Phosphorylation of Kanamycin, Lividomycin A, and Butirosin B by *Providencia stuartii*

PAUL B. MARENGO, MAYMIE E. CHENOWETH, GARY D. OVERTURE, AND JEANETTE WILKINS

Department of Pediatrics, Los Angeles County/University of Southern California Medical Center; Department of Biological Sciences, University of Southern California; and the Hastings Infectious Disease Laboratory, Los Angeles, California 90033

Received for publication 18 April 1974

The isolation of *Providencia stuartii* resistant to multiple aminoglycoside antibiotics prompted an investigation into the mechanism of their resistance. Crude enzyme extracts of a strain of *P. stuartii* inactivated kanamycin, lividomycin A, and butirosin B in the presence of adenosine 5'-triphosphate (ATP), as measured by a microbiological assay. The occurrence of inhibitory concentrations of 500 μ g or greater per ml against kanamycin, lividomycin A, and butirosin B, coupled with the inactivation of these antibiotics in the presence of ATP, suggested enzymatic phosphorylation. This was documented by the transfer of the γ -phosphate of [γ - 32 P]ATP. In contrast, the inability to inactivate gentamicin or tobramycin by the crude enzyme extracts in the presence of ATP suggests another enzymatic mechanism of resistance for these antibiotics, such as adenylation or acetylation. Of importance is the fact that amikacin, a semisynthetic analogue of kanamycin A which is resistant to inactivation by most resistance transfer factor enzymes, was found to inhibit the growth of *P. stuartii* at low concentrations.

Resistance of bacteria to the several classes of antimicrobial agents may be due to a number of mechanisms (2, 4, 18). Currently, enzymatic inactivation of antibiotics by resistance transfer factor enzymes is being recognized more frequently in clinical isolates of resistant bacteria (2, 4). Cross-resistance of bacteria to the aminoglycosidic aminocyclitol antibiotics (14) may emerge because of structural similarities within this class of antibiotics that permit inactivation by common enzyme systems or by simultaneous occurrence of several enzyme systems within a single organism. The emergence of resistance among isolates of *Providencia stuartii* to several aminoglycoside antibiotics in our institution (11) prompted an investigation into the mechanism for their resistance.

MATERIALS AND METHODS

Bacterial strain. A representative strain of *P. stuartii* (no. 73-197) resistant to multiple antibiotics was isolated from a patient in the burn unit of Los Angeles County/University of Southern California Medical Center, Los Angeles, Calif. The isolate was checked for purity and identity and tested for antimicrobial susceptibility in the Hastings Infectious Disease Laboratory, Los Angeles.

Antibiotic susceptibility testing. Minimal inhibitory concentrations were determined against kanamycin (Bristol Laboratories), neomycin (Upjohn), livi-

domycin A (Bristol Laboratories), neomycin (Upjohn), butirosin B (Bristol Laboratories), gentamicin C components (Schering Corp.), tobramycin (Lilly Research Laboratories), and amikacin (Bristol Laboratories), utilizing serial twofold dilutions of each antibiotic in Mueller-Hinton broth. Inocula were standardized to contain 10^8 bacteria per ml. The minimal inhibitory concentration was defined as the lowest concentration of drug in which no visible turbidity was present after overnight incubation at 37 C. In addition, susceptibility testing was carried out in increasing concentrations (7×10^{-5} to 1.1×10^{-3} M) of ethylenediaminetetraacetic acid (EDTA) against kanamycin, gentamicin, tobramycin, and amikacin.

Preparation of enzyme extract. *P. stuartii* 73-197 was grown in 200 ml of Mueller-Hinton broth on a gyratory (model G-25) shaker for 4 h at 37 C. Cells were harvested in the mid-logarithmic phase of growth by centrifugation at $4,500 \times g$ for 10 min., and were washed three times with equal volumes of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0). Washed cells were resuspended in 20 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) and disrupted in an MSE ultrasonic disintegrator (Instrumentation Associates, Inc., New York) for 6 min at 4 C. The suspension of disrupted cells was centrifuged at $30,000 \times g$ for 30 min at 4 C, and the supernatant fluid was sterilized by membrane filtration (Millipore Corp.). Protein concentration of the prepared supernatant was estimated by the method of Lowry et al., with crystalline bovine serum as the standard (9).

Inactivation of aminoglycoside antibiotics. Adenosine 5'-triphosphate (ATP) was utilized as the substrate for the enzymatic reactions. For the microbiological assay, various concentrations (0.1 to 1,000 $\mu\text{g/ml}$) of the selected antibiotics were added to a reaction mixture containing ATP (10 μmol), dithiothreitol (Cleland reagent, 1 μmol), MgCl_2 (8 μmol), tris(hydroxymethyl)aminomethane-hydrochloride buffer at pH 7.0, 7.5, or 8.0 (50 μmol), and 0.01 to 0.5 ml of crude enzyme extract calculated to contain 3.5 mg of protein per ml in a total reaction mixture of 1.0 ml. Reaction mixtures were incubated in a water bath at 37 C for 4 and 22 h.

For the enzymatic phosphorylation incorporation assay, $[\text{U-}^{14}\text{C}]\text{ATP}$, $[\text{U-}^{14}\text{C}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ with specific activities of 0.6 μCi and 6 $\mu\text{Ci}/\mu\text{mol}$ of ATP, respectively, were utilized for each reaction mixture. For the individual assay, 15 μg of kanamycin, lividomycin A, or butirosin B was added to the reaction mixture containing 0.05 μmol of ATP, 0.05 μmol of Cleland reagent, 0.4 μmol of MgCl_2 , 2.5 μmol of tris(hydroxymethyl)aminomethane-hydrochloride buffer at pH 7.5, and 15 μl of protein, in a total volume of 55 μl . Reaction mixtures were incubated in a water bath at 30 C for 4 h.

Microbiological assay. Bioassays of kanamycin, lividomycin A, and butirosin B were performed by a modification of the method of Bennett et al. (1). *Bacillus subtilis* spores (Difco) were used as the test microorganism. Forty milliliters of "seeded" nutrient agar (Difco nutrient agar, 1.5%) was poured into plastic plates (150 by 15 mm; Falcon) to provide a layer which was uniformly 4 mm deep. Wells 5.5 mm in diameter and 4 mm deep were made with a metal cylinder in the solidified, seeded agar. The complete enzyme reaction mixture containing standard antibiotic concentrations was delivered by 50- μg capillary pipettes (Corning) to the agar wells. The assay plates were incubated in an upright position at 30 C and read at 16 to 18 h. The diameter of the zones of inhibition surrounding the agar wells were read to the nearest 0.5 mm with a calibrated hand lens (Bausch & Lomb).

Enzymatic phosphorylation incorporation assay. The enzyme assay was performed with the phosphocellulose paper-binding assay described by Ozanne et al. (12). Twenty microliters of the complete enzyme reaction mixture described above and containing either kanamycin, lividomycin A, or butirosin B was placed on squares (1 cm^2) of phosphocellulose paper (Whatman P-81). After it was washed with distilled water at 70 to 80 C, the dried square of paper was placed in toluene-based scintillant and counted with a Beckman scintillation counter, model LS-233.

RESULTS

Results pattern of *P. stuartii*. *P. stuartii* 73-197 was shown to be resistant to multiple aminoglycoside antibiotics (Table 1). Indeed, a concentration of $\geq 500 \mu\text{g}$ of kanamycin, neomycin, lividomycin A, or butirosin B per ml was required to inhibit in vitro growth. Resistance to gentamicin and tobramycin was less marked.

TABLE 1. Inhibitory concentrations of aminoglycoside antibiotics against *P. stuartii* 73-197

Antibiotic	Minimal inhibitory concentration ($\mu\text{g/ml}$)
Kanamycin	1,400
Lividomycin A	>500
Butirosin B	500
Neomycin	>500
Gentamicin	50
Tobramycin	50
BB-K8 (Amikacin)	0.8

Only amikacin demonstrated inhibitory activity (minimal inhibitory concentration, 0.8 $\mu\text{g/ml}$) at a concentration clinically achievable in serum. Increasing concentrations of EDTA failed to lower the minimal inhibitory concentration to each of the aminoglycoside antibiotics.

An enzymatic mechanism for resistance to some of the selected aminoglycoside antibiotics was found to occur. Crude extracts of *Providencia* strain 73-197 in the presence of ATP inactivated kanamycin, lividomycin A, and butirosin B (Table 2), but failed to inactivate gentamicin or tobramycin at pH 7.5 or 7.0. Of importance is the fact that antibiotic inactivation, as in the case of kanamycin, did not occur with levels of ATP below 10 mM nor in controls without the crude extract of *Providencia*. Up to 200 μg of kanamycin or lividomycin A per ml and 100 μg of butirosin B per ml could be completely inactivated. The optimal pH for enzymatic inactivation of the three antibiotics was found at pH 7.5, as compared with assays at pH 7.0 and 8.0. The use of radiolabeled ATP as the substrate further documented enzymatic phosphorylation of kanamycin, lividomycin A, and butirosin B (Table 3).

DISCUSSION

The emergence of strains of *P. stuartii* resistant to multiple aminoglycoside antibiotics colonizing and infecting patients in the burn unit at our institution has been documented previously (11). Strain 73-197 with marked resistance to kanamycin, neomycin, lividomycin A, butirosin B, gentamicin, and tobramycin was selected as a representative isolate for more extensive evaluation into the mechanism of resistance. EDTA (9, 18) has been shown to alter the permeability of *Escherichia coli* to a variety of unrelated compounds, including some antibiotics. The binding of divalent ions in the cell wall by EDTA, which leads to disruption of its lipopolysaccharide layer, has been proposed as the mechanism for this enhanced permeability. By

TABLE 2. Inactivation of kanamycin, lividomycin A and butirosin B by crude extracts of *Providencia stuartii* at pH 7.5

Kanamycin (200 µg/ml)			Lividomycin A (200 µg/ml)			Butirosin B (100 µg/ml)		
Protein concn ^a	Inactivation (%)		Protein concn ^a	Inactivation (%)		Protein concn ^a	Inactivation (%)	
	4 h	20 h		4 h	20 h		4 h	20 h
35	50	81	35	100	100	35	63	63
175	99.6	99.4				175	88	88
350	98.5	98.5	350	100	87.5	350	88	88
700	95.5	83	750	100	100	700	99	99
1,050	75	94	1,050	100	100	1,050	88	88
1,750	25	0	1,750	100	100	1,750	75	75

^a Concentration expressed as micrograms per milliliter.TABLE 3. Transfer of radioactivity from [¹⁴C]ATP and [³²P]ATP by enzymatic extract of *P. stuartii*

Antibiotic	Counts/min incorporated in assays	
	Phosphorylation ([³² P]ATP)	Adenylylation ([¹⁴ C]ATP)
Kanamycin	6,135	0
Lividomycin A . . .	20,220	0
Butirosin B	6,971	0

analogy with *E. coli*, the failure of EDTA treatment to reduce the inhibitory concentrations of the above antibiotics against *P. stuartii* suggests that decreased permeability is not the mechanism for its resistance.

Resistance to high concentrations of kanamycin, neomycin, lividomycin A, and butirosin B was demonstrated. Resistance transfer factor enzyme phosphorylation, involving several phosphotransferases, has been responsible for resistance to high concentrations of aminoglycoside antibiotics in *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (3, 4, 7, 8, 20). Neomycin-kanamycin phosphotransferase I has been shown to phosphorylate the 3'-hydroxyl group of the aminohexose moiety of kanamycin and neomycin (5, 15, 16) and the 5''-hydroxyl group of the ribose moiety of lividomycin A (17). Lividomycin phosphotransferase, an enzyme distinct from phosphotransferase I, has been shown to phosphorylate lividomycin on the 5''-hydroxyl group of the ribose moiety but is incapable of phosphorylating kanamycin (20). In addition, neomycin-kanamycin phosphotransferase II, has been shown to phosphorylate also the 3'-hydroxyl group of the aminohexose moiety of kanamycin and the 3'-hydroxyl group of the 2,6-diaminod-glucose moiety of butirosin (3, 19). The optimal pH for inactivation of these antibiotics has been shown to occur between pH 7.0 and

7.5 (5, 8, 19). Therefore, the high inhibitory concentrations against kanamycin, lividomycin A, and butirosin B, coupled with their inactivation in the presence of ATP at an optimum pH of 7.5, strongly suggested *O*-phosphorylation in *P. stuartii* 73-197. This was confirmed by the preferential transfer of the γ -phosphate of [³²P]ATP. The presence of more than one phosphorylating enzyme in *P. stuartii* could explain the resistance to kanamycin, neomycin, lividomycin, and butirosin.

Lower resistance to gentamicin and tobramycin, as compared with the other antibiotics studied, suggests partial antibiotic inactivation by enzymatic modification. However, the failure to inactivate tobramycin and gentamicin in the presence of ATP suggests enzymatic acetylation rather than phosphorylation. Other observers have noted that acetylation of aminoglycoside antibiotics tends to correlate with lower inhibitory concentrations (4), plus, the inactivation of gentamicin C components and tobramycin have not been mediated by phosphorylating enzymes previously (12). Amikacin, a derivative of kanamycin acylated with L(-)-amino- α -hydroxybutyric acid at the C1 amino group of the 2-deoxystreptamine moiety, effectively inhibits the growth of *P. stuartii* and is not inactivated by the known aminoglycoside inactivating enzymes with the exception of kanamycin acetyltransferase (2, 15).

Few reports have documented R-factor-mediated antibiotic resistance in *Providencia* from clinical isolates. However, Hedges (6) has recently extensively reviewed R-factors in this group. The demonstration of the phosphorylation of multiple aminoglycoside antibiotics by *P. stuartii* isolated from a patient in the burn unit demonstrates the role that intensive antibiotic therapy may play in the selection of potential pathogens with marked antibiotic resistance. It is apparent that this resistance may be

stable and persist for long periods of time within the nosocomial environment (11). Continued efforts to elucidate the mechanism of antibiotic resistance among bacteria may serve to direct appropriate chemical modifications of antibiotics, thereby changing their susceptibility to inactivating enzymes. The development of BB-K8 (amikacin) exemplifies the favorable experience that molecular modifications may have in rendering antibiotics resistant to enzymatic destruction (13). This continued effort, coupled with appropriate restraint with antibiotics and surveillance of changing patterns of antibiotic resistance, may allow for the continued availability of effective antimicrobial agents.

ACKNOWLEDGMENTS

We wish to thank Arnold Dunn for the use of his laboratory for the determination of enzymatic incorporation assays.

We also thank Bristol Laboratories for their Grant-in-Aid to support this research.

LITERATURE CITED

- Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. M. Kirby. 1966. Simplified accurate method for antibiotic assay of clinical specimens. *Appl. Microbiol.* 14:170-177.
- Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. *Annu. Rev. Biochem.* 42:471-506.
- Brzezinska, M., and J. Davies. 1973. Two enzymes which phosphorylate neomycin and kanamycin in *Escherichia coli* strains carrying R factors. *Antimicrob. Ag. Chemother.* 3:266-269.
- Davies, J., M. Brzezinska, and R. Benveniste. 1971. R-factors: biochemical mechanisms of resistance to aminoglycoside antibiotics. *Ann. N.Y. Acad. Sci.* 182:226-233.
- Doi, O., M. Ogura, N. Tanaka, and H. Umezawa. 1968. Inactivation of kanamycin, neomycin, and streptomycin by enzymes obtained in cells of *Pseudomonas aeruginosa*. *Appl. Microbiol.* 16:1276-1281.
- Hedges, R. W. 1974. R-factors from Providence. *J. Gen. Microbiol.* 81:171-181.
- Kobayashi, F., T. Koshi, J. Eda., Y. Yoshimura, and S. Mitsuhashi. 1973. Lividomycin resistance in staphylococci by enzymatic phosphorylation. *Antimicrob. Ag. Chemother.* 4:1-5.
- Kobayashi, F., M. Yahaguchi, and S. Mitsuhashi. 1972. Activity of lividomycin against *Pseudomonas aeruginosa*: its inactivation by phosphorylation induced by resistant strains. *Antimicrob. Ag. Chemother.* 1:17-21.
- Leive, L. 1965. A nonspecific increase in permeability in *Escherichia coli* produced by EDTA. *Proc. Nat. Acad. Sci. U.S.A.* 53:745-750.
- Lowry, O. H., N. J. Rosenbough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Overturf, G. D., J. Wilkins, and R. Ressler. 1974. Emergent resistance of *Providencia stuartii* to multiple antibiotics: speciation and biochemical characterization of *Providencia* microorganisms. *J. Infect. Dis.* 129:353-357.
- Ozanne, B., R. Benveniste, D. Tipper, and J. Davies. 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. *J. Bacteriol.* 100:1144-1146.
- Price, K. E., D. R. Chisholm, M. Misiak, F. Leitner, and Y. H. Tasi. 1972. Microbiological evaluation of BB-K8, a new semisynthetic aminoglycoside. *J. Antibiot.* 25:709-731.
- Price, K. E., T. A. Pursiano, M. D. DeFuria, and G. E. Wright. 1974. Activity of BB-K8 (amikacin) against clinical isolates resistant to one or more aminoglycoside antibiotics. *Antimicrob. Ag. Chemother.* 5:143-152.
- Umezawa, H., O. Doi, M. Ogura, S. Kondo, and N. Tanaka. 1968. Phosphorylation and inactivation of kanamycin by *Pseudomonas aeruginosa*. *J. Antibiot.* 21:154-155.
- Umezawa, H., M. Okanishi, R. Utahara, K. Maeda, and S. Kondo. 1967. Isolation and structure of kanamycin inactivated by a cell-free system of kanamycin-resistant *E. coli*. *J. Antibiot.* 20(Ser. A):136-141.
- Umezawa, H., H. Yamamoto, M. Yagisawa, S. Kondo, and T. Takeuchi. 1973. Kanamycin phosphotransferase I. Mechanism of cross resistance between kanamycin and lividomycin. *J. Antibiot.* 26:407-411.
- Weiser, R., A. W. Asscher, and J. Wimpenny. 1968. In vitro reversal of antibiotic resistance by ethylenediaminetetraacetic acid. *Nature (London)* 219:1365-1366.
- Yagisawa, M., H. Yamamoto, H. Naganawa, S. Kondo, T. Takeuchi, and H. Umezawa. 1972. A new enzyme in *Escherichia coli* carrying R-factor phosphorylating 3'-hydroxyl of butirosin A, kanamycin, neamine and ribostamycin. *J. Antibiot.* 25:748-750.
- Yamaguchi, M., F. Kobayashi, and S. Mitsuhashi. 1972. Antibacterial activity of lividomycin toward R factor-resistant strains of *Escherichia coli*. *Antimicrob. Ag. Chemother.* 1:139-142.
- Yamaguchi, M., T. Koshi, F. Kobayashi, S. Mitsuhashi. 1972. Phosphorylation of lividomycin by *Escherichia coli* carrying an R factor. *Antimicrob. Ag. Chemother.* 2:142-146, 1972.