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Synergism Between Aminoglycosides and Cephalosporins with Antipseudomonal Activity: Interaction Index and Killing Curve Method

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Combinations of gentamicin with cefotaxime, moxalactam, and ceftazidime were tested against 43 bacterial strains, most of them blood isolates. With an interaction index of ≤0.5 as borderline, synergism was demonstrated against 30 to 40% of the strains by the fractional inhibitory concentration index and against 50 to 70% by the fractional bactericidal concentration index. The reproducibility of the index was within ±0.2 for two-thirds of 40 repetitive assays and within ±0.4 to 0.5 for all of these assays. Similar results were obtained when netilmicin was substituted for gentamicin. The killing curve system for studying antibiotic synergism was standardized to give results comparable to those obtained with the interaction index. This was achieved when one-half of a previously determined minimum bactericidal concentration was used for single drugs and the amount of antibiotic was at least halved again when drugs were used in combination. An initial bacterial concentration of 10^5 to 10^6 colony-forming units per ml is recommended. Given these conditions, synergism could be defined as a 2-$\log_{10}$ or more decrease in viable count given by both drugs together, as compared with the more active of the pair after 24 h. Prediction of killing curve results could then be obtained with the fractional bactericidal concentration index. When cephalosporins and gentamicin were combined from the start, the beta-lactam antibiotics were less susceptible to inactivation, as demonstrated in time-killing assays. If one of the antibiotics were added after 24 h, synergism was not demonstrable. The results indicate that the new cephalosporins may be advantageously combined with aminoglycosides.

Evidence is accumulating that infections in immunocompromised patients may cause fewer deaths if they are treated with synergistic combinations of antibiotics rather than nonsynergistic combinations (12, 14, 31, 33). Such combinations are usually comprised of a penicillin or a cephalosporin and an aminoglycoside.

The development of highly active beta-lactam antibiotics with broad antibacterial spectra, including activity against *Pseudomonas aeruginosa* (5, 10, 23), has led to a tendency to replace the former combinations by single-drug therapy. It is therefore essential that the efficacy of these drugs used singly be weighed against their potential synergistic capacity.

Most data on synergistic action are based on in vitro observations. The time-kill curve, determining lethal effect, and the checkerboard titration method, giving bacteriostatic as well as bactericidal results, are two basic techniques for quantitative assessment of synergism (2, 8, 15, 21, 32). In a modification of the checkerboard method proposed by Berenbaum (3), the two drugs are mixed in fractions of their minimum inhibitory concentrations (MICs) for each bacterial strain and serially diluted.

The results of synergism testing are variable, however, depending on differences in technique and a multiplicity of laboratory definitions of synergism. Norden et al. (22) demonstrated a lack of correlation between different methods for assaying synergy against *Klebsiella* strains. Similar data were reported for enterococci by Ryan et al. (27). On the other hand, Weinstein et al. (30) found excellent correlation between the methods in question.

The present study concerned the in vitro effect of cefotaxime, ceftazidime, and moxalactam, singly and in combination with gentamicin or netilmicin. For some bacterial strains, calculations of interaction index were compared with results obtained from killing curves. Factors influencing results and comparability were analyzed.

MATERIALS AND METHODS

Bacterial strains. Forty-three strains were used: 22 *P. aeruginosa*, 1 *Pseudomonas maltophilia*, 4 *Escherichia coli*, 1 *Enterobacter* species, 1 *Citrobacter* species, 1 *Proteus mirabilis*, 3 *Serratia* species, 1 *Providencia* species, and 9 *Staphylococcus* species. Most were blood isolates from patients with septicemia, e.g., *P. maltophilia* SI164 and *P. aeruginosa* SI188. Some were selected strains of *P. aeruginosa*, representing various types of enzymatic resistance to aminoglycosides. Strain PAO1 (9) was used as a plasmid-free, genetically well-characterized strain of *P. aeruginosa*. As reference strains, *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used. The MICs and minimum bactericidal concentrations (MBCs) for three strains studied in more detail in supplemented broth are shown in Table 1.

Antibiotics. Cefotaxime was kindly supplied by Hoechst AG (Frankfurt, West Germany), moxalactam by Eli Lilly & Co. (Indianapolis, Ind.), ceftazidime by Glaxo (Greenford, England), and gentamicin and netilmicin by Schering Corp. (Bloomfield, N.J.).

Medium. Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.; batch GODGOG 7/81), nonsupplemented or supplemented with 25 mg of Mg^{2+} and 50 mg of Ca^{2+} per liter (26, 29), was used.

The respective final concentrations of Ca^{2+} and Mg^{2+} were 0.07 and 0.11 mmol/liter in nonsupplemented broth and 1.44 and 1.08 mmol/liter in supplemented broth, as determined by atomic absorption spectrometry.

Determination of MIC and MBC. The broth dilution method as described by Ericsson and Sherris (4) was used to determine MIC and MBC. An inoculum prepared from an overnight broth culture was diluted and added to twofold serial dilutions of the antibiotic (0.5 + 0.5 ml) to provide approximately 5×10^5 colony-forming units (CFU) per ml in each tube. The tubes were incubated at 37°C for 18 to 20 h. The MIC was defined as the lowest concentration of antibiotic giving complete inhibition of visible growth. When tests against ceftazidime and moxalactam were read, a very faint haziness generally was disregarded.

From each tube, 10 μl was inoculated onto an agar plate, and the MBC was determined after overnight incubation. The MBC was defined as the lowest concentration of antibiotic giving 99.9% killing (24).

Assay of antibiotic concentration. The samples from vials with and without bacteria were kept frozen for a few days before the tests, all of which were concurrent. The cells were not removed before the assays.

The concentration of antibiotic in the samples was monitored by microbiological assay, by using the agar well method with DST agar (Oxoid Ltd., London, England). The indicator strains were *E. coli* ATCC 25922 for cefotaxime, *Klebsiella pneumoniae* ATCC 10031 for ceftazidime and moxalactam, and *Staphylococcus warneri* (BAB 40) for gentamicin. The latter was resistant to all cephalosporins. Gentamicin was eliminated from the mixtures by cellulose phosphate powder (28).

When the plates had been flooded with indicator strain, wells were punched out and filled with standards or samples in duplicate. After overnight incubation at 37°C, the zones were measured. Standard curves were constructed by relating the inhibition zone to the concentrations. The interassay variation was about 10 to 15%.

Synergy studies. (i) Determination from FIC or FBC in liquid medium. The two antibiotics in each combination were mixed to give an initial concentration of two times the MIC of the respective antibiotics alone, in accordance with previous determinations (3). The mixtures were serially diluted twofold in a single row, and fractions (0.5 ml each) were inoculated with 0.5 ml of an overnight culture diluted to give a final concentration of approximately 5×10^5 CFU/ml. After 16 to 18 h at 37°C, the fractional inhibitory concentration (FIC) was determined as the combined concentration divided by the single concentration. The combination value was derived from the highest dilution of the antibiotic combination permitting no visible growth. The fractional bactericidal concentration (FBC) was determined from the highest dilution giving 99.9% killing when 10 μl was subcultured on agar.

The interaction index was calculated as follows:

$$\Sigma FIC = \frac{MIC_{combination\ a}}{MIC_{alone\ a}} + \frac{MIC_{combination\ b}}{MIC_{alone\ b}}$$

$$\Sigma FBC = \frac{MBC_{combination\ a}}{MBC_{alone\ a}} + \frac{MBC_{combination\ b}}{MBC_{alone\ b}}$$

where a and b are two different antibiotics. A sum of 0.5 or less was tentatively used as the border line for synergism.

(ii) Determination from killing curves. The final concentration of each antibiotic was half the previously determined MBC or fractions of that concentration. In earlier experiments, MIC was used. When two drugs were tested in combination, one-quarter or less of the MBC of each was used. The flasks were incubated on a shaker for 24 h at 37°C. Samples were taken for viable count and antibiotic concentration assays after 0, 4, 6, and 24 h. Synergism was defined as

TABLE 1. MICs and MBCs of three study strains

Antibiotic	MIC (MBC)[a]		
	P. aeruginosa PAO1	*P. maltophilia* SI164	*E. coli* ATCC 25922
Cefotaxime	16 (128)	64 (128)	0.064 (0.125)
Moxalactam	8 (32)	32 (64)	0.25 (0.25)
Ceftazidime	2 (16)	32 (64)	0.125 (0.25)
Gentamicin	8 (16)	4 (8)	2 (4)
Netilmicin	16 (32)	8 (32)	2 (2)

[a] Mean values in micrograms per milliliter.

a 2-$\log_{10}$ decrease in CFU per milliliter between the combination and its most active constituent after 24 h.

RESULTS

MIC and MBC values. The MIC and MBC ranges and the MICs and MBCs for 50 and 90% of the strains are shown in Table 2. That the new cephalosporins have low MICs when used alone was evident from these data, which included 23 strains of pseudomonas. Ceftazidime was the most active agent.

When the aminoglycosides were tested in nonsupplemented broth, significantly lower MICs and MBCs were found for strains of *P. aeruginosa*. For gentamicin, a MIC of 1.0 μg/ml and an MBC of 4.0 μg/ml were found for 56 and 51%, respectively, of all strains.

Interaction between gentamicin and cefotaxime, moxalactam, or ceftazidime. The observed interaction indices are presented in four groups as cumulative percentages (Table 3). The range is from synergism toward antagonism. For example, with gentamicin-cefotaxime, for 4.7% of the strains ΣFIC was 0.25 or less. The corresponding figure for ΣFBC was 45%. Using MIC, an additional 35.3% of the strains showed values between 0.25 and 0.5, giving a total of 40% of the strains with interaction indices of 0.5 or less.

With gentamicin-ceftazidime, the MBC was reduced to one-eighth or less (ΣFBC ≤ 0.25) for 20% of the strains. The MIC was not reduced to this level with any of the strains. If an interaction index of 0.5 is accepted as borderline, synergism was demonstrated against 30 to 40% of the strains by MIC and against 50 to 70% by MBC with the new beta-lactam antibiotics. If ΣFBC = 0.75 is accepted as the borderline value, a synergistic effect was obtained against 77 to 93% of the strains. Synergism was observed on all levels of susceptibility.

Interaction between netilmicin and cefotaxime, moxalactam, or ceftazidime. When netilmicin was substituted for gentamicin, the pattern of results was similar. Synergism, demonstrated by ΣFBC ≤ 0.5, was found with 50 to 60% of the bacterial strains. Here again, synergy was more easily demonstrated with MBC than with MIC values.

Reproducibility of ΣFIC and ΣFBC testing. Test values for repetitive assays with gentamicin-cefotaxime and three strains of pseudomonas and an *E. coli* strain are shown in Table 4. The reproducibility was within ±0.2 for two-thirds of the assays and within ±0.4 to 0.5 for all of them. With this error of method, ΣFIC or ΣFBC = 0.5 would amount to synergism. A value of 0.75 might indicate synergism, thus suggesting a need for further investigation.

Only small discrepancies (≤0.25) were found between interaction indices obtained from testing in supplemented as compared with nonsupplemented broth (Table 4).

Correlation between interaction indices and results from the killing curve method. To compare the modified checkerboard technique with the killing curve method, three strains were selected to represent low (PAO1), moderate (*E. coli* ATCC 25922), or high (SI164) ΣFBC for the combination gentamicin-cefotaxime (Table 5). With this combination, the interaction indices FIC and FBC for PAO1 were 0.5 and 0.19, respectively. In a second and a third assay, ΣFBC was 0.19 and 0.28, respectively. In one killing curve experiment, synergism was demonstrated with both drugs at one-eighth of the MBC per ml (Fig. 1b). In another experiment, this result was not obtained (Fig. 2). The synergy-negative result may be in agreement with ΣFBC = 0.28 and the positive with ΣFBC = 0.19. For *E. coli* ATCC 25922, ΣFIC was calculated to be 0.92 as a mean of three experiments and ΣFBC was calculated as 0.67, which means that the combination had bactericidal action when one-quarter of the MBC of each drug was used. In the killing curve, consequently, synergism was demonstrated when one-quarter, but not one-eighth, of the MBC of each drug was used.

With strain SI164, the modified checkerboard technique gave results indicating only additive, or even antagonistic, effect (ΣFBC = 0.75, 1.1, 1.13, and 1.25). In time-killing assays, we used

TABLE 2. MICs and MBCs of 43 strains[a]

Antibiotic	MIC			MBC		
	Range	50%	90%	Range	50%	90%
Ceftazidime	0.032–128	4	32	0.125–512	16	128
Cefotaxime	0.064–512	8	128	0.064–>512	32	>512
Moxalactam	0.064–128	16	64	0.125–>512	32	>512
Gentamicin	0.125–>512	8	256	0.5–>512	16	>512
Netilmicin	0.25–>512	16	64	0.25–>512	32	512

[a] Medium was Mueller-Hinton broth supplemented with 25 mg of Mg^{2+} and 50 mg of Ca^{2+} per liter. Values are in micrograms per milliliter.

TABLE 3. Cumulative interaction indices between gentamicin or netilmicin and cephalosporins in supplemented broth for 43 strains

Antibiotic combination	Index	Cumulative % strains with interaction index of:			
		≤0.25	≤0.5	≤0.75	≤1.5
Gentamicin plus					
Cefotaxime	ΣFIC	4.7	40	60	95
	ΣFBC	45	70	77	95
Moxalactam	ΣFIC	12	40	63	93
	ΣFBC	27	63	93	100
Ceftazidime	ΣFIC	0	30	63	95
	ΣFBC	20	49	83	98
Netilmicin plus					
Cefotaxime	ΣFIC	12	30	51	95
	ΣFBC	35	59	76	100
Moxalactam	ΣFIC	4.7	35	63	98
	ΣFBC	23	51	77	100
Ceftazidime	ΣFIC	0	19	60	95
	ΣFBC	13	53	80	100

one-half the MBC as well as three-quarters of the MBC, i.e., one-quarter plus one-quarter and three-eighths plus three-eighths in the respective combinations. Since SI164 is a fairly slow-growing strain, the experiment was extended over 48 h. No synergism was found with the smaller amounts of antibiotic. An antibiotic concentration corresponding of ΣFBC = 0.75 gave 1 log difference between the combination curve and the gentamicin curve after 6 and after 24 h, but after 48 h, there was regrowth in all vials. The other combinations gave similar results, although only single experiments were performed.

Factors influencing the outcome of the time-killing assay. Initial antibiotic concentration, inoculum size, presence of divalent cations, and definition of synergism in relation to time were factors studied in more detail.

(i) Level of antibiotic concentration. The level of antibiotic concentration is obviously crucial for synergism, defined as a detectable reduction of CFU effected by the combination, as compared with each constituent drug. This is exemplified by the combination gentamicin-cefotaxime against *P. aeruginosa* PAO1 (Fig. 1a to c). Concentrations that were too low resulted in regrowth in the presence of each drug, as well as with the combination (Fig. 1a), and concentrations higher than the MBC gave bacterial killing throughout (Fig. 1c). The initial concentration of the individual drugs should therefore be close to the expected inhibitory level that still permits regrowth (Fig. 1b). In this study, consistent results were obtained with one-half the MBC of the drugs when used alone.

(ii) Relationship between concentration in single-drug use and in combinations. Most investigators have combined two antibiotics in concentrations corresponding to $(A + B)$ per milliliter, where A and B are the respective concentrations per milliliter when the drugs are tested separately (2, 15). However, in a situation in which A =

TABLE 4. Reproducibility of interaction index in nonsupplemented and supplemented broth (gentamicin plus cefotaxime) with three strains of pseudomonas and *E. coli* ATCC 25922

Strain	Index	Interaction index value									
		Nonsupplemented broth				Supplemented broth					
		Assay 1	Assay 2	Assay 3	Mean	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Mean
P. aeruginosa											
PAO1	ΣFIC	0.38	0.5		0.44	0.75	0.38	0.5			0.54
	ΣFBC	0.16	0.14		0.15	0.28	0.19	0.19			0.22
P. maltophilia											
SI164	ΣFIC	1.5	1.0		1.25	0.75	1.25	1.25	0.75		1
	ΣFBC	1.5	0.64		1.07	1.1	1.13	1.25	0.75		1.06
P. aeruginosa											
SI188	ΣFIC	0.75	0.75		0.75	0.38	0.56	0.38	0.3	0.38	0.67
	ΣFBC	0.05	0.16	0.38	0.20	0.19	0.09	0.16	0.25	0.16	0.17
E. coli											
ATCC 25922	ΣFIC	0.75	1.5	1.25	1.17	1.0	0.75	1.0			0.92
	ΣFBC	0.32	1.0	1.13	0.82	0.75	0.7	0.5			0.67

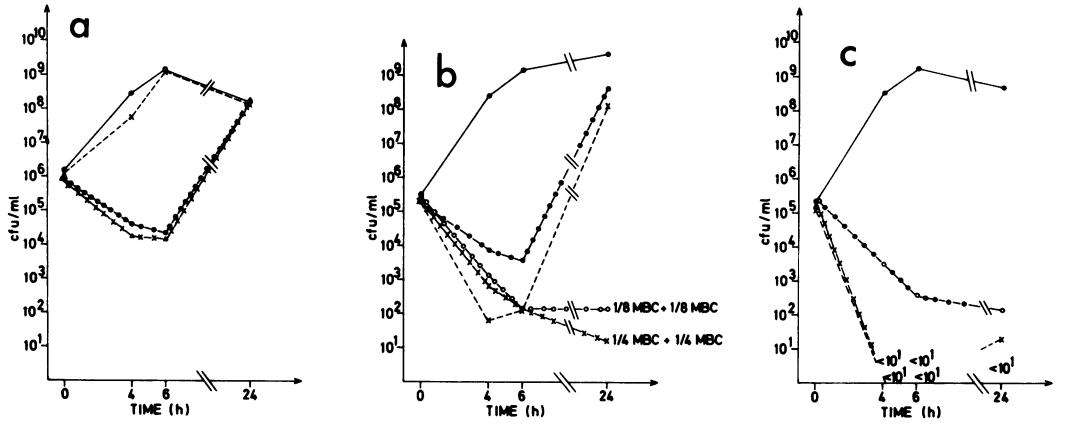

FIG. 1. Killing curves for *P. aeruginosa* PAO1 in supplemented broth with various concentrations of gentamicin and cefotaxime. (a) Gentamicin, 1/16 MBC (1.0 μg/ml); cefotaxime, 1/16 MBC (8.0 μg/ml); gentamicin plus cefotaxime, 1/32 MBC + 1/32 MBC (0.5 + 4.0 μg/ml). (b) Gentamicin, 1/2 MBC (8.0 μg/ml); cefotaxime, 1/2 MBC (64 μg/ml); gentamicin plus cefotaxime, 1/4 MBC + 1/4 MBC (4.0 + 32 μg/ml) and 1/8 MBC + 1/8 MBC (2.0 + 16 μg/ml). (c) Gentamicin, 2 × MBC (32 μg/ml); cefotaxime, 2 × MBC (256 μg/ml); gentamicin plus cefotaxime, MBC + MBC (16 + 128 μg/ml). Symbols: ●—●, control; X---X, gentamicin; ○-●-●-○, cefotaxime; △-X-X-△ and ○-○-○-○, gentamicin plus cefotaxime.

B, such a combination may give enhanced killing simply as a drug-response effect (3).

The example in Fig. 2 shows *P. aeruginosa* PAO1 ($\sum$FBC = 0.29) grown in the presence of gentamicin and cefotaxime at 4 and 32 μg/ml, respectively (one-quarter of their MBCs). When the same concentrations were used in combination, the reduction of growth compared with the most efficient single substance was more than 8 $\log_{10}$ after 24 h, indicating synergism. Reduction

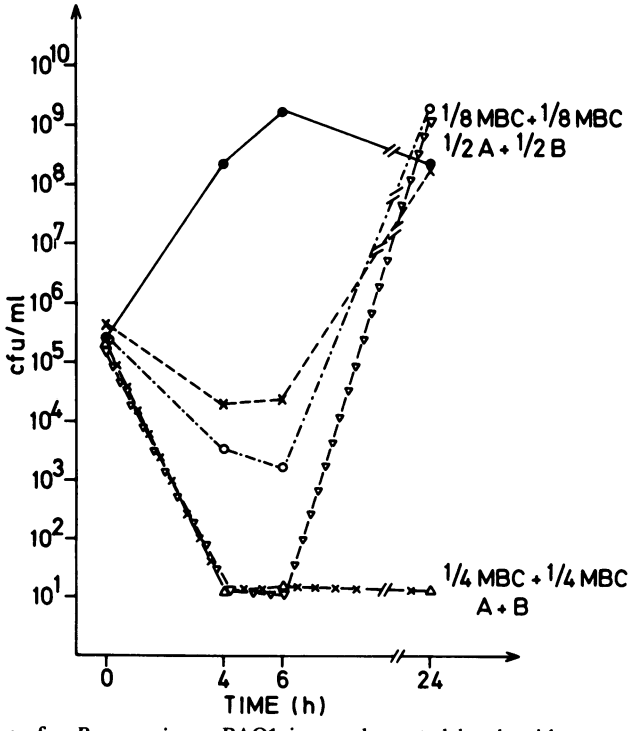

FIG. 2. Killing curves for *P. aeruginosa* PAO1 in supplemented broth with gentamicin and cefotaxime. Symobls: ●—●, control; X---X, gentamicin, 1/4 MBC (4.0 μg/ml); ○-·-·-○, cefotaxime, 1/4 MBC (32 μg/ml); △-X-X-△, gentamicin plus cefotaxime, 1/4 MBC + 1/4 MBC (4.0 + 32 μg/ml); ▽-▽-▽-▽, gentamicin plus cefotaxime, 1/8 MBC + 1/8 MBC (2.0 + 16 μg/ml).

TABLE 5. Synergism between gentamicin in combination with cefotaxime, moxalactam, or ceftazidime in supplemented broth, determined by interaction index and killing curve method[a]

Strain	Gentamicin plus:											
	Cefotaxime				Moxalactam				Ceftazidime			
	ΣFIC	ΣFBC	KC1[b]	KC2[c]	ΣFIC	ΣFBC	KC1[b]	KC2[c]	ΣFIC	ΣFBC	KC1[b]	KC2[c]
P. aeruginosa PAO1	0.54	0.22	+	±	1.0	0.31	+	ND	1.0	0.56	+	ND
E. coli ATCC 25922	0.92	0.67	+	−	0.75	0.75	−	−	0.5	0.5	+	−
P. maltophilia SI164	1.0	1.06	−[d]	−[b]	0.19	0.09	ND[d]	ND[b]	0.63	0.75	ND[d]	ND[b]

[a] KC, Killing curve; +, synergy demonstrated; −, synergy not demonstrated; ND, not done.
[b] ¼ + ¼ MBC in combination.
[c] ⅛ + ⅛ MBC in combination.
[d] ⅜ + ⅜ MBC in combination.

of the respective antibiotic concentrations to 2 and 16 μg/ml, however, eliminated the differences at 24 h, even though the drugs were still effective after 4 and 6 h. To ensure that the observed effect is not only an additive effect, the amount of each constituent in the combination should be no more than one-half the concentration used for that drug alone.

(iii) Effect of inoculum size. The initial inoculum was varied from 10^2 to 10^3 to 10^6 to 10^7 CFU/ml (Fig. 3a to c). With the lower inoculum, the killing effect of single antibiotics was so pronounced that synergism could not be demonstrated. With an inoculum of 10^6 to 10^7 CFU/ml, on the other hand, conditions for regrowth seemed to be established when the drugs were used separately at the MIC (Fig. 3c). Also, 10^5 to 10^6 CFU/ml and one-half the MBCs for the drugs alone were shown to be adequate (Fig. 1b and 4a and b).

(iv) Effect of divalent cations in the medium. In synergy tests with *P. aeruginosa* PAO1, no difference in results was found in supplemented as compared with nonsupplemented medium, provided that the antibiotic concentrations were adjusted in accordance with the MBCs obtained from susceptibility testing in the respective media.

(v) Definition of synergism in relation to time. In our experiments, it was seldom possible to discern synergism after 4 to 6 h, even in runs with clear evidence of synergism after 24 h. (Fig. 1 to 4). Inhibition of regrowth in the combination as compared with the most effective constituent after 24 h seems to be the most reliable indicator of synergism with the killing curve method.

(vi) Reproducibility of killing curves. Reproducibility of synergism measured by the killing curve method is affected by the amount of antibiotics used in the combination. With con-

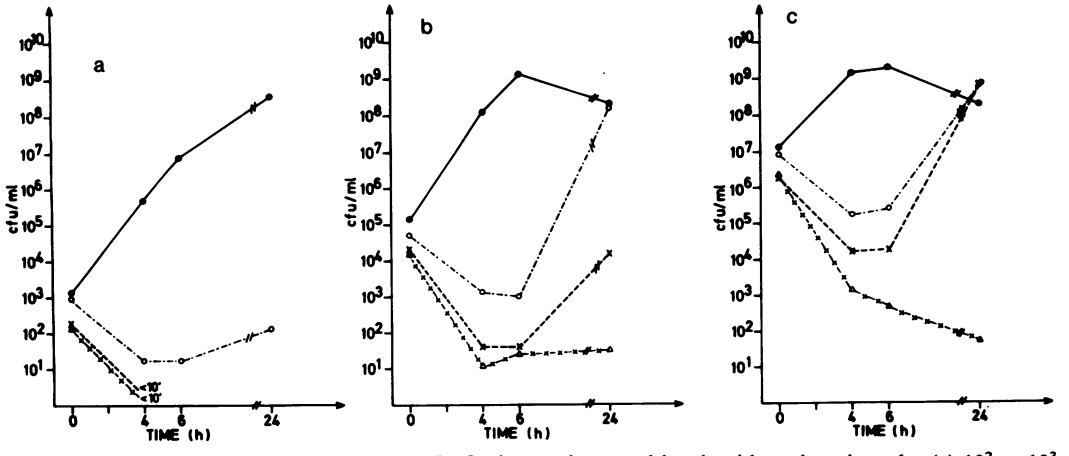

FIG. 3. Killing curves for *P. aeruginosa* PAO1 in supplemented broth with various inocula. (a) 10^2 to 10^3 CFU/ml. (b) 10^4 to 10^5 CFU/ml. (c) 10^6 to 10^7 CFU/ml. Symbols: ●—●, control; X---X, gentamicin, MIC (8.0 μg/ml); O---O, cefotaxime, MIC (16 μg/ml); Δ-X-X-X-Δ, gentamicin plus cefotaxime, 1/2 MIC + 1/2 MIC (4.0 + 8.0 μg/ml).

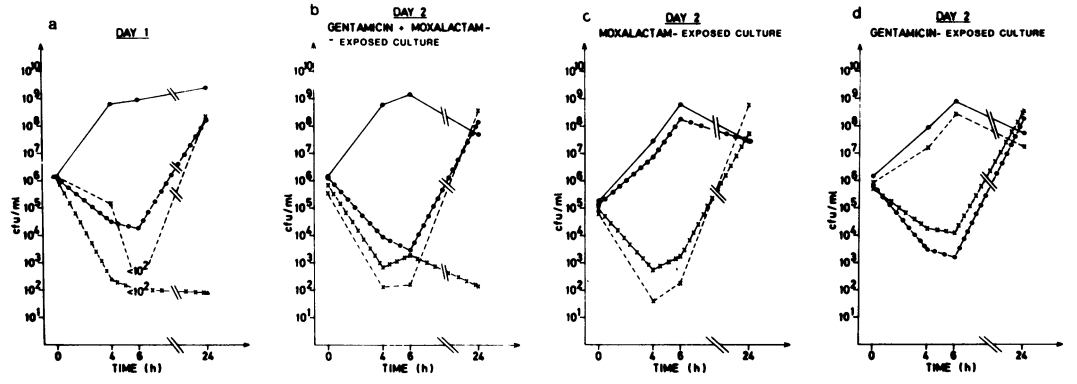

FIG. 4. Killing curves for *P. aeruginosa* PAO1 in supplemented broth with gentamicin-moxalactam. (a) Nonexposed culture. (b), (c), (d) Exposed culture. Symbols: ●—●, control; X---X, gentamicin, 1/2 MBC (8.0 μg/ml); ●-●-●-●, moxalactam, 1/2 MBC (16 μg/ml); △-X-X-△, gentamicin plus moxalactam, 1/4 MBC + 1/4 MBC (4.0 + 8.0 μg/ml).

centrations well within the limits of lethal activity, the reproducibility is good (Table 6). When low concentrations close to the endpoints of synergistic capacity are chosen, the reproducibility is more uncertain (compare Fig. 1b and 2). Repeated experiments with PAO1 and the combination gentamicin-cefotaxime, however, indicate that a 2-$\log_{10}$ difference or more between the combination and its most active constituent after 24 h may be used as an indicator of synergism.

Second-dose effect: exposed and nonexposed cultures. The laboratory model does not take into account the fact that the drugs are given repeatedly over several days. Furthermore, therapy is often initiated with one antibiotic, and others are added later.

(i) Synergism. The situation in which therapy is begun with one antibiotic and then others are added was mimicked in killing curve experiments with gentamicin plus cefotaxime, moxalactam, or ceftazidime. *P. aeruginosa* PAO1 was grown with the antibiotic separately and in combinations. Similar results were obtained with all three combinations. The gentamicin-moxalactam combination (ΣFBC = 0.51) (Fig. 4) illustrates the steady bacterial decrease that occurred over 24 h in response to gentamicin-moxalactam, whereas with each antibiotic alone there was regrowth between 6 and 24 h (Fig. 4a).

After 24 h, bacteria were sampled as inocula for new, identical runs (Fig. 4b to d). To obtain appropriate inocula (10^5 to 10^6 CFU/ml) from the combinations, however, bacteria from such vials were sampled at 6 h and then were grown in 1/16 MBC plus 1/16 MBC before use in the day 2 experiment. Bacteria exposed to moxalactam (Fig. 4c) or to gentamicin (Fig. 4d) were no longer inhibited by the respective antibiotics, although they remained susceptible to the other

TABLE 6. Reproducibility of killing curves[a]

Drug concn			Log_{10} reduction	
Gentamicin	Cefotaxime	Gentamicin + cefotaxime	6 h	24 h
½ MBC	½ MBC	¼ + ¼ MBC[b]	0.5	8
½ MBC	½ MBC	¼ + ¼ MBC[b]	0	7
½ MBC	½ MBC	¼ + ¼ MBC[b]	0	6.5
¼ MBC	¼ MBC	⅛ + ⅛ MBC[c]	0	2
¼ MBC	¼ MBC	⅛ + ⅛ MBC[c]	2	0
MIC	MIC	½ + ½ MIC[d]	0	7
MIC	MIC	½ + ½ MIC[d]	0	2
MIC	MIC	½ + ½ MIC[d]	1	6
MIC	MIC	½ + ½ MIC[d]	0	3

[a] *P. aeruginosa* PAO1 in Mueller-Hinton broth supplemented with 25 mg of Mg^{2+} and 50 mg of Ca^{2+} per liter was used.
[b] 4.0 + 32 μg/ml.
[c] 2.0 + 16 μg/ml.
[d] 4.0 + 8.0 μg/ml.

drug and to the combination, after 4 and 6 h. After 24 h, however, there was regrowth in all vials, indicating no synergism. On the other hand, bacteria exposed to both antibiotics together (Fig. 4b) remained synergistically inhibited by the combination after 4 h and after 24 h (Table 7). They were also susceptible after 4 and 6 h to both constituents tested separately.

(ii) Antibiotic inactivation. Parallel with colony counting, samples were taken for assay of residual amounts of antibiotic (Table 7). In general, inactivation of antibiotics was followed by regrowth. With moxalactam, there was a reduction in the concentration sufficient for regrowth, but not total inactivation, except when the moxalactam-exposed culture had been grown with moxalactam alone on day 2. Cefotaxime, on the other hand, was completely inactivated, except after 24 h, when used in combination with gentamicin and after 48 h in the same combination against bacteria exposed to cefotaxime plus gentamicin. The results may suggest differences in killing effect between the cephalosporins. However, the experiments were not identical with regard to inocula and initial antibiotic concentrations. Moreover, gentamicin activity was reduced and regrowth observed when the drug was used separately against a culture exposed to gentamicin for 24 h (Table 7). Corresponding figures for gentamicin with cefotaxime and moxalactam were 42 and 47%, respectively.

DISCUSSION

The results from this in vitro study with 43 strains of different bacterial species indicate a synergistic potential for combinations of gentamicin or netilmicin with cefotaxime, moxalactam, or ceftazidime. Similar results have been reported with other aminoglycoside-cephalosporin combinations against pathogens such as *P. aeruginosa* and *Serratia marcescens* (7, 13, 16–20, 25).

The findings in the killing curve experiments further suggest that the combined effect operates from the start of therapy. Cultures exposed to only one of the antibiotics over a 24-h period readily adapted to that antibiotic by the end of the period. If the other drug of a pair was thereafter added, the lethal effect of the drug combination was less than after simultaneous exposure of the culture to both drugs, with inactivation also of cephalosporins in combinations. These findings may have practical implications.

General recommendations on antibiotic combinations are difficult to formulate, however. In a clinical situation, each isolate must be judged separately. There is thus a need for simple and appropriate methods to measure synergism. The modified checkerboard technique of Berenbaum (3) is less laborious than other techniques and therefore more convenient for routine work. It also permits rapid and economical testing of large numbers of combinations with multiple components.

The concept of interaction index, representing the sum of the FICs of the drugs in combination, was also introduced by Berenbaum (3). When broth medium is used, the sum of the FBCs can also be calculated. Theoretically, an interaction index of 1 indicates additive effect, and any value less than 1 denotes synergism. The final definition, however, is necessarily dependent on the day-to-day variations in MIC and MBC testing. $\sum FBC = 0.5$ was confirmed to be a reliable borderline value for synergism that could be reproduced in killing curves. This means that inhibition by the combined agents must occur at one-quarter or less of the MBC of each of the drugs. At this level, positive interaction was expressed for 50 to 70% of the strains.

TABLE 7. Killing curve experiments showing synergistic effect of gentamicin-cephalosporin combinations on nonexposed bacteria and bacteria exposed to either the combination or the single drugs and loss of antibiotic activity[a]

Time and exposure	Single drug or combination	Cefotaxime		Moxalactam		Ceftazidime		Gentamicin[b]
		Synergism	% Loss	Synergism	% Loss	Synergism	% Loss	% Loss
0 to 24 h; nonexposed culture	Alone		100 (13)		57 (22)		82 (20)	13 (0)
	With gentamicin	+	3 (18)	+	36 (22)	+	19 (19)	0 (0)
24 to 48 h; culture exposed to combination	Alone		100 (25)		63 (23)		18 (12)	3 (11)
	With gentamicin	+	18 (9)	+	22 (8)	+	2 (18)	5 (0)
24 to 48 h; culture exposed to gentamicin	Alone		100 (25)		27 (23)		14 (12)	19 (11)
	With gentamicin	−	100 (9)	−	48 (8)	−	7 (18)	0 (0)
24 to 48 h; culture exposed to respective cephalosporin	Alone		100 (25)		100 (23)		100 (12)	10 (11)
	With gentamicin	−	100 (9)	−	30 (8)	−	51 (18)	0 (0)

[a] Numbers in parentheses are concentrations from controls incubated without cells.
[b] From ceftazidime experiment.

Such interaction may be clinically useful, particularly in situations in which host defense mechanisms are depressed, when bacterial killing may depend wholly on the antimicrobial potency of the antibiotics administered. In the present study, the MBCs for 50% of strains were 16 to 32 μg/ml when the antibiotics were tested individually, but these concentrations could be reduced to 4 or 8 μg/ml in synergistic action.

When the MIC was used for calculating the interaction index, only 30 to 40% of the strains showed synergism, and ΣFIC did not predict results in time-kill assays. In the checkerboard technique, however, the endpoint is usually defined as the MIC after overnight incubation. This may be one explanation of the discrepancies described by Norden et al. (22). A few investigators have used the MBC to study antibiotic synergism (1, 13, 30).

Since the time-kill assay measures bactericidal activity in relation to time, it is logical that results obtained with this method after overnight incubation should be viewed in relation to interaction indices calculated from MBCs. King et al. (11) advocated the use of a growth rate constant obtained from empirical dose-response curves.

To provide optimal and comparable results, the killing curve method must be adequately standardized. In the literature, various criteria for synergism in time-killing assays have been used, from 1 $\log_{10}$ between the combination and the most effective constituent after 4 h (6, 19) to 2 $\log_{10}$ (22) or more than 3 $\log_{10}$ (30) increased killing after 24 h. Data presented in this report support a difference of 2 $\log_{10}$ or more after 24 h with one-half the MBC of the drugs used alone and the amount of antibiotic at least halved in the combination. An incubation time of 24 h and an inoculum of 10^5 to 10^6 CFU/ml are similar to conditions used in susceptibility testing and with the checkerboard technique.

One of the most intriguing questions is whether in vitro synergism is associated with improved clinical outcome. Young (31) reported such a positive association from a prospective, randomized study on gentamicin-carbenicillin versus amikacin-carbenicillin in neutropenic patients. Klastersky and Zinner (14) reviewed data showing a favorable clinical response in 113 (79%) of 149 cancer or neutropenic patients when synergistic combinations of antibiotics were used against severe infections, but in only 64 (45%) of 143 patients when the combinations were nonsynergistic. A combination of the new broad-spectrum cephalosporins with aminoglycosides may be advantageous. Further studies must be undertaken, however, to determine whether these new antibiotics also give better clinical results in combination than when used separately.

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