data help in explaining differences in resistance

Table 1. Number of strains examined and percentage of gentamicin resistant cultures of clinical isolates of gram-positive and gram-negative bacteria.

Bacteria	Relation to hospitalization[a]	1974 (May-December)		1975		1976	
		No. of strains	Gen^{R}[b] %	No. of strains	Gen^{R}[b] %	No. of strains	Gen^{R}[b] %
Staphylococcus aureus	Outpatients	170	0	406	0	459	0
	Hospitalized pat.	1,355	0	2,685	0	2,639	1
Staphylococcus epidermidis	Outpatients	168	1	247	0	183	0
	Hospitalized pat.	1,239	1	1,899	0	1,114	2
Escherichia coli	Outpatients	450	0	1,130	0	1,199	0
	Hospitalized pat.	2,215	0	4,742	1	4,724	2
Citrobacter freundii	Outpatients	11	0	12	0	13	0
	Hospitalized pat.	54	0	96	5	83	6
Klebsiella spp.	Outpatients	92	0	200	1	191	4
	Hospitalized pat.	761	6	1,886	12	1,875	18
Enterobacter spp.	Outpatients	37	0	72	0	98	1
	Hospitalized pat.	230	5	524	8	571	9
Serratia spp.	Outpatients	2	0	6	17	13	31
	Hospitalized pat.	31	35	145	59	529	80
Proteus mirabilis	Outpatients	101	0	219	1	239	0
	Hospitalized pat.	548	1	1,166	1	1,154	3
Proteus spp. indole-positive	Outpatients	14	7	51	2	61	0
	Hospitalized pat.	150	5	291	5	383	6
Proteus inconstans	Outpatients	—	—	2	50	3	33
	Hospitalized pat.	15	53	24	62	27	63
Salmonella spp.	Outpatients	57	0	48	0	46	0
	Hospitalized pat.	79	0	68	1	71	7
Pseudomonas spp.	Outpatients	86	3	223	3	212	9
	Hospitalized pat.	1,069	9	2,143	9	2,231	14
Further non-fermentative gram-negative rods	Outpatients	61	7	87	2	96	15
	Hospitalized pat.	176	10	302	9	311	15
Total	Outpatients	1,249	1	2,703	1	2,813	2
	Hospitalized pat.	7,921	4	15,971	6	15,712	9

a: Status of prior hospitalization of outpatients not determined.
b: Gen^{R} = gentamicin resistance.

to gentamicin, sisomicin, tobramycin, netilmicin and amikacin existing in our collection of strains.

Materials and Methods

Bacterial Strains

These were isolated in 1976 from different clinical materials. They were comprised of *Staphylococcus aureus* (435); *Escherichia coli* (114); *Klebsiella pneumoniae* (71); *Klebsiella ozaenae* (4); *Serratia marcescens* (43); *Enterobacter cloacae* (36); *Enterobacter agglomerans* (1); *Citrobacter freundii* (6); *Proteus vulgaris* (10); *Proteus mirabilis* (9); *Proteus rettgeri* (3); *Proteus morganii* (3); *Proteus inconstans* (3); *Salmonella* spp. (15); *Shigella* spp. (2); *Pseudomonas aeruginosa* (57); *Pseudomonas maltophilia* (3); *Pseudomonas* spp. (2); *Acinetobacter* spp. (5); *Alcaligenes* spp. (5).

All strains were identified according to standard procedures.

Staph. aureus 5532 was a gentamicin-resistant strain, isolated by PORTHOUSE *et al.*[1] in England.

Staph. aureus S25 was a gentamicin-susceptible mutant of 5532. Both strains were provided by Dr. BROWN. *E. coli* W677 (pHJR66) was a gift of Dr. SMITH.[2] This strain is the source of the adenylylating enzyme, widely used for enzymatic assay of gentamicin. All the rest of the strains were of wild type, isolated at our institution.

Antibiotics and Chemicals.

Laboratory standard preparation of gentamicin C complex, sisomicin and netilmicin were generously supplied by Schering Corp. (Bloomfield, N.Y.); tobramycin by E. Lilly and Co. (Indianapolis, Ind.), and amikacin by Bristol Lab. (Syracuse, N. Y.).

Radio-labelled chemicals were bought from the Radiochemical Centre (Amersham, England). Dithiothreitol, ATP and acetyl-CoA were obtained from Calbiochem (Los Angeles, Calif.), and lysostaphin from Becton, Dickinson and Co. (Orangeburg, N. Y.). Inorganic salts of analytical grade were bought from Merck (Darmstadt, Germany), and agarose from Behringwerke AG (Marburg, Germany).

Antibiotic Susceptibility Testing.

Disc susceptibility testing was performed by the slightly modified single-disc method of BAUER *et al.*[3] and the U.S. Food and Drug Administration,[4,5] using MUELLER-HINTON agar of Baltimore Biological Laboratories (BBL) (Cockeysville, Md.) supplemented with 5% (v/v) old human blood from the blood bank. An inhibition zone of 15 mm or more around the 10 μg gentamicin disc, for a given strain, was interpreted as being susceptible to the drug.[6] The minimal inhibitory concentration (MIC) of drugs was determined on MUELLER-HINTON agar (BBL) according to the standard methods proposed by ERICSSON and SHERRIS.[7]

Production of Enzyme.

The shock method of NOSSAL and HEPPEL[8] was used for gram-negative organisms. The bacterial strains under study were serially subcultured on Brain Heart Infusion (BHI) agar (Difco), containing 10 μg gentamicin/ml, and inoculated into a medium composed of Bacto-tryptone (Difco), 10 g; Bacto yeast extract (Difco), 50 g; NaCl, 10 g; glucose, 4 g; and an amount of water sufficient to make 1 liter. The pH was adjusted to 7.2. A hundred ml of the medium was inoculated with one ml of a 24-hours-old culture and incubated in a shaking water bath at 37°C until the end of logarithmic growth phase. Cells were harvested and washed twice with 40 ml of a buffer containing 0.01 M Tris-HCl and 0.03 M NaCl, pH 7.8 and were suspended in 20 ml of 0.033 M Tris-HCl buffer, pH 7.3 containing 0.003 M EDTA and 20% (w/v) sucrose. After it stood at room temperature for 10 minutes, the suspension was stirred with a magnetic stirrer for 5 minutes and centrifuged for 15 minutes at 10,000$\times g$ and 4°C. The supernatant was discarded and the remaining sucrose was removed carefully from the wall of tubes with cotton swabs. The pellet of cells was suspended in 4 ml of an ice-cold solution of 0.0005 M $MgCl_2$, stirred for 5 minutes and centrifuged for 15 minutes at 22,000$\times g$ and 4°C. The supernatant (osmotic shockate) was carefully decanted to a flask and stabilized with 0.001 M dithiothreitol. Enzymatic extracts of staphylococci were obtained either by the shock method or by lysing the cells with lysostaphin as previously described.[9]

Enzymatic Assays.

These were similar to those described by BENVENISTE *et al.*[10], DAVIES *et al.*[11] and HAAS and DAVIES.[12] Inactivation by adenylylation of aminoglycosides was tested in a reaction mixture consisting of 40 μl enzymatic extract; 3 μmol of Tris-maleate buffer, pH 7.4; 1.4 μmol $MgCl_2$; 0.1 μmol dithiothreitol; 40 nmol (2-^{3}H)ATP (specific activity, 60 μCi/μmol) and 1 nmol of drug base in a total volume of 130 μl. Inactivation by phosphorylation was tested in a reaction mixture containing 40 nmol of (γ-^{32}P)ATP (specific activity, 1~4 mCi/mmol). Acetylation was examined in tests comprising 5.5 nmol (1-^{14}C)acetyl-CoA (specific activity, 36.3 mCi/mmol) and 0.4 nmol drug base. Appropriate controls were devoid of either the labelled coenzymes, or the drug base or the enzymatic extract. After incubating for 2.5 hours at 35°C, 50 μl were assayed for residual antibiotic by agar diffusion[13] in Antibiotic Medium 5 (Difco) containing agarose instead of agar. *Bacillus subtilis* ATCC 6633 was used as test organism, in some cases, and the enzyme-producing strain was used in certain others. Another 50 μl were examined for the extent of adenylylation, phosphorylation or acetylation by mea-

suring the radioactivity incorporated by the phosphocellulose paper binding assay described by DAVIES and coworkers.[11] Radioactivity was counted in a Packard Tri-Carb scintillation spectrometer (Packard Instr., Downers Grove, Ill.) in 10 ml of a toluene based scintillant.

For determining the relative efficiency of the 5 aminoglycosides as substrates, enzymatic extracts were appropriately diluted and the reaction mixtures incubated for 2.5 and 5 minutes at 35°C, after which 50 μl were assayed by the phosphocellulose paper binding method mentioned above. These conditions ensured that linear assays were always occurring.

Elimination.

Attempts to obtain enzyme-negative variants of *Staphylococcus* strains were made as described previously[14]. To eliminate R-plasmids from gram-negative bacteria, the sodium dodecyl sulfate method was used.[15]

Results

Antimicrobial Activity of Aminoglycosides

The *in vitro* activity of 5 aminoglycosidic antibiotics against 395 gram-negative organisms, selected from our collection of fresh clinical isolates, are summarized in Table 2. The minimal inhibitory concentration (MIC) of the drugs against susceptible strains are similar to those reported by other investigators[16~20]. Resistant strains were found among bacterial species as outlined in Table 3. No strain of *Salmonella, Shigella, Enterobacter* and *Citrobacter* in the collection was observed to be resistant to the examined drugs. Resistance to gentamicin in these species, however, do occur in our area (see Table 1).

The susceptibility pattern of 435 *Staphylococcus aureus* strains is shown in Table 4. At the time of collection of these strains (January to March 1976), no isolate resistant to gentamicin was observed. Since that time, however, gentamicin-resistant *Staphylococcus aureus* strains appeared in Zurich (see Table 1).

Aminoglycoside Modifying Enzymes

In Tables 5~7, data on tests with strains capable of modifying aminoglycoside antibiotics are shown. The enzymatic nomenclature and the abbreviations were taken from the Plasmid Nomenclature Group[21] and from the commission on Chemoresistance, International Society of Chemotherapy (S. MITSUHASHI, personal communication, Gunma University, Maebashi, Japan).

The APH(3′) type 4 was recently found in *Staph. aureus* and *Staph. epidermidis* strains.[22,23] This enzyme phosphorylates the hydroxyl group in position 3 of ring *I* of aminoglycosides (see Fig. 1). In contrast to the 3′-phosphotransferases of gram-negative organisms, amikacin, also, is phosphorylated and inactivated by the staphylococcal enzyme (see Table 5). The drug, however, is a weak substrate, and strains producing APH(3′)-4, therefore, were phenotypically susceptible to amikacin. Some strains of staphylococci that were resistant or moderately resistant to amikacin (*i.e.* FK170) possessed an inherent mechanism of resistance still unknown. The APH(2″) was recently observed in gentamicin-resistant staphylococci in England.[1,24] The enzyme phosphorylates the hydroxyl group in position 2 of ring *III* of aminoglycosides.[25] (see Fig. 1) Strains producing APH(2″) exhibited resistance to gentamicin, sisomicin and tobramycin, and intermediate resistance to netilmicin and amikacin (see Table 5). In addition to APH(2″), strain 5532 produced an acetylating enzyme with low affinity to tobramycin and amikacin.[26] The enzyme was still produced by the phosphotransferase-negative mutant. This is the reason for the rather high MIC of tobramycin and amikacin against the mutant strain (see Table 5).

Table 2. Summary of antimicrobial activity of five aminoglycoside antibiotics against 395 gram-negative bacteria (Zurich, 1976).

Antibiotic	Susceptible strains[a]				Resistant strains			
	No. of strains	% of strains	Geometric mean of MICs (μg/ml)	Range of MICs (μg/ml)	No. of strains	% of strains	Geometric mean of MICs (μg/ml)	Range of MICs (μg/ml)
Gentamicin	325	82.3	0.93	0.2~6.25	70	17.7	41.8	12.5~>200
Sisomicin	341	86.3	0.56	0.1~6.25	54	13.7	31.1	12.5~>200
Tobramycin	332	84.1	0.80	0.1~6.25	63	15.9	31.9	12.5~>200
Netilmicin	366	92.7	0.84	0.1~6.25	29	7.3	50.0	12.5~>200
Amikacin	387	98.0	1.66	0.4~12.5	8	2.0	77.1	25~>200

[a] Strains were regarded as susceptible, when the MICs of gentamicin, sisomicin, tobramycin and netilmicin were 6.25 μg/ml or less, and of amikacin 12.5 μg/ml or less.

Table 3. Summary of aminoglycoside-antibiotic resistant gram-negative organisms.

Organism	No. of strains	No. of strains resistant to				
		Gen	Sis	Tob	Net	Ami
Escherichia coli	114	4	1	3	1	0
Klebsiella spp.	75	14	6	14	0	0
Serratia spp.	43	27	24	26	5	1
Proteus mirabilis	9	1	1	0	0	0
Proteus spp. (indole positive)	19	8	8	7	7	0
Pseudomonas spp.	62	14	12	11	14	5
Acinetobacter spp. and *Alcaligenes* spp.	10	2	2	2	2	2
Total	332	70	54	63	29	8

Strains were regarded as resistant, when the MICs of gentamicin (Gen), sisomicin (Sis), tobramycin (Tob) and netilmicin (Net) were 12.5 μg/ml or greater, and of amikacin (Ami) 25 μg/ml or more.

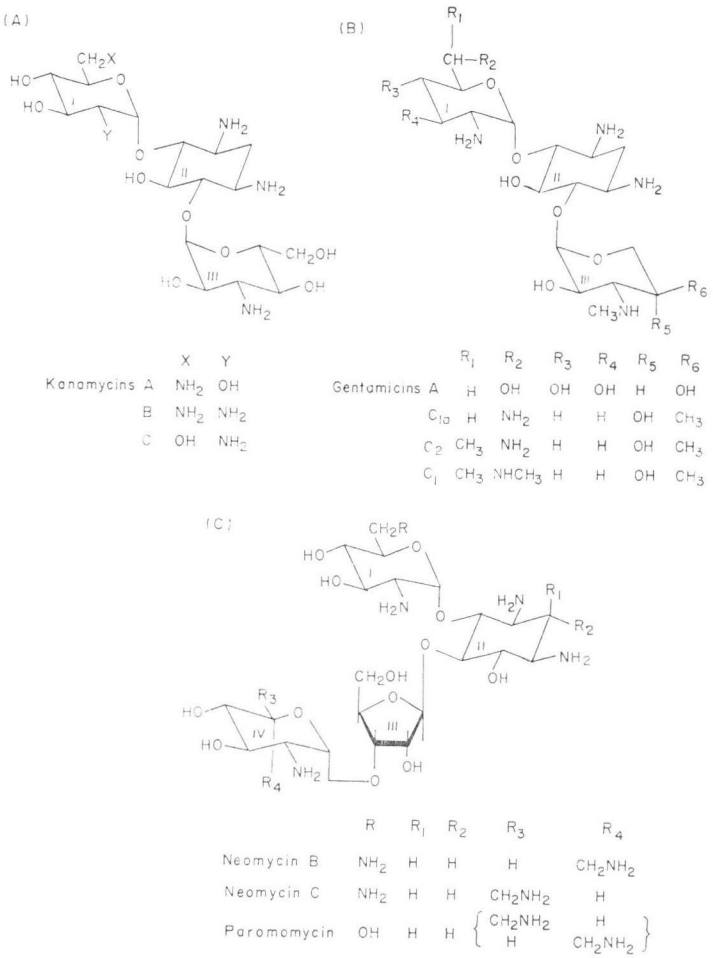
Table 4. Summary of antimicrobial activity of five aminoglycoside antibiotics against 435 *Staphylococcus aureus* strains (Zurich, 1976).

Antibiotics	Susceptible strains[a]				Resistant strains			
	No. of strains	% of strains	Geometric mean of MICs (μg/ml)	Range of MICs (μg/ml)	No. of strains	% of strains	Geometric mean of MICs (μg/ml)	Range of MICs (μg/ml)
Gentamicin	435	100	0.044	0.015~1.6	0	0	—	—
Sisomicin	435	100	0.026	0.007~1.6	0	0	—	—
Tobramycin	435	100	0.047	0.03~6.25	0	0	—	—
Netilmicin	435	100	0.071	0.03~1.6	0	0	—	—
Amikacin	433	99.4	0.422	0.1 ~6.25	2	0.6	50	50

[a] Strains were regarded as susceptible, when the MICs of gentamicin, sisomicin, tobramycin and netilmicin were 6.25 μg/ml or less, and of amikacin 12.5 μg/ml or less.

The AAC(2′) type 2[26] transfers the acetyl group from acetyl-CoA to the amino group in position 2 of ring *I* of aminoglycosides (see Fig. 1). This enzyme was produced by the *Proteus vulgaris* strain HK231. It efficiently acetylated and inactivated gentamicin, sisomicin, tobramycin and netilmicin, but not amikacin (see Table 6). In contrast, the aminoglycoside 3-N-acetyltransferase[27] was only capable of acetylating the deoxystreptamine moiety (ring *II* in Fig. 1) of gentamicin and, slowly, of

Fig. 1.



(A) The structures of the kanamycins. Positions in ring *I* are numbered 1′–6′ (clockwise), in ring *II* 1–6 (counterclockwise) and in ring *III* 1″–6″ (counterclockwise). Position Y is the 2′ position. The hydroxyl group in ring *II* is in position 5. Tobramycin is 3′-deoxykanamycin B. Amikacin is 1-hydroxybutyric acid-kanamycin A.

(B) The structures of the gentamicins. Numbering is as indicated for the kanamycins. The hydroxyl group in ring *III* is in position 2″. Sisomicin is 4′,5′-dehydrogentamicin C_{1a}. Netilmicin is 1-N-acetyl-4′,5′-dehydrogentamicin C_{1a}.

(C) The structures of the neomycins. Positions are numbered as in the kanamycins.

netilmicin. Strains producing this enzyme, therefore, were susceptible to tobramycin, netilmicin and amikacin (see Table 6).

Resistance to tobramycin and weak resistance to amikacin, but susceptibility to gentamicin and sisomicin was recently observed in *Staph. epidermidis*[9] and *Staph. aureus*.[28] This unusual phenotype was due to the new aminoglycoside 4′-nucleotidyltransferase, adenylylating the OH-group in position 4 of ring *I* and also perhaps of ring *III* (see Fig. 1) of tobramycin, amikacin and other drugs (U. SCHWOTZER, F. H. KAYSER and W. SCHWOTZER, in preparation). Since the gentamicins, sisomicin and netilmicin do not contain reactive groups in the corresponding position, strains were susceptible to these drugs (see Table 7).

The aminoglycoside 2″-nucleotidyltransferase[29] is widely observed in gentamicin-resistant members of *Enterobacteriaceae* and also in *Pseudomonas aeruginosa*. The enzyme adenylylates the hydroxyl group in position 2 of ring *III* (see Fig. 1). Accordingly, gentamicin, sisomicin, tobramycin and netilmicin were substrates for this enzyme, but amikacin was not. Strains producing this enzyme, however, were phenotypically susceptible to netilmicin. The low level of resistance of the *Pseudomonas* strain

Table 5. Relation of aminoglycoside-phosphorylating enzymes and inactivation of antibiotics to MIC.

Enzyme	Strain	Antibiotic[a]	MIC (μg/ml)		Inactivation[b] (zone diameter in mm)	Extent of phosphorylation[c] (cpm)	Efficiency of phosphorylation[d] (%)
			Wild type strain	Enzyme-negative variant			
Aminoglycoside 3′-phosphotransferase type 4 [APH(3′)-4]	*Staph. aureus* FK170	Gen	0.8	0.4	18/18	130	0
		Sis	0.4	0.4	22/22	20	0
		Tob	0.8	0.4	16/16	0	0
		Net	0.2	0.4	22/23	20	0
		Ami	12.5	6.25	6/24	6,060	18
		Neo	>200	1.6	6/12	9,800	100
Aminoglycoside 2″-phosphotransferase [APH(2″)]	*Staph. aureus* 5532	Gen	50	0.8	6/21	2,500	100
		Sis	50	0.8	6/18	2,800	93
		Tob	50	3.1	6/17	1,600	27
		Net	12.5	0.8	6/21	3,200	132
		Ami	6.25	6.25	18/23	1,500	12

[a] Abbreviations see legend of Table 3; Neo=neomycin B.
[b] Zone diameter of assayed sample/zone diameter of control sample. A value of 6 mm means no inhibition zone around well.
[c] Counts per minute incorporated and assayed after 2.5 hours incubation at 35°C.
[d] The percent is relative to the phosphorylation of neomycin or gentamicin.

Table 6. Relation of aminoglycoside-acetylating enzymes and inactivation of antibiotics to MIC.

Enzyme	Strain	Antibiotic[a]	MIC (μg/ml)		Inactivation[b] (zone diameter in mm)	Extent of acetylation[c] (cpm)	Efficiency of acetylation[d] (%)
			Wild type strain	Enzyme-negative variant			
Aminoglycoside 2′-N-acetyltransferase type 2 [AAC(2′)-2]	*Proteus vulgaris* HK231	Gen	50	ND	6/16	1,730	100
		Sis	50	ND	12/15	5,040	170
		Tob	50	ND	6/14	1,750	149
		Net	>200	ND	6/18	4,700	196
		Ami	6.25	ND	24/24	0	0
Aminoglycoside 3-N-acetyltransferase type 2 [AAC(3)]	*Proteus mirabilis* HK238	Gen	25	3.1	6/16	2,300	100
		Sis	25	1.6	6/15	4,900	155
		Tob	3.1	0.4	12/14	490	0
		Net	0.8	0.8	17/17	3,300	3
		Ami	1.6	3.1	24/24	48	0

[a] Abbreviations see legend of Table 3.
[b] Zone diameter of assayed sample/zone diameter of control sample. A value of 6 mm means no inhibition zone around well.
[c] Counts per minute incorporated and assayed after 2.5 hours of incubation at 35°C.
[d] The percent is relative to the phosphorylation of gentamicin.
ND=not done.

to netilmicin and amikacin (see Table 7) was due to a mechanism other than enzymatic modification, inherent in this strain. Variants, which had lost the ability to produce AAD(2″), did not differ from the wild type culture in the degree of resistance to netilmicin and amikacin.

Discussion

The antimicrobial activity of the five aminoglycoside antibiotics examined against susceptible strains are in agreement with other reports[16~20]. On the basis of micrograms per milliliter, amikacin

Table 7. Relation of aminoglycoside-adenylylating activity and inactivation of antibiotics to MIC.

Enzyme	Strain	Antibiotic[a]	MIC (μg/ml)		Inactivation[b] (zone diameter in mm)	Extent of adenylylation[c] (cpm)	Efficiency of adenylylation[d] (%)
			Wild type strain	Enzyme-negative variant			
Aminoglycoside 4′-nucleotidyltransferase [AAD(4′)]	*Staph. epidermidis* FK109	Gen	0.1	0.05	22/22	50	0
		Sis	0.02	0.05	17/18	0	0
		Tob	200	0.05	6/16	4,300	100
		Net	0.1	0.1	22/23	100	0
		Ami	12.5	0.4	6/24	3,900	43
Aminoglycoside 2″-nucleotidyltransferase [AAD(2″)]	*E. coli* W677 (pHJR66)	Gen	25	ND	6/23	1,870	100
		Sis	25	ND	6/18	2,900	258
		Tob	25	ND	6/16	3,600	183
		Net	0.2	ND	6/23	4,020	81
		Ami	1.6	ND	23/23	0	0
	Pseudomonas aeruginosa HK232	Gen	>200	0.8	7/21	1,540	100
		Sis	100	0.4	6/18	2,300	830
		Tob	25	0.2	6/18	2,300	292
		Net	6.25	3.1	19/21	1,300	109
		Ami	3.1	6.25	22/22	0	0

[a] Abbreviations see legend of Table 3.
[b] Zone diameter of assayed sample/zone diameter of control sample.
[c] Counts per minute incorporated and assayed after 2.5 hours of incubation at 35°C.
[d] The percent is relative to the adenylylation of tobramycin or gentamicin.
ND=not done.

is the least active agent, especially against *Staphylococcus aureus*. However, this *in vitro* disadvantage may be offset clinically by the fact that amikacin reaches higher levels in human serum than the other drugs.[30]

Amikacin is the most effective drug against gentamicin-resistant strains, followed by netilmicin, sisomicin and tobramycin (see Tables 2 and 4). This discrepancy can be partly explained by the differences in efficiency of these drugs as substrates of the aminoglycoside-modifying enzymes, and by the frequencies of the occurrence of the enzymes among aminoglycoside-resistant strains. In Tables 5~7, the activity of such enzymes *in vitro* and the phenotypic character of the corresponding strain are given. As can be seen, the data of test tube experiments correlated well with the susceptibility or resistance of some of the strains. In certain other cases, however, the results of experiments *in vitro* and *in vivo* were not in concurrence.

The APH(3′)-4 of staphylococci, for instance, phosphorylated amikacin. Strains producing this enzyme, however, were phenotypically susceptible to the drug.[22] (Strain FK170 exhibits an unknown intrinsic mechanism of resistance to amikacin). A simple explanation would be that—because of a slow rate of phosphorylation[22]—sufficient molecules of the drug pass through the cell wall and membrane and reach the cytoplasm to exert an antibacterial effect. A similar cause could explain the susceptibility to netilmicin of strains producing the AAC (3). In addition, the 3-N-acetylated netilmicin still had antimicrobial activity, since no inactivation in the microbiological assay was observed.

It is more difficult to understand, why strains producing the AAD(2″) were susceptible to netilmicin (see Table 7). The enzyme isolated from the *E. coli* strain efficiently adenylylated the drug. In addition, inactivation *in vitro* also occurred, but the strain was highly susceptible to netilmicin. On the other hand, the strain of *Pseudomonas aeruginosa* was inhibited only by 6.25 μg/ml of netilmicin. Enzyme-negative variants, however, did not differ in the level of resistance to this drug.

The examples demonstrate that the resistant or susceptible phenotypic expression of bacterial strains do not depend exclusively on the presence or absence of aminoglycoside-modifying enzymes.

The amount of such enzymes within the bacterial cell, the kinetic constants at the site of their location, and the antibacterial activity of modified aminoglycosides may all play a significant role. In addition, certain intrinsic mechanisms of resistance in these strains can contribute to resistance. Thus, an assessment of the degree of influence by the aminoglycoside-modifying enzymes on the phenotypic expression of a given strain can be made only by the comparative examination of the wild type strain and its corresponding enzyme-negative variant.

As mentioned above, the differences in resistance of gram-negative strains to the five antibiotics (see Table 2) are mainly due to the occurrence of various aminoglycoside-modifying enzymes. Out of 15 randomly selected gentamicin-resistant cultures from our collection, nine produced the AAD(2″) and, all understandably were susceptible to netilmicin and amikacin. Five strains produced the AAC-(2′)-2, which modifies all the drugs except amikacin. Only one strain produced the AAC(3), rendering the strain resistant to gentamicin and sisomicin. To our knowledge, there is no enzyme known to inactivate selectively gentamicin only, and not sisomicin. The differences in the frequency of resistance against these two drugs are due to a slightly better antimicrobial activity of sisomicin. Thus, some strains with a low degree of resistance to gentamicin may show susceptibility to sisomicin.

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