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Antimicrobial Activities of Beta-Lactam Antibiotics and Gentamicin against Penicillin-Susceptible and Penicillin-Resistant Pneumococci

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The MICs of penicillin and cefotaxime for a range of penicillin-susceptible and penicillin-resistant isolates of *Streptococcus pneumoniae* were unchanged by the addition of gentamicin. In time-kill studies the rate of killing was greater for 18 of 20 isolates in the presence of gentamicin. However, mean differences in killing after 6 h of incubation were modest, not exceeding 1 $\log_{10}$ unit.

In an era of increasing resistance of *Streptococcus pneumoniae* to most antibiotics, it seems reasonable to reexamine all potentially available modalities for treating pneumococcal infections. A synergistic bactericidal effect between penicillin and an aminoglycoside is regularly demonstrable in the case of certain pathogenic gram-positive cocci such as *Enterococcus faecalis*, *Staphylococcus aureus*, and the viridans group streptococci (2). Until recently there has been little need to enhance the antimicrobial effect of penicillin against *S. pneumoniae*, and few studies of the possible synergy between a beta-lactam antibiotic and an aminoglycoside against pneumococci have been reported (4, 5). We now present data on the effect of penicillin or cefotaxime together with gentamicin against penicillin-susceptible and penicillin-resistant *S. pneumoniae*.

Twenty clinical isolates of *S. pneumoniae* obtained from Texas Children's Hospital, the Veterans Affairs Medical Center, Houston, Tex., and the Kentucky Department for Health Services were studied. Strains were selected to represent various degrees of penicillin susceptibility such that 6 were penicillin susceptible (MICs, <0.1 μg/ml), 10 were intermediately resistant to penicillin (MICs, ≥0.1 to ≤1 μg/ml), and 4 were highly resistant to penicillin (MICs, ≥2.0 μg/ml). Reference strain ATCC 49619, a moderately penicillin-resistant strain which has been proposed to be a quality control organism (7), was included in all determinations of MICs. Pneumococci were defined as susceptible, intermediately resistant, or resistant to cefotaxime if MICs for the organisms were ≤0.5, 1.0, or ≥2.0 μg/ml, respectively. The means and ranges of the MICs of penicillin or cefotaxime for the isolate studied are reported in Table 1. Strains were serotyped with appropriate antisera (Statens Seruminstitut, Copenhagen, Denmark) and were found to be types 6 ($n = 5$), 8 ($n = 1$), 12 ($n = 1$), 14 ($n = 5$), 19F ($n = 4$), and 23F ($n = 4$).

Todd-Hewitt medium supplemented with 0.5% yeast extract (THY; Difco, Detroit, Mich.) was chosen for use in MIC and MBC determinations. This clear broth is easy to prepare; it reliably supports the growth of *S. pneumoniae*, and its use yields reproducible MIC results (9). Penicillin G (Pfizer, New York, N.Y.), cefotaxime (Hoechst-Roussel, Sommerville, N.J.), and gentamicin (Schering, Kenilworth, N.J.) were dissolved in phosphate-buffered saline, stored at −80°C, and used within 3 months. Penicillinase type 1 from *Bacillus cereus* (1,500 to 3,000 U/mg; Sigma, St. Louis, Mo.) was dissolved in 0.1 M Tris (pH 7.0) with 0.5% bovine serum albumin, and the mixture was filter sterilized. One hundred units was added to the surfaces of blood agar plates to subculture suspensions containing penicillin, and 1,000 U was added to the surfaces of blood agar plates to subculture suspensions containing cefotaxime.

MICs were determined by the broth macrodilution method for penicillin G (32 to 0.008 μg/ml), cefotaxime (40 to 0.01 μg/ml), and gentamicin (256 to 0.25 μg/ml). The bacterial inoculum was standardized to yield between 5×10^5 and 1×10^6 CFU/ml. Tubes were incubated at 35°C for 20 to 24 h. MICs were read as the lowest concentration of antibiotic at which there was no visible growth. Two to three determinations were made for each isolate, and the geometric mean was determined. At the time that the MIC was assessed, 10 μl from each clear tube was streaked onto blood agar to which penicillinase had been added. The MBC was defined as the lowest concentration of antibiotic that caused a ≥99.9% reduction in the number of CFU from that of the original inoculum.

The rate of killing by penicillin or cefotaxime at a concentration of four times the MIC, with or without gentamicin at 1 μg/ml, was studied for each isolate. Tubes containing THY with appropriate concentrations of antibiotic(s) and a control tube with no antibiotic were inoculated to yield an estimated 10^7 CFU of log-phase *S. pneumoniae* per ml, and the tubes were incubated in a shaking water bath at 35°C. To quantitate the bacteria, aliquots were removed at time zero and after 2, 4, and 6 h and were serially diluted; 10-μl aliquots were streaked onto blood agar plates to which penicillinase had been added; this was followed by incubation overnight at 35°C. At 6 h, 0.1-ml aliquots were also subcultured. If the 6-h culture yielded <10 CFU/ml, the experiment was repeated with a higher initial inoculum. Quantitative subcultures were not done at 18 to 24 h because, with pneumococci, results are likely to be confounded by autolysis.

MICs are reported as means ± standard errors of the means. Data were normalized to a uniform starting inoculum by reporting the results as the difference between the $\log_{10}$ CFU per

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TABLE 1. MICs of penicillin and cefotaxime for 20 pneumococcal isolates and RDCs after addition of gentamicin

Isolate no.	Penicillin MIC (μg/ml)	RDC for penicillin plus gentamicin	Cefotaxime MIC (μg/ml)	RDC for cefotaxime plus gentamicin
1	0.008	1.4	<0.01	0.9
2	0.03	−0.3	<0.01	0.3
3	0.04	0.5	0.04	0.7
4	0.06	0.3	0.35	0.7
5	0.06	1.0	0.05	0.2
6	0.06	−0.7	0.05	−0.6
7[a]	0.13	0.7	0.08	0.9
8[a]	0.25	0.9	0.15	0.4
9[a]	0.44	0.2	0.32	0.7
10	0.5	0.9	0.63	0.8
11[a]	0.77	2.0	0.31	1.7
12[a]	1	1.7	0.32	1.1
13	1	0.5	0.63	0.2
14	1	1.3	0.63	1.8
15	1	0.8	0.63	0.9
16	1	1.4	1.5	0.7
17[b]	2	1.5	1.3	1.3
18[b]	2	2.2	1.25	0.1
19	4	2.1	2.5	0.2
20	16	0.5	14.65	0.1

[a] Isolate is intermediately resistant to penicillin and susceptible to cefotaxime.
[b] Isolate is resistant to penicillin and intermediately resistant to cefotaxime.

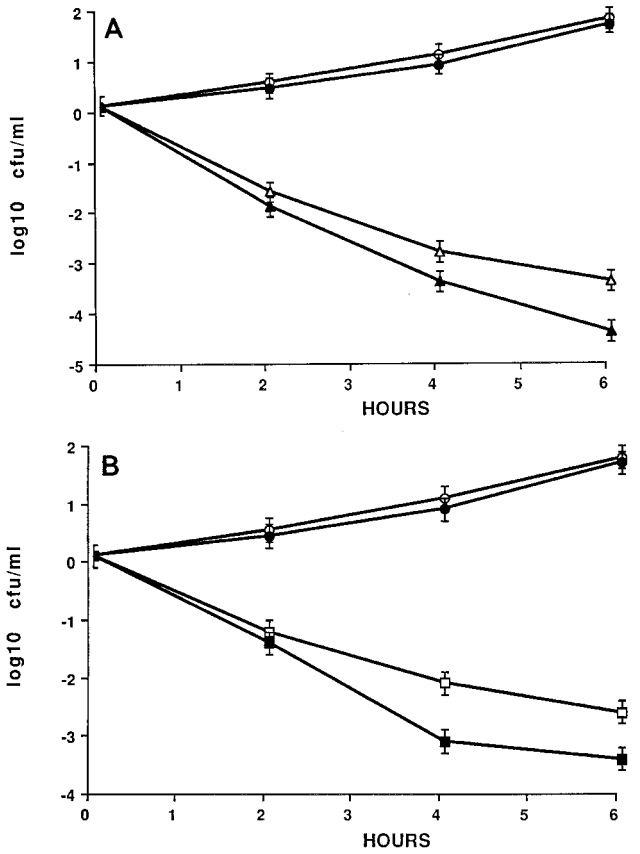

FIG. 1. (A) Mean $\log_{10}$ adjusted CFU of *S. pneumoniae* per milliliter after incubation with gentamicin at 1 μg/ml (●), penicillin at four times the MIC (△), penicillin plus gentamicin at the same concentrations (▲), or no antibiotic (control; ○). (B) Mean $\log_{10}$ adjusted CFU of *S. pneumoniae* per milliliter after incubation with gentamicin at 1 μg/ml (●), cefotaxime at four times the MIC (□), cefotaxime plus gentamicin at the same concentrations (■), or no antibiotic (control; ○).

milliliter at each time point and the $\log_{10}$ of the initial CFU per milliliter. The relative decrease in concentration (RDC) with the beta-lactam antibiotic plus gentamicin versus that with the beta-lactam alone was calculated by subtracting the number of $\log_{10}$ CFU per milliliter with the beta-lactam antibiotic plus gentamicin from that with the beta-lactam antibiotic alone after 6 h of incubation.

The MICs of penicillin and cefotaxime showed a significant correlation ($r > 0.99$; $P < 0.001$), as has been demonstrated previously (3). As shown in Table 1, the same classification (susceptible, intermediately resistant, resistant) described the effects of penicillin and cefotaxime for 13 isolates. Five isolates that were intermediately resistant to penicillin were susceptible to cefotaxime. Two isolates that were resistant to pencillin were intermediately resistant to cefotaxime. None of the isolates demonstrated high-level gentamicin resistance (MICs, >100 μg/ml); the mean ± standard error gentamicin MIC for all isolates studied was 6.4 ± 0.8 μg/ml (range, 4 to 16 μg/ml).

Antibiotic interaction was studied initially by determining the MICs and MBCs of penicillin and cefotaxime in the presence or absence of a fixed, subinhibitory concentration of gentamicin (1 μg/ml); identical results were obtained with or without gentamicin. Time-kill assays were then performed. Gentamicin alone at 1 μg/ml had no effect on the growth of any isolate when compared with the growth of the antibiotic-free control isolate. Penicillin at four times the MIC resulted in a substantial decline in the mean number of CFU per ml: 1.7 ± 0.2 $\log_{10}$ CFU/ml at 2 h, 2.9 ± 0.2 $\log_{10}$ CFU/ml at 4 h, and 3.4 ± 0.2 $\log_{10}$ CFU/ml at 6 h (Fig. 1A). The addition of gentamicin at 1 μg/ml to penicillin modestly enhanced the observed bactericidal effect, such that the number of CFU at 2, 4, and 6 h had declined to 1.9 ± 0.2, 3.4 ± 0.2, and 4.3 ± 0.2 $\log_{10}$ CFU/ml, respectively. Similar results were observed when cefotaxime alone was compared with cefotaxime used with gentamicin (Fig. 1B). RDCs for all isolates are given in Table 1. For 18 of 20 isolates, including all penicillin-resistant ones, the decline in the number of CFU per milliliter at 6 h in the presence of penicillin or cefotaxime together with gentamicin was greater than that in the presence of either beta-lactam alone. The mean RDCs for all strains were 1.02 ± 0.16 for penicillin and 0.76 ± 0.11 for cefotaxime, indicating the presence of a modest synergistic effect, on average. As shown in Table 1, the addition of gentamicin to penicillin appeared to cause greater bactericidal activity for resistant isolates (RDC = 1.7) than for susceptible ones (RDC = 0.4). The RDC had a negative value for penicillin and gentamicin for two beta-lactam-susceptible strains (−0.3 and −0.7, respectively [mean of three determinations]) and a negative value for cefotaxime and gentamicin in the case of one of these two strains (−0.6 [mean of three determinations]).

Previous reports have demonstrated a limited degree of synergy between penicillin and gentamicin against *S. pneumoniae*. Frimodt-Moller and Thomsen (4) showed synergy by time-kill analysis and the use of a mouse model for a small number of penicillin-susceptible strains, and Haynes et al. (5) documented synergy in a minority of penicillin-susceptible isolates and intermediately penicillin-resistant isolates using the checkerboard technique. Motivated by the recent emergence of penicillin-resistant isolates, we addressed the issue of synergy between beta-lactam antibiotics and an aminoglycoside using standard tube dilutions as well as the time-kill method for

pneumococcal isolates with a wide range of penicillin susceptibilities. The fixed ratio of beta-lactam at four times the MIC was chosen to provide adequate killing while allowing comparison among the isolates; in most instances, this approach resulted in antibiotic levels which were well below those that would be found in serum (1) but which were similar to those that might readily be achieved in the cerebrospinal fluid of infected children or adults (6, 8, 10).

The addition of gentamicin to penicillin or cefotaxime resulted in increased rates of killing, with differences in the number of CFU at 6 h averaging 1.02 and 0.83 $\log_{10}$ CFU for penicillin and cefotaxime, respectively. A synergistic effect, as defined by a $\geq$2-$\log_{10}$-unit increase in killing by the two drugs together, was observed for only three isolates; however, this definition has generally been used for studies that use overnight incubation, whereas the autolytic capacity of *S. pneumoniae* renders readings after such prolonged incubations unreliable (9). In the case of two isolates, a slight degree of antagonism was demonstrable.

It is unclear whether these modest differences would translate into an enhanced outcome in the treatment of infected patients. The absence of a more sizable enhancement of killing by the addition of an aminoglycoside, together with the occasional occurrence of antagonism, probably argue against the routine empiric use of a beta-lactam antibiotic together with an aminoglycoside in treating pneumococcal infections. However, a 1 $\log_{10}$ difference at 6 h might translate into much greater differences with more prolonged incubation, and the use of the two drugs together might be considered in individual cases if synergy is demonstrable, especially if resistance to vancomycin begins to appear.

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