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Presence of *Clostridium difficile* and Antibiotic and β-Lactamase Activities in Feces of Volunteers Treated with Oral Cefixime, Oral Cefpodoxime Proxetil, or Placebo

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Three groups of six healthy adult volunteers were randomly assigned to a treatment with 400 mg of oral cefpodoxime proxetil, oral cefixime, or placebo per day for 10 days. Informed consent was obtained from all volunteers. *Clostridium difficile* was not detected in the feces of any subject before treatment or at any time in the subjects in the placebo group. *C. difficile* was, however, detected in all subjects treated with cefpodoxime proxetil and in five of six treated with cefixime. Genomic DNA restriction patterns showed that the strains of *C. difficile* differed from one volunteer to another. Two subjects both shed different strains at different times during the 25-day surveillance period. All isolates were resistant to cefixime and cefpodoxime (MIC for 90% of strains, 256 and 512 mg/liter, respectively). Antibiotic activity was found in the feces of one volunteer treated with cefpodoxime proxetil and of four volunteers treated with cefixime. It was inversely correlated with the presence of fecal β-lactamase activity. Intestinal side effects were limited to modifications of stool consistency, which occurred in only 3 of the 12 treated volunteers and did not lead to cessation of treatment. These modifications were significantly associated with the presence of fecal antibiotic activity ($P < 0.05$) but not with the shedding of toxigenic or nontoxigenic strains of *C. difficile* or with the presence of toxin A in feces, which was detected only in one perfectly healthy treated volunteer.

Clostridium difficile is a sporulating gram-positive bacillus which can cause pseudomembranous colitis in patients given antibiotic treatment (2). It is associated with the production of an enterotoxin (toxin A) and a cytotoxin (toxin B) (16), both of which are usually present in the feces of patients with antibiotic-associated colitis (17).

Several authors have recently stressed the risk of nosocomial acquisition and transmission of *C. difficile* (11, 19). *C. difficile* has also been isolated from healthy infants (29), as well as from healthy adults (29), although to a lesser extent. However, few data are available about the mechanisms of the acquisition of *C. difficile* or the number and characteristics of the strains that colonize the subjects.

Besides pseudomembranous colitis, *C. difficile* has been associated with postantibiotic diarrhea (1). Although this condition is less severe and is often benign, its physiopathology is unclear (1).

Treatment with beta-lactam antibiotics is a frequent cause of postantibiotic diarrhea. We have shown that modifications in the composition of the intestinal flora occurred in volunteers after treatment with oral (5) or parenteral (15) cephalosporins but were more marked in those who had no *Bacteroides*-type β-lactamase activity in their feces. In gnotobiotic mice experimentally infected with a complex human fecal flora and treated with ceftriaxone, these β-lactamases minimized modifications of the intestinal flora and minimized colonization by *Candida albicans* and *Enterobacter cloacae* (15). However, in that study we did not investigate colonization by *C. difficile*. Others have shown that hydrolysis of ampicillin by fecal β-lactamases promoted the overgrowth of *C. difficile* in hamsters, suggesting that intestinal β-lactamases did not afford protection against *C. difficile* disease (26).

Recently, oral broad-spectrum cephalosporins have been marketed to replace ampicillin, because of the growing number of infections caused by resistant microorganisms. Of these cephalosporins, cefixime and cefpodoxime have high in vitro antibacterial activity (13). In various series of patients treated with cefixime, changes in stool consistency were observed in 11% (10) and 13% (12) and diarrhea was noted in 14% (12) and 15% (10). On the other hand, in some series of patients treated with cefpodoxime proxetil, stool changes were observed in only 0.7% (23, 27) and diarrhea was noted in 4.6% (27) and 12% (23).

Cefixime is an active compound, and cefpodoxime proxetil is a prodrug without any antibacterial activity before its ester bond is hydrolyzed; this occurs either during its passage through the wall of the small intestine or inside the intestinal lumen itself (22). Although these drugs have relatively high bioavailability, they undergo only partial intestinal absorption (31). As regards cefpodoxime proxetil, no information is available about the proportions of prodrug and active drug that reach the colon after oral absorption. However, in a study of human volunteers given cefotiam hexetil, a prodrug closely related to cefpodoxime proxetil, we could not detect any prodrug in the feces (5). Therefore it can be expected that even after oral absorption of prodrugs, a significant amount of active drug reaches the colon and affects the intestinal flora, just as an active compound would do.

In the present study, we tested this hypothesis by investigating the *C. difficile* colonization of community-living healthy volunteers to see whether it was different after treatment with either cefixime or cefpodoxime proxetil. We

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also investigated the possible relationships between this colonization and fecal excretion of antibiotic and β-lactamase activities. We used genomic typing to differentiate the isolates of *C. difficile*.

MATERIALS AND METHODS

Eighteen healthy adult volunteers aged from 24 to 42 years (median, 30 years), were included in the study. None had taken antibiotics during the preceding month. They were divided into three groups of six subjects each, and the groups were randomly assigned to oral treatment with cefpodoxime proxetil (200 mg twice daily), cefixime (400 mg once daily), or placebo for 10 days. The volunteers were not institutionalized, and they were asked to continue their normal lifestyle and food preferences. They lived separately, except for two (one from the cefpodoxime proxetil group and one from the placebo group) who shared the same accommodation. During the experiment, all volunteers were interviewed daily by a physician to obtain information about intestinal symptoms and stool consistency. Fecal samples were obtained before treatment, daily during treatment, and every other day for the 2 weeks after treatment. All samples were coded blindly before transport to the laboratory, and the code was not broken before all clinical and microbiological data had been entered into a computerized data base. Informed consent to participate in the trial was obtained from all volunteers, and the study protocol was approved by the local Ethics Committee.

Fecal samples were streaked on Wilkins-Chalgren agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood, 1 g of taurocholate (Sigma, Saint Louis, Mo.) per liter (30), 16 mg of cefoxitin per liter, and 500 mg of cycloserine per liter (supplement SR 96; Oxoid) and incubated for 48 h in anaerobic jars (Anaerocult A system; Merck, Darmstadt, Germany). Fluorescent colonies were identified as *C. difficile* by microscope examination, the shape and position of spores, and the API profile of the colonies (An Ident System; API System SA, La Balmes-Grottes, France). Preliminary experiments (results not shown) involving fecal samples reconstructed in the laboratory showed that the detection limit of this technique for identifying *C. difficile* was 10^3 to 10^4 CFU/gram of feces. For further typing, a single colony of *C. difficile* from each positive fecal sample obtained from the volunteers was purified and then stored at room temperature in beef liver agar (Diagnostics Pasteur, Marnes-la-Coquette, France).

The various isolates of *C. difficile* were grown in 10 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 5 days in an anaerobic glove box and centrifuged at 7,000 × *g* for 5 min. The supernatants were kept at 4°C and tested within 48 h for toxin B in a cytotoxicity assay with Chinese hamster ovary cells (CHO-K1; Flow Laboratories Inc., McLean, Va.) grown on 96-well microtiter plates (Nunc, Roskilde, Denmark), as previously described (7).

Aliquots of culture supernatants and of fecal samples obtained from the volunteers were kept frozen at −20°C until assay for toxin A by a commercially available enzyme immunoassay (Premier *C. difficile* toxin A; Meridian Diagnostics, Inc., Cincinnati, Ohio) as recommended by the manufacturer. The accuracy of this technique in detecting toxin A is close to that of reference tissue culture assay methods for detection of toxin B (8).

The chromosomal restriction endonuclease digestion patterns of the *C. difficile* isolates were compared by pulsed-field gel electrophoresis of large chromosomal fragments. For this purpose *C. difficile* strains were grown under anaerobic conditions to the midexponential phase in thioglycolate broth (Hoffman-La Roche, Basle, Switzerland). Washed cells were mixed with an equal volume of 2% low-melting-temperature agarose (FMC Bioproducts, Rockland, Maine) and allowed to solidify into a 200-μl plug mold (Bio-Rad, Richmond, Calif.). Genomic DNA was prepared in agarose plugs as previously described (3). Plugs were placed for 18 h at 37°C in 10 ml of lysis solution (6 mM Tris-HCl [pH 7.6], 0.5% Sarkosyl, 460 U of lysozyme per ml), transferred to a solution of 0.5 M EDTA and 10 mg of proteinase K (Boehringer, Mannheim, Germany) per ml, and incubated at 50°C for 48 to 72 h. The plugs were then washed twice with 10 mM Tris-HCl–1 mM EDTA (1× TE) medium, incubated twice for 1 h at 50°C with 400 μg of phenylmethylsulfonyl fluoride per ml in 1× TE medium, washed in 0.5× TE medium, and stored at 4°C in 0.2 M EDTA until digestion for 3 h with 20 U of either *Nru*I at 37°C or *Sma*I at 25°C in 700 μl of the buffer specified by the manufacturer (Boehringer). Digested plugs were inserted into the wells of a 1.5% agarose gel (Appligene, Illkirch, France) containing 0.5× TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA [pH 8.3]). This preparation was subjected to pulsed-field gel electrophoresis with a contour-clamped homogeneous electric field device (CHEF-DRII; Bio-Rad). Electrophoresis was run for 30 h at 14°C with a field of 200 V. Pulse times were increased linearly from 5 to 50 s. Lambda concatemers (Bio-Rad) were used as size standards. After electrophoresis, the gels were stained with ethidium bromide, destained overnight in distilled water, and photographed. Preliminary experiments (not shown) demonstrated the good reproducibility of the technique.

The MICs of cefixime and cefpodoxime were determined by the method of Steers et al. (28) by using Wilkins-Chalgren agar supplemented with 5% of defibrinated horse blood and incubated in anaerobic jars for 48 h.

Concentrations of cefpodoxime or cefixime in feces were measured in an agar diffusion assay (4) with antibiotic medium 5 at pH 8.0 (Difco) and with *Escherichia coli* VC1894 as the indicator strain.

We determined β-lactamase activity in the supernatants of fecal samples diluted in 0.1 M phosphate buffer (pH 7) and centrifuged at 7,000 × *g* for 20 min with nitrocefin (kindly provided by Glaxo Laboratories, Paris, France) as substrate. Readings were made at 486 nm on a Dacos analyzer (Coultronics, Inc., Hialeah, Fla.) and 1 U of β-lactamase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of nitrocefin per min at 37°C. The limit of detection was 10 mU of β-lactamase per g of feces.

Differences in proportions among groups were assessed by using Fisher's exact test.

RESULTS

The median number of specimens analyzed from each volunteer was 16 (range, 12 to 17). *C. difficile* was not isolated from any of the 18 pretreatment samples (one per volunteer) or from any sample from the 6 volunteers given placebo. It was, however, isolated from at least one sample from five of the six volunteers taking cefixime (83%), and from all six taking cefpodoxime proxetil (100%). The initial detection of *C. difficile* did not occur significantly earlier in the volunteers taking cefpodoxime than in those taking cefixime ($P = 0.06$; log rank test) (Fig. 1).

In 8 of the 11 colonized volunteers (73%), several samples were positive for *C. difficile*. The intervals between the first

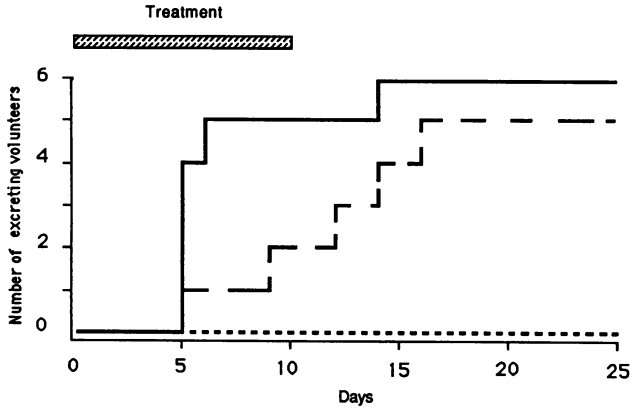

FIG. 1. Excretion of *C. difficile* by healthy volunteers treated for 10 days with placebo (- - - -), cefixime (— —), or cefpodoxime proxetil (——).

and last isolations ranged from 2 to 20 days (median, 11 days). Of the 11 volunteers, 4 were still shedding *C. difficile* at the end of the 25-day surveillance period.

Seventeen clones of *C. difficile* from 10 volunteers were tested in vitro for production of toxins A and B (Table 1). Eight volunteers shed at least one toxigenic strain of *C. difficile* (Table 1). In volunteer 5, both toxigenic and nontoxigenic strains were present on different days (Table 1).

Only one volunteer (volunteer 2) had detectable toxin A in the feces. This occurred on days 9 and 16 after the beginning of treatment. Toxigenic strains of *C. difficile* were isolated from the feces of the same volunteer on and after day 12. No toxin A was detected in any other fecal sample from any volunteer in any group, even when some individuals were shedding strains of *C. difficile* which were toxigenic in vitro.

Pulsed-field gel electrophoresis of the restricted DNA from 24 strains of *C. difficile* isolated from eight different volunteers (three per volunteer) showed that *Sma*I generated seven to nine DNA fragments (Fig. 2). Strain patterns differed among volunteers, except for the isolates from volunteers 6 and 17, which displayed the same *Sma*I patterns. However, analysis of the patterns after digestion with *Nru*I revealed differences between the strains isolated from the feces of these two volunteers (Fig. 2). In six subjects, three strains isolated on different days had identical *Sma*I patterns (Fig. 2) and *Nru*I patterns (data not shown). By contrast, two strains from volunteer 8 and three strains from volunteer 5 displayed different *Sma*I patterns (Fig. 2) and *Nru*I patterns (data not shown).

Some β-lactamase activity was present in the pretreatment samples from all the volunteers, at concentrations which were not significantly different in the three groups (Fig. 3). No antibiotic activity was detected in any pretreatment sample or in samples from 7 of the 12 patients (58%) taking either cefixime or cefpodoxime proxetil. The remaining five treated volunteers (42%), four of whom were treated with cefixime and one who was treated with cefpodoxime proxetil, displayed some antibiotic activity during treatment. The fecal β-lactamase activity, which had been present in the feces of all five before treatment, disappeared during treatment (Fig. 3), either before or concomitantly with the detection of antibiotic activity. Three fecal samples with detectable levels of cefixime (in two cases) or cefpodoxime (in one case) were positive for *C. difficile*.

The MIC of cefixime and cefpodoxime for 90% of strains (MIC_{90}) for the 10 strains of *C. difficile* tested were 256 and 512 mg/liter, respectively.

No major intestinal symptom such as pseudomembranous colitis or severe diarrhea was observed in any volunteer. Three of the 12 volunteers taking antibiotics (25%) and none

TABLE 1. In vitro toxinogenesis and DNA restriction patterns of *C. difficile* strains isolated from treated volunteers

Treatment	Volunteer designation	Time required for isolation of *C. difficile* (days)[a]	Characteristics of isolates:	
			Toxigenesis in vitro[b]	DNA restriction pattern[c]
Cefpodoxime proxetil	1	7	+	ND
	6	5	+	ND
		14	+	E e
		16	ND[d]	E e
		23	ND	E e
	10	14	ND	ND
	12	5	+	I i
		8	ND	I i
		16	+	I i
	16	6	ND	J j
		7	+	ND
		9	ND	J j
		10	ND	ND
		16	ND	J j
	17	5	ND	E h
		7	+	E h
		16	+	E h
Cefixime	2	12	+	A a
		18	ND	A a
		23	+	A a
	5	14	+	B b
		16	−	C c
		21	−	D d
	8	9	−	F f
		12	ND	F f
		16	−	G g
	9	5	ND	H h
		7	−	H h
		9	ND	H h
	18	16	+	ND

[a] After beginning of treatment.
[b] All toxigenic strains produced both toxins A and B.
[c] Each capital letter characterizes a specific pattern after digestion with *Sma*I, and each small letter characterizes a specific pattern after digestion with *Nru*I.
[d] ND, not done.

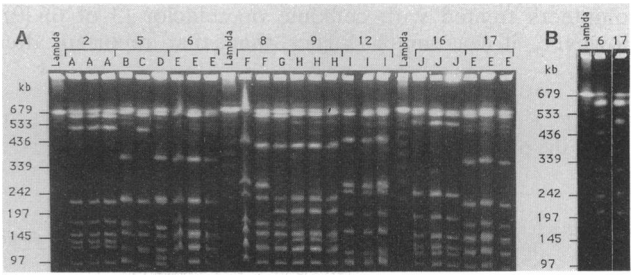

FIG. 2. Pulsed-field gel electrophoresis of various *C. difficile* genomic DNAs restricted by *Sma*I (A) and *Nru*I (B). Each number characterizes a volunteer from whom three *C. difficile* strains were isolated, and each letter characterizes a specific pattern. Bacteriophage lambda concatemers were used as size standards.

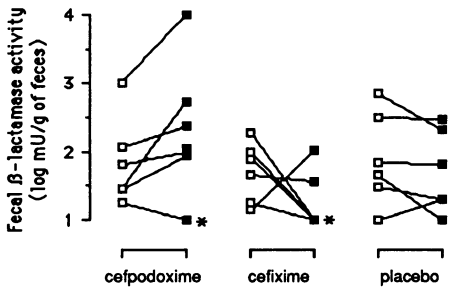

FIG. 3. Fecal β-lactamase activity (nitrocefin assay) in the feces of 18 volunteers before (□) and during (■) 10 days of treatment with cefpodoxime proxetil, cefixime, or placebo. Asterisks designate the four subjects from the cefixime group and the one from the cefpodoxime group whose feces contained detectable drug during treatment.

of the 6 taking placebo (0%) had loose stools for 1 or 2 days toward the end of the treatment. The symptoms were always mild and did not cause any volunteers to withdraw from the study. The three volunteers with loose stools were among the five whose feces contained detectable levels of antibiotic activity. Thus, loose stools were significantly associated with the presence of fecal antibiotic activity (Table 2). By contrast, all fecal samples from these volunteers with loose stools were negative for toxin A. As mentioned above, only volunteer 2 had toxin A in feces, although at the time (days 9 and 16 of treatment) the stools were perfectly formed.

C. difficile was present in the feces of the three volunteers with loose stools. Two of these isolates were toxigenic in vitro. However, six volunteers with perfectly formed stools also excreted *C. difficile*, and at least one strain from each of them was toxigenic in vitro. Therefore, in the present study, we did not find any statistical association between the passage of loose stools and the shedding of toxigenic or nontoxigenic strains of *C. difficile* (Table 2).

DISCUSSION

Our data show no significant difference between the results of treatments with cefixime and with cefpodoxime proxetil, either in the proportion of volunteers excreting *C. difficile* or in the time that elapsed before the excretion was detected. Strikingly, at least 11 of the 12 volunteers taking one of the antibiotics (92%) excreted *C. difficile*, but the organism was not detected in the feces of any of those taking placebo. The proportion of subjects excreting *C. difficile* observed in this study was not significantly different from the 4 of 6 (9) or the 5 of 10 (21) previously reported for volunteers treated with cefixime or cefaclor (3 of 6) (9). However, it was much higher than that reported after administration of cefuroxime axetil (none of 10 volunteers) (14), although differences between the protocols of the latter study and the present one make comparisons unreliable. For instance, the high rate of detection of *C. difficile* observed in our study might occur, at least in part, because the feces of the volunteers were assayed daily.

TABLE 2. Relationship between the passage of loose stools and the presence of *C. difficile* and of antibiotic activity in the feces of 12 treated volunteers

Characteristic detected in feces	Presence in volunteers		*P* value
	With loose stools (n = 3)	Without loose stools (n = 9)	
C. difficile[a]	3	8	1.0
Toxigenic *C. difficile* strains	2	6	1.0
Antibiotic activity	3	2	0.05

[a] Either toxigenic or nontoxigenic.

Analysis of DNA restriction patterns combined with a study of the in vitro toxigenesis of the *C. difficile* isolates showed that different toxigenic or nontoxigenic strains could be isolated on different days from the feces of the same volunteer. However, since we did not analyze several of the *C. difficile* clones isolated from the same volunteer on the same day, we cannot conclude that this suggests either repeat colonization or cocolonization by different strains. Also, digestion of DNA with more than two restriction enzymes might have generated different profiles for strains considered to be identical. However, most investigators use only one or two enzymes for pulsed-field gel electrophoresis (20, 24). Our result confirmed that the use of two enzymes instead of one only increased the discriminant power of the technique to a limited extent. In addition, because the detection limit of the isolation technique used was around 10^3 CFU of *C. difficile* per g of feces, it is not possible to conclude either that antibiotic treatments promoted the overgrowth of strains colonizing the volunteers before the start of the experiment (but in counts below the detection limit of the technique) or that colonization from the environment occurred during treatment.

The intestinal side effects observed in the volunteers were confined to modifications of stool consistency that never led to the cessation of treatment. There was no statistically significant association between the passage of loose stools and the carriage of toxigenic or nontoxigenic strains of *C. difficile* or the presence of toxin A in the feces. This is in agreement with the finding by others that the incidence of excretion of toxigenic *C. difficile* was not significantly different in hospitalized patients with or without diarrhea (18).

We found, however, a significant correlation between the passage of loose stools and the presence of fecal antibiotic activity. Others postulated that antibiotic-associated diarrhea results from a reduced metabolism of the colonic microflora (6, 25). We reported previously that during treatment with cephalosporins, antibiotic activity in the feces seriously disrupted the composition of the anaerobic flora (15) but not when β-lactamase activity was present in the feces during treatment (15). The present study confirmed this; it also confirmed that, as suggested by others (26), fecal β-lactamases did not prevent colonization by *C. difficile*, because all but one of our treated volunteers were colonized by *C. difficile*, irrespective of the presence or absence of antibiotic or β-lactamase activity in their fecal samples.

Taken together, our results show that treatments with oral cephalosporins frequently give rise to the excretion of *C. difficile* but that this excretion is not statistically associated with the passage of loose stools. Hydrolysis of antibiotic residues by fecal β-lactamases apparently afforded protection against such side effects.

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