Selective Antimicrobial Modulation of the Intestinal Tract by Norfloxacin in Human Volunteers and in Gnotobiotic Mice Associated with a Human Fecal Flora

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Intestinal endogenous members of the family *Enterobacteriaceae* were eliminated in 12 human volunteers treated with 400 or 800 mg of oral norfloxacin per day for 5 days. No clones resistant to quinolone derivatives were isolated. Counts of aerotolerant streptococci were affected to various degrees, depending on their susceptibility to norfloxacin. During treatment, counts of anaerobes remained above $9.8 \log_{10}$ CFU/g of feces. A total of 932 anaerobic isolates from the predominant flora (over 10^9 CFU/g) in fecal samples obtained before or during norfloxacin treatment were classified by a simple morphological and physiological scheme. The composition of this flora was fairly stable from one sample to another before treatment and was not substantially modified by norfloxacin. Intestinal resistance to colonization by exogenous microorganisms was studied in gnotobiotic mice associated with a human fecal flora. The composition of the fecal flora of the human donor and the fecal concentrations of norfloxacin in the volunteers were reproduced in the intestine of the mice. Resistance to colonization by exogenous microorganisms was reduced by norfloxacin for only 2 of 14 (14%) of the strains tested. These results suggest that norfloxacin is a good candidate for selective antimicrobial modulation of the intestinal tract in humans.

Intestinal colonization is the major harbinger of gram-negative bacteremia in neutropenic patients with hematological malignancies (23). Selective antimicrobial modulation of the intestinal tract has been proposed and successfully used to prevent such colonization and subsequent infections (12). Drugs potentially useful for such modulation should be active in vitro against aerobic gram-negative bacteria and in vivo should reduce the number of endogenous members of the family *Enterobacteriaceae* in the intestinal lumen. They should be as little active as possible against the anaerobic endogenous microflora, because studies in animals suggest that resistance of the gut to colonization by exogenous microorganisms may be related to the activity of the anaerobic flora (24). Polymyxin (17), nalidixic acid (25), and trimethoprim-sulfamethoxazole (25) have been proposed for antimicrobial modulation of the intestinal tract, and more recently, erythromycin (2), aztreonam (9), pipemidic acid (19), and ciprofloxacin (6).

A new quinolone derivative, norfloxacin, is also a potential candidate for this modulation because in vitro it has an expanded spectrum against gram-negative and gram-positive aerobic bacteria and displays little activity against anaerobes (20). We therefore assessed the effect of norfloxacin on the fecal flora of human volunteers. Ethical and cost considerations prevented us from challenging the volunteers with live potentially pathogenic microorganisms. Gnotobiotic mice, on the other hand, can be associated with strains pathogenic for humans without experiencing any clinical symptom of disease (for a recent review, see reference 3). Thus, in several instances, these mice have been associated with a human fecal flora for the study of enteric bacterial interactions (1, 2, 18). Consequently, we studied the effects of norfloxacin treatment on the resistance of human intestinal flora to colonization by exogenous bacteria, when these flora were associated with gnotobiotic mice.

MATERIALS AND METHODS

Human volunteers. Twelve healthy, fully informed adult volunteers were included in the study. None had taken antibiotics for at least 1 month before the study. Oral norfloxacin was administered twice daily for 5 days in doses of 200 or 400 mg. Six subjects were randomly assigned to each of the two regimens. Blood samples were drawn 2 h after drug absorption. Freshly passed fecal samples were obtained once before treatment, daily during treatment, and 1 week thereafter.

Gnotobiotic mice. Adult germfree C3H mice (Centre de Sélection des Animaux de Laboratoire, Orléans, France) were maintained in plastic Trexler-type isolators. They were fed a locally prepared diet (2) sterilized by gamma irradiation. Autoclaved drinking water at pH 3 prepared by the addition of hydrochloric acid to deionized water was given ad libitum. Where indicated, 4 or 0.4 g of norfloxacin per liter was added to the drinking water. Bottles were changed every week. Antibiotic activity remained stable between the changes (data not shown).

Some of the mice were used in the germfree state. The remainder were given a complex human fecal flora by intragastric and intrarectal inoculation of a dilution of the original flora, as previously described (2). The human donor of the flora was chosen from among the volunteers on the basis of the absence of strains of *Enterobacteriaceae* resistant to quinolone derivatives in a series of fecal samples obtained during the days preceding the transfer of the flora to the mice. Germfree and human-flora-associated (HFA) mice were each divided into two groups. One germfree and one HFA group were left untreated, and one group of each type was treated with norfloxacin; for the treated HFA group 2 weeks were allowed to elapse between the introduction of the fecal flora and the beginning of norfloxacin administration. Both treated and untreated mice were challenged with various strains 2 weeks after norfloxacin ingestion started in the treated groups. The challenge was performed as previ-

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FIG. 1. Concentrations of norfloxacin in serum and feces of human volunteers treated with 400 or 800 mg of norfloxacin per day for 5 days and in pellets of mice treated with 0.4 or 4 g of norfloxacin per liter in drinking water.

ously described, by intragastric inoculation of 1 ml of a culture containing 10^8 CFU of one of the challenge strains per ml (2).

Challenge strains. *Escherichia coli* IGR46 was the predominant endogenous coliform of the human flora transferred to the mice. The strains *E. coli* IGR48, *E. coli* IGR49, *Providencia stuartii* IGR51, *Morganella morganii* IGR52, *Staphylococcus aureus* IGR53, *Pseudomonas aeruginosa* IGR54, *Candida albicans* IGR41, and *Campylobacter coli* IGR6 were isolated in our laboratory from human feces. *S. aureus* MSD4310 and *P. aeruginosa* MSD4385 were from Merck Pharmaceuticals. *Vibrio cholerae* 569B, *Shigella flexneri* DKR115, and *Vibrio parahaemolyticus* J525C were isolated from diarrheic patients. The MICs of norfloxacin and nalidixic acid for the challenge strains are given in Table 3.

Counts and identification of bacteria. Total counts of anaerobes were performed on fecal samples from the volunteers and on mouse pellets in an anaerobic glove box as described previously (2). The clones isolated inside the glove box from the petri dishes inoculated with the 10^{-8} and 10^{-9} dilutions of the original flora were further analyzed as follows. Each isolate was inoculated into 5 ml of the liquid medium of Aranki et al. (4) and incubated for 48 h at 37°C inside the glove box. Samples of 100 µl from each culture were then transferred to separate wells in a round-bottomed sterile microtiter plate (Dynatech, Paris, France). A multiple inoculator device was used to inoculate simultaneously up to 96 cultures on solid media poured into petri dishes (8 by 12 cm) (Dynatech); 10 to 100 CFU per spot were obtained. By this technique, the MICs of norfloxacin were determined

inside the glove box by the method of Steers et al. (22) on Aranki agar (4). An inoculated petri dish was incubated outside the glove box to confirm that the isolates studied were strict anaerobes. Susceptibility to 1 h of exposure to atmospheric oxygen was studied as described previously (2). Strains killed by such exposure are referred to as oxygen sensitive. The shape and Gram stain properties of bacteria were studied on the first day on which the colonies grown on Aranki agar inside the glove box were visible. Kopeloff's modification of the Gram stain procedure (8) was used; reading was done as described previously (8). The shape and position of spores were determined by direct phase-contrast microscopic examination of a wet-mounted preparation from colonies grown inside the glove box on Aranki agar.

Group D streptococci from four volunteers were counted on bile-esculin agar (Difco Laboratories, Detroit, Mich.) without antibiotic or supplemented with 1, 10, 100, or 1,000 µg of norfloxacin (Merck) per ml. Endogenous enterobacteriaceae were counted on Drigalski agar (IPP, Paris, France) without antibiotic or supplemented with 16 µg of norfloxacin per ml or 4 or 40 µg of nalidixic acid (Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) per ml.

E. coli IGR46 was counted on Drigalski agar, and *E. coli* IGR49 was counted on Drigalski agar with 4 µg of nalidixic acid per ml. *E. coli* IGR48, *P. stuartii* IGR51, and *M. morganii* IGR52 were counted on Drigalski agar with 40 µg of nalidixic acid per ml. *S. aureus* IGR53 and MSD4310 were counted on Chapman agar (Difco). *S. flexneri* DKR115 was counted on salmonella-shigella agar (Difco) or detected after enrichment in Mueller-Kauffmann broth (IPP). *P. aeruginosa* IGR54 and MSD4385 were counted on cetrimide agar (IPP), *V. cholerae* 569B and *V. parahaemolyticus* J525C were counted on thiosulfate-citrate-bile-sucrose agar (Difco), *C. coli* IGR6 was counted on Butzler agar (Oxoid Ltd., London, England), and *C. albicans* IGR41 was counted on Sabouraud agar with 10 µg of gentamicin (Bio-Mérieux, Charbonnières-les-Bains, France) per ml. Isolates were biotyped according to the API system (API System S.A., La Balme-les-Grottes, France). Strain discrimination was based on biochemical and growth characteristics. The MICs of nalidixic acid and norfloxacin were determined on Mueller-Hinton agar.

Norfloxacin assays. The norfloxacin concentrations in hu-

TABLE 1. Bacterial counts in the feces of human volunteers and the pellets of HFA mice before, during, and after norfloxacin treatment^a

Source of bacteria	Phase of treatment	Enterobacteriaceae	Total anaerobes
Human volunteers			
400 mg/day (<i>n</i> = 6 ^b)	Before	7.15 ± 0.68	10.59 ± 0.25
	During ^c	<2.00	10.17 ± 0.31
	After ^d	7.30 ± 0.55	10.27 ± 0.28
800 mg/day (<i>n</i> = 6)	Before	7.08 ± 1.59	10.42 ± 0.38
	During	<2.00	9.86 ± 0.55
	After	6.95 ± 0.27	10.32 ± 0.23
HFA mice (<i>n</i> = 6) ^e			
	Before	6.35 ± 0.77	10.88 ± 0.27
	During ^f	<2.00	10.21 ± 0.32

^a Data are mean ± standard deviation ± log CFU/per gram.

^b *n*, Number of individuals.

^c Four days after the beginning of treatment.

^d Five days after the end of treatment.

^e Norfloxacin (4 g/liter) in drinking water.

^f Treatment began 2 weeks after the association of the mice with the human flora.

FIG. 2. Effect of norfloxacin treatment on the respective importance of different groups of bacteria from the predominant anaerobic flora ($>10^9$ CFU/g of feces) of human volunteers and HFA mice. Column 1, Gram-positive bacilli without visible spores; column 2, gram-positive bacilli with visible spores; column 3, gram-negative bacilli; column 4, gram-positive cocci; column 5, extremely oxygen-sensitive strains. Symbols: ●, samples obtained before treatment; ○, samples obtained during treatment. Lines indicate paired samples from the same human volunteer (—) or the same gnotobiotic mouse (---). Treatments were 800 mg/day for 5 days in humans and 4 g/liter of drinking water in mice.

man serum and feces and in mouse pellets were measured by the agar diffusion assay (7). Antibiotic medium 5 (Difco) at pH 8.0 and *E. coli* IPP7624 were used. Experimental samples and control norfloxacin were diluted in saline.

Statistical analysis. Bacterial counts were converted into common logarithms because the log distribution of intestinal bacterial counts has been shown to be normal (5). Counts of less than $2.00 \log_{10}$ CFU/g of feces were converted to 2.00 for calculation of the mean values given in Table 3. Wilcoxon's test was used for statistical analysis of bacterial counts because of the above conversion and of differences in the variance of count distributions. Paired or unpaired Student's *t* tests were used for comparison of intestinal concentrations of norfloxacin.

RESULTS

In the human volunteers, mean ($\pm$ standard deviation) peak norfloxacin concentrations in serum were 1.0 ± 0.3 and 1.9 ± 0.9 $\mu\text{g/ml}$, respectively, after absorption of 400 or 800 mg of norfloxacin per day ($P < 0.01$) (Fig. 1). Mean fecal concentrations were much higher than those observed in serum but were not significantly different for the two regimens (Fig. 1). On day 2 of treatment, the fecal concentration of norfloxacin was 537 ± 626 $\mu\text{g/g}$ of feces. The fecal concentrations of the drug peaked at day 5 ($2,271 \pm 859$ $\mu\text{g/g}$ of feces). Antibiotic activity was still present 5 days after the end of norfloxacin ingestion (462 ± 570 $\mu\text{g/g}$ of feces) but was undetectable 2 days later. In gnotobiotic mice fecal concentrations were 180 ± 80 $\mu\text{g/g}$ of feces when 0.4 g of norfloxacin per liter was added to the drinking water of the

TABLE 2. MICs of norfloxacin for intestinal bacteria isolated from human volunteers and HFA mice

Source	MIC ($\mu\text{g/ml}$) for:							
	<i>Enterobacteriaceae</i>		Anaerobic gram-positive bacilli		Anaerobic gram-negative bacilli		Anaerobic gram-positive cocci	
	MIC ₅₀	MIC ₉₀	Without visible spores	With visible spores	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Human volunteers								
Before treatment	0.125 ($n = 95$) ^a	0.250 ($n = 95$)	32 ($n = 244$)	128 ($n = 244$)	32 ($n = 28$)	128 ($n = 32$)	128 ($n = 32$)	16 ($n = 79$)
During treatment ^b	ND ^c	ND	128 ($n = 270$)	256 ($n = 270$)	128 ($n = 33$)	64 ($n = 52$)	256 ($n = 52$)	8 ($n = 20$)
HFA mice								
Before treatment	0.06 ($n = 95$)	0.125 ($n = 95$)	64 ($n = 67$)	128 ($n = 67$)	128 ($n = 14$)	8 ($n = 6$)	64 ($n = 6$)	128 ($n = 1$)
During treatment ^d	ND	ND	512 ($n = 75$)	1,024 ($n = 75$)	512 ($n = 12$)	1,024 ($n = 12$)	ND	256 ($n = 8$)

^a *n*, Number of strains tested.

^b 800 mg/day.

^c ND, Not done (no strain isolated).

^d 4 g/liter of drinking water.

FIG. 3. Effect of norfloxacin treatment (800 mg/day from day 0 to day 4) on the mean fecal counts of enterococci resistant to various concentrations of norfloxacin from four human volunteers. Total counts (●—●) and counts of enterococci resistant to 1 (○—○), 10 (●—●), 100 (○—○), or 1,000 (×) µg/ml. Arrows indicate that no strain was detected.

animals. At a dosage of 4 g of norfloxacin per liter, mean fecal concentrations were $2,690 \pm 1,520$ µg/g, i.e., equivalent to those observed in the volunteers (Fig. 1). Consequently, only the 4-g/liter of drinking water regimen was used in all further experiments with gnotobiotic mice.

Before treatment, counts of enterobacteriaceae were similar in both groups of volunteers (Table 1). The MICs of norfloxacin for these endogenous enterobacteriaceae were low (Table 2). Four days after norfloxacin treatment had started enterobacteriaceae were not detectable in the feces of any volunteer (Table 1). Counts again rose to pretreatment levels within 10 days of the end of treatment (Table 1). No enterobacteriaceae resistant to nalidixic acid or norfloxacin were isolated from the 72 human samples analyzed.

In HFA mice, MICs of norfloxacin for enterobacteriaceae were low (Table 2), and elimination of these bacteria was also observed during treatment (Table 1).

Total counts of anaerobes were greater than $9.8 \log_{10}$ CFU/g of feces during treatment in both volunteers and HFA mice (Table 1). However, these counts were lower than those observed before and after treatment. This decrease reached the significance level ($P < 0.01$) for both the volunteers who took 800 mg of norfloxacin per day and the HFA mice. The composition of the predominant anaerobic fecal flora (in counts greater than 10^9 CFU/g of feces) of five volunteers and two gnotobiotic mice was studied before and during norfloxacin treatment. A total of 932 anaerobic isolates (80 ± 12 per sample) were classified into five different groups according to morphological and physiological criteria. The relative importance of each of these groups in the fecal samples is presented in Fig. 2. Before treatment, the differences observed between the flora predominating in the HFA mice and in the human volunteers were not greater than the variations among the volunteers. Modifications induced by norfloxacin treatment were also within this range, except for the disappearance of gram-negative bacilli from the predominant flora in the HFA mice (Fig. 2). MICs of norfloxacin for anaerobes isolated from the dominant flora of the volunteers and HFA mice were not substantially altered by norfloxacin treatment except for gram-positive bacilli which were markedly more resistant in HFA mice during treatment (Table 2). However, the effect of norfloxacin on the MICs for anaerobic gram-negative bacilli was not measured in HFA mice because no strains of this group were isolated in these animals.

TABLE 3. Fecal counts of challenge strains in gnotobiotic mice and MICs of nalidixic acid and norfloxacin

Strains	MIC (µg/ml)		Fecal counts ^a			
	Nalidixic acid	Norfloxacin	Monoassociated mice ^b		HFA mice ^c	
			Before treatment	During treatment ^{d,e}	Before treatment	During treatment
<i>E. coli</i> IGR46	1	0.06	9.5 ± 0.22	<2.00	6.35 ± 0.77	<2.00
<i>E. coli</i> IGR48	64	0.250	9.65 ± 0.36	<2.00	<2.00	<2.00
<i>E. coli</i> IGR49	8	0.25	9.55 ± 0.44	<2.00	<2.00	<2.00
<i>P. stuartii</i> IGR51	>1,024	64	9.44 ± 0.23	7.81 ± 1.36	<2.00	<2.00
<i>M. Morganii</i> IGR52	>1,024	16	9.52 ± 0.39	9.12 ± 0.2	<2.00	<2.00
<i>S. aureus</i> IGR53	8	0.06	9.14 ± 0.17	<2.00	<2.00	<2.00
<i>S. aureus</i> MSD4310	64	1	9.23 ± 0.13	7.76 ± 0.74	2.84 ± 0.81	2.34 ± 0.68
<i>P. aeruginosa</i> IGR54	>1,024	64	8.68 ± 0.28	8.52 ± 0.36	2.2 ± 0.35	3.02 ± 0.91
<i>P. aeruginosa</i> MSD4385	1,024	4	8.66 ± 0.25	<2.00	<2.00	<2.00
<i>C. albicans</i> IGR41	64	16	7.13 ± 0.3	7.3 ± 0.2	<2.00	3.55 ± 1.45
<i>V. cholerae</i> 569B	1	0.03	9.45 ± 0.35	<2.00	<2.00	<2.00
<i>V. parahaemolyticus</i> J525C	1	0.06	8.88 ± 0.47	<2.00	<2.00	<2.00
<i>S. flexneri</i> 115DKR	1	0.125	9.45 ± 0.22	<2.00	<2.00	<2.00
<i>C. coli</i> IGR6	4	0.06	8.96 ± 0.57	<2.00	<2.00	<2.00

^a Data are mean $\pm$ standard deviation log counts per gram.

^b Mean of three animals.

^c Mean of six animals.

^d Two weeks after challenge.

^e Dosage of 4 g/liter in drinking water.

The effects of norfloxacin on counts of group D streptococci susceptible, or resistant, to different concentrations of norfloxacin are shown in Fig. 3. Total counts and counts of strains resistant to 1 µg of norfloxacin per ml were equivalent, while counts of strains resistant to 10 µg of norfloxacin per ml were 10 times lower. All these counts dropped by 2 logs during treatment. By contrast, counts of strains resistant to 100 µg of norfloxacin per ml were low before treatment but were not affected by norfloxacin. No clone resistant to 1,000 µg of norfloxacin per ml was isolated.

In the intestine of monoassociated mice, all the challenge strains were individually established at high concentrations (Table 3). These were stable with time (data not shown). No sign of sickness or disease was observed in any animal. During treatment, all the strains with MICs below 1 µg of norfloxacin per ml, as well as strain *P. aeruginosa* MSD4385, were eliminated; counts of *P. stuartii* IGR51 and *S. aureus* MSD4310 diminished slightly but significantly ($P < 0.01$). Counts of *M. morganii* IGR52, *P. aeruginosa* IGR54, and *C. albicans* IGR41 were not affected (Table 3). The MICs of norfloxacin for clones of the challenge strains isolated from mouse pellets during treatment were not different from those for the clones isolated from untreated animals (data not shown).

The role of the fecal anaerobic flora in the resistance to colonization by the challenge strains was demonstrated by comparing the population densities of the challenge strains in HFA and monoassociated mice (Table 3). In HFA mice, all the challenge strains except *E. coli* IGR46 were either not detectable or present at low concentrations 2 weeks after the challenge. *E. coli* IGR46 was eliminated from HFA mice by norfloxacin.

The colonization resistance exerted toward *E. coli* IGR48 and IGR49, *P. stuartii* IGR51, *M. morganii* IGR52, *S. aureus* IGR53 and MSD4310, *P. aeruginosa* MSD4385, *V. cholerae* 569B, *V. parahaemolyticus* J525C, *S. flexneri* DKR115, and *C. coli* IGR6 was not altered by norfloxacin, since counts for these strains were similar in HFA mice before and during treatment. Counts of *P. aeruginosa* IGR54 and *C. albicans* IGR41 were higher in HFA mice during norfloxacin treatment than before ($P = 0.02$ and $P < 0.01$, respectively). However, norfloxacin did not abolish all resistance to colonization by these two strains, since during treatment their counts were lower in HFA than in monoassociated mice ($P < 0.01$ in both cases).

DISCUSSION

The results of this study show that norfloxacin compares favorably with other drugs advocated for intestinal antimicrobial modulation in neutropenic patients, since selective decontamination was achieved in all the volunteers. Endogenous enterobacteriaceae were eliminated, counts of aerotolerant streptococci were not much affected, and counts of anaerobes remained high. No selection of gram-negative aerobic bacilli resistant to nalidixic acid or norfloxacin was observed (Table 2), and no major qualitative disturbance of the predominant anaerobic flora was induced by norfloxacin (Fig. 2). Although there was a significant reduction in anaerobes in recipients of the 800-mg/day dose and in mice, this reduction had little biologic significance in terms of colonization resistance as shown by the mouse studies.

The scheme used to classify the anaerobes was simple but allowed a large number of isolates to be tested. Provided that staining and reading procedures were carried out meticu-

lously, the results were reproducible from one volunteer to another (Fig. 2). The percentage of oxygen-sensitive isolates was determined, because these bacteria were considered to be major determinants of colonization resistance (11). Although rather large variations in the percentage of oxygen-sensitive strains were observed (Fig. 2), they were present in the predominant flora of all the samples tested, even during norfloxacin treatment.

Comparison of MIC determinations with the results observed in monoassociated mice showed that norfloxacin was more active in vitro than in the intestinal lumen. Although intestinal concentrations of norfloxacin (Fig. 1) were much higher than the MICs for the challenge strains (Table 3), not all these strains were eliminated from monoassociated mice during treatment. No strict parallel could be drawn between the activity of norfloxacin in vivo and in vitro. However, the general tendency was that during norfloxacin treatment in mice, the strains with the lowest MICs were more frequently eliminated.

In HFA mice, norfloxacin did not greatly alter colonization resistance (Table 3) since microbial antagonisms were only modified in 2 of 14 (14%) of the challenge strains. HFA mice have previously been used, both by ourselves (2, 18) and others (13, 14), when ethical and cost limitations prevented the challenge of volunteers with potentially pathogenic microorganisms. In the present study, the adequacy of the HFA mice model was supported by the similarities observed between humans and HFA mice as regards fecal concentrations of norfloxacin and bacterial counts and composition of the flora before and during norfloxacin treatment. We chose HFA mice to study colonization resistance because the differences in the intestinal flora of humans and conventional rodents (10) might have made it difficult to extrapolate to humans the results obtained in conventional mice (15). The measurement of beta-aspartylglycine in fecal samples has also been proposed as a global indicator of colonization resistance and has been used in human volunteers (19). We did not use it because it would not have revealed strain-to-strain variations such as those observed in HFA mice with norfloxacin (Table 3), erythromycin (2), and nifurzide (18). However, final appreciation of changes in colonization resistance during antibiotic treatments will only be possible by observing the actual colonization of patients during clinical trials. Such appreciation is important, because antibiotic treatments are associated with increased susceptibility to intestinal pathogens (16, 21) and might be a risk factor associated with antibiotic-resistant gram-negative bacteremia in neutropenic patients.

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