Potentialiation of Antibiotic Bactericidal Activity by Normal Human Serum

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Combinations of certain antibiotics and normal human serum at concentrations at which there was no killing by the agents when used alone were found to be bactericidal for *Escherichia coli* K-12 cells. This effect was observed with tetracycline, streptomycin (SM), trimethoprim, and ampicillin, but not with chloramphenicol or nalidixic acid. Synergy between SM and human serum was also observed against four of nine smooth strains of *E. coli*. A plasmid-bearing strain of *E. coli* K-12 was also killed by combinations of tetracycline or SM plus serum, even though the plasmid conferred resistance to tetracycline and SM. Evidence is presented that the synergy between antibiotics and serum is due to a complement-mediated effect on the bacterial cells that makes the cells more susceptible to the bactericidal effects of the antibiotics.

Measurement of the bactericidal effects of antibiotics in human serum as devised by Schlichter and MacLean (23) and modified by Jawetz (12) and many others has been performed for years, yet there have been only a few reports concerning the bactericidal effects of combinations of antibiotics and serum. It is well established that polymyxin B and serum, both of which have direct effects on the envelope of gram-negative cells, are synergistic (19, 27) or additive (26) when used in combination. However, there have been less consistent reports about other antibiotics. Chloramphenicol and serum have been reported to have an additive effect in killing *Salmonella typhosa* (28), whereas both chloramphenicol and tetracycline have been found to interfere (7, 8, 15, 30) with serum in killing of *Escherichia coli*. Ampicillin, though its action is directed against the bacterial cell wall, has been reported (27) to have no effect on serum bactericidal action. Kanamycin (27) had either no effect or an additive effect on serum bactericidal activity, depending on experimental conditions.

The paucity of reports on the interactions between antibiotics and serum is, in part, the result of many tests being run with heat-inactivated serum, thereby precluding any possible combined activity. Furthermore, many tests do not include the controls of antibiotic alone and serum alone that are necessary for demonstration of synergy or interference with combinations. The lack of consistency in results is probably due to the large number of experimental

variables which must be manipulated in experiments with combinations of antibiotics and serum.

As part of a program (20; A. M. Reynard and B. S. Dutcher [Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, New York, Abstr. no. 304, 1977]) to investigate the various factors that affect the killing of bacterial cells, experiments are described here in which conditions were arranged to permit the possibility of observing interactions between serum and antibiotics. Several antibiotics, including streptomycin and tetracycline, were found to be synergistic with normal human serum in the killing of various strains of *E. coli*, including a plasmid-bearing strain which was resistant to streptomycin and tetracycline.

(A preliminary report of some of these findings has been presented [Reynard and Dutcher, 17th ICAAC, Abstr. no. 304].)

MATERIALS AND METHODS

Reagents. Tetracycline hydrochloride (TC), streptomycin sulfate (SM), and puromycin dihydrochloride (PU) were purchased from Nutritional Biochemicals Corp.; actinomycin D (AM-D) was purchased from Sigma Chemical Co.; and chloramphenicol (CM) and rifampin (RI) were purchased from Calbiochem. Ampicillin trihydrate (AM) was a gift from Bristol Laboratories. Nalidixic acid (NA) was a gift from Sterling Winthrop Research Institute. Trimethoprim (TM) was a gift from Burroughs Wellcome Research Laboratories. *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from

ICN Pharmaceuticals, Inc. Penassay agar, Penassay broth, and MacConkey agar were purchased from Difco. Serum was collected from normal human volunteers, pooled, and kept at -70°C until used. The volunteers had taken no drugs for at least a week prior to collection of the serum.

Bacterial strains and plasmids. *E. coli* J6-2 (F^{-} , *pro*, *try*, *his*, *lac*) was provided by J. T. Smith; *E. coli* J6-2R100 was from the laboratory collection. The plasmid, R100, provides resistance to TC, SM, CM, and sulfonamides. *E. coli* CSH-2 (F^{-} , *trp*, *lacZ*, *strA*, *thi*) came from Cold Spring Harbor Laboratories. Smooth strains of *E. coli* were kindly provided by Betty Davis, Center for Disease Control, Atlanta, Ga, and Konrad Wicher, E. J. Meyer Memorial Hospital, Buffalo, N. Y.

Serum bactericidal assay for rough strains. The bactericidal assay used was similar to that of Taylor (25) except for the use of BES and HEPES to avoid the metal ion interaction and other difficulties encountered with tris(hydroxymethyl)aminomethane (Tris) buffer. Cells grown to exponential phase in Penassay broth were washed three times with 0.05 M NaCl and resuspended in 0.05 M NaCl to 9×10^6 cells per ml. The incubation mixture consisted of 0.5 ml of cell suspension; 0.25 ml of 0.1 M BES, pH 8.4, or 0.25 ml of 0.1 M HEPES, pH 7.4; 0.36 ml of 0.36 M NaCl; 0.25 ml of human serum diluted in 0.05 M NaCl; and water to a final volume of 2 ml. The use of broth was avoided because of the anti-complementary effects of broth components (15). Additions to the incubation mixture were prepared in water, except where specified. No alteration of the antibody content of the serum was made. The reaction mixtures were incubated at 37°C for 2 h by which time bactericidal effects would be observed even with strains which had delayed susceptibility (24). Samples taken at 0 and 2 h were diluted in Penassay broth and plated on Penassay agar for overnight incubation at 37°C . Percent survival was calculated as follows:

$$\text{Percent survival} = \frac{\text{Colonies at 2 h}}{\text{Colonies at 0 h}} \times 100$$

Serum bactericidal assay for smooth strains. Since smooth strains are often more resistant than rough strains to the bactericidal effects of serum (22, 24), the incubation mixture was modified. The procedures were identical except for the incubation mixture as follows. Washed cells were resuspended in 0.05 M NaCl to 1.2×10^7 cells per ml. The reaction mixture consisted of 0.1 ml of cell suspension; 0.6 ml of undiluted human serum; and 0.1 ml of streptomycin in 0.1 M HEPES, pH 7.4. When the serum was omitted, it was replaced by 0.6 ml of 0.18 M NaCl, and when the streptomycin was omitted, it was replaced by 0.1 ml of 0.1 M HEPES, pH 7.4.

Complement hemolytic assays. Total hemolytic complement assay was essentially as described by Levine (14). Briefly, washed sheep erythrocytes were sensitized with an optimal dilution of rabbit hemolytic antibody (amboceptor) and made to 5×10^7 cells per ml in isotonic Tris buffer, pH 7.4. These cells were used to estimate the dilution of serum that contained 1 50% hemolytic complement unit for the human serum pool used in these experiments.

To evaluate the effect of tetracycline and streptomycin on total hemolytic complement activity, a dilution of serum that contained 1 50% hemolytic complement unit in 1 ml was added to 4 ml of Tris buffer followed by 1 ml of antibiotic solution. Both antibiotics were tested at 50 and 5 $\mu\text{g}/\text{ml}$. After antibiotic and serum were incubated for 30 min at room temperature, 1 ml of sensitized sheep erythrocytes was added, and the tubes were incubated for an additional 60 min at 37°C . At the end of incubation, the tubes were chilled to 4°C for 5 min and centrifuged to sediment ghosts and lysed cells. Each supernatant fluid was then measured for optical density at 413 nm. Controls were run similarly to correct for contribution to the optical density by the antibiotic.

RESULTS

Bactericidal activity of human serum and SM or TC on *E. coli* K-12. The results of experiments in which cells of *E. coli* J6-2 were incubated with human serum (final dilution, 1:320) and several concentrations of TC or SM are shown in Fig. 1. Incubation of cells with serum alone or antibiotic alone produced no bactericidal effect. However, there was a marked decrease in percent survival of the cells when

FIG. 1. Bactericidal activity of human serum plus SM or TC on *E. coli* K-12. Bar at left is result of experiments at pH 7.4 in which cells were incubated with human serum alone (cross-hatched). Other pairs of bars represent incubations with antibiotic alone (dotted) or with antibiotic plus serum (striped). Vertical lines on tops of bars represent the standard error of the mean. All incubations were for 2 h at 37°C .

the cells were incubated with both antibiotic and serum. In all cases, the percent survival with antibiotic plus serum was different from antibiotic alone at the 0.05 level of significance as estimated by the two-tailed Student's *t* test. These results show that there is synergism between human serum and TC or SM in the killing of *E. coli* K-12. SM was more potent than TC in producing a bactericidal effect in the presence of serum. Serum complement, inactivated by heating at 56°C for 30 min, had no bactericidal effect when combined with antibiotic at 50 µg/ml (data not shown), suggesting that the synergism between antibiotic and serum is complement dependent.

E. coli J6-2R100 is strain J6-2 bearing the plasmid R100, which confers resistance to both TC and SM. In the presence of serum, both TC and SM exhibited marked bactericidal effects on J6-2R100 (Fig. 1). TC alone or SM alone had no bactericidal effect on J6-2R100. Thus, the antibiotic resistance of this strain can be overcome by exposing the cells to antibiotic in the presence of serum.

In experiments performed at pH 8.4, there was greater bactericidal activity than at 7.4. This was probably due to the increased activity of complement at pH 8.4, although certain antibiotics, such as SM, have increased activity at pH 8.4. With both TC and SM, bactericidal activity in combination with serum was dependent on the concentration of antibiotic. For example, in experiments performed at pH 8.4 with TC at concentrations of 0, 0.4, 2, 5, 10, and 50 µg/ml, there was 71, 36, 23, 16, 8, and 2% survival, respectively.

The bactericidal activity of the combination of serum plus antibiotic also depends on the dilution of the serum. This was demonstrated at pH 7.4, where serum alone at 1:240 produced 75% survival, and at dilutions of 1:320 (and greater), serum alone exhibited no bactericidal activity. With the addition of SM at 2 µg/ml, there was 2, 4, 6, 36, and 58% survival at serum dilutions of 1:240, 1:320, 1:480, 1:640, and 1:800, respectively.

Potentiation of bactericidal activity of SM by human serum with smooth strains of *E. coli*. Bactericidal assays were performed with nine smooth strains of *E. coli*, using SM (5 µg/ml) alone, human serum (final dilution, 3:4) alone, and both together. All nine strains were resistant to human serum alone and were not killed by SM at 5 µg/ml alone. With four of the nine strains, there was an appreciable bactericidal activity with human serum plus SM (percent survival ranged from 0 to 70). With the other five strains, there was no bactericidal activity with the combination of human serum

plus SM. Of the four strains in which potentiation was demonstrated, two were serotype O75 and two were serotype O1.

Bactericidal activity of human serum plus various antibiotics. The results of experiments in which cells of *E. coli* J6-2 were incubated with human serum (final dilution, 1:320) and various antibiotics are shown in Fig. 2. There was no bactericidal effect of serum alone (see Fig. 1). When AM (50 µg/ml) was combined with serum, there was a marked increase in bactericidal activity. With PU and AM-D, there were slight increases in bactericidal activity, but only at concentrations that produced bactericidal activity of the agents alone. With AM (5 µg/ml), CM (50 µg/ml), and NA (50 µg/ml), there was no significant increase in bactericidal activity. It proved to be impossible to test RI in this system because of strong binding of the drug to serum. At concentrations of RI that were bactericidal when the drug was added alone, the addition of fresh serum at a final dilution of 1:320 prevented bactericidal activity.

For comparison, the minimum bactericidal concentration for each antibiotic was determined under the same conditions used for determination of potentiation by serum but with no added serum. For purposes of this study, the minimum bactericidal concentration was determined as the concentration of antibiotic which resulted in less than 30% survival of the original inoculum. The range of concentrations used was 25, 50, 100, 200, 500, and 1,000 µg/ml. The minimum bactericidal concentrations were (in

FIG. 2. Bactericidal activity of human serum plus various antibiotics with *E. coli* J6-2. Pairs of bars represent experiments at pH 7.4 in which cells were incubated with antibiotic alone (dotted) or with antibiotic plus serum (striped). Vertical lines on top of bars represent the standard error of the mean. All incubations were for 2 h at 37°C.

micrograms per milliliter): 25 for SM, 200 for TC, 100 for AM, >500 for PU, 50 for TM, 200 for AM-D, >1,000 for CM, and 500 for NA. It can be seen that for certain antibiotics such as SM, TC, AM, and TM, there is significant bactericidal activity at concentrations of 200 $\mu\text{g}/\text{ml}$ or less. For PU and NA, on the other hand, there was appreciable bactericidal activity only at 500 $\mu\text{g}/\text{ml}$ or greater, and for CM, bactericidal activity could not be found even at 1,000 $\mu\text{g}/\text{ml}$.

Bactericidal activity of serum plus antibiotic with *E. coli* CSH-2. *E. coli* CSH-2 is a high-level, SM-resistant mutant of *E. coli* K-12. Resistance is due to a ribosomal alteration which produces resistance to SM at >1,000 $\mu\text{g}/\text{ml}$ but no resistance to TC. Table 1 shows that SM at 50 $\mu\text{g}/\text{ml}$ had no effect either alone or in combination with human serum on CSH-2. In contrast, other experiments (data not shown) had demonstrated that against the SM-susceptible strain, J6-2, SM alone at 50 $\mu\text{g}/\text{ml}$ produced considerable killing and in combination with human serum gave less than 0.1% survival. With CSH-2 (Table 1), there was no bactericidal activity of TC alone at 50 $\mu\text{g}/\text{ml}$, but the addition of human serum reduced survival to 6%. These results demonstrate that inhibition of protein synthesis by SM is necessary for the synergism of SM with human serum.

Effects of antibiotics on hemolytic complement activity. To test the possibility that SM and TC affect complement activity directly, thereby increasing the bactericidal activity of complement, antibiotic and a dilution of human serum containing 1 50% hemolytic complement unit were incubated for 30 min. Amboceptor-sensitized sheep erythrocytes were then added and incubated for a further 60 min. Neither SM nor TC had any effect on complement-mediated lysis of sheep erythrocytes at either 2.5 or 50 $\mu\text{g}/\text{ml}$.

Time course of bactericidal effects of serum and SM. Although neither serum nor SM alone exhibited bactericidal activity at the con-

centrations used, there was a marked bactericidal effect when they were used together (Fig. 3). With serum and SM combined, there was a delay of 15 to 30 min before a significant bactericidal effect was observed, after which there was a rapid loss of viable cells. The experiments illustrated in Fig. 4 were designed to determine whether human serum and the antibiotic acted in concert or sequentially. The lag period previously observed (Fig. 3) suggested that one agent may act prior to the other to produce the augmented killing. In these experiments, the cells were preincubated with SM for 30 min prior to addition of the serum or were preincubated with serum for 30 min prior to addition of the SM. Preincubation with SM followed by addition of serum resulted in very little killing of the cells during a 30-min incubation. In contrast, preincubation with serum followed by addition of SM produced a significant killing even by 15 min. These results indicate that serum treatment prepares the cells for antibiotic killing, whereas antibiotic treatment has little effect on killing when serum is added later.

DISCUSSION

Incubation of *E. coli* K-12 (a rough strain) with normal human serum results in rapid killing of the cells, but if the serum is diluted suffi-

TABLE 1. Bactericidal activity of serum plus SM or TC with *E. coli* CSH-2

Addition ^a	% Survival ^b
None	91 $\pm$ 2
HS	77 $\pm$ 11
SM	100 $\pm$ 17
TC	136 $\pm$ 21
HS + SM	92 $\pm$ 1
HS + TC	6 $\pm$ 2

^a Human serum (HS) was at a dilution of 1:400. SM and TC were at a final concentration of 50 $\mu\text{g}/\text{ml}$. Experiments were performed at pH 8.4.

^b Results are means and standard errors of means.

FIG. 3. Time course of bactericidal effect of serum and SM. At time zero, serum (circles), SM (triangles), or serum plus SM (squares) was added to the cell suspension. Concentration of SM was 2.5 $\mu\text{g}/\text{ml}$, and serum was diluted 1:320 in the incubation mixture. Incubations were carried out at pH 8.4, using *E. coli* J6-2, at 37°C.

FIG. 4. Time course of bactericidal effect of serum and SM. Cells were preincubated with SM for 30 min prior to addition of the serum (circles) or were preincubated with serum for 30 min prior to addition of the SM (squares). Time zero was taken as the time of addition of the second component. Concentrations of streptomycin and serum and other conditions were the same as in Fig. 3.

ciently, there is no observable killing. Similarly, many antibiotics that are bactericidal at high concentrations are bacteriostatic but do not kill when diluted. We report here results of experiments which show that *E. coli* K-12 cells are efficiently killed by combinations of human serum and antibiotic at concentrations which, by themselves, produce no bactericidal activity. The participation of complement in this synergistic action is suggested by the lack of bactericidal effect when the same serum dilution is heated at 56°C for 30 min. Furthermore, either Mg ethyleneglycol-bis (β -aminoethyl ether)-*N,N*-tetraacetic acid or anti-C4 completely block killing by the combination of antibiotic and diluted serum (unpublished observations).

Bactericidal action of the combination depends on both the antibiotic concentration and serum dilution. Although 200 μ g of TC per ml is required for bactericidal activity when used alone, as little as 0.4 μ g of TC per ml produced bactericidal activity in combination with serum. Similarly, significant bactericidal activity of serum alone was not observed at dilutions greater than 1:160, but in the presence of SM (2 μ g/ml), killing occurred at dilutions as high as 1:800. The concentrations of TC or SM at which synergy is observed are thus in the range of therapeutic serum concentrations.

Smooth strains of *E. coli* are relatively resistant to the bactericidal activity of normal serum (22, 24). Of nine smooth strains that were completely resistant to normal human serum (final serum dilution of 3:4), four became susceptible to bactericidal activity when SM (5 μ g/ml) was added, although SM alone at this concentration had almost no bactericidal effect.

Bactericidal activity of SM or TC plus serum was also measured with *E. coli* CSH-2, a high-level, SM-resistant mutant of K-12. SM at 50 μ g/ml with or without serum exhibited no bactericidal effect on CSH-2, whereas TC plus serum was bactericidal. SM resistance by CSH-2 is due to a ribosomal alteration that prevents inhibition of protein synthesis by SM at concentrations less than 1,000 μ g/ml, while not affecting the action of TC. These experiments demonstrated that the bactericidal activity of SM plus serum depends on inhibition of protein synthesis by SM and eliminate the possibility that such activity is due to membrane damage by SM. They also suggest that SM does not increase the bactericidal activity of complement.

Other antibiotics such as AM and TM also exhibit bactericidal activity when used in combination with human serum. PU and AM-D have some bactericidal activity when combined with serum, but only at concentrations at which they show some bactericidal activity when used alone. A bactericidal effect could not be demonstrated for either CM or NA in combination with human serum at concentrations up to 50 μ g/ml.

The outer membrane of gram-negative organisms is a partial permeability barrier. Decad and Nikaido (3) showed that saccharides with molecular weights greater than approximately 600 are excluded. When the outer membrane is disrupted by ethylenediaminetetraacetic acid treatment, the penetration of various molecules, including some antibiotics, increases (13, 31). It seems likely that serum, when used in combination with certain antibiotics, acts to disrupt the permeability barrier of the outer membrane, thereby increasing the intracellular concentration of antibiotic to a bactericidal level. This interpretation is supported by the demonstration that TM, TC, and SM were bactericidal alone at concentrations of 50, 200, and 50 μ g/ml, respectively. CM was not potentiated by serum and was not bactericidal alone, even at 1,000 μ g/ml, whereas NA was bactericidal only at 500 μ g/ml. This is also consistent with the observation that the outer membrane is the site of initial attack by complement (5, 6, 32). An energy-dependent uptake in *E. coli* has been described for both TC (1, 4, 9, 21) and SM (2), presumably located at the plasma membrane. It is possible

that a complement-mediated reduction in the permeability barrier of the outer membrane results in presentation of the antibiotics to the plasma membrane in increased concentration.

This interpretation is also supported by measurement of the time course of killing. When SM and serum were added together, 15 to 30 min were required before killing began. When cells were preincubated with SM for 30 min and then serum was added, there was also a 30-min delay after serum addition before killing. However, when the cells were preincubated with serum and then SM was added, killing began promptly. These results suggest that the serum was affecting the cells in a way that made them more susceptible to SM. If this is correct, then synergism as demonstrated here represents an action of complement on the bacterial cell envelope which may be a preliminary to the killing event.

The possibility that TC and SM act by enhancing the effects of complement was tested by incubating antibiotic with serum and then measuring the lytic power of the serum on sensitized sheep erythrocytes. Neither TC nor SM had an effect on lysis of sheep erythrocytes by serum, suggesting that the antibiotic had no effect on classical complement activity. Furthermore, the alternative pathway of complement activity is affected by dilution such that at the high dilution of serum used here, alternative pathway activity is essentially eliminated, and hence an effect of antibiotic to potentiate the alternative pathway directly seems unlikely.

It is also possible that high dilutions of serum produce lesions in the cell envelope which are repaired rapidly enough to avoid cell death, but that a repair is inhibited by TC and SM. The fact that CM is not synergistic with serum militates against this possibility. There are differential effects of antibiotics on bacterial protein synthesis (10), and it may be, though it appears unlikely, that TC and SM affect a repair mechanism and CM does not. The fact that preincubation of cells with SM did not prepare the cells for serum also makes this possibility unlikely.

The bactericidal effects of TC or SM in combination with serum occurred with both antibiotic-susceptible and -resistant strains of *E. coli* K-12. With *E. coli* J6-2R100 (minimum inhibitory concentrations for both TC and SM, about 250 µg/ml), there was marked bactericidal activity with TC or SM at 50 µg/ml when human serum was present. The mechanisms of resistance to TC and SM mediated by R100 are quite different. Resistance to TC is due to inhibition of uptake of the drug by the bacterial cells (4, 9, 11, 21). Resistance to SM is due to enzymatic inactivation of the drug (17, 29). In both cases, the effect is to decrease the concentration of

drug at its target site, the 30S ribosomal subunit. Because the mechanisms of resistance are so different, it seems unlikely that serum overcomes resistance by a direct effect on the resistance mechanisms. A possible explanation, analogous to that offered for the susceptible strains, is that resistance is by-passed by a decrease in permeability due to complement action. The observed synergy between serum and certain antibiotics reported here is contrary to other reports (7, 8, 30), but variations in the test methods may account for this. For example, Traub and Sherris (27) found no synergism and some antagonism between AM or kanamycin and serum.

Their tests differed from ours in that serum dilutions of 1:2 and 1:10 in nutrient broth were used. Too little is known about the effects of broth to comment, but serum at those relatively low dilutions produced considerable bactericidal effect alone, perhaps making it more difficult to observe an effect of the antibiotic. In addition, serum proteins at these dilutions appeared to bind the drugs. The use of extremely serum-susceptible rough strains in the experiments reported here may have helped avoid some of these effects. Nevertheless, synergy was demonstrated with four of nine smooth strains at a serum dilution of 3:4. Reports of interference with serum bactericidal action by TC (7, 8) are less easy to evaluate, although bacterial strain differences and serum dilutions may play a role. Pien and Vosti (18) have recently pointed out the marked variability of ways in which bactericidal tests are carried out in the clinical laboratory. More than 30 years after the first description of a serum bactericidal test for antibiotic effectiveness (23), it is obvious that there is still the need to standardize such tests and perform them in a way which will provide the best correlation with clinical effectiveness of the drug.

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