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Resistance Mechanisms of Multiply Resistant Pneumococci: Antibiotic Degradation Studies

R. M. ROBINS-BROWN,[1]* M. N. GASPAR,[1] J. I. WARD,[2] I. K. WACHSMUTH,[2] H. J. KOORNHOF,[1] M. R. JACOBS,[1] AND C. THORNSBERRY[3]

Department of Microbiology, School of Pathology of the South African Institute for Medical Research and the University of the Witwatersrand, Johannesburg, Republic of South Africa[1]; and Bacterial Disease Division, Bureau of Epidemiology[2] and Bacteriology Division, Bureau of Laboratories,[3] Center for Disease Control, Atlanta, Georgia 30333

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Strains of *Streptococcus pneumoniae* resistant to penicillin have been reported from several countries around the world. Many South African isolates, in addition, exhibit resistance to tetracycline, chloramphenicol, erythromycin, clindamycin, and cotrimoxazole in varying patterns. A qualitative test of the ability of antibiotic-resistant pneumococci to inactivate penicillin, oxacillin, cephalothin, cefoxitin, chloramphenicol, tetracycline, minocycline, erythromycin, clindamycin, streptomycin, gentamicin, and cotrimoxazole revealed that only chloramphenicol was degraded. This finding was confirmed in a quantitative test in which the residual antimicrobial activity of broth containing chloramphenicol in subinhibitory concentrations was determined after incubation with antibiotic-resistant bacteria. Chloramphenicol resistance was shown to be associated with the production of inducible chloramphenicol acetyltransferase. No beta-lactamase activity was demonstrated. Plasmid deoxyribonucleic acid was not demonstrable in partially purified lysates of antibiotic-resistant strains of *S. pneumoniae*.

Isolates of *Streptococcus pneumoniae* relatively nonsusceptible to penicillin have been reported from Australia, New Guinea, Great Britain, Canada, the United States, and South Africa (8). Most South African strains have shown higher minimal inhibitory concentrations (MICs) of penicillin (up to 4 μg/ml) than have previously been reported for pneumococci (1, 13). Many strains, moreover, have been multiply resistant, exhibiting resistance to tetracycline, chloramphenicol, erythromycin, clindamycin, and cotrimoxazole, in addition to penicillin (13). The uniqueness of these bacteria suggested a novel mechanism of resistance.

Accordingly we undertook an investigation of the antibiotic-degrading ability of 10 strains of resistant pneumococci recovered from South African and North American patients. Penicillin G, oxacillin, cephalothin, cefoxitin, chloramphenicol, tetracycline, minocycline, erythromycin, clindamycin, streptomycin, gentamicin, and cotrimoxazole were tested under various conditions, but only chloramphenicol was inactivated. The ability to degrade chloramphenicol was linked to the production of inducible chloramphenicol acetyltransferase (CAT) by chloramphenicol-resistant strains.

MATERIALS AND METHODS

Bacteria. Ten clinical isolates of *S. pneumoniae* with different resistance patterns were investigated. One strain, no. 3, was recovered from the blood of a child with septicemia in Durban (case no. 5 in ref. 2). Two strains, no. 9 and 10, were recovered from patients in the United States (3) and Canada (7), respectively. The rest were isolated from nasopharyngeal swabs of patients at Baragwanath Hospital, Johannesburg, during a survey for carriers of resistant pneumococci (13). The bacteria were identified by standard laboratory methods (2). Capsular typing was performed by R. Austrian, University of Pennsylvania Medical School, Philadelphia, Pa. The South African strains were either type 19A or type 6A (Danish Classification); the North American strains, no. 9 and 10, were types 14 and 23, respectively.

Antibiotic susceptibility testing. MICs were determined by the tube dilution technique. Approximately 10^5 colony-forming units were inoculated into beef infusion broth containing 10% horse serum (serum broth) and varying dilutions of antibiotic. Beef infusion broth was prepared from 500 g of lean beef, 15 g of Neopeptone (Difco Laboratories, Detroit, Mich.), and 5 g of NaCl in 1 liter of water, then infused at 60°C for 1 h, and the pH was adjusted to 7.6. Tubes were incubated in ambient atmosphere at 37°C overnight, and the least concentration showing no turbidity was read as the MIC.

Antimicrobial agents used were benzylpenicillin, oxacillin, cephalothin, cefoxitin, chloramphenicol, tetracycline, minocycline, erythromycin, clindamycin, streptomycin, gentamicin, and cotrimoxazole. All were obtained commercially as diagnostic powders.

Antibiotic degradation studies. A qualitative test to screen cultures for antibiotic-inactivating enzymes was developed by modifying the disk method

reported by El Ghoroury for the detection of penicillinase-producing staphylococci (9). Plates (150 mm in diameter) containing Todd-Hewitt agar (40 ml of Todd-Hewitt broth plus 1.5% agar [Difco]) were flooded with 20 ml of Todd-Hewitt agar seeded with 2×10^5 colony-forming units of an antibiotic-susceptible "indicator" bacterium: *Sarcina lutea, Bacillus subtilis, Bacillus cereus, Pasteurella multocida,* or *Staphylococcus aureus,* according to the antibiotic under test. The agar was allowed to harden, and a heavy streak (approximately 2 cm in length) of the test bacterium was made on its surface. A filter paper disk containing the appropriate antibiotic (BBL, Cockeysville, Md.) was placed adjacent to the streak, and the plates were incubated overnight under carbon dioxide. The inhibition zones of the indicator strain around antibiotic disks in contact with resistant strains of pneumococci were compared with those around an uninoculated control disk included on each plate.

Quantitative studies were undertaken to investigate the kinetics of chloramphenicol degradation. For these experiments, bacteria representing different resistance patterns (strains no. 1, 3, 4, and 5) were selected for cultivation in Todd-Hewitt broth containing 8 μg of chloramphenicol per ml. An uninoculated tube served as a control. After incubation periods of 5, 24, or 48 h at 37°C, each tube was centrifuged at 8,000 × *g*, and the supernatant fluid was filtered through a 0.22-μm membrane (Millipore Corp., Bedford, Mass.) to remove bacteria. The antimicrobial activity of the filtrate was determined microbiologically by diffusion with the large plate method (20). *S. lutea* was used as the test organism. Each assay was performed in quadruplicate. The zone diameters were measured with calipers and read off a standard curve constructed from duplicate measurements of six known concentrations of antibiotic diluted in Todd-Hewitt broth.

Assays for CAT. CAT activity in crude bacterial extracts was assayed spectrophotometrically and by direct measurement of [*acetyl*-^{14}C]chloramphenicol (18). Bacteria were cultivated in 100 ml of serum broth for 16 h at 37°C in an orbital shaker set at 180 rpm. For the enzyme induction experiments, chloramphenicol, in a final concentration of 0.5 μg/ml, was added to the culture medium. After incubation, bacteria were removed by centrifugation at 12,000 × *g*. The pellets were washed three times in phosphate-buffered saline (pH 7.2) and resuspended in distilled water. This procedure yielded 100 to 150 mg of bacteria (wet weight) per 100 ml of broth.

Cell lysates were prepared in a Braun cell homogenizer with glass beads of ca. 0.1-mm diameter. Lysates were centrifuged at 60,000 × *g* to remove the debris and stored at −70°C until required.

CAT activity was assayed spectrophotometrically by using 5′,5′-dithiobis-2-nitro-benzoic acid (Sigma Chemical Co., St. Louis, Mo.) in a Gilford model 300T recording spectrophotometer with the cuvette chamber set at 37°C. The final concentrations of reagents in the reaction mixture were: 100 mM tris-(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 0.1 mM acetyl-coenzyme A (Sigma), 1 mM 5′,5′-dithiobis-2-nitro-benzoic acid, and 0.1 mM chloramphenicol. One unit of CAT activity was defined as the amount of enzyme required to catalyze the acetylation of 1 μmol of chloramphenicol per min at 37°C (18).

CAT activity was also determined by the direct measurement of [*acetyl*-^{14}C]chloramphenicol (18). The 250-μl reaction mixture, prior to the addition of chloramphenicol, contained 100 μl of enzyme extract, 100 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 6 mM magnesium chloride, 3 mM adenosine triphosphate (Boehringer Mannheim, Germany), 20 mM phosphoenolpyruvate (Boehringer), 0.4 mM coenzyme A (Sigma), 0.4 mM sodium [1-^{14}C]acetate (specific activity, 59 mCi/mmol) (Radiochemical Centre, Amersham, England), and 1 unit each of acetate kinase (Sigma), pyruvate kinase (Sigma), and phosphotransacetylase (Sigma). The reaction was started by the addition of 5 μl of 5 mM chloramphenicol and terminated after 20 min by the addition of 25 μl of 1.1 M sodium arsenate. ^{14}C radioactivity extracted with benzene was determined in a Packard liquid scintillation spectrometer. Results were expressed as net counts per minute after subtraction of radioactivity in controls which contained enzyme extract but no chloramphenicol. In all determinations of CAT activity, a homogenate of an *Escherichia coli* strain carrying an R factor for chloramphenicol, ampicillin, and streptomycin was included as a positive control.

To facilitate comparison of enzyme activity in the various preparations examined, enzyme activity was expressed as units per gram of protein. The protein content of the cell-free homogenates was determined by the method of Lowry et al. with bovine serum albumin as the standard (14).

Assays for penicillinase. Whole bacteria and cell lysates were examined for penicillinase (beta-lactamase) activity by means of an iodometric screening test (4) and chromogenic cephalosporin (Glaxo Research Ltd., Greenford, England) (17). Both tests were performed on uninduced cultures and after induction with benzylpenicillin in a final concentration of 1 μg/ml.

Plasmid analysis. Lysates of 30-ml cultures of antibiotic-susceptible and antibiotic-resistant pneumococci were prepared by dodecyl sulfate-salt precipitation (11) or by treatment with Triton X-100 (5) or 10% Desoxycholate. Crude lysates from all procedures were divided into two aliquots for partial purification. One portion was processed by phenol extraction and ethanol precipitation at −20°C overnight (15, 21); the second was ethanol precipitated only.

All deoxyribonucleic acid (DNA) preparations, including standard known molecular weight plasmids, were analyzed by vertical slab agarose-gel electrophoresis as previously described (15, 21). Gels were stained with ethidium bromide (5 μg/ml) and viewed with long-wave ultraviolet illumination.

RESULTS

Antibiotic susceptibility. The MICs of the pneumococcal strains examined are listed in Table 1. The fully susceptible strain, no. 1, served as a negative control in the studies of antibiotic degradation. The remaining strains all exhibited resistance to penicillin, with MICs ranging from 0.25 to 4 μg/ml. Three of these, no. 6, 8, and 10, were resistant to penicillin only, and the remainder were resistant to chloramphenicol, tetracycline, minocycline, erythromycin,

clindamycin, and cotrimoxazole in varying patterns. Many strains, moreover, also demonstrated far greater levels of resistance to streptomycin than is usually encountered in pneumococci (10).

Antibiotic degradation. In the test for diffusible inactivating enzymes using antibiotic-containing disks, only chloramphenicol was shown to be inactivated by chloramphenicol-resistant pneumococci (Fig. 1). None of the other antimicrobial agents was inactivated by resistant bacteria, irrespective of their resistance pattern (Fig. 1).

The timed quantitative study of chloramphenicol degradation revealed that although some chloramphenicol was inactivated within the first 5 h of incubation, a longer period was required for complete inactivation (Table 2).

CAT activity. The spectrophotometric and [*acetyl*-^{14}C]chloramphenicol assays correlated closely (correlation coefficient: $r = 0.87$). Both methods revealed minimal constitutive CAT activity in pneumococci cultivated in the absence of chloramphenicol. The addition of 0.5 μg of chloramphenicol per ml to the culture medium, however, resulted in a marked increase in CAT synthesis by chloramphenicol-resistant bacteria (Table 3). The yield of enzyme, even from the induced cultures, however, was low (approximately 0.2 units/liter), compared with 160 units/liter obtained from *E. coli*. Chloramphenicol-susceptible strains were consistently negative for CAT regardless of exposure to chloramphenicol during growth.

Penicillinase activity. The iodometric assay and the chromogenic cephalosporin test failed to reveal beta-lactamase activity in either penicillin-resistant pneumococci or bacterial lysates.

Plasmid analysis. No plasmid DNA was evident in electrophoretically analyzed lysates of either the antibiotic-susceptible or the antibiotic-resistant strains of *S. pneumoniae*. The presence of a distinct band of DNA corresponding to chromosomal gel mobility in all preparations indicated that lysis and DNA extraction had been accomplished.

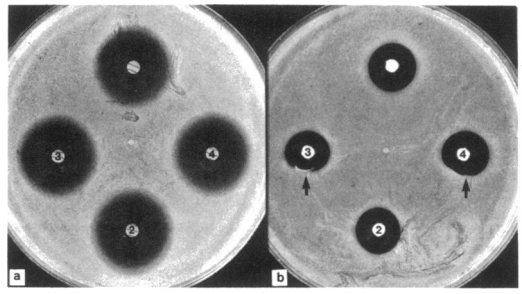

FIG. 1. *Assay for diffusible degradation enzymes. A heavy inoculum of S. pneumoniae strains no. 2, 3, and 4 was made adjacent to filter paper disks containing benzylpenicillin (a) or chloramphenicol (b), and the fourth disk on each plate served as an uninoculated control. Distortion and narrowing of the zone of inhibition of the indicator organism (arrows) indicates antibiotic degradation.*

TABLE 2. *Residual chloramphenicol content of Todd-Hewitt broth plus chloramphenicol (8 μg/ml) incubated with S. pneumoniae*

Strain no.	Chloramphenicol MIC (μg/ml)	Chloramphenicol concn (μg/ml) at time (h)[a]:			
		0	5	24	48
1	1	8.0	6.5	6.5	6.5
2	16	8.0	4.5	<2.0	<2.0
3	32	8.0	6.0	<2.0	<2.0
4	16	8.0	5.5	<2.0	<2.0
5		8.0			

[a] Mean of triplicate determinations.

TABLE 1. *Serotypes and antimicrobial susceptibility of 10 clinical isolates of S. pneumoniae*

Strain no.	Capsular type	MIC (μg/ml)[a]											
		Pen	Ox	Ceph	Cfx	Chlor	Tet	Min	Ery	Clin	Str	Gent	Co-T[b]
1	19A	*0.01*	*< 0.1*	*< 0.2*	*1*	*1*	*0.1*	*0.2*	*<0.1*	*<0.1*	32	16	*2.4/0.12*
2	6A	1	16	4	64	*4*	64	64	*<0.1*	*<0.1*	>256	16	19/1
3	19A	4	16	16	64	16	*1*	*0.2*	*<0.1*	*<0.1*	>256	8	38/2
4	19A	4	16	16	64	32	32	32	32	64	>256	16	19/1
5	19A	4	16	8	64	16	32	32	16	64	>256	16	19/1
6	19A	0.25	2	*0.5*	*2*	*2*	*0.2*	*<0.2*	*<0.1*	*<0.1*	32	8	19/1
7	19A	4	16	8	32	16	32	16	*<0.1*	*<0.1*	>256	8	19/1
8	6A	0.25	2	*0.5*	*2*	*2*	*0.2*	*<0.2*	*<0.1*	*<0.1*	16	8	*4.8/0.25*
9	14	4	16	16	64	*2*	64	16	*<0.1*	*<0.1*	>256	4	*2.4/0.12*
10	23	1	8	*2*	*8*	*2*	*0.5*	*<0.2*	*<0.1*	*<0.1*	32	4	*9.5/0.5*

[a] Pen, Penicillin G; Ox, oxacillin; Ceph, cephalothin; Cfx, cefoxitin; Chlor, chloramphenicol; Tet, tetracycline; Min, minocycline; Ery, erythromycin; Clin, clindamycin; Str, streptomycin; Gent, gentamicin; Co-T, cotrimoxazole. Values in regular type are resistant to the agent concerned; those in italics are susceptible.

[b] Cotrimoxazole MICs are expressed as a ratio of sulfamethoxazole to trimethoprim.

TABLE 3. *CAT activity in cell-free homogenates of five strains of S. pneumoniae and an E. coli control, with and without prior induction by chloramphenicol (0.5 μg/ml)*

Strain no.	Chloramphenicol MIC (μg/ml)	Spectrophotometric assay (units/g of protein)		Radioisotopic assay (10^7 cpm/g of protein)	
		Uninduced	Induced	Uninduced	Induced
S. pneumoniae	1	0.7	1.4	1.0	0
1	4	1.6	0.25	0.4	0.12
2	16	1.9	18.0	10.1	51.1
3	32	1.3	23.3	2.6	34.7
4	16	1.2	11.1	1.1	47.8
5					
E. coli	128	130	137	181	207

DISCUSSION

Qualitative microbiological determinations of antibiotic degradation, as performed in this study, are extremely sensitive when cultures are screened for the presence of degrading enzymes. Demonstration of the loss of the antibiotic activity of penicillin, for example, is at least 20 times more sensitive in detecting beta-lactamase than the widely used microiodometric assay (5). Thus, the persistence of penicillin activity in broth inoculated with resistant pneumococci confirms previous reports of failure to demonstrate beta-lactamase activity in pneumococci nonsusceptible to penicillin (2, 13).

In a similar fashion the inability of resistant bacteria to inactivate tetracycline, erythromycin, and streptomycin indicated the absence of degrading enzymes for these drugs. This finding was not unexpected, since degrading enzymes for these drugs have not been reported among clinical isolates of streptococci. Chloramphenicol, on the other hand, was inactivated by resistant organisms, and this property was linked to the production of inducible CAT. Inducible CAT has previously been detected in chloramphenicol-resistant pneumococci from France and Japan (6, 16).

The yield of CAT from pneumococci was at the lower limit of detection by the 5',5'-dithiobis-2-nitro-benzoic acid method. The use of a spectrophotometer with a three-decimal digital readout, however, permitted highly reproducible quantification of enzyme activity, which was corroborated by the [*acetyl*-^{14}C]chloramphenicol assay. Enzyme yields from chloramphenicol-resistant pneumococci could presumably be improved by repeated addition of small amounts of chloramphenicol to the medium during incubation, as has been reported for strains of *S. aureus* producing inducible CAT (19).

In gram-negative bacilli and staphylococci, the production of CAT is frequently plasmid mediated (19). This fact, taken with the resistance patterns of our bacteria, suggested that plasmids may have been responsible for the multiple drug resistance of South African pneumococci. Plasmid DNA, however, was not demonstrable in partially purified lysates prepared from these bacteria. This does not entirely rule out a plasmid-mediated mechanism of antibiotic resistance, since plasmid-degrading enzymes or extraction procedures may have accounted for the apparent absence of plasmid DNA.

The lack of extracellular degrading enzymes for penicillin, tetracycline, erythromycin, and streptomycin in multiply resistant bacteria points to the presence of a permeability barrier to these drugs as the likely mechanism of resistance. The barrier may, in addition, limit the entry of chloramphenicol in chloramphenicol-resistant strains, thus accounting for the low yields of CAT obtained from these bacteria. Studies using radiolabeled antibiotics are currently in progress so that the barrier may be defined more clearly.

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