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Mechanism of Chloramphenicol-Cephaloridine Synergism on *Enterobacteriaceae*

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A synergistic in vitro bactericidal effect of combinations of chloramphenicol and beta-lactams on strains of *Enterobacteriaceae* is described. The synergism is seen with strains which are resistant to the beta-lactam and is due to chloramphenicol-induced inhibition of beta-lactamase production.

The increasing resistance of gram-negative bacilli to anti-bacterial agents (6), including gentamicin (12) and the cephalosporins (19), raises the possibility of having to resort to a synergistic combination of drugs in the therapy of infections caused by these bacteria.

Synergism between chloramphenicol and several beta-lactam antibiotics on strains of *Klebsiella-Enterobacter-Serratia*, which are resistant to the beta-lactam alone, has recently been described as a not uncommon event (13). The present study was undertaken to examine the occurrence of this synergism with gram-negative bacilli belonging to other genera, and to elucidate its mechanism.

MATERIALS AND METHODS

Bactericidal effect of combinations of chloramphenicol and three beta-lactams. Ninety-four strains of *Enterobacteriaceae* isolated in the diagnostic laboratory of the Department of Clinical Microbiology, Hadassah University Hospital, from sputum, infected wounds, stools, and blood cultures were studied. These strains were identified according to Ewing (5) as *Escherichia coli* (15 strains), *Shigella* (15 strains), *Citrobacter* (3 strains), *Salmonella* (12 strains), *Proteus* (13 strains), *Providence* (6 strains), *Klebsiella* (11 strains), *Enterobacter* (9 strains), and *Serratia* (10 strains). The cellophane transfer technique of Chabbert (1) as modified by Cluzel et al. (4) was used to determine a synergistic bactericidal effect. The principles and techniques of this method have recently been described and illustrated in detail (3, 7, 8, 16). The results were interpreted according to previously reported criteria and were classified as synergism, antagonism, or indifference (3, 7, 8, 16). The beta-lactams used were ampicillin, cephaloridine, and carbenicillin.

Mechanism of synergistic effect. For these experiments the following strains of beta-lactamase-producing *Enterobacteriaceae* were used: *Enterobacter cloacae* NCTC 10005, *Klebsiella pneumoniae* K 1296 obtained from John Matsen, University of Minnesota (14), and eight strains (see Table 2) isolated from clinical material at the Hadassah University Hospital and identified according to Ewing (5). Each strain was tested for beta-lactamase production by the iodometric method as described by Workman and Farraz (22).

Susceptibility to single antibiotics. The susceptibility of these strains to chloramphenicol and cephaloridine was determined by preparing doubling dilutions of the antibiotic in Trypticase soy broth (TSB; Difco). The inoculum was 0.1 ml of a suitably diluted overnight culture in TSB to give a final concentration in the test dilutions of approximately 10^5 bacteria/ml. The minimal inhibitory concentration (MIC) was expressed in terms of the lowest concentration of antibiotic that totally inhibited visible growth after 24 h at 37 C. The minimal bactericidal concentration was expressed as the lowest concentration showing no visible growth on subculture to blood agar plates.

Bactericidal effect of combinations of chloramphenicol and cephaloridine. Box titrations were performed with doubling dilutions of chloramphenicol and cephaloridine and every combination of the various concentrations of each antibiotic. The bacterial inoculum was 0.1 ml of a suitably diluted overnight culture in TSB, to give a final concentration of approximately 10^5 bacteria/ml. The tubes were incubated at 37 C and samples were taken for viable counts at time intervals from 0 to 24 h.

Influence of chloramphenicol on cephaloridine inactivation by the test strains. In the previous experiments, at the same time as viable counts were performed, starting from time zero, samples were taken for assay of cephaloridine activity. These were centrifuged at 11,000 $\times$ *g* at 4 C for 20 min, and the supernatant fluid was passed through a membrane filter (Millipore Corp.) (0.22 μm). The cephaloridine activity was assayed biologically using the agar diffusion method and a locally isolated strain of *Staphylococcus aureus* resistant to chloramphenicol (MIC > 100 μg/ml) as indicator strain. The medium used was Oxoid DST agar. When the cephaloridine activity was being measured in samples from tubes containing chloramphenicol as well, the reference standard solutions of cephaloridine were prepared in TSB containing the same concentration of chloramphenicol as in the sample being assayed. There was neither synergism nor antagonism between chloramphenicol and cephaloridine on this strain of *S. aureus*. As a control

of the stability of the antibiotic over a 24-h period at 37 C, twofold dilutions of cephaloridine without a bacterial inoculum were incubated and remaining cephaloridine activity was assayed at intervals up to 24 h.

Influence of chloramphenicol on preformed beta-lactamase. *E. cloacae* NCTC 10005 was used as the source of the enzyme. Crude beta-lactamase was obtained using the technique described by Goldner et al. (9). TSB was inoculated from nutrient agar (Difco) slants and incubated with shaking for 24 h at 37 C and then centrifuged at 11,000 × *g* at 4 C for 10 min. The supernatant fluid was passed through a membrane filter (Millipore Corp.) (0.22 μm), and the filtrate, which constituted the crude beta-lactamase activity, was assayed immediately. Chloramphenicol was added to a portion of this preparation at a final concentration of 100 μg/ml, and another portion was used as control. After 30 min at 37 C, beta-lactamase activity was measured on both portions, using the timed iodometric method described by Citri (2). The test was performed at 37 C using a freshly prepared solution of cephaloridine (3 μg/ml in 0.1 M phosphate buffer at ph 7.0) as substrate.

RESULTS

The results of the combinations between chloramphenicol and the three beta-lactams using the cellophane transfer technique showed a surprisingly high frequency of synergism. Of the 282 combinations tested (94 strains tested against chloramphenicol in combination with each of 3 beta-lactams), 41 (15%) showed synergism, 61 (22%) antagonism, and 180 (63%) indifference. There was no correlation between the frequency of synergism and the species or genus of bacteria. There was, however, a striking relationship between the outcome of the combined action of chloramphenicol and beta-lactam and the sensitivity to the beta-lactam alone. On 83 occasions chloramphenicol was combined with a beta-lactam that was active on its own; in 61 (73%) antagonism was found, and on only one occasion (1.2%) synergism; indifference was seen in the remaining 21 combinations. Chloramphenicol was combined with a beta-lactam that was itself inactive on 199 occasions, and synergism was seen on 40 (20%), indifference on 159 (80%), and antagonism not once. The results obtained in this qualitative test for synergism with each of the 3 beta-lactams on the 84 strains are summarized in Table 1.

To elucidate the mechanism of this synergistic effect, 10 strains of beta-lactamase-producing gram-negative bacteria were studied with combinations of chloramphenicol and cephaloridine. The enzyme was constitutive in eight strains and inducible in two. Table 2 shows the susceptibility of these strains to cephaloridine and chloramphenicol. They were all highly resistant to cephaloridine, whereas the MIC of chloramphenicol varied from 8 μg/ml to more than 128 μg/ml. A synergistic bactericidal effect was observed with all of the strains except the *K. pneumonia* K 1296 which was very resistant to chloramphenicol. The other nine strains showed a synergistic effect with concentrations of chloramphenicol lower than (seven strains) or equal to the MIC (two strains). The concentrations of cephaloridine necessary for the bactericidal effect varied from 12.5 to 500 μg (Table 2). In all of the experiments showing synergism, a constant finding was the inhibition in the presence of chloramphenicol of cephaloridine inactivation by the test strain. Representative detailed results are shown in Fig. 1 and in Tables 3 and 4.

Figure 1 illustrates the influence of a subinhibitory concentration of chloramphenicol on the inactivation of cephaloridine by the type strain of *E. cloacae* and the accompanying synergistic bactericidal effect of the mixture of antibiotics. Table 3 shows that with this strain the synergistic effect was achieved with as little as 12.5 μg of cephalorodine per ml, and that it was always associated with inhibition of cephaloridine inactivation. Table 4 shows the pattern of results achieved with the strain of *Serratia liquefaciens*, where a synergistic bactericidal effect was seen only with chloramphenicol at the MIC and 500 μg of cephaloridine per

TABLE 1. *Results of combinations of chloramphenicol with ampicillin, carbenicillin, or cephaloridine on 94 strains of Enterobacteriaceae*[a]

Combination of chloramphenicol with:	Strains susceptible to beta-lactams				Strains resistant to beta-lactams			
	Strains (no.)	Synergism (no.)	Antagonism		Strains (no.)	Synergism		Antagonism (no.)
			No.	%		No.	%	
Ampicillin	17	0	12	71	77	14	18	0
Carbenicillin	37	0	25	68	57	7	12	0
Cephaloridine	29	1	24	83	65	19	29	0

[a] Cellophane transfer technique.

TABLE 2. *Susceptibility of 10 strains of Enterobacteriaceae to cephaloridine, chloramphenicol, and to the combination of the two drugs*

Strains	Source	Susceptibility to:			Minimal concentrations showing a bactericidal effect in combination		Nature of beta-lactamase
		Cephaloridine MIC[a]	Chloramphenicol MIC[a]	Chloramphenicol MBC[a, b]	Cephaloridine[a]	Chloramphenicol[a]	
Salmonella typhimurium	Blood	250	32	>64	50	20	C[e]
Klebsiella pneumoniae	K1296	>1,000	>128	NT[c]	NSE[d]	NSE[d]	C
K. pneumoniae	Sputum	>1,000	16	>64	50	8	C
Enterobacter cloacae	NCTC 10005	>1,000	8	>32	12.5	6	C
E. cloacae	Wound	>1,000	64	>128	250	10	I[f]
Serratia marcescens	Blood	>500	8	32	125	6	I
S. liquefaciens	Sputum	>1,000	8	32	500	8	C
Proteus rettgeri	Blood	>500	16	64	50	16	C
Providencia	Blood	>1,000	32	>64	50	25	C
Yersinia enterocolitica	Wound	>500	8	>64	50	6	C

[a] Micrograms per milliliter.
[b] MBC, Minimal bactericidal concentration.
[c] NT, Not tested.
[d] NSE, No synergistic effect.
[e] C, Constitutive.
[f] I, Inducible.

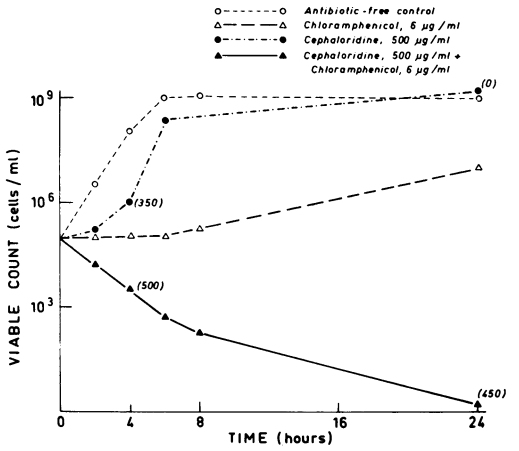

FIG. 1. *Synergistic bactericidal effect of chloramphenicol and cephaloridine on E. cloacae (NCTC 10005). (Figures in parentheses indicate the residual cephaloridine activity in micrograms per milliliter.)*

ml. With lower concentrations of cephaloridine in the presence of chloramphenicol there was no bactericidal effect, although there was inhibition of inactivation of the beta-lactam by the test organism.

Chloramphenicol at a concentration of 100 μg/ml failed to produce any significant change in the activity of the beta-lactamase obtained from the *E. cloacae* type strain NCTC 10005. A preparation showing activity of 250 U/ml showed a drop in activity to 230 U/ml after incubation at 37 C for 30 min both with and without chloramphenicol.

TABLE 3. *Inhibition of cephaloridine (CD) inactivation by chloramphenicol and consequent synergistic effect on E. cloacae NCTC 10005*[a]

CD concn at time zero (μg/ml)	Chloramphenicol concn of 0 μg/ml		Chloramphenicol concn of 6 μg/ml	
	CD concn at 24 h (μg/ml)	Viable counts/ml at 24 h	CD concn at 24 h (μg/ml)	Viable counts/ml at 24 h
500	0	10^9	450	0
100	0	10^9	50	0
50	0	10^9	40	0
25	0	10^9	20	0
12.5	0	10^9	5	0
0	NT[b]	10^9	NT	10^7

[a] Inoculum at time zero: 10^5/ml. MIC of chloramphenicol for test strain: 8 μg/ml.
[b] NT, Not tested.

DISCUSSION

This study confirms the previous report (13) of synergism between chloramphenicol and beta-lactams on certain strains of gram-negative bacteria. When the strain is susceptible to both drugs antagonism is common, and this conforms to the general rule about combinations of bacteriostatic and bactericidal drugs (11, 15).

TABLE 4. *Inhibition of cephaloridine* (CD) *inactivation by chloramphenicol and consequent synergistic effect only at high concentration of cephaloridine (Serratia liquefaciens)*[a]

CD concn at time zero (μg/ml)	Chloramphenicol concn of 0 μg/ml		Chloramphenicol concn of 8 μg/ml	
	CD concn at 24 h (μg/ml)	Viable counts/ml at 24 h	CD concn at 24 h (μg/ml)	Viable counts/ml at 24 h
500	0	10^9	500	0
100	0	10^9	100	10^4
50	0	10^9	45	10^5
25	0	10^9	20	10^5
12	0	10^9	10	10^5
0	NT[b]	10^9	NT	10^5

[a] Inoculum at time zero: 10^5/ml. MIC of chloramphenicol for test organism: 8 μg/ml.
[b] NT, Not tested.

When, however, the strain is resistant to the beta-lactam and susceptible to chloramphenicol, a synergistic bactericidal effect is not uncommon. The findings reported here suggest a possible mechanism for this synergism. The resistance of gram-negative bacilli to beta-lactams is known to be related to several possible mechanisms, including the presence of a permeability barrier and/or enzyme degradation by a beta-lactamase produced by the resistant strain (17, 20). Chloramphenicol is an inhibitor of bacterial protein synthesis (10) and, as such, could inhibit the production of beta-lactamase. We have demonstrated a synergistic effect between chloramphenicol and cephaloridine, in strains which are beta-lactamase producers. This synergism is accompanied by inhibition of destruction of the cephaloridine by these strains in the presence of chloramphenicol. We also demonstrated that chloramphenicol has no effect on preformed beta-lactamase. In many cases the concentration of chloramphenicol needed to inhibit cephalosporinase production is lower than the MIC, suggesting that since the bacteriostatic effect is presumably due to inhibition of essential protein synthesis this occurs at a higher concentration of chloramphenicol than inhibition of beta-lactamase production. In some strains the synergistic effect occurs at low concentrations of cephaloridine that can easily be achieved in the serum on standard dosage schedules, suggesting that the effect might be of clinical value. In other cases, although there is inhibition of beta-lactamase production in the presence of chloramphenicol, the synergistic bactericidal effect is only seen at high concentrations of cephaloridine. In these cases the resistance to cephaloridine is probably related to both beta-lactamase production and the presence of a permeability barrier which is only overcome by very high concentrations of cephaloridine. The strain of *Klebsiella* which did not show the synergistic bactericidal effect was resistant to high concentrations of chloramphenicol. Onishi et al. (18) recently reported the susceptibility of beta-lactamase induction in a single strain of *E. coli* and one of *Klebsiella* to chloramphenicol in final concentrations of 250 μg/ml. They did not examine lower concentrations, or for possible synergistic effects. Traub (21) described the synergistic effect of a combination of chloramphenicol and cephalothin on strains of *S. epidermidis* and speculated that the effect might be due to alteration of the cell wall by cephalothin facilitating the uptake of chloramphenicol, or that cephalothin might inhibit bacterial protein synthesis at the ribosomal level and thus have a synergistic effect with chloramphenicol.

It remains to be seen whether the synergistic mechanism based on inhibition of enzyme production by chloramphenicol, which we have described, is a general phenomenon applicable to other beta-lactam antibiotics and other bacterial species. It is, however, a phenomenon with possible clinical significance and we have recently used the combination successfully in two cases of septicemia caused by strains of gram-negative bacteria resistant to all available antibiotics, but in which the combination of chloramphenicol and cephaloridine showed a synergistic bactericidal effect in vitro (manuscript in preparation).

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