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Increased Phagocytosis and Killing of *Escherichia coli* Treated with Subinhibitory Concentrations of Cefamandole and Gentamicin in Isolated Rat Livers

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Our purpose was to study whether treatment of *Escherichia coli* with subinhibitory concentrations of either cefamandole or gentamicin could change bacterial susceptibility to the serum bactericidal effect and to the phagocytic and killing activity of the rat liver reticuloendothelial system. Bacteria were grown overnight with 1/5 or 1/10 of the MIC of each antibiotic. At one-fifth of the MIC, cefamandole induced filamentous elongated bacteria whose viability was decreased by 75%. The susceptibility of control and antibiotic-treated bacteria to serum was tested by measuring the survival of organisms exposed to different concentrations of rat serum in vitro. Susceptibility of bacteria to hepatic macrophage activity was tested by following the hepatic clearance of bacteria after they were added to the perfusate of the isolated rat liver. *E. coli* treated with subinhibitory concentrations of cefamandole or gentamicin appeared somewhat more resistant to the lytic activity of serum at a concentration of 4%, but not at 20%. Bacteria treated with 1/5 or 1/10 of the MIC of cefamandole or with 1/5 of the MIC of gentamicin were significantly more susceptible to phagocytosis and to the bactericidal activity of liver macrophages. Cefamandole appeared more potent than gentamicin in inducing these effects. The results suggest that subinhibitory levels of antibiotics may alter bacterial cell surface (cefamandole) or may impair the expression of antiphagocytic material (gentamicin), thus favoring phagocytosis and killing by macrophages. Our study provides evidence that antibiotics at subinhibitory concentrations may cooperate with host defence mechanisms against bacterial infections.

It has been demonstrated that antibiotics at concentrations below the MIC, here referred to as subinhibitory concentrations (sub-MICs), are still capable of altering bacterial form, growth rate, and adhesiveness to mucosal surfaces (7, 11, 12, 20). Based on this evidence, it has been suggested that sub-MICs of antibiotics may also determine changes of bacteria invasiveness (4, 18). To establish whether sub-MICs of antibiotics have some effect on the course of infection, it appears necessary to verify first whether they also alter bacterial susceptibility to serum factors and to phagocytic cell activity, which play a major role in eradicating the infection. Data on these questions are so far contradictory since they show either a greater or a lower susceptibility of sub-MIC-treated bacteria to serum bactericidal activity or to phagocytic cell activity as compared with untreated microorganisms (2, 8, 9, 13).

Accordingly, this work was designed to study whether pretreatment of *Escherichia coli* with sub-MICs of cefamandole, a β-lactam antibiotic, or gentamicin, an aminoglycoside, alters its susceptibility to serum factors and to the phagocytic and bactericidal activities of macrophages. These two antibiotics were chosen because they are widely used in clinical practice and have different antibacterial mechanisms. For a better-controlled approach, we employed isolated and perfused rat liver. This system afforded the advantages of allowing us to study the activity of hepatic macrophages, which play a crucial role in defence against gram-negative bacteria (16), and to evaluate separately the effects of cellular and humoral factors in bacterial clearance by the liver (5, 10, 15).

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MATERIALS AND METHODS

Chemicals. Cefamandole nafate, lot P67888, was kindly provided by Eli Lilly & Co., Indianapolis, Ind., and gentamicin sulfate was purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade and were obtained from commercial sources.

Bacteria. *E. coli* ATCC 25922 was used as the test organism. The strain was maintained on tryptic soy agar (Difco Laboratories) slants at 4°C after overnight growth at 37°C and was subcultured weekly.

Determination of MIC. The MICs for cefamandole and gentamicin versus *E. coli* were determined by a microtiter broth dilution method with serial twofold dilutions of the antibiotic in brain heart infusion (Difco) inoculated with 10^5 *E. coli* cells (from a 4-h broth culture) per 0.1 ml. Microtiter plates were incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of antibiotic that resulted in complete inhibition of bacterial growth. Under these circumstances, the MIC of cefamandole for the test organism was 0.5 μg/ml, and the MIC of gentamicin was 1 μg/ml.

Conditions of growth of bacteria. *E. coli* cells were inoculated in 50 ml of control brain heart infusion or brain heart infusion containing either 1/5 or 1/10 of the MIC of the antibiotic used. After 18 h of growth, bacteria were harvested, washed twice, and suspended in phosphate-buffered saline at pH 7.4. The bacterial concentration was adjusted

photometrically at 660 nm by using a Klett-Summerson colorimeter (Klett Manufacturing Co., New York, N.Y.) to a Klett value of 200, which corresponded to 2×10^9 CFU/ml in control cultures. The viability of cultures with or without the antibiotic was determined by plate counts of appropriate dilutions in triplicate on tryptic soy agar plates. Alteration of bacterial form was evaluated by examining Gram-stained smears of bacterial suspensions from either control or antibiotic-containing broths.

Blood serum. Homologous rat serum was obtained by sterile cardiac puncture under ether anesthesia. Serum samples were then pooled and stored in aliquots at −80°C in plastic tubes. Serum pools were thawed by placing them in a water bath at 37°C; the serum was then immediately used for experiments.

Liver perfusion technique. Livers were isolated from male Sprague-Dawley rats, 250 to 300 g (S. Morini Co., S. Polo D'Enza, R.E., Italy) and perfused as previously described (10). Perfusing medium consisted of 100 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 200 mg of glucose, 3 g of bovine serum albumin (Sigma), and 4% homologous serum. Perfusions were run under sterile conditions in a perfusion chamber at 37°C.

Susceptibility of bacteria to serum factors and to hepatic phagocytes. For each experiment, the susceptibility of the test inoculum to the serum present in the perfusate was assessed separately by measuring bacterial survival after exposure in vitro under continuous mixing to the perfusate alone containing serum at a 4% concentration. All other experimental conditions were similar to those used for liver perfusions. In other experiments, the effects of rat serum on bacterial survival at both 1 and 20% concentrations in the Krebs-Ringer bicarbonate-albumin buffer were also evaluated.

In perfusion experiments, after an initial 15-min equilibration, 1 ml of the *E. coli* suspension from either control or antibiotic-containing cultures was added to the perfusate. Perfusions were then prolonged for 30 min at a perfusate rate of 3 ml/min per g of liver. Samples (0.5 ml each) of perfusate were taken at 0, 10, 20, and 30 min after the addition of bacteria to the medium. At the end of perfusion, the liver was washed with sterile saline to remove bacteria trapped intrasinusoidally, and the effluent was kept at 4°C. Then the liver was weighed, and a sample of approximately 3 g was excised and homogenized in sterile 10% (wt/vol) saline for 2 min at 21,000 rpm in a blender homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). Samples of perfusate, liver homogenate, and effluent wash were sonicated at 21 kc/s at 4°C for 20 s, diluted, and plated in triplicate on tryptic soy agar for determination of bacterial concentrations.

For liver perfusions, the phagocytic index (K) was calculated according to the formula $K = (\log C_0 - \log C_1)/(t_1 - t_0)$, where C_0 and C_1 are the concentrations of bacteria in the perfusate at times t_0 and t_1, respectively (3). At the end of perfusion, the phagocytic activity of hepatic macrophages was calculated by subtracting the amount of bacteria recovered in both the perfusate and the effluent wash at 30 min from the amount inoculated at time zero. The value was then corrected for the bactericidal activity of the serum-containing perfusate. The amount of phagocytized bacteria that could not be recovered in the liver at the end of perfusion was considered to represent the amount of bacteria killed by Kupffer cells after phagocytosis (15). Both phagocytosis and killing are expressed as percentages of the initial inoculum.

Statistics. The student t test was used to compare means, and a level of 5% was taken to be significant (17).

RESULTS

Bacterial form and growth. The growth in the presence of one-fifth of the MIC of cefamandole produced filamentous, elongated forms of *E. coli*. No morphological changes were observed under light microscopy when bacteria were grown in either 1/10 of the MIC of cefamandole or in presence of 1/5 or 1/10 of the MIC of gentamicin.

When bacteria from either control or antibiotic-containing cultures were adjusted photometrically to the same density, bacterial viability was decreased by 75% in the group of cultures grown in one-fifth of the MIC of cefamandole as compared with the control group. No changes in viability were observed in the groups of bacteria grown with 1/10 of the MIC of cefamandole or in the presence of sub-MICs of gentamicin. Several colonies isolated from cultures of bacteria grown in the presence of sub-MICs of either antibiotic were tested again for MIC. In all cases, the MIC of the antibiotic tested was the same as that previously obtained for control bacteria.

Susceptibility to serum factors. The bactericidal effects of the serum at different concentrations against untreated and sub-MIC-treated *E. coli* are shown in Table 1. In general, we found that the bactericidal activity of rat serum increased with serum concentration. The in vitro incubation of the test inoculum in serum at a 4% concentration showed that bacterial recovery after 30 min was 89 ± 2% for untreated *E. coli* and 112 ± 4% for cells grown in the presence of one-fifth of the MIC of cefamandole ($P < 0.001$), suggesting an increased resistance of treated bacteria to serum bacteriolysis. This phenomenon was also observed for gentamicin-treated bacteria exposed to 4% serum. With serum concentrations of 1 and 20%, no difference in susceptibility to serum was observed between treated and control cells.

Susceptibility to hepatic phagocytes. In perfusion experiments, we used a serum concentration of 4% in the circulating medium since this concentration has little or no direct bactericidal activity (Table 1), yet it was found to ensure an adequate opsonization of bacteria (15, 16, 19). Thus, by minimizing the extracellular interference of serum, the disappearance of bacteria from the perfusate was found to be primarily due to the activity of hepatic macrophages per se.

The addition of 1 ml of the standardized bacterial suspension to the circulating medium of the isolated rat liver preparation provided an initial bacterial concentration of 2×10^7 CFU/ml in all experiments, except in those in which *E. coli* exposed to one-fifth of the MIC of cefamandole was used. In these latter experiments, the viability of the inoculum was only 25% of that predicted by turbidity, and therefore, the initial viable count was 5×10^6 CFU/ml.

TABLE 1. Percentage of survival of *E. coli* treated with sub-MICs of cefamandole or gentamicin after in vitro incubation in rat serum for 30 min.

Antibiotic	Fraction of MIC	% Survival (mean ± SE) at serum concn[a]: 1%	4%	20%
None		100 ± 2 (4)	89 ± 2 (11)	74 ± 3 (7)
Cefamandole	1/5	104 ± 3 (4)	112 ± 4[b] (8)	80 ± 3 (4)
	1/10	101 ± 4 (4)	82 ± 5 (6)	80 ± 6 (4)
Gentamicin	1/5	100 ± 2 (4)	106 ± 3[b] (6)	70 ± 2 (4)
	1/10	98 ± 3 (4)	101 ± 4[b] (4)	78 ± 3 (4)

[a] Numbers of experiments are given in parentheses.
[b] $P < 0.001$ versus controls.
[c] $P < 0.05$ versus controls.

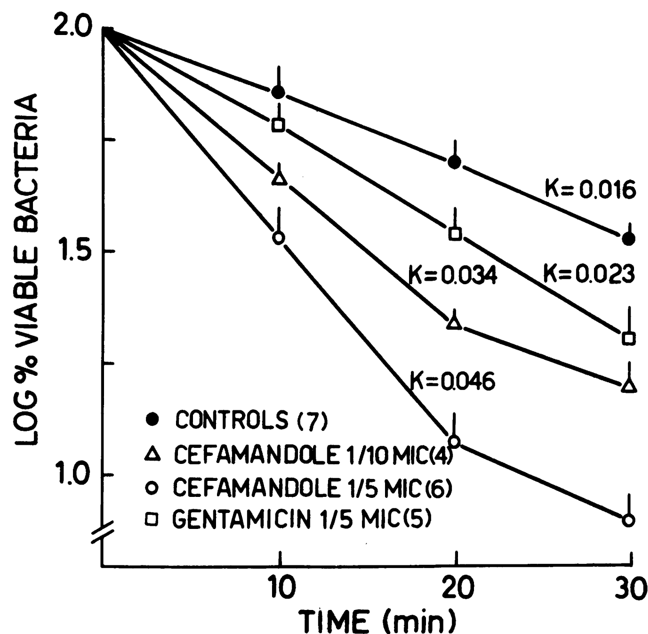

FIG. 1. Kinetics of disappearance of control and sub-MIC-treated *E. coli* from medium during isolated rat liver perfusions. Data are means ± standard errors. Numbers of experiments are in parentheses.

During the 30-min experimental period, an exponential hepatic clearance of bacteria was observed in control experiments with a K value of 0.016 ± 0.002 (Fig 1). At the end of control experiments, 34 ± 4% of the initial inoculum (mean, 6.8×10^6 CFU/ml) was recovered in the perfusate.

In contrast, hepatic clearances of *E. coli* treated with cefamandole were exponential only during the initial 20 min, with K values of 0.034 ± 0.005 for bacteria treated with 1/10 of the MIC and 0.046 ± 0.005 for bacteria treated with 1/5 of the MIC ($P < 0.001$ versus controls for both values). In these experiments, the 30-min recovery of the initial inoculum in the perfusate was 16 ± 5% (mean, 3.2×10^6 CFU/ml) for bacteria treated with 1/10 of the MIC of cefamandole and 8 ± 3% (mean, 4×10^5 CFU/ml) for bacteria treated with 1/5 of the MIC ($P < 0.001$ versus controls; $P < 0.05$ versus bacteria treated with 1/10 of the MIC). *E. coli* cells treated with 1/5 of the MIC of gentamicin were phagocytized exponentially for 30 min with a K of 0.023 ± 0.005; at the end of perfusion, 20 ± 5% (mean, 4×10^6 CFU/ml) of the initial inoculum was recovered in the perfusate.

By taking into account the bactericidal activity of serum present in the perfusate, the amount of bacteria present in the effluent wash (<1%), and the amount of bacteria recovered in the system at the end of perfusions, it was possible to calculate the fraction of bacteria phagocytized and killed by the liver (Fig. 2). In control experiments, 56 ± 6% of the inoculum was phagocytized, and 12 ± 4% was killed. Pretreatment of *E. coli* with sub-MICs of cefamandole resulted in increased phagocytosis and killing of bacteria by the liver. With 1/10 of the MIC, phagocytosis was 71 ± 5% ($P < 0.05$ versus controls) and killing was 14 ± 5%; with 1/5 of the MIC, the respective values were 91 ± 4% ($P < 0.001$ versus controls) and 28 ± 3% ($P < 0.05$ versus controls). Bacteria treated with 1/5 of the MIC of gentamicin also were significantly more susceptible to phagocytosis (75 ± 8%; $P < 0.01$ versus controls) and killing (21 ± 3%; $P < 0.05$ versus controls) by hepatic macrophages.

DISCUSSION

Our study demonstrates that *E. coli* grown overnight in the presence of sub-MICs of either cefamandole or gentamicin become more susceptible to the phagocytic and bactericidal activities of isolated rat liver. Moreover, the study shows that the test organism grown in the presence of 1/5 of the MIC of cefamandole or 1/5 or 1/10 of the MIC of gentamicin appeared resistant to the lytic effect of rat serum at a 4% concentration. At higher serum concentration (20%), resistances of untreated and treated bacteria to serum were similar. These results on serum susceptibility agree with those of Lorian and Atkinson (13), who showed that in most instances, several gram-negative bacteria treated with sub-MICs of ampicillin or mecillinam were more resistant to serum bactericidal effect than control cells were. In contrast, *E. coli* pretreated with sub-MICs of cyclacillin was shown to become more susceptible to lysis by immune serum (9).

In other studies, *Staphylococcus aureus* treated with sub-MICs of nafcillin, but not of methicillin and oxacillin, was found to be more susceptible to in vitro phagocytosis by mouse peritoneal cells (8). On the other hand, filamentous forms of *E. coli* induced by sub-MICs of cefalexin were less efficiently phagocytized by mouse peritoneal macrophages than by control cells (2). All these studies indicate that different antibiotic molecules, even at sub-MICs, may affect bacterial susceptibility to host humoral or cellular factors in different ways. Differences in results might be accounted for by differences in the bacterial strain, antibiotic tested, source of serum, phagocytes to which organisms were exposed, and experimental procedures.

The mechanisms by which sub-MICs of cefamandole and gentamicin enhance phagocytosis and killing of *E. coli* by hepatic macrophages and modify bacterial susceptibility to serum remain subject to speculation. Of the two antibiotics tested at sub-MICs, cefamandole appeared more efficient than gentamicin in causing these effects.

In the perfusions with bacteria grown in the presence of one-fifth of the MIC of cefamandole, it is important to discuss whether the reduced viability of the inoculum injected into the perfusate could account for the increased rate of

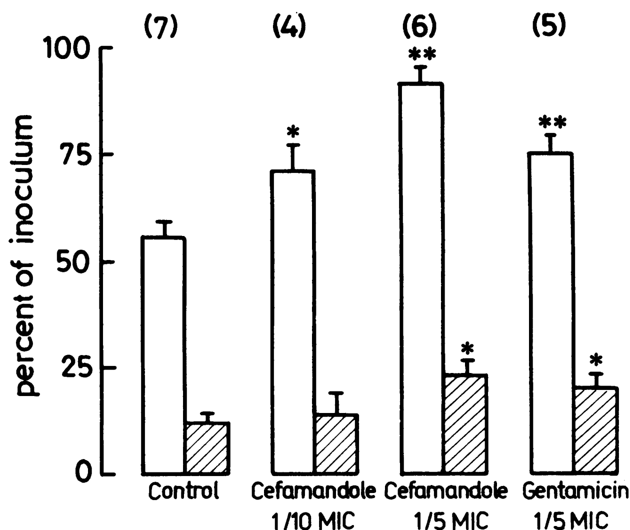

FIG. 2. Percentage of phagocytosis (open bars) and killing (shaded bars) of control and sub-MIC-treated *E. coli* by isolated and perfused rat liver. Data are means ± standard errors. Numbers of experiments are shown in parentheses.

phagocytosis and killing by hepatic macrophages as compared with controls. This seems unlikely since in the cefamandole experiments the inoculum, although containing 2.5% fewer viable cells than controls, did not differ in the total number of cells as determined by turbidity, and therefore it was not adjusted for reduced viability to avoid an alteration of the initial number of cells. Previous studies (3) with amounts of bacteria comparable to ours demonstrated that the in vivo clearance of live and killed *E. coli* occurs at similar rates. Furthermore, the increase in phagocytosis and killing which we observed was seen also at 1/10 of the MIC of cefamandole, a dose which had no noticeable effect on bacterial growth.

At one-fifth of the MIC, cefamandole, like other β-lactam antibiotics (2, 11, 12), induced elongation of bacteria by virtue of its effect on cell wall synthesis. Since the filamentous forms of *E. coli* were phagocytized at the highest rates, it seems attractive to speculate that elongation per se could be responsible at least in part for the increased phagocytosis. However, a higher susceptibility to the phagocytic activity of Kupffer cells was also observed for bacteria treated with a lower concentration of cefamandole or with one-fifth of the MIC of gentamicin, which did not modify cell form. This suggests that different factors other than cell elongation may affect the susceptibility to phagocytic activity of bacteria grown in the presence of sub-MICs of antibiotics. The enhanced phagocytosis and intracellular killing of sub-MIC-treated *E. coli* which we observed might be accounted for either by the loss of antiphagocytic cellular material caused by the β-lactam antibiotic through an alteration of the membrane surface or by the lack of its expression induced by the aminoglycoside through the inhibition of protein synthesis. Eisenstein et al. have demonstrated that the inability of sub-MIC-treated bacteria to express specific lectin-like ligand on their surface mediates the suppression of bacterial adherence to epithelial cells (7). Furthermore, it has also been suggested that an alteration of bacterial surface may modulate cell lysis by serum factors (9).

Low doses of antibiotics may be useful in long-term prophylaxis of recurrent infections such as those of the urinary tract (2, 18). Furthermore, it has been shown that sub-MIC levels of antibiotics may have some therapeutic effects. In fact, experimentally infected animals treated with low doses of β-lactam antibiotics and gentamicin which achieved subinhibitory plasma levels showed a prolonged survival and a lower mortality than untreated controls (21).

In the light of our results, it may be suggested that this response is mediated by an enhancing effect of antibiotics on bacterial phagocytosis. This effect has been observed in vitro (1, 14) but cannot be easily demonstrated in vivo. Only recently, Butler et al. showed an accelerated clearance of *Borrelia* spirochetes in infected patients after antibiotic therapy (6).

The demonstration of a cooperating activity between host defense mechanisms and antibacterial drugs may explain why some antibiotics exert an in vivo efficacy greater than their in vitro activity and why they may have a therapeutic efficacy even when their plasma concentration is below the MIC. On the basis of these observations, one may suggest that the rationale in choosing an antibiotic treatment should be based not only on the bactericidal in vitro activity of the drug but also on its ability to modulate host phagocytic activity.

The clinical usefulness of low levels of antibiotics remains to be established. However, our results seem to provide data that justify further research in this field.

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