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Paradox Between the Responses of *Escherichia coli* K1 to Ampicillin and Chloramphenicol In Vitro and In Vivo

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We evaluated the activity of ampicillin and chloramphenicol in vitro and in vivo against an *Escherichia coli* K1 strain. In vitro, the strain was relatively susceptible to both antibiotics (MIC and MBC of ampicillin, 2 and 4 μg/ml; MIC and MBC of chloramphenicol, 4 and 64 μg/ml). Checkerboard determinations of MBCs of drug combinations were consistent with antibiotic antagonism. Killing curves with concentrations of antibiotics similar to in vivo levels in blood and cerebrospinal fluid of infected rats indicated antagonism within the first 4 h and an indifferent effect of the combination at 24 h. Paradoxically, the combination was significantly more effective than ampicillin or chloramphenicol alone in vivo in infant rats. This was shown by (i) more rapid bacterial clearance from the blood and cerebrospinal fluid, (ii) a decreased incidence of meningitis in bacteremic animals, and (iii) improved survival. These findings illustrate a divergence between the effects of ampicillin and chloramphenicol against *E. coli* in vitro and in vivo and suggest that this combination is an effective synergistic regimen in this experimental model of *E. coli* bacteremia and meningitis.

Meningitis of the newborn caused by gram-negative bacilli responds more slowly and less predictably to antimicrobial therapy than meningitis caused by gram-positive organisms (18). Because the clinical outcome of bacterial meningitis in this age group can be directly correlated with the persistence of bacteria in the cerebrospinal fluid (CSF; 18, 19), rapid bacteriological cure is a requirement for effective therapy.

The infant rat has been used previously to reproduce bacteremia and meningitis with *Haemophilus influenzae* type b, *Escherichia coli*, and group B streptococci in studies of the pathogenesis and immunology of these infections (8, 13, 25). These studies have illustrated important similarities between *E. coli* infections in infant rats and those in human infants: age dependency, susceptibility to strains bearing the K1 capsular polysaccharide, hematogenous infection of the meninges without the need for adjuvants or direct inoculation, and high mortality. With serial atraumatic collection of blood and CSF specimens, we have used this model to study the efficacy of antimicrobial agents in terms of rates of bacterial clearance and mortality (17). In the course of these studies, we observed and report here a striking paradox between the in vitro and in vivo responses of *E. coli* K1 to combinations of ampicillin and chloramphenicol. In vitro, the combination exerted an antagonistic or indifferent effect, whereas the in vivo findings were consistent with bona fide synergism.

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MATERIALS AND METHODS

Organism. A serum-resistant *E. coli* K1 strain, C5 (serotype O18ac:K1:H7), was kindly provided by Robert Bortolussi (Dalhousie University, Halifax, N.S., Canada). This strain was isolated from the CSF of a newborn infant with meningitis (7). Logarithmic-phase cultures of the C5 strain in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) were stored at −70°C in aliquots.

In vitro tests. Frozen cultures were thawed and transferred to MacConkey agar plates (Clinical Standards, Carson, Calif.) for incubation overnight at 37°C. Four colonies were inoculated into 10 ml of Mueller-Hinton (MH) broth (Difco), incubated for approximately 4 h in a 37°C water bath to match the turbidity of a MacFarland no. 0.5 standard (22), and diluted 1:500 in fresh MH broth. This final dilution was shown to contain 1.1×10^5 to 7.7×10^5 (mean, 4.6×10^5) CFU/ml.

Ampicillin trihydrate (Bristol Laboratories, Syracuse, N.Y.) in a potency of 832 μg/mg and chloramphenicol (Parke, Davis & Co., Morris Plains, N.J.) were both reconstituted to 6.4 mg/ml in 0.1 M phosphate buffer (pH 8.0) and ethanol-distilled water, respectively (2), filtered through 0.45-μm membranes (Gelman Instrument Co., Ann Arbor, Mich.), stored at −70°C in aliquots, and used within 4 weeks. Antibiotic solutions were thawed immediately before use and serial twofold dilutions were made in MH broth. Control tubes of MH broth without antibiotics were included in each series of dilutions.

Equal volumes (0.5 ml) of antibiotic and bacterial dilutions were mixed in tubes (13 by 100 mm) and incubated at 37°C for 24 h. The MIC was the lowest antibiotic concentration resulting in no visual turbidity. From each tube, 50 μl was transferred to sheep blood agar and incubated at 37°C for 24 h to determine the MBC, the lowest concentration with ≥99.9% killing of the original inoculum.

The MICs and MBCs of ampicillin-chloramphenicol combinations were measured in MH broth (1). Test tubes in a checkerboard fashion received 0.5 ml of ampicillin or chloramphenicol or both and 0.5 ml of final bacterial dilution. Ampicillin was tested in doubling concentrations of 0.125 through 64 μg/ml and chloramphenicol was tested at 0.5 through 256 μg/ml. After 24 h of incubation at 37°C, MIC and MBC endpoints were read as above for each antibiotic alone and in various combinations. The results were also expressed as the fractional bactericidal concentration index, which was calculated by the following formula (5, 21): (MBC of ampicillin in combination/MBC of ampicillin alone) + (MBC of chloramphenicol in combination/MBC of chloram-

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phenicol alone). When the fractional bactericidal concentration index was <0.5, the combination was considered to have a synergistic effect; when the index was >2, the combination was considered antagonistic; and when the index was 0.5 to 2, the combination was considered indifferent.

Time-kill curves were performed with 4 or 40 μg of ampicillin and 16 μg of chloramphenicol per ml, singly and in combination, in MH broth. At 0, 2, 4, and 24 h of incubation at 37°C, aliquots of 1 ml were removed and diluted 10-fold in sterile distilled water. A 100-μl amount of each dilution was spread on blood agar with a glass rod to determine the surviving CFU.

In vivo studies. *E. coli* bacteremia and meningitis were induced in infant rats by a modification of the method of Bortolussi et al. (8). Outbred, pathogen-free Sprague-Dawley pregnant rats with timed conception (Hilltop Laboratories, Chatsworth, Calif.) gave birth 5 to 7 days after arrival in our vivarium. Each adult rat and her pups (average litter size, 10) were housed in a separate solid polypropylene opaque cage with a filter hood.

A total of 93 newborn rats from 10 litters were used in the primary experiment. At 5 days of age (2 p.m.), all members of each litter were inoculated intraperitoneally with 100 CFU of the C5 strain. As determined in previous experiments (17), this inoculum produces nonlethal bacteremia (with or without meningitis) in 100% of animals within 18 h of inoculation. Eighteen hours after inoculation and daily thereafter for 4 days, 0.1 ml of blood and 0.01 ml of CSF were obtained as described previously (17) for quantitative cultures. Blood was diluted 10-fold and CSF was diluted 100-fold in brain heart infusion broth and further diluted 10-fold in sterile distilled water; 0.1 ml of each dilution was spread on MacConkey agar. The lowest dilution was also incubated overnight at 37°C, and a loopful was streaked on MacConkey agar to detect bacteremia of $<10^2$ CFU/ml and CSF counts of $<10^3$ CFU/ml. Immediately after the first blood and CSF specimens were obtained, each litter was randomly divided into four groups to receive ampicillin, chloramphenicol, a combination of the two, or saline (control). Both drugs were given subcutaneously in a dose of 50 mg/kg, twice daily (9 a.m. and 7 p.m.). Animals that died during therapy were removed and postmortem blood specimens were obtained by cardiac puncture. Blood cultures obtained from animals that died more than 1 h before daily culture were excluded from the analysis of results. CSF specimens could not be obtained in dead animals. Therapeutic efficacy was determined from rates of mortality, bacterial clearance from blood and CSF, and development of meningitis during therapy among the four groups.

Additional experiments were performed with 36 pups from three litters to further delineate the differences between ampicillin and ampicillin plus chloramphenicol. Since experimental procedures were the same as those given above, data from this experiment were combined with those of the preceding experiments.

Both blood and CSF for determination of drug levels were collected from most animals 2 h after antibiotic administration on therapy day 3 and blood alone was collected at 6 h. Serum and CSF antibiotic levels were measured by an agar disk diffusion method, using *Bacillus subtilis* (ATCC 6633) for ampicillin and *Beneckea natrigenes* (ATCC 14048) for chloramphenicol (3).

Statistical methods. The chi-square test (with the Yates correction for small samples) or Student's *t* test was used (9). *P* values of ≤0.05 were considered significant.

RESULTS

In vitro findings. The MIC and MBC of ampicillin were 2 and 4 μg/ml and those of chloramphenicol were 4 and 64 μg/ml for the C5 strain. Checkerboard combinations of ampicillin and chloramphenicol revealed that chloramphenicol interfered with the bactericidal effect of ampicillin. The ampicillin MBC was increased from 4 to 8 to 32 μg/ml in the presence of chloramphenicol concentrations of 2 to 32 μg/ml. The fractional bactericidal concentration index was 2.1, indicating an antagonistic effect.

Figure 1 shows a time-kill study of the C5 strain in the presence of 4 and 40 μg of ampicillin and 16 μg of chloramphenicol per ml, singly and in combination. These antibiotic concentrations were chosen because they approximated the mean serum and CSF levels in vivo 2 h after injection (Table 1). The bactericidal activity of chloramphenicol alone was incomplete and clearly less than that of ampicillin. During the first 4 h, killing by the combinations was less by ≥1 logarithm than with ampicillin alone in the same concentration, but the counts at 24 h were not different between the combinations and ampicillin in the same concentrations.

Pharmacology of ampicillin and chloramphenicol in infant rats. Table 1 summarizes the levels of ampicillin and chloramphenicol in serum and CSF of infected animals. Serum and CSF from untreated healthy and infected animals did not inhibit the assay organisms. The mean serum levels of both drugs were maintained above the respective MICs against the C5 strain for a 6-h period. The mean 2-h CSF levels also

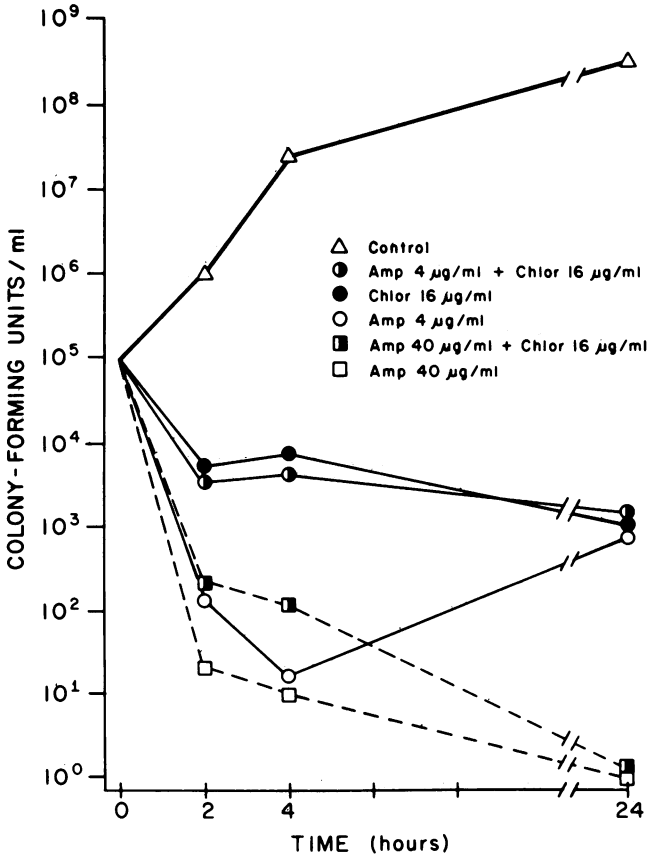

FIG. 1. Time-kill curves of *E. coli* in the presence of ampicillin (4 and 40 μg/ml) and chloramphenicol (16 μg/ml), alone and in combination. Control values are for cultures without antibiotics.

TABLE 1. Serum and CSF levels[a] of ampicillin and chloramphenicol after subcutaneous administration of 50 mg of each antibiotic per kg to 5-day-old newborn rats with *E. coli* bacteremia and meningitis

Antibiotic	Serum level (μg/ml) at:		CSF level (μg/ml) at 2 h	Ratio (%) of CSF serum levels at 2 h
	2 h	6 h		
Ampicillin (n = 14)	41.6 ± 19.1	2.7 ± 1.4	3.2 ± 1.2	7.7
Chloramphenicol (n = 8)	20.0 ± 10.6	4	15.5 ± 1.7	77.5

[a] Expressed as mean ± standard deviation.

exceeded the respective MICs. The mean ratios of CSF/serum levels of ampicillin and chloramphenicol at 2 hours after injection were 7.7 and 77.5%, respectively.

In vivo efficacy of ampicillin and chloramphenicol. *E. coli* bacteremia was present in 129 (100%) and meningitis (positive CSF culture) was present in 45 (35%) animals 18 h after infection and before the beginning of therapy. At this time, the prevalence of meningitis and the bacterial counts in blood and CSF were not significantly different among the four treatment groups (Table 2).

Table 2 compares the overall mortality and bacterial clearance from blood and CSF among the four treatment groups. Overall, antimicrobial therapy significantly decreased the mortality rates (50% of 106 animals receiving antibiotics versus 100% of 23 animals receiving saline; $P < 0.001$). The differences between ampicillin and chloramphenicol were not significant ($P > 0.1$). However, the mortality with ampicillin-plus-chloramphenicol therapy (21%) was significantly less ($P < 0.001$) than that with ampicillin (63%) or chloramphenicol (82%) alone.

TABLE 3. Development of meningitis in bacteremic animals free of meningitis at the beginning of therapy

Therapeutic regimen	No. of animals that developed meningitis during 4 days of therapy/total (%)
Ampicillin	11/23 (48)[a]
Chloramphenicol	13/14 (93)
Ampicillin plus chloramphenicol	5/28 (18)[b]
Saline (control)	5/5 (100)

[a] Significantly less than chloramphenicol ($\chi^2 = 10.1$; $P < 0.01$).
[b] Significantly less than ampicillin ($\chi^2 = 3.96$; $P < 0.05$) or chloramphenicol ($\chi^2 = 18.1$; $P < 0.001$).

The bacterial clearances from blood of the four treatment groups were compared by determining bacterial counts of animals with positive blood cultures at 1, 2, and 3 days of completed therapy (Table 2). The number of animals available for these observations decreased with deaths in every group and this attrition rate varied among the groups. Treatment with ampicillin or with ampicillin plus chloramphenicol promoted bacterial clearance from the blood of surviving animals, whereas bacterial counts in blood increased in animals treated with chloramphenicol or saline. These differences were significant ($P < 0.001$) at 1, 2, and 3 days of completed therapy. With combination therapy the bacterial clearance was significantly greater ($P < 0.01$) and the incidence of bacteremia was significantly less ($P < 0.01$) than with ampicillin alone at the end of 1 day of treatment; later differences between these groups were insignificant.

After 1 day of treatment, relatively few animals with meningitis (positive CSF culture) before therapy were alive for repeated cisternal puncture, but the data suggested more rapid clearance of *E. coli* from CSF in animals receiving combination therapy than in those receiving ampicillin alone

TABLE 2. Comparison of mortality and bacterial clearance in blood and CSF among the four groups of animals treated with ampicillin, chloramphenicol, ampicillin plus chloramphenicol, or saline

Therapy regimen	No. of animals	Treatment days	Deaths	Overall mortality [no. (%)]	Mean bacterial counts ± SD ($\log_{10}$ CFU/ml)[a] in:	
					Blood	CSF
Ampicillin	41	0			4.95 ± 1.18 (41/41)	3.15 ± 2.08 (15/15)
		1	10		3.98 ± 1.60[b] (24/32)[c]	7.28 ± 0.90[d] (7/8)
		2	12		3.45 ± 2.16 (10/20)	1.50 (1/2)
		3	4	26 (63)	3.52 ± 2.50 (4/18)	0 (0/2)
Chloramphenicol	22	0			4.73 ± 1.13 (22/22)	3.18 ± 1.90 (6/6)
		1	7		5.06 ± 1.58 (21/21)	8.30 (1/1)
		2	5		5.17 ± 1.01 (12/12)	NA[e]
		3	6	18 (82)	5.50 ± 1.01 (12/12)	NA
Ampicillin plus chloramphenicol	43	0			4.81 ± 1.48 (43/43)	4.18 ± 2.06 (14/14)
		1	8		2.17 ± 1.03[b] (12/36)[c]	3.81 ± 2.84[d] (2/7)
		2	1		2.78 ± 1.37 (9/34)	4.73 ± 0.74 (2/6)
		3	0	9 (21)[f]	3.25 ± 1.13 (5/34)	4.43 ± 0.12 (2/6)
Saline	23	0			5.25 ± 1.04 (23/23)	4.53 ± 1.44 (10/10)
		1	18		5.39 ± 1.83 (5/5)	NA
		2	4		5.04 (1/1)	NA
		3	1	23 (100)	NA	NA

[a] Numbers in parentheses indicate number of animals positive for culture after completion of treatment day/number of animals positive for culture before therapy and available for subsequent determination of bacterial counts.
[b] Bacterial acounts in blood after 1 day of therapy are significantly less ($P < 0.01$) with ampicillin and chloramphenicol than with ampicillin alone.
[c] The incidence of bacteremia after 1 day of therapy is significantly less ($\chi^2 = 10.1$; $P < 0.01$) with ampicillin and chloramphenicol than with ampicillin alone.
[d] Bacterial counts in CSF after 1 day of therapy are significantly less ($P < 0.02$) with ampicillin and chloramphenicol than with ampicillin alone.
[e] NA, No survivors available.
[f] Significantly less than ampicillin ($\chi^2 = 15.5$; $P < 0.001$) or chloramphenicol ($\chi^2 = 19.7$; $P < 0.001$).

(Table 2). In the former group, two of seven (29%) had positive CSF cultures with bacterial counts (mean ± standard deviation) of 3.81 ± 2.84 $\log_{10}$ CFU/ml; in the latter group, seven of eight (88%) had positive cultures with counts of 7.28 ± 0.90 $\log_{10}$ CFU/ml. The differences in the bacterial counts of these two groups were significant ($P < 0.02$). Only one animal survived in the chloramphenicol group at this time and it had a bacterial count of 8.30 $\log_{10}$ CFU per ml of CSF. Subsequently, there were too few survivors in any groups for valid comparisons.

Table 3 compares the cumulative rates at which meningitis developed in the four treatment groups over the course of the experiments. Among 84 bacteremic animals that were free of meningitis at the beginning of therapy, 70 survived 1 or more days of treatment and were available for this comparison. All such animals in the control group developed meningitis. This complication developed significantly more often in animals on chloramphenicol than in those on ampicillin therapy ($P < 0.01$). Combination therapy appeared to be significantly more effective in preventing meningitis than did single therapy with ampicillin ($P < 0.05$) or chloramphenicol ($P < 0.001$).

DISCUSSION

The clinical use of some antimicrobial combinations in serious infections can be supported by the demonstration that they are synergistic in vitro against the infecting bacteria. However, careful studies comparing the in vitro and in vivo effects of drug combinations have been limited (11). The activity of combinations of penicillin and chloramphenicol is a subject of considerable debate. In vitro, such combinations have been shown to be antagonistic, indifferent, or synergistic against several gram-negative and gram-positive organisms (6, 12, 14, 20, 26, 30). The in vivo effects of such combinations in experimental infections have been related to drug administration: antagonism occurred when chloramphenicol was given before but not after penicillin administration (15, 29).

The present study was undertaken to evaluate and compare the activities of ampicillin, chloramphenicol, and a combination of the two in vitro and in vivo against an *E. coli* K1 strain. The results document a surprising paradox between the in vitro and in vivo effects of ampicillin and chloramphenicol against *E. coli*. In vitro, the combination appeared to be antagonistic (by checkerboard determinations of MBCs, fractional bactericidal concentration index, and 4-h killing rates) or indifferent (by 24-h killing studies). However, when ampicillin and chloramphenicol were given simultaneously, the combination was clearly beneficial in vivo and significantly more effective than either drug alone. Significant differences were noted in survival, bacterial clearance from the blood and CSF, and prevention of the development of meningitis during therapy. The levels of ampicillin and chloramphenicol obtained in the blood and CSF specimens exceeded the respective MICs against the infecting strain and were comparable to those seen in patients on therapy (10, 16, 27).

The finding that both normal and infected human CSF lack measurable opsonic and bactericidal activity against the common pathogens in bacterial meningitis (24, 28) suggests a need for bactericidal antimicrobial regimens. In this study, only the combination of ampicillin and chloramphenicol reduced the *E. coli* counts in the CSF after 1 day of therapy, whereas the bacterial counts in the CSF actually increased after 1 day of ampicillin or chloramphenicol therapy. These findings are quite different from those of Beam and Allen (4), who reported significant reduction of *E. coli* counts in the CSF of rabbits by treatment with ampicillin or chloramphenicol. However, their results were based on a brief period of observation and continuous infusion with much higher doses of antibiotics in adult rabbits infected by direct instillation of bacteria in CSF. The rabbit model differs in several respects from disease in the infant rat and in humans, including spontaneous resolution of infection in some rabbits (4).

The reasons for a discrepancy between in vitro and in vivo findings are not completely understood. Host factors may interact in vivo with antimicrobial agents and microorganisms. Our preliminary studies have suggested a possible role of a heat-labile serum factor(s). Studies are in progress to further understand the interactions of serum factors with antimicrobial agents.

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