IN VIVO AND *IN VITRO* CROSS-RESISTANCE OF KANAMYCIN-RESISTANT MUTANTS OF *E. COLI* TO OTHER AMINOGLYCOSIDE ANTIBIOTICS

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Cross resistance of kanamycin-resistant mutants of *E. coli* Q13 to other aminoglycosides (streptomycin, neomycin, gentamicin and dibekacin) was demonstrated *in vivo* (growth) and *in vitro* (polyphenylalanine synthesis, codon misreading and translocation on the ribosomes). Kanamycin-resistant mutants, R1–4, R2–1, R2–2, R3–3 and R3–5 showed various degrees of cross-resistance to streptomycin, gentamicin, neomycin and dibekacin *in vivo*. *In vitro*, polyphenylalanine synthesis was more resistant to kanamycin, streptomycin, neomycin and gentamicin on the ribosomes of the kanamycin-resistant mutants than on those of the parental strain. In the presence of kanamycin, neomycin or gentamicin, less degrees of [^{14}C]isoleucine uptake with poly[U] (codon misreading) were observed on the ribosomes obtained from the resistant mutants than on the sensitive cell ribosomes. The N-acetyl-[^{14}C]phenylalanyl-puromycin synthesis enhanced by an elongation factor, EF-G and GTP (translocation) was more resistant to kanamycin and dibekacin on the mutant ribosomes than on the parental ribosomes. The results indicate that the cross-resistance to other aminoglycoside antibiotics, as well as the kanamycin resistance, are attributed to mutational alterations of the ribosomes in these mutants.

Kanamycin, neomycin and gentamicin have been observed to bind to both large and small subunits of *Escherichia coli* ribosomes and to inhibit translocation of peptidyl-tRNA. Contrary to these aminoglycoside antibiotics, streptomycin binds to the small ribosomal subunit, and does not affect translocation[1]. Several kanamycin-resistant mutants of *E. coli* Q13 have been isolated, in which the resistance was attributed to alterations of the small ribosomal subunit in some of the mutants, and to the large ribosomal subunit in the others[2].

We have further studied the cross-resistance of the kanamycin-resistant mutants to other aminoglycoside antibiotics (streptomycin, neomycin, gentamicin and dibekacin) *in vivo* and *in vitro*, using polyphenyalanine synthesis, codon misreading, and translocation of peptidyl-tRNA on the ribosomes. The results are presented in this publication.

Materials and Methods

Chemicals

[^{14}C]Phenylalanine (513 mCi/mmol) and [^{14}C]isoleucine (360 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. Kanamycin, dibekacin, neomycin and streptomycin were products of Meiji Seika Kaisha, Ltd., Tokyo, and gentamicin of Schering Corporation, Bloomfield, New Jersey. Phosphoenolpyruvate, ATP, GTP, poly[U], *E. coli* tRNA, and pyruvate kinase were purchased from Boehringer, Mannheim, Germany. Other reagents were of the highest grade available.

Kanamycin-resistant mutants were isolated after treating *E. coli* Q13 with N-methyl-N′-nitro-N-nitrosoguanidine[2]. The preparation of S100 fractions, washed ribosomes, and N-acetyl-[^{14}C]Phe-tRNA was according to the procedures described previously[2,3].

Poly[U]-dependent [^{14}C]polyphenylalanine synthesis on the ribosomes

The reaction mixture, in 0.2 ml, contained: 50 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 8 mM magnesium acetate, 6 mM 2-mercaptoethanol, 2 mM ATP, 5 mM phosphoenolpyruvate, 4 μg pyruvate kinase, 0.2 mM GTP, 20 μg *E. coli* tRNA, 20 μg poly[U], 25.7 pmoles ribosomes, 160 μg S100 fraction, 0.06 μCi [^{14}C]phenylalanine, and the antibiotic. The mixture was incubated at 37°C for 20 minutes. The reaction was terminated by addition of 2 ml of 5% trichloroacetic acid, and heating at 90°C for 20 minutes. The radioactivity, collected on glass filters, was determined in a liquid scintillation counter.

Codon misreading

[^{14}C]Isoleucine uptake with poly[U] was examined as a model of codon misreading in the presence of aminoglycosides by the same method as used for polyphenylalanine synthesis, except that [^{14}C]phenylalanine was replaced by 0.04 μCi [^{14}C]isoleucine and 51.4 pmoles ribosomes were used. The incubation period was 30 minutes.

Translocation of N-acetyl-[^{14}C]Phe-tRNA from the acceptor site to the donor site

The reaction mixture, in 0.2 ml, contained: 50 mM Tris-HCl, pH 7.8, 150 mM NH_4Cl, 20 mM magnesium acetate, 6 mM 2-mercaptoethanol, 20 μg poly[U], 51.4 pmoles *E. coli* ribosomes, and 30 μg N-acetyl-[^{14}C]Phe-tRNA. The mixture was incubated at 37°C for 40 minutes, and cooled in an ice-bath. Then 0.2 mM puromycin and the antibiotic with or without 50 μg EF-G and 0.2 mM GTP were added to the mixture. It was further incubated at 37°C for 10 minutes, and extracted with 1.5 ml of ethyl acetate by adjusting pH to 5.5 with 1 ml of 0.1 M sodium acetate. The difference of N-acetyl-[^{14}C]Phe-puromycin in the presence and absence of EF-G and GTP was taken as translocation of N-acetyl-[^{14}C]-Phe-tRNA from the acceptor site to the donor site[4].

Results

Growth Inhibition of *E. coli* Strains by Aminoglycoside Antibiotics

The effects of kanamycin, streptomycin, neomycin, gentamicin and dibekacin on the growth of *E. coli* Q13 and its kanamycin-resistant mutants were observed by a disc diffusion method, and the minimal growth-inhibitory concentrations (MIC) of the antibiotics determined by an agar dilution procedure. As presented in Table 1, MIC's of the aminoglycosides were 1.6~3.1 μg/ml for the parental strain and 12.5~200 μg/ml for the resistant mutants: R1–4, R2–1, R2–2, R3–3 and R3–5. The difference was *ca.* 4~65 fold. The results are in accord with those obtained by the diffusion method (data are not shown), indicating that the kanamycin-resistant mutants showed various degrees of cross-resistance to streptomycin, gentamicin, neomycin and dibekacin.

Effects of Antibiotics on Polyphenylalanine Synthesis on the Ribosomes Derived from the Parental and Resistant Strains of *E. coli*

Table 1. Minimal inhibitory concentrations (MIC in μg/ml) of aminoglycoside antibiotics for kanamycin-resistant mutants and the parental strain Q13 of *E. coli*.
Medium: heart infusion agar.

Antibiotic	Parent	Resistant mutants				
		R1-4	R2-1	R2-2	R3-3	R3-5
Kanamycin	3.1	100	50	100	100	100
Dibekacin	3.1	100	50	100	100	100
Gentamicin	1.6	50	25	25	25	25
Neomycin	3.1	200	50	50	50	50
Streptomycin	3.1	50	12.5	25	50	25

Table 2. Effect of antibiotics on poly[U]-dependent [^{14}C]polyphenylalanine synthesis on the ribosomes derived from the parental and kanamycin-resistant strains of *E. coli*.*

Antibiotic (μM/ml)		Parent	Resistant mutants				
			R1-4	R2-1	R2-2	R3-3	R3-5
None		100 (40)	100 (52)	100 (47)	100 (44)	100 (53)	100 (55)
Kanamycin	3	59	99	94	105	92	94
	30	41	74	84	72	79	87
Streptomycin	3	59	97	92	106	98	85
	30	39	95	51	80	81	66
Neomycin	0.3	66	100	83	83	92	84
	3	41	93	72	71	63	58
Gentamicin	0.3	68	98	99	96	96	91
	3	42	92	81	90	91	79
Viomycin	0.3	53	48	47	53	51	53
	3	18	14	26	24	19	19

* Expressed as percent incorporation of [^{14}C]phenylalanine. Actual accounts in dpm$\times 10^{-3}$ of incorporated [^{14}C]phenylalanine are in parentheses.

Table 3. Codon misreading induced by aminoglycoside antibiotics on the ribosomes from parental and kanamycin-resistant strains of *E. coli* Q 13. Poly[U]-dependent incorporation of [^{14}C]isoleucine.

Antibiotic (μM/ml)		Parent	Resistant mutants				
			R1-4	R2-1	R2-2	R3-3	R3-5
None		1.0 (10)	1.0 (12)	1.0 (9)	1.0 (11)	1.0 (12)	1.0 (11)
Kanamycin	3	4.6	1.7	1.8	1.5	1.8	1.7
	30	12.0	5.3	4.9	3.8	5.6	4.2
	300	69.0	33.0	30.0	29.0	24.0	31.0
Streptomycin	300	7.5	3.3	3.3	2.8	3.9	4.4
Neomycin	30	16.0	8.2	9.2	7.3	9.5	7.4
Gentamicin	30	58.0	14.0	21.0	17.0	18.0	22.0

* Expressed as percent uptake of [^{14}C]isoleucine. Actual accounts in dpm$\times 10^{-2}$ of incorporated [^{14}C]isoleucine are given in parentheses.

Since the kanamycin resistance is due to alterations of the ribosomes in these mutants[2], effects of various antibiotics on polypeptide synthesis were examined, using the ribosomes obtained from the parental and resistant strains. Kanamycin, streptomycin, neomycin, gentamicin and viomycin were observed to inhibit poly [U]-dependent polyphenylalanine synthesis in a cell-free system derived from the parental cells. The relative uptakes of [^{14}C]phenylalanine are shown in Table 2. The polyphenylalanine synthesis was more resistant to kanamycin, streptomycin, neomycin and gentamicin on the ribosomes of R1–4, R2–1, R2–2, R3–3 or R3–5 mutants than on the those of the parental strain. In a simultaneous experiment, viomycin was taken as a control drug; ribosomal polypeptide synthesis in the parent was sensitive to viomycin to the same extents as in the mutants.

Comparison of Codon Misreading Induced by Aminoglycosides on the Parental and Resistant Ribosomes

In vitro codon misreading was studied by the incorporation of [^{14}C]isoleucine into polypeptide with

poly[U] on the ribosomes derived from parental and resistant cells, and the results are summarized in Table 3. Kanamycin, neomycin and gentamicin markedly enhanced the isoleucine uptake in the extract of the parental strain. Less degrees of codon misreading were observed on the ribosomes obtained from R1–4, R2–1, R2–2, R3–3 or R3–5 resistant mutants. The cross-resistance was again demonstrated with *in vitro* codon misreading.

Effects of Antibiotics on Peptidyl Transferase Reaction and Translocation of Peptidyl-tRNA on the Ribosomes Obtained from the Parental and Resistant Cells

N-Acetylphenylalanyl-puromycin synthesis by the ribosomes with N-acetyl-[^{14}C]phenylalanyl-tRNA and puromycin in the absence of EF-G and GTP were employed as a model reaction for peptidyl transferase; and the effects of antibiotics was studied. The puromycin reaction was not significantly affected by 30 μM kanamycin, 10 μM dibekacin or 3 μM viomycin on the ribosomes derived from the parental and resistant cells (data are not shown).

The translocation of N-acetylphenylalanyl-tRNA from the acceptor site to the donor site on the ribosome was observed by the puromycin reaction stimulated by the addition of EF-G and GTP[4]. As presented in Table 4, the enhanced puromycin reaction was definitely blocked by kanamycin, dibekacin and viomycin, indicating that translocation of N-acetylphenylalanyl-tRNA was prevented by these antibiotics on the parental ribosome.

Lesser degrees of inhibition by kanamycin and dibekacin of EF-G- and GTP-stimulated puromycin reaction (translocation) were observed on the resistant ribosomes of R1–4, R2–1, R2–2, R3–3 and R3–5 than on the sensitive ribosome. On the contrary, viomycin affected the translocation at the same level on both parental and resistant ribosomes. The cross-resistance between kanamycin and dibekacin was also found in the inhibition of translocation of N-acetylphenylalanyl-tRNA.

Table 4. Effects of antibiotics on translocation—N-acetyl-[^{14}C]phenylalanyl-puromycin synthesis enhanced by EF-G and GTP—on the ribosomes obtained from the parental and kanamycin-resistant strains of *E. coli* Q 13.*

Antibiotic (μM/ml)		Parent	Resistant mutants				
			R1-4	R2-1	R2-2	R3-3	R3-5
None		100 (15)	100 (12)	100 (12)	100 (14)	100 (14)	100 (14)
Kanamycin	3	56	95	85	95	92	94
	30	28	57	60	63	56	65
Dibekacin	1	60	90	80	94	91	89
	10	17	63	59	57	57	56
Viomycin	3	10	19	16	18	11	16

* Expressed as percent acetyl-[^{14}C]Phe-puromycin synthesis stimulated by EF-G and GTP. Actual accounts in dpm$\times 10^{-2}$ of acetyl-[^{14}C]Phe-puromycin formed are given in parentheses.

Discussion

The current experiments present some aspects of cross-resistance of kanamycin-resistant *E. coli* mutants to aminoglycosides (streptomycin, neomycin, gentamicin and dibekacin). The cross-resistance seems to be due to alterations of the ribosomes, because the cross-resistance is also observed in the ribosomal systems: polyphenylalanine synthesis, codon misreading, and translocation. Since the resistance to kanamycin is caused by reduced affinity of the drug for the mutant ribosomes[2], the cross-

resistance to the other aminoglycosides may be also attributed to decreased affinity of the mutant ribosomes for the antibiotics.

The resistance is due to changes of the 30S ribosomal subunit in the 3 kanamycin-resistant mutants, and to those of the 50S ribosomal subunit in the other mutants[2]. We had initially attempted, by using both types of mutants, to determine which ribosomal subunit participates in codon misreading and translocation. However, the present results fail to answer the question, because codon misreading and translocation are resistant to kanamycin and other aminoglycosides in both types of resistant mutants.

Since the parental strain is considerably resistant to viomycin, the sensitivity or resistance of the mutants to viomycin has not been studied in details.

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