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ORIGINAL

CEFOTAXIME: RATE OF BACTERIAL KILLING AND INTERACTIONS WITH SERUM AND LEUKOCYTE ACTIVITY

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SUMMARY

Cefotaxime (HR 756) was compared with cefazolin and cephaloridine with regard to the rate of bacterial killing and to the influence on the bactericidal activity of human serum and polymorphonuclear leukocytes. At concentrations equal to 1-2×MIC cefotaxime showed a more rapid and complete bactericidal activity on *E. coli K 12* than cephaloridine and cefazolin. In presence of the latters in fact, bacterial regrowth was observed after 24 hours. On *S. aureus* a complete killing activity of cefotaxime appeared at concentrations of 8×MIC. A synergism seemed to exist between the complement dependent bactericidal activity of normal human serum and cephalosporins. *E. coli K 12* (a rough strain) was killed by the combination of human serum and both cefotaxime and cefazolin at concentrations that were not bactericidal by themselves. Cephaloridine on the contrary showed an additive activity with normal human serum. Finally evidence that cefotaxime enhanced the bactericidal activity of human polymorphonuclear leukocytes against *S. faecalis*, is reported.

RESUMO

Cefotaxima: Poder Bactericida e Interacção com a Actividade Leucocitária e do Soro.

Comparou-se o poder bactericida e a interferência da cefotaxima (HR 756) na actividade bactericida dos leucocitos polimorfonucleares e do soro com os parametros equivalentes referentes à cefazolina e à cefaloridina. A concentrações equivalentes a 1-2 vezes a concentração Inibitória Mínima (CIM) a cefotaxima revelou uma acção bactericida mais rápida e completa sobre o *E. Coli K12* que a cefaloridina e a cefazolina. De facto, na presença destes últimos antibióticos o crescimento das bactérias voltou a verificar-se ao fim de 24 horas. No referente ao S. aureus, a actividade bactericida total da cefotaxima revelou-se à concentração de 8×CIM: Verificou-se a existência de um sinergismo entre a actividade bactericida complemento-dependente do soro humano normal e das cefalosporinas. O *E. Coli K 12* (uma estirpe rugosa) foi aniquilado por uma associação de soro humano e cefotaxima e cefazolina em concentrações que só por si não seriam bactericidas. A cefaloridina por outro lado evidenciou uma actividade aditiva com o soro humano normal. Por fim fez-se referência ao facto de a cefatoxima potenciar a actividade bactericida dos leucocitos polimorfonucleares humanos em relação ao S. faecalis.

INTRODUCTION

Cefotaxime (HR 756) is a parenteral semisynthetic derivate of 7-aminocephalosporanic acid,[2] representative of a new group of cephalosporins which show relative resistance to beta-lactamases of Gram-negative bacilli.[1, 7, 13, 15, 19] The antibiotic presents a broad spectrum of activity, including Gram-positive and negative aerobic and anaerobic bacteria.[3, 9, 10, 11, 14, 17, 18, 20, 23] The determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of antibiotics *in vitro* cannot give a complete understanding of their activity; for this purpose it seems to us that some more informations can be drawn from the determination of the rate of bacterial killing Moreover the aspects of the *in vivo* activity of antibiotics cannot be fully understood if the integration of their activity with the mechanisms of specific and aspecific natural defence against infection is not determined.

Among the aspecific system of organic defence, bactericidal activity of serum and polymorphonuclear (PMN) leukocytes are particularly relevant.

Serum bactericidal activity is a reaction which occurs through the activation of the classical or the alternative pathways of complement. There is a great deal of indirect evidences on the implication of this process in host defence mechanisms against Gram-negative infections. In fact resistance to serum is an important determinant of virulence of Gram-negative organisms in mouse and rabbit as well of *E. coli* in humans; failure of antibiotic therapy is often associated with isolation from blood cultures of organisms resistant to serum. Finally, in humans Gram-negative infections are generally associated with defects of the alternative pathway of complement.[16, 21, 22]

The importance of the role of PMN in the outcome of infections is documented by the final stage of phagocytic functions that leads to the killing of ingested organisms. A

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defect in this function can have too an important impact in the results of chemotherapy.

The purpose of this study was to compare cefotaxime with two widely used cephalosporins, cefazolin and cephaloridine, with regard to some aspects of their *in vitro* activity that could possibly allow a better differentiation among them and a better understanding of their therapeutic action. In detail, the following experiences were performed: a) determination of the rate of bacterial killing against *E coli* and *S. aureus* b) determination of interactions with the bactericidal activity of normal (NHS) and Ethyleneglycol 2 (2-aminoethyl) tetracetic acid (EGTA) — chelated Mg^{++} — treated human serum (CHS) c) determination of interactions with bactericidal activity of human polymorphonuclear leukocytes.

MATERIALS AND METHODS

Bacterial strains and killing curves. *S. aureus P 209, S. faecalis, E. coli K 12* and the *E. coli K 12* derivate J6-2 *pro, trp, lac, str* were used. MIC_s and MBC_s are reported in Table 1. *E. coli K 12* and *S. aureus P 209* strains, grown overnight in Penassay broth at 37 °C, were diluted in fresh medium, with and without antibiotics to a final concentration of 10^5 cells/ml. Incubation was carried out in shaking water bath at 37 °C. Samples, removed at different times were immediately diluted in cold saline and plated on Penassay agar to determine the CFU present.

Serum source and treatment. The blood of the 10 healthy donors was allowed to clot for 1 hour at room temperature; the sera were separated by centrifugation, pooled, divided into 0.2 ml samples, and stored in liquid nitrogen (NHS). A single preparation lasted throughout the experiments. When indicated, heat-labile components were inactivated by incubation at 56 °C for 30 minutes (H56). In order to test the bactericidal activity only through the activation of the alternative pathway of complement EGTA and Mg^{++} were added to human serum at final concentrations respectively of 9 and 4 mM (CHS).

Serum bactericidal activity. Cells of the *E. coli K 12* derivative *J6-2* grown overnight in Penassay broth at 37 °C, were diluted 1:40 in fresh broth and grown to 1×10^8 cells/ml. The bacteria, harvested by centrifugation, were washed twice in Phosphate Buffered Saline Oxoid (PBS) and diluted to a final concentration of 1×10^6 cells/ml for bactericidal assay. Bacterial suspensions were added to antibiotic alone, NHS alone, antibiotic plus NHS, CHS alone and CHS plus antibiotic. The incubation was carried out at 37 °C with agitation for 60'. Viable counts were obtained by plating suitable dilutions in cold PBS on Penassay agar plates.

Bactericidal activity of human leukocytes. *S. faecalis*, grown in Penassay broth to mid-log phase, was washed in PBS and opsonized before use. For the assay, 0.1 ml of a diluted bacterial suspension (10^5/ml) was added to 0.85 ml of whole blood in a capped plastic test tube. Final volume of each sample was adjusted to 1 ml with PBS or drug dilutions.

The ratio of number of bacteria to number of leukocytes was about 1:10, in order to ensure a virtually complete phagocytic uptake of the organisms. Incubation was made at 37 °C with shaking, samples, removed at indicated times, were diluted in sterile distilled water. After thorough agitation on a vortex mixer to obtain lysis of both red and white cells, the total number of viable bacteria present was determined.

RESULTS

Bactericidal activity of cefotaxime against E. coli and S. aureus

Killing curves of cefotaxime, cefazolin and cephaloridine on *E. coli K12* and *S. aureus P 209* were comparatively determined. Cefotaxime showed a more rapid and complete bactericidal effect than cefazolin and cephaloridine against *E. coli K 12* at concentrations of 1-2 × MIC. In fact with the latter derivatives regrowth was observed after 24 hours of incubation (Fig. 1); on the contrary a complete killing activity of cefotaxime against *S. aureus 209 P* was achieved only at concentrations of 8 × MIC, while cephaloridine and cefazolin were active also at 2 × MIC (Fig. 2).

Effect of cephalosporins on bactericidal activity of human serum on E. coli K 12

Experiments in which cells of *E. coli K 12* were incubated with NHS (final dilution 1:400) and different concentrations of each cephalosporin were performed. Diluted human serum alone did not produce any significative bactericidal effect (Survival %, 96 ± 6 %). The addition of cefotaxime and cefazolin but not cephaloridine at concentrations from 0.01 μg/ml induced the appearance of a killing activity that was superior to the one shown by the antibiotic alone (Fig. 3). In all cases the percent of survival with antibiotic plus serum was different from antibiotic alone at the level of significance as estimated by the two tailed Student's test.

The addition of antibiotics to H56 or to CHS did not induce any significant killing activity.

TABLE 1 **Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs) of the tested cephalosporins**

Strain	Cefotaxime		Cefazolin		Cephaloridine	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus P.209	2.00	4.00	0.50	1.00	0.50	1.00
E. coli K 12	0.12	0.12	4.00	8.00	4.00	8.00
E. coli K 12 J6-2	0.10	0.10	2.00	2.00	2.50	5.00
S. faecalis	1.00	—	10.00	—	10.00	—

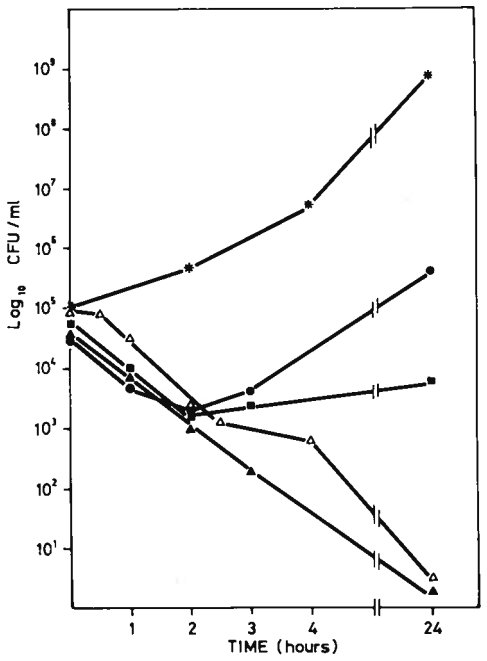

Fig. 1 Bactericidal activity of cephalosporins against *E. coli K 12* (10^5 cells/ml)

* control
△, ▲ plus cefotaxime respectively 1 and 2×MIC
■ plus cephaloridine 2×MIC
● plus cefazolin 2×MIC

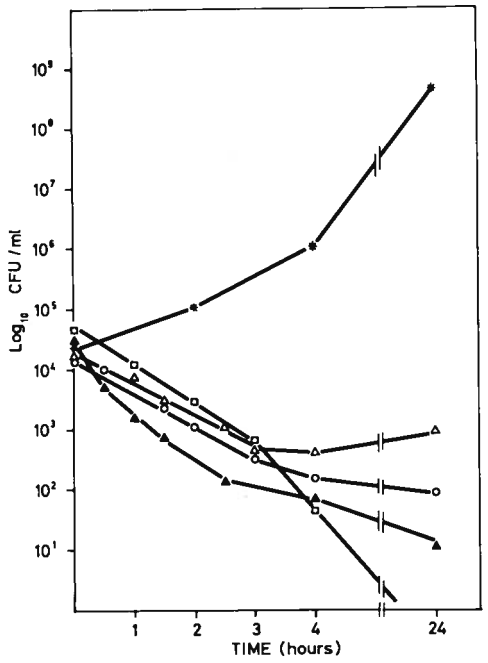

Fig. 2 Bactericidal activity of cephalosporins against *S. aureus P 209* (10^5 cells/ml)

* control
△, ▲ plus cefotaxime respectively 4 and 8×MIC
□ plus cephaloridine 4×MIC
○ plus cefazolin 4×MIC

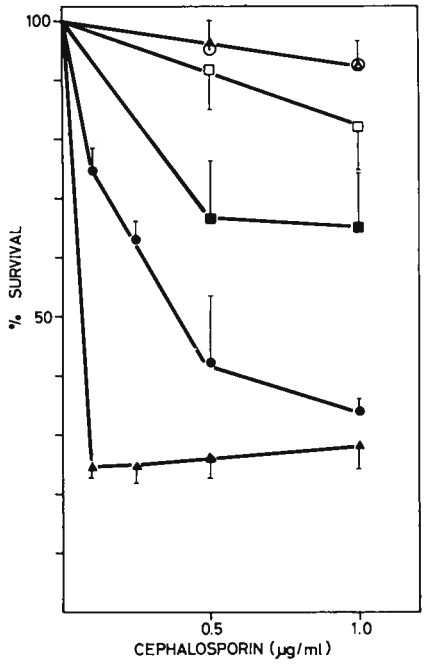

Fig. 3 Bactericidal activity of normal human serum plus cephalosporins on *E. coli J 62*

△ cefotaxime alone or plus H56 (1/400)
○ cefazolin alone or plus H56 (1/400)
□ cephaloridine alone or plus H56 (1/400)
▲ cefotaxime plus NHS (1/400)
● cefazolin plus NHS (1/400)
■ cephaloridine plus NHS (1/400)

Vertical lines represent the standard deviations from the means. All determinations were performed in quadruplicate.

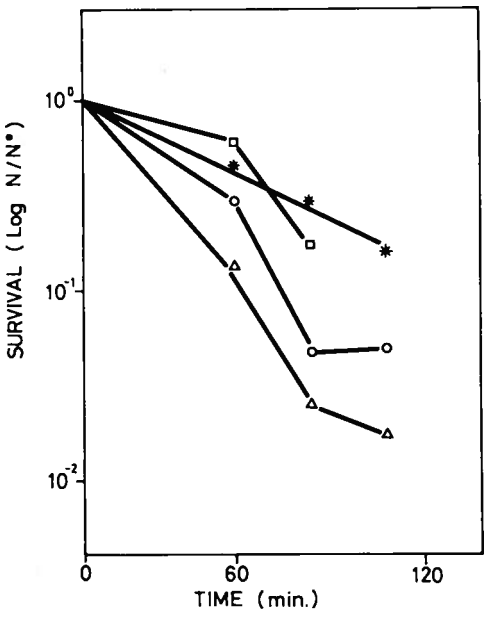

Fig. 4 Effect of cephalosporins on the bactericidal activity of human PMN

* control
△ plus 1 µg/ml cefotaxime
□ plus 1 µg/ml cefazolin
○ plus 1 µg/ml cephaloridine

All the experiments were done in duplicate

Effect of cephalosporins on intraleukocytic killing

The effect of the cephalosporins on the bactericidal activity of human polymorphonuclear leukocytes against *S. faecalis* was tested. Bactericidal activity, using either whole blood or purified leukocytes, was determined with a very low bacteria /PMN ratio, to ensure total phagocytosis. Cefotaxime and at certain extent cephaloridine, but not cefazolin, appeared to enhance the killing of *S. faecalis* which was phagocytised by human polymorphonuclear leukocytes (Fig. 4). *S. faecalis*, in the same experimental conditions of test, was not affected by the adopted doses of antibiotics.

DISCUSSION

A more efficient and rapid killing activity of cefotaxime than that of cefazolin and cephaloridine against *E. coli*, but not against *S. aureus*, was shown. This was in agreement with results obtained by *in vitro* determination of MIC and MBC [3, 9, 10, 11, 12, 17] which demonstrated a stronger activity of this antibiotic on Gram-negative bacilli.

Incubation of *E. coli K 12* (a rough strain) with NHS results in rapid killing of the cells[6] but, if serum was diluted sufficiently, the effect was no longer observed. If the inactive dilution of serum was added to cefotaxime or cefazolin (not to cephaloridine) at concentrations that by themselves produced no or little bactericidal effect an efficent killing of *E. coli K 12* could be achieved. This synergistic action is dependent on the integrity of complement. In fact no bactericidal effect was shown when serum was heated at 56 °C for 30 min. Killing was also blocked by the treatment with EGTA-Mg^{++}, that is when only the alternative pathway of complement was functioning. An hypothesis that can be put forward is that serum can disrupt or modify the permeability barrier of the outer membrane of Gram-negative organisms, allowing an increase of the intracellular concentration of antibiotics to a bactericidal level or rendering bacteria more susceptible to the killing activity of antibiotic.

In fact the outer membrane of Gram-negative bacteria is the final locus of the lethal event in serum bactericidal reaction [4, 5] and it is probable that an interaction at this level can produce phenomena of synergy or antagonism.

Finally we showed an enhancement of intraleukocytic killing of *S. faecalis* by cefotaxime and cephaloridine, not by cefazolin. It seems therefore, that every cephalosporin derivative has a peculiar behaviour with respect to different expressions of the mechanisms of organic defence.[8]

This behaviour seems to be quite unpredictable on the basis of the present knowledges. In conclusion three important properties of cefotaxime emerge out of these studies: a) a more efficient and rapid killing in vitro activity than that of cefazolin and cephaloridine against *E. coli*. b) a sinergy with complement-dependent bactericidal activity of normal human serum c) an enhancement of microbicidal activity of human PMN leukocytes against *S. faecalis*. These properties of cefotaxime, in addition to its wellknown higher antibacterial activity, may be relevant for its efficacy in some clinical situations.

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