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Development of Resistance to Fleroxacin during Therapy of Experimental Methicillin-Susceptible *Staphylococcus aureus* Endocarditis

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The efficacy of fleroxacin was compared with that of vancomycin by using the rabbit model of methicillin-susceptible *Staphylococcus aureus* endocarditis. Animals received intravenous therapy with fleroxacin, 30 mg/kg every 8 h, or vancomycin, 17.5 mg/kg every 6 h, for 4 days. Both antimicrobial agents effectively cleared bacteremia and significantly reduced bacterial counts in vegetations and tissues compared with those in untreated controls. However, resistance to fleroxacin at 5- and 10-fold the MIC arose in the test strain of *S. aureus* in 73 and 27%, respectively, of animals that received the drug. Resistant isolates were found mainly in vegetations and were composed of up to 7% of the residual population recovered from that site. We conclude that fleroxacin is as effective as vancomycin in this model of a serious systemic *S. aureus* infection, but resistance to the drug may develop during therapy. If similar results are found with other strains of *S. aureus* during therapy with this or other fluoroquinolones, such data, when they are combined with the high incidence of fluoroquinolone resistance among *S. aureus* isolates being reported from selected institutions, would support the contention that these drugs should not be used as first-line therapeutic agents for *S. aureus* infections.

Fleroxacin is a new trifluorinated quinolone that has significant in vitro activity versus gram-negative and gram-positive pathogens, including methicillin-susceptible and -resistant strains of *Staphylococcus aureus* (1, 13). Using the rabbit model of endocarditis, we have shown the drug to be as effective as standard therapy versus a methicillin-resistant test strain of *S. aureus* (6). However, we found that modest resistance (MIC, ≥3.0 μg/ml) developed in a small proportion of surviving organisms recovered from the vegetations of 8% of animals that received the drug. In order to assess further the in vivo activity of this compound against *S. aureus* and to determine the frequency at which resistance to it develops during therapy with a second test strain, we again used the rabbit model of endocarditis and a methicillin-susceptible strain of *S. aureus* as the test organism. Alternative therapeutic options for serious staphylococcal infections are needed, especially in light of recent reports of clinically relevant glycopeptide resistance in *Staphylococcus haemolyticus* and *S. aureus* (8, 16). However, it is important to have some insight into the likelihood of resistance arising during fluoroquinolone therapy of infections caused by such organisms.

MATERIALS AND METHODS

Organism. The methicillin-susceptible strain of *S. aureus* used in this study (MSSA-1199) was a bloodstream isolate from a patient with endocarditis.

In vitro studies. The MICs and MBCs of methicillin, vancomycin, and fleroxacin for MSSA-1199 at inoculum sizes of 5×10^5 (standard inoculum) and 5×10^7 (high inoculum) CFU/ml were determined in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), which was cation adjusted with calcium and magnesium according to the guidelines of the National Committee for Clinical Laboratory Standards (10).

The frequency at which MSSA-1199 developed spontaneous mutational resistance to 2-, 5-, and 10-fold the fleroxacin MIC for a standard inoculum was determined by exposing exponential-growth-phase organisms (~10^{10} CFU) to the appropriate concentration of fleroxacin incorporated into Mueller-Hinton agar (Difco). Colonies were counted 48 h later.

Animal studies. All studies were done with male New Zealand White rabbits (weight, 2 to 3 kg). Left-sided endocarditis was established as described previously by using an intravenous bacterial inoculum of 10^6 CFU (4). Eighteen hours later 1 ml of blood was withdrawn from all animals for culture. Serial dilution and plating techniques were used to determine the number of CFU per milliliter of blood. Inclusion in the study required that this blood culture be positive and that the catheter be positioned properly across the aortic valve at autopsy. Rabbits then were randomized to receive 4 days of fleroxacin (30 mg/kg of body weight intravenously every 8 h), vancomycin (17.5 mg/kg of body weight intravenously every 6 h), or no treatment (controls). The dose administered was adjusted for weight on a daily basis. Controls were sacrificed at the time therapy was begun in animals receiving antimicrobial agents, followed by the determination of bacterial counts in vegetations and tissues (see below).

Serum samples for the measurement of peak (obtained 1 h

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TABLE 1. Vegetation and tissue bacterial counts

Treatment	No. of animals	Mean ± SD $\log_{10}$ CFU/g (no. of culture-negative specimens) in:		
		Vegetation	Kidney	Spleen
Control (no therapy)	10	10.29 ± 0.46 (0)	5.09 ± 0.84 (0)	6.13 ± 0.39 (0)
Vancomycin	14	7.08 ± 1.81 (0)	1.19 ± 0.04 (13)	1.28 ± 0.16 (12)
Fleroxacin	15	5.66 ± 1.73 (1)	1.30 ± 0.29 (10)	1.49 ± 0.49 (11)

postdose) and trough (obtained just before a scheduled dose) antibiotic content were collected from all animals at the time of the first dose on day 2. Blood for culture was obtained before the first dose on day 3.

Following 4 days of therapy, all animals were sacrificed 8 to 10 h (for vancomycin) or 10 to 12 h (for fleroxacin) following the final dose and autopsied in an aseptic manner. Terminal blood cultures were obtained, and vegetations and a 500-mg (mean weight) section of left kidney and spleen were removed for culture. These specimens were weighed, suspended in 0.85% NaCl (final volume, 1 ml), and homogenized. Quantitative bacterial counts, determined by serial dilution and plating techniques, were expressed as the $\log_{10}$ CFU/gram (sensitivity limit, 10 CFU per vegetation or tissue section; culture-negative specimens were considered to contain 10 CFU for numerical and statistical purposes). The effect of antibiotic carryover on cultured material was minimized by the volume of agar used in culture plates. The dilution effect for cultured material was at least 100-fold.

Antibiotic content of serum. Fleroxacin concentrations in serum were determined by bioassay by using an agar well diffusion method (14); *Escherichia coli* 1346 was used as the indicator organism. Vancomycin concentrations were determined by fluorescence polarization immunoassay (TDx; Abbott Diagnostics, Irving, Tex.) (17). Pooled normal rabbit serum was used to prepare standards and dilute unknowns as needed.

Resistance to fleroxacin. Isolates of MSSA-1199 recovered from blood, vegetations, or tissues of animals that had received fleroxacin were screened for the emergence of resistance to the drug by replica plating them from their plates of original growth onto tryptic soy agar (Difco) containing 5- and 10-fold the MIC for a standard inoculum of the organism. These plates were examined for growth 48 h later.

Statistical analysis. Comparisons of blood, vegetation, and tissue bacterial counts and vegetation weights were made by one-way analysis of variance. Comparisons of the frequencies of sterilization of blood, vegetations, and renal and splenic tissues were made by use of the Fisher exact test. A P value of <0.05 was considered significant.

RESULTS

In vitro studies. The standard inoculum MICs and MBCs of methicillin, vancomycin, and fleroxacin for MSSA-1199 were 1.5 and 1.6, 0.5 and 0.5, and 0.4 and 0.8 μg/ml, respectively. MICs did not change with the high inoculum, but MBCs did rise twofold for methicillin, fourfold for vancomycin, and eightfold for fleroxacin. The frequency of spontaneous mutational resistance for the test organism to 2-fold its fleroxacin MIC for a standard inoculum was 1.65×10^{-8}; a frequency of $<1 \times 10^{-10}$ was found at 5- and 10-fold the MIC.

Animal studies. The aggressive nature of untreated MSSA-1199 endocarditis has been established previously, with death occurring in less than 2 days (7). Vegetation and tissue bacterial counts in animals allowed to succumb to the infection were 0.5 to 1.0 $\log_{10}$ CFU/g higher than those found in controls sacrificed at the initiation of antimicrobial therapy (data not shown). These data establish the fact that this test strain produces a lethal and non-self-curing infection.

No differences were found in the intensity of pretreatment bacteremia (mean $\log_{10}$ CFU per milliliter ± standard deviation) for animals receiving vancomycin (3.84 ± 0.86) or fleroxacin (3.93 ± 1.10) sacrificed after 4 days of therapy or controls sacrificed 18 h after bacterial challenge (3.78 ± 0.57).

Peak and trough concentrations of vancomycin in serum were 30.40 ± 6.26 and 3.97 ± 1.87 μg/ml (mean ± standard deviation), respectively. For fleroxacin, the corresponding values were 14.16 ± 3.01 and 1.21 ± 0.66 μg/ml. This peak concentration of fleroxacin in serum is similar to that observed in humans following multiple oral doses of 800 mg (11).

There were no differences in the frequencies of blood culture sterilization during therapy with either antimicrobial agent. For animals receiving vancomycin, cultures were sterile for 78 and 100% of animals after 2 and 4 days, respectively. For those treated with fleroxacin, the corresponding values were 80 and 93%.

Quantitative bacterial counts found in vegetations and tissues are given in Table 1. Compared with control animals, significant reductions in these counts were noted at each cultured site in rabbits treated with either drug ($P < 0.0001$ for all comparisons). There were no differences between groups receiving either vancomycin or fleroxacin with respect to reductions in renal or splenic bacterial counts. However, reductions in vegetation bacterial counts favored fleroxacin ($P < 0.04$). Both drugs produced an equal proportion of culture-negative vegetations and tissues.

Resistance to fleroxacin. Isolates of MSSA-1199 able to grow on agar containing fivefold the fleroxacin MIC for a standard inoculum were recovered from 11 of 15 animals that received the drug (73%). Isolates able to grow on agar containing 10-fold the MIC were recovered from 4 animals (27%); all of these animals also harbored additional organisms resistant at only fivefold the MIC and were included in the group of 11 animals noted above. The majority of resistant organisms were recovered from vegetations; only two rabbits yielded such organisms from both vegetations and other cultured sites (spleen in both). At 5-fold the MIC, the resistant organisms made up 0.2 to 7.3% (mean, 1.8%) of the total number of CFU recovered, and at 10-fold the MIC, the corresponding range was 1.0 to 5.7% (mean, 3.3%).

DISCUSSION

In general, many fluoroquinolones have been shown to compare favorably with standard therapy in animals with experimental *S. aureus* infections (2, 4, 5, 7, 19). In our hands, fleroxacin, a new trifluorinated quinolone, also dem-

onstrated good efficacy in the rabbit model of methicillin-resistant *S. aureus* endocarditis (6). Unfortunately, some of these studies did not address the issue of the emergence of resistance to the test drug during therapy. Having found in our previous study with fleroxacin that 8% of rabbits with endocarditis caused by a methicillin-resistant test strain harbored organisms for which the fleroxacin MICs were at least fivefold higher following therapy with the drug, we felt that further evaluation of this phenomenon using a different test strain was necessary.

Vancomycin and fleroxacin cleared strain MSSA-1199 bacteremia at equivalent rates. Additionally, both drugs were effective in reducing bacterial counts in vegetations and tissues compared with those in untreated controls. Both drugs were equivalent with respect to reducing renal and splenic bacterial counts, but fleroxacin achieved a somewhat greater reduction in vegetation bacterial counts. All of these findings, when combined with similar results that we found with a methicillin-resistant test strain (6), suggest that this fluoroquinolone might be a reasonable alternative to standard therapy for serious systemic *S. aureus* infections. However, a high proportion of rabbits were found to harbor fleroxacin-resistant organisms, some of which were resistant at a level of at least 10-fold the MIC. The presence of resistant organisms was not clinically apparent in any animal; only one was bacteremic at the time of sacrifice, and that organism remained fleroxacin susceptible. A longer treatment course may have allowed these organisms to become clinically important. Of note, however, was the fact that the vegetations found in rabbits having resistant organisms contained more residual CFU (means, 6.52 versus 3.30 $\log_{10}$ CFU/g; $P < 0.0001$) and were significantly larger (mean weights, 100 versus 42 mg; $P = 0.03$) than those found in rabbits lacking such organisms. It is conceivable that fleroxacin penetrated these larger vegetations to a lesser extent, resulting in favorable conditions for bacterial survival and the development of resistance by prolonged exposure to subinhibitory concentrations of the drug. Alternatively, the increase in MBC demonstrated for MSSA-1199 at a higher inoculum may have played a role in bacterial survival and the emergence of resistant organisms. Both of these phenomena may be important, and the determination of intravegetation drug concentrations would help to define the relative importance of each.

Recently, there have been a number of reports from various institutions worldwide of a high incidence of fluoroquinolone resistance among *S. aureus* isolates, especially methicillin-resistant strains (15, 18). These occurrences, when combined with our results with methicillin-susceptible and -resistant test strains of *S. aureus*, as well as those of a previous study (5) demonstrating the emergence of resistance to ciprofloxacin during therapy of experimental *S. aureus* endocarditis, may have important implications with respect to the use of certain fluoroquinolones for therapy of human *S. aureus* infections. Indeed, the development of fluoroquinolone resistance during therapy of such human infections has already been described (3, 12).

We have investigated the mechanisms by which strain MSSA-1199 developed fluoroquinolone resistance during our earlier study in which we evaluated the efficacy of ciprofloxacin for therapy of experimental endocarditis (5), and our findings have been published elsewhere (9). In brief, this strain has been found to possess an augmented active efflux system for fluoroquinolones. It also may possess a species of DNA gyrase that is less susceptible to the inhibitory effects of fluoroquinolones. From our current results we can conclude that, for MSSA-1199, fleroxacin has no advantage over ciprofloxacin with respect to the likelihood of in vivo development of resistance. Whether or not the mechanism(s) by which resistance to fleroxacin arose in both MSSA-1199 and our methicillin-resistant test strain (MRSA-494) is the same as those described above for MSSA-1199 and ciprofloxacin remains to be determined.

Compared with results found in our earlier study with strain MRSA-494 (6), posttreatment vegetation bacterial counts in the current study were reduced to a lesser extent by both vancomycin and fleroxacin. This finding may be explained by the generally larger vegetations produced by MSSA-1199 and the resultant larger initial bacterial burden seen with this organism compared with that seen with MRSA-494 (data not shown). As noted above, drug penetration into larger vegetations may be less efficient. We consistently have seen greater reductions in vegetation bacterial counts using MRSA-494 as the test organism compared with those seen with MSSA-1199 with each fluoroquinolone we have studied in this animal model (4–7).

In conclusion, we found fleroxacin to have activity equivalent to that of vancomycin in this model of a serious methicillin-susceptible *S. aureus* infection. This drug may serve as an alternative to vancomycin in humans with similar infections, but resistance to the drug may develop during therapy. If additional work demonstrates that other strains of *S. aureus* have a similar predisposition to the development of resistance during therapy with this or other fluoroquinolones, these drugs would not be a viable first-line therapeutic option for human *S. aureus* infections. This especially would be true in those institutions with a high incidence of fluoroquinolone resistance among *S. aureus* isolates.

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