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Evaluation of the In Vitro Bactericidal Action of Ciprofloxacin on Cells of *Escherichia coli* in the Logarithmic and Stationary Phases of Growth

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Cells of *Escherichia coli* Neumann and *E. coli* KL16 were suspended in phosphate-buffered saline pH 7.4 and allowed to reach stationary growth conditions. Ciprofloxacin was added at different concentrations, and time-kill curves were constructed. It could be demonstrated that the number of viable cells was reduced quickly by several logs for *E. coli* Neumann, whereas a weak and slow killing effect was observed with *E. coli* KL16. When ciprofloxacin or norfloxacin was added to logarithmically growing cultures of *E. coli* Neumann or *E. coli* KL16, no principal differences in the killing rate for the two strains could be observed. Ciprofloxacin, however, was more bactericidal than norfloxacin. It was also demonstrated that the bactericidal action of ciprofloxacin on cells in the stationary growth phase was better at pH 7.4 than at pH 8.6. This dependence is different from that observed in MIC studies, in which the MIC were lower at pH 8.0 than at pH 7.2. It was also found that the bactericidal action of ciprofloxacin or norfloxacin on cells of *E. coli* Neumann in the stationary phase of growth could not be reduced by the addition of chloramphenicol, whereas under conditions of logarithmic growth the rapid killing effect of ciprofloxacin was reduced in the presence of chloramphenicol.

Ciprofloxacin, a new quinolone derivative with a broad antibacterial spectrum (1, 3, 5, 7, 9), has become the subject of many in vitro studies. Ciprofloxacin has been characterized by its extraordinary killing effect on bacteria which are in a stationary phase of growth (10). Furthermore, it has been shown that the killing effect of ciprofloxacin or norfloxacin on actively growing cells is antagonized in the presence of chloramphenicol or rifampin (4, 7, 8). It was also reported that ciprofloxacin or ofloxacin must have an additional bactericidal action, which makes these two quinolones more independent from the antagonistic influence of chloramphenicol or rifampin (8). This paper reports results that concern the killing activity of ciprofloxacin on cells of different strains of *Escherichia coli* in the stationary phase of growth. The effect of pH and the influence of chloramphenicol under such conditions are also described and compared with those under normal growth conditions in broth.

MATERIALS AND METHODS

Organisms. The test strains used were isolates from the strain collection of the Institute of Chemotherapy, Bayer AG, Wuppertal, Federal Republic of Germany. *E. coli* KL16 (6) was originally obtained from J. T. Smith, School of Pharmacy, University of London, London, England.

Antibacterial agents. The test substances were ciprofloxacin, norfloxacin (synthesized at Bayer AG), and chloramphenicol (Sigma Chemical Co.).

MICs. MICs were measured by broth dilution techniques in microtiter trays (Greiner). Serial dilutions of 0.1 ml of the agents used were prepared in Isosensitest broth (Oxoid). To these were added 0.1 ml of a 1:1,000 dilution of an overnight culture of organisms, ending in a final inoculum of 1×10^5 to 2×10^5 CFU/ml. The microtiter plates were incubated at 37°C for 18 h. MICs were determined by visual inspection for turbidity. The pH was adjusted by adding small amounts of sterile 0.1 N NaOH or HCl.

Time-kill curves. Logarithmically growing bacteria in Isosensitest broth were centrifuged (10 min, 6,000 rpm) and washed twice with phosphate-buffered saline (pH 7.4). The pellet was suspended in phosphate-buffered saline and adjusted to the desired inoculum. This suspension was incubated for 1 h at 37°C so that stationary growth conditions were achieved. After that time the different drug concentrations were added, and the number of CFU was determined at different time points by plating serial dilutions of the culture on Isosensitest agar plates which were then incubated for 18 h at 37°C. For tests with exponentially growing bacteria the cells were diluted in Isosensitest broth and incubated at 37°C on a minishaker rotating at 150 rpm.

RESULTS

The bactericidal effect of ciprofloxacin on bacteria in the stationary phase of growth was studied in vitro with *E. coli* Neumann and *E. coli* KL16 as test organisms. The resulting time-kill curves are shown in Fig. 1 and 2. It could be demonstrated that for *E. coli* Neumann the reduction in the number of CFU after addition of 1 or 0.1 mg of ciprofloxacin per liter was rapid and that more than 99% of the initial number of viable bacteria were inactivated within the first 30 min. With *E. coli* KL16 a rather slow killing and poor bactericidal effect under stationary growth conditions were observed. It was interesting to see that high concentrations of ciprofloxacin, for example 10 mg/liter did not increase the killing effect. On the other hand it could be demonstrated that there were no significant differences in the susceptibility of these two strains to ciprofloxacin when normal MIC tests were performed in a microdilution assay (Table 1). When ciprofloxacin or norfloxacin was added to logarithmically growing cultures of the two test strains (Fig. 3 and 4), no differences in the killing rates for *E. coli* Neumann and *E. coli* KL16 were observed. However, ciprofloxacin was more effective than norfloxacin.

E. coli Neumann was used as a test organism to study the influence of pH on the killing effect of ciprofloxacin on cells in the stationary growth phase. The results indicate that the killing effect with ciprofloxacin was best at pH 7.4, whereas

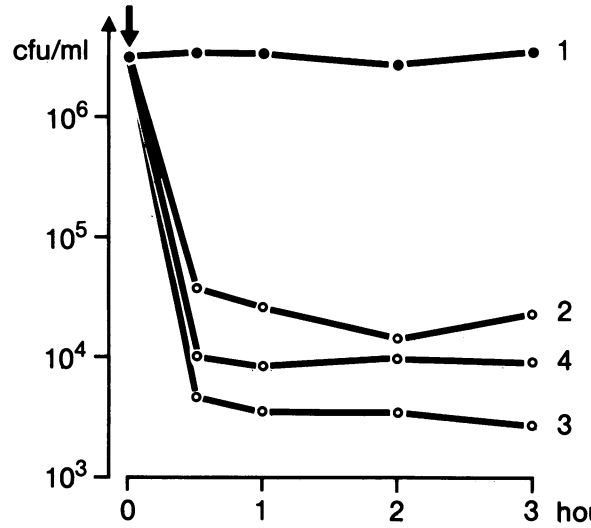

FIG. 1. Bactericidal action of ciprofloxacin on *E. coli* Neumann cells in the stationary phase of growth. Curves: 1, control; 2, ciprofloxacin (0.1 mg/liter); 3, ciprofloxacin (1 mg/liter); 4, ciprofloxacin (10 mg/liter).

at pH 8.6 the bactericidal activity was reduced (Fig. 5). On the other hand, the results of MIC studies, in which *E. coli* Neumann cells were cultivated in Isosensitest broth, show that the MIC for ciprofloxacin were lower at pH 8.0 than at pH 7.2 (Table 1).

In another experiment the influence of chloramphenicol on the killing effect of ciprofloxacin and norfloxacin on cells in the stationary phase of growth was studied. The results show that addition of chloramphenicol directly after addition of ciprofloxacin did not influence the killing effect of either quinolone (Fig. 6). However, it could be demonstrated that the bactericidal activity of ciprofloxacin on a logarithmically growing culture of *E. coli* Neumann could be reduced when chloramphenicol was added at a concentration of 4 mg/liter at the same time (Fig. 7). Even the addition of chloramphenicol 15 min after the addition of ciprofloxacin immediately resulted in a slower killing rate compared with that due to ciprofloxacin alone. Furthermore, we found that the degree of the antagonistic effect depended on the concentration of chloramphenicol added.

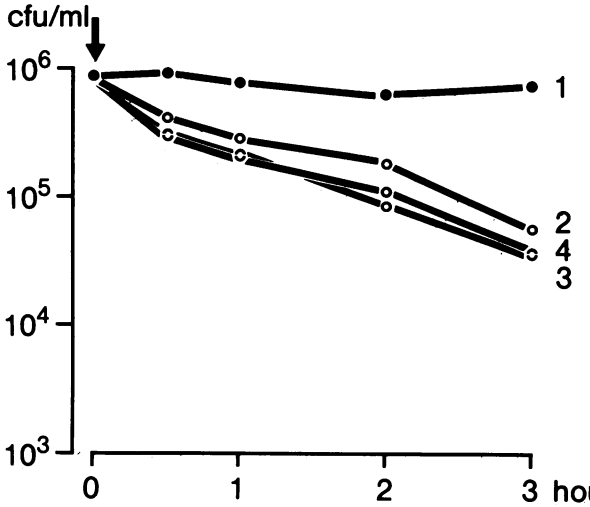

FIG. 2. Bactericidal action of ciprofloxacin on *E. coli* KL16 cells in the stationary phase of growth. Curves: 1, control; 2, ciprofloxacin (0.1 mg/liter); 3, ciprofloxacin (1 mg/liter); 4, ciprofloxacin (10 mg/liter).

TABLE 1. MICs for ciprofloxacin at various pH

pH	MIC (mg/liter) for:	
	E. coli Neumann	*E. coli* KL16
6.5	0.015	0.03
7.2	0.008	0.008
8.0	0.002	0.002

DISCUSSION

Killing of bacteria that are in a stationary phase of growth has been described as a specific characteristic of the bactericidal action of ciprofloxacin (10). In this study it could be demonstrated that different strains of *E. coli* react differently with respect to this special killing mechanism, whereas killing curves with logarithmically growing bacteria did not indicate any differences among the strains. Therefore, *E. coli*

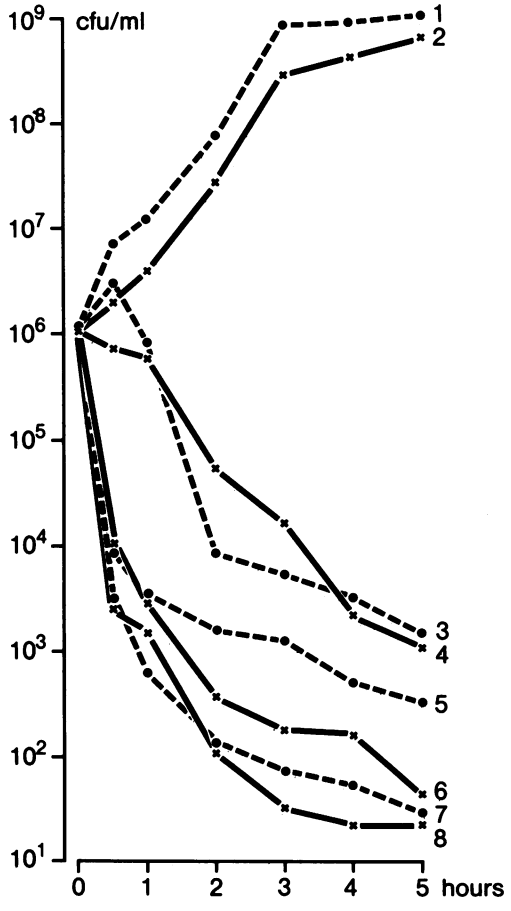

FIG. 3. Bactericidal activity of ciprofloxacin on actively growing cells of *E. coli* Neumann (dashed lines) and *E. coli* KL16 (solid lines) in Isosensitest broth. Curves: 1 and 2, controls; 3 and 4, ciprofloxacin (0.01 mg/liter); 5 and 6, ciprofloxacin (0.1 mg/liter); 7 and 8, ciprofloxacin (1 mg/liter).

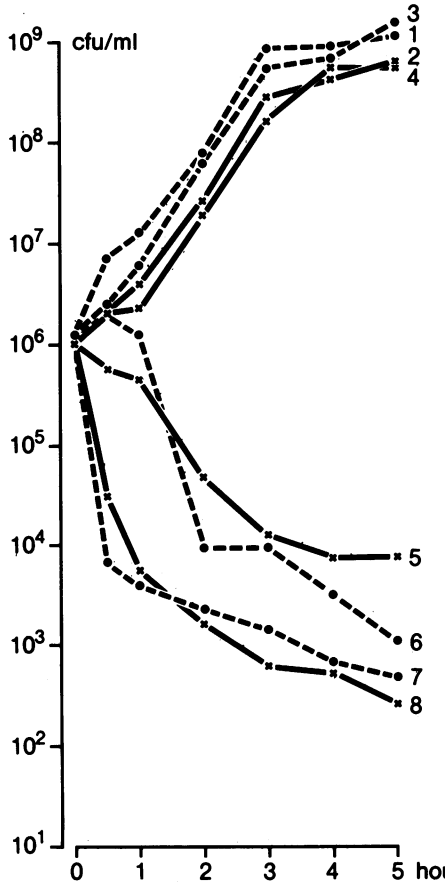

FIG. 4. Bactericidal activity of norfloxacin on actively growing cells of *E. coli* Neumann (dashed lines) and *E. coli* KL16 (solid lines) in Isoscensitest broth. Curves: 1 and 2, controls; 3 and 4, ciprofloxacin (0.01 mg/liter); 5 and 6, ciprofloxacin (0.1 mg/liter); 7 and 8, ciprofloxacin (1 mg/liter).

Neumann represents a species in which more than 99% of viable bacteria are killed rapidly within the first 30 min under stationary conditions, whereas *E. coli* KL16 showed a rather slow reaction to the bactericidal activity of ciprofloxacin. In both cases it was interesting to see that very high concentrations of ciprofloxacin (e.g., 10 mg/liter) did not increase the bactericidal effect. Since these preliminary studies were carried out we have studied a larger number of clinical isolates of *E. coli*. The results obtained so far show that most of these isolates were similar to *E. coli* Neumann, whereas the pattern seen with *E. coli* KL16 seems to be rare. It was also surprising that an alkaline pH reduced the killing effect of ciprofloxacin on cells in the stationary phase, whereas our MIC studies and those of others (2, 3, 7), as well as time-kill curves (10) with actively growing cultures, indicate that ciprofloxacin has a greater effect under alkaline conditions. The effect of acidic pH on the killing action of ciprofloxacin with cells in the stationary phase could not be studied, because it was not possible to keep the cells viable at low pH. There was a significant decline in the number of CFU within the test period of 3 to 5 h.

In recent publications it has been shown that the addition of chloramphenicol or rifampin reduces the bactericidal action of ciprofloxacin or norfloxacin (4, 7, 8). Furthermore, it was found that ciprofloxacin and ofloxacin, in contrast to norfloxacin, nalidixic acid, or flumequine, must have an additional killing mechanism which cannot be blocked by the addition of chloramphenicol or rifampin (8).

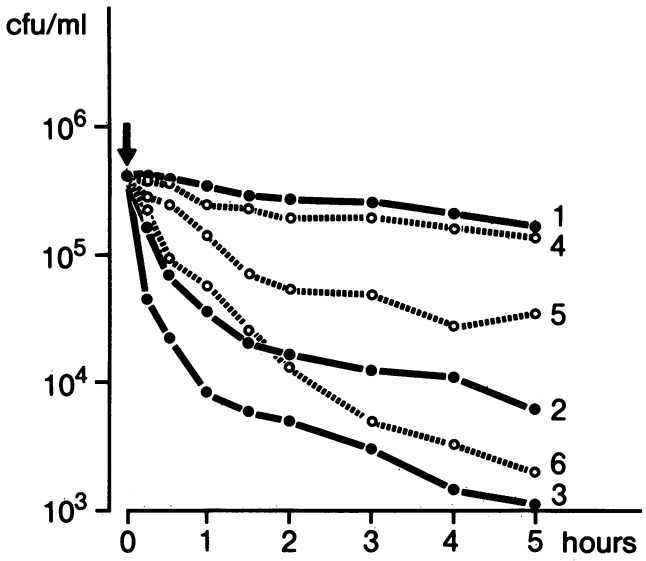

FIG. 5. Bactericidal activity of ciprofloxacin on *E. coli* Neumann cells in the stationary phase of growth at pH 7.4 and 8.4. Curves: 1, control (pH 7.4); 2, ciprofloxacin (0.1 mg/liter, pH 7.4); 3, ciprofloxacin (1 mg/liter, pH 7.4); 4, control (pH 8.4); 5, ciprofloxacin (0.1 mg/liter, pH 8.4); 6, ciprofloxacin (1 mg/liter, pH 8.4).

From our results we conclude that ciprofloxacin has a greater killing effect on stationary cells of *E. coli* Neumann than does norfloxacin. One could assume that this stronger killing activity will also contribute to the rapid initial killing

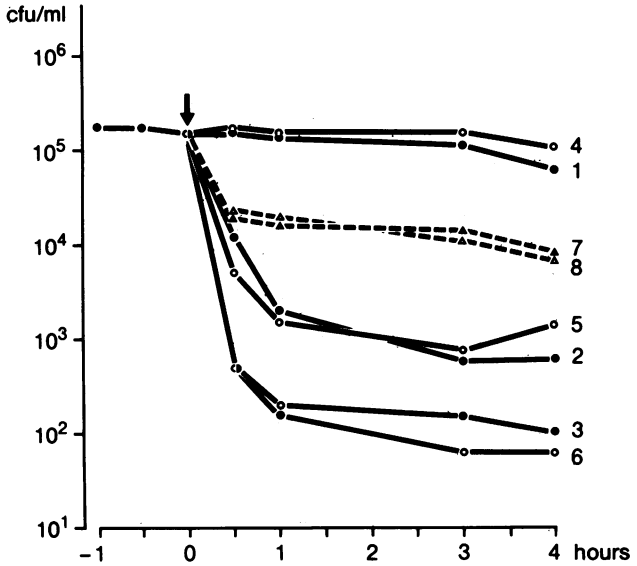

FIG. 6. Influence of chloramphenicol on the bactericidal action of ciprofloxacin and norfloxacin on cells of *E. coli* Neumann in the stationary phase of growth. Curves: 1, control; 2, ciprofloxacin (0.1 mg/liter); 3, ciprofloxacin (1 mg/liter); 4, control + chloramphenicol (4 mg/liter); 5, ciprofloxacin (0.1 mg/liter) + chloramphenicol (4 mg/liter); 6, ciprofloxacin (1 mg/liter) + chloramphenicol (4 mg/liter); 7, norfloxacin (1 mg/liter); 8, norfloxacin (1 mg/liter) + chloramphenicol (4 mg/liter).

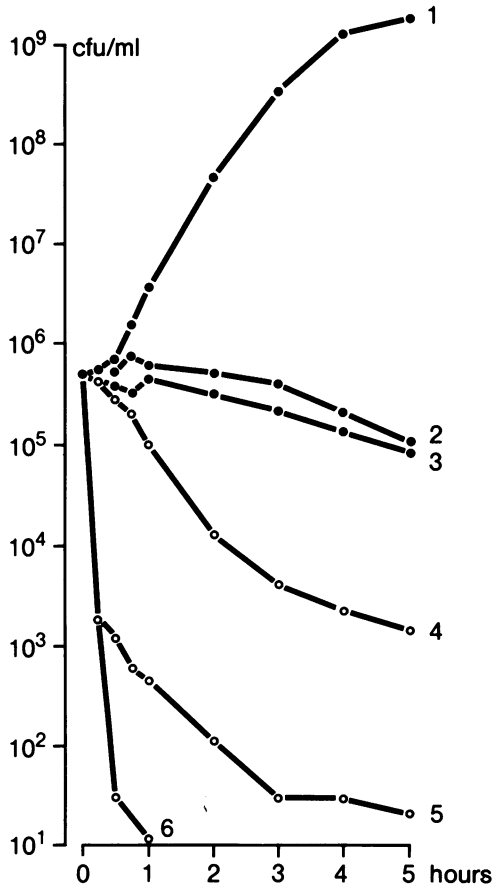

FIG. 7. Influence of chloramphenicol (4 mg/liter) on the bactericidal action of ciprofloxacin (0.1 mg/liter) on cells of *E. coli* Neumann in the logarithmic phase of growth. Curves: 1, control; 2, chloramphenicol (t = 15 min); 3, chloramphenicol (time zero); 4, ciprofloxacin (time zero) + chloramphenicol (time zero); 5, ciprofloxacin (time zero) + chloramphenicol (t = 15 min); 6, ciprofloxacin (time zero).

effect of ciprofloxacin, seen with logarithmically growing cells at concentrations of 1 or 0.1 mg/liter. At lower concentrations which are nearer to the MIC, killing is slower and may be more correlated to the concentrations which have been reported to inhibit DNA gyrase (R. D. Ronnlund, S. A. Chartrand, and J. W. Gaubatz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A26, p. 5). One could speculate that the killing activity of ciprofloxacin observed with cells in the stationary phase may be due to a different mode of action, because it could not be inhibited by the addition of chloramphenicol. This could explain why ciprofloxacin and even ofloxacin (which also has a stronger killing effect on cells in the stationary phase [Zeiler, unpublished results]) are less influenced by the antagonistic effect of chloramphenicol or rifampin described by Smith (8).

Further studies are necessary to clarify the extent to which the weak bactericidal effect of ciprofloxacin, observed with cells of *E. coli* KL16 in the stationary phase, contributes to the killing profile seen with actively growing cells, which was not different from that observed with *E. coli* Neumann.

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