Effects of Antibiotics and Other Drugs on Toxin Production in *Clostridium difficile* In Vitro and In Vivo

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In an attempt to understand more completely why patients treated with phenothiazines (chlorpromazine and cyamemazine), methotrexate, and certain antibiotics such as clindamycin have an increased risk of developing pseudomembranous colitis, the production of toxins A and B by *Clostridium difficile* in the presence of these drugs was measured in vitro as well as in vivo by using axenic mice. None of the drugs tested increased the production of toxins either in vitro or in vivo.

*Clostridium difficile* is widely incriminated in cases of pseudomembranous colitis (PMC) and postantibiotic diarrhea (15, 20, 21). The pathogenicity of this organism is due to the production of two toxins: toxin A (enterotoxin), which has strong enterotoxic activity and weaker cytotoxic activity and toxin B, which is strongly cytotoxic. Both toxins are lethal to tissue culture cells and animals (16, 19, 23, 25, 32). It has been known for some time that antibiotics such as clindamycin can lead to both PMC and postantibiotic diarrhea (1–5, 11–14, 27, 31). It is suspected that antibiotics promote the overgrowth of *C. difficile* in these patients, leading to development of the toxin-mediated diseases. In addition, other, nonantibiotic drugs have also been suspected of inducing these conditions. Neuroleptics, such as cyamemazine and chlorpromazine (6), and methotrexate, which is used in the long-term treatment of cancer (8, 10, 22, 27), have been reported to induce PMC and postantibiotic diarrhea. The purpose of the present work was to determine the action of such drugs on the production of toxins A and B by *C. difficile* in vitro. We also examined the production of toxins in germfree mice treated with drugs and inoculated with *C. difficile*. By using germfree mice, we attempted to determine directly whether certain drugs have an influence on the production of toxins A and B in the absence of interference from the normal intestinal flora.

The toxigenic strain *C. difficile* VPI 10463 was kindly provided by T. D. Wilkins (32). All cultures were incubated under anaerobic conditions in an anaerobic chamber, and all growth media were reduced prior to use. The media used were brain heart infusion (Pasteur Diagnostic, Paris, France) and GAPTT medium (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) (26). The strain was cultured and stored on a semisolid GAPTT medium (26).

For toxin production in vitro, the MICs of drugs were determined according to established European standards (Comité de l’Antibiogramme). The drugs were diluted in sterile water and then filtered (Minisart membrane; Sartorius, Paris, France). Twofold serial dilutions of drugs were made in brain heart infusion and inoculated with 10⁶ CFU of bacteria per ml. The results were read after a 48-h incubation at 37°C. The MIC was defined as the lowest drug concentration giving visible inhibition of growth and was found to be 4 μg/ml for clindamycin hydrochloride, 1,024 μg/ml for cyamemazine tartrate, and 64 μg/ml for chlorpromazine hydrochloride.

To determine the effects of various drugs on expression of *C. difficile* toxins in vitro, the following concentrations were used at one-half and one-fourth the MIC, respectively: clindamycin, 2 and 1 μg/ml; chlorpromazine, 32 and 16 μg/ml; and cyamemazine, 512 and 256 μg/ml. Methotrexate was used at 5, 2.5, and 1.25 μg/ml, corresponding to 2, 1, and 0.5 times the blood levels encountered in human treatment, respectively. A 100-ml volume of brain heart infusion containing the drugs listed above at different concentrations was seeded with 1 ml of broth from an 18-h culture of *C. difficile* (final inoculum, 10⁶ CFU/ml). Brain heart infusion medium containing the same drugs at the MICs was also seeded with *C. difficile* and served as a control. Media were then distributed into 10-ml tubes and incubated under anaerobic conditions at 37°C in an anaerobic chamber. A drug-free control tube was included in each batch. After a 6-day incubation period, bacteria were counted by making 10-fold serial dilutions (10⁶ to 10⁻⁶) of each culture and subsequently seeding 1 ml of each dilution in duplicate in GAPTT medium (26). Bacteria were counted 48 h later after incubation at 37°C.

To determine the effect of drugs on toxin production in vitro, the 6-day cultures described above were centrifuged (3,000 × g) and the supernatants were stored at 4°C for the cytotoxin assay and at −20°C for the enterotoxin assay.

Toxin A was assayed by using a double sandwich enzyme-linked immunosorbent assay by the method of Lyerly et al. (17) as modified by Corthier et al. (9). Threelfold serial dilutions of the test samples and control were made in phosphate-buffered saline–Tween. Toxin A present in the sample was captured by two antitoxin antibodies, prepared by immunization of swine and rabbits (Institut National de la Recherche Agronomique, Jouy-en-Josas, France). An antirabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma) was then added, followed by detection with a substrate (p-nitrophenyl phosphate disodium hexahydrate; Sigma). The results are expressed as log_{10} concentrations of toxin A.

The level of toxin B production was assayed by determining its effect on Chinese hamster ovary cells (CHO-K1) cultured in microtiter plates (3 × 10⁴ cells per well) (9). Fivefold serial dilutions of the toxin samples were incubated
for 18 h with the cells at 37°C in an atmosphere containing 5% CO2. After fixation and coloration of the culture cells with methylene blue, toxin B activity was determined, and the titer was defined as the log10 of the highest dilution at which 100% of cells were rounded.

For the assays of toxins in vivo, 6-week-old (25- to 30-g) male and female germfree C3H mice (CSEAL, Orléans, France) were maintained in sterile isolators. The mice were given food sterilized by irradiation (Ro3 40 UAR) and water sterilized by autoclaving. Groups of five mice each were treated with clindamycin, methotrexate, or the phenothiazines. The drugs were given in drinking water every 2 days; five untreated mice served as a control.

One group received a dose of 75 mg of clindamycin per kg of body weight per day, corresponding to five times the human daily dose. On day 10, the control and experimental animals were gastrically intubated with 0.5 ml of an 18-h culture of *C. difficile* (5 × 107 CFU). Drug treatment was continued for an additional 7 days. One group of mice received 250 mg of methotrexate per kg per day (twice the human daily dose) in their drinking water. On day 10, the treated and control mice were challenged with *C. difficile* (5 × 107 CFU) as described above, and drug treatment was continued until the animals died. Four groups of mice were treated for a period of 4 weeks with phenothiazines: group 1 received 10 mg of chlorpromazine per kg per day (five times the human daily dose) and 20 mg of mecamylamine per kg per day (mecamylamine, which is a powerful β-adrenergic agent that slows intestinal transit, was given intraperitoneally to enhance intestinal absorption of the two phenothiazines on intraintestinal levels), group 2 received 35 mg of cyamemazine per kg per day (five times the human daily dose) and 20 mg of mecamylamine per kg per day, group 3 served as a control (the mice were given only 20 mg of mecamylamine per kg per day), and group 4 consisted of untreated mice. After 4 weeks of treatment, the mice were challenged with *C. difficile*, and the drug treatment was continued until the animals died.

For the toxin assays, the feces or the ceca of the dead or moribund animals were removed, weighed, homogenized, and diluted 10 times in LCY (0.2% casein hydrolysate, 0.5% NaCl, 0.1% KH2PO4, 0.2% yeast extract in water [pH 7.0]). After centrifugation (3,000 × g), 1 ml of trypsin inhibitor per ml (type I-S from soybean; Sigma) was added to the supernatants, which were then stored for assay as described above. Bacteria in the feces were counted as described above. For statistical analysis of the results, the variance analysis and Student's *t* test were used to compare the mean value of log10 bacterial counts, log10 cytotoxin titers, and log10 nanograms of enterotoxin per milliliter.

As shown in Table 1, addition of different concentrations of drugs did not affect bacterial growth at day 6. Clindamycin at subinhibitory concentrations had no effect on cytotoxin production or enterotoxin production. There was no significant difference between the control group and the experimental group (*P* < 0.05). The same was true for methotrexate (*P* < 0.05). With chlorpromazine and cyamemazine, however, a significant decrease in enterotoxin and cytotoxin production was seen when chlorpromazine (32 μg/ml) (*P* < 0.01 for toxin A; *P* < 0.01 for toxin B) and cyamemazine (32 μg/ml) (*P* < 0.01 for toxin A; *P* < 0.01 for toxin B) were added. For other concentrations, there was no significant difference (*P* < 0.05).

In studies assessing the effects of clindamycin treatment, results in vivo showed that the untreated control animals challenged with *C. difficile* died within 48 h. In the ceca of these animals, we detected 10⁶ CFU of *C. difficile* per g with high toxin concentrations. The mice treated with clindamycin survived through day 2 with no detectable toxins and a significantly reduced organism count in the feces (Fig. 1). All the animals died within 4 days after termination of treatment with toxin levels in their ceca identical to those in untreated controls (Fig. 1). The mice treated with methotrexate for 10 days died within 48 h of *C. difficile* challenge, and they had bacterial counts and toxin concentrations comparable to those in untreated controls (Fig. 2). The same was true for chlorpromazine plus mecamylamine, cyamemazine plus mecamylamine, and mecamylamine alone. Counts of bacteria and toxins similar to those in controls were observed (Fig. 3).

The analysis of the results indicates that there is no increase in toxin production by *C. difficile* after treatment with various drugs either in vitro or in vivo. In fact, in vitro

| Table 1. Toxin production in *C. difficile* following drug exposure* |
|---------------------------------------------|
| Treatment group and concn (µg/ml) | Cytotoxin (log10 titer) | Enterotoxin (log10 ng/ml) | *C. difficile* (log10 CFU/g) |
| Control | 5.6 ± 0.1 | 2.5 ± 0.2 | 7.5 ± 0.4 |
| Clindamycin | | | |
| 1 | 5.3 ± 0.2 | 1.9 ± 0.2 | 7.4 ± 0.3 |
| 2 | 5.0 ± 0.1 | 1.6 ± 0.3 | 7.5 ± 0.2 |
| Chlorpromazine | | | |
| 16 | 4.8 ± 0.1 | 1.5 ± 0.1 | 7.5 ± 0.5 |
| 32 | 4.8 ± 0.2 | 1.1 ± 0.2 | 7.4 ± 0.4 |
| Cyamemazine | | | |
| 256 | 5.5 ± 0.4 | 1.7 ± 0.3 | 7.6 ± 0.3 |
| 512 | 4.6 ± 0.2 | 1.3 ± 0.1 | 7.5 ± 0.2 |
| Methotrexate | | | |
| 1.25 | 5.9 ± 0.1 | 2.6 ± 0.2 | 7.5 ± 0.3 |
| 2.5 | 5.9 ± 0.1 | 2.5 ± 0.2 | 7.6 ± 0.2 |
| 5 | 5.9 ± 0.2 | 2.2 ± 0.3 | 7.5 ± 0.2 |

* All values are means ± standard errors.

FIG. 1. *C. difficile* count and toxin levels in axenic mice treated with clindamycin (75 mg/kg) after *C. difficile* challenge. The bars at the top of the columns indicate standard deviations.
at day 6, chlorpromazine and cyamemazine led to a slight reduction in the levels of both toxins. Methotrexate and clindamycin had no effect on toxin production. George et al. (12) reported that clindamycin increased production of cytoxin in six strains of C. difficile. Honda et al. (14) found that clindamycin increased the production of enterotoxin by a strain isolated from an infected patient. Nakamura et al. (24), on the other hand, found that toxin production in response to drug treatment tended to vary depending on the particular toxigenic strain used; these researchers studied 80 clinical isolates of C. difficile, and only six clindamycin-susceptible strains showed high levels of cytoxin production. Our results with clindamycin cannot be directly compared with those of previous investigators, since in the present work we used only one strain of C. difficile that is highly toxigenic (32), and there appears to be variation among the strains studied.

In vivo, clindamycin was found to protect mice after challenge with C. difficile, and the mice died after therapy was stopped. This can be accounted for by the antibacterial action of clindamycin, which prevents the growth of C. difficile in the intestine at the doses used in this study. In fact, the C. difficile strain studied is susceptible to clindamycin, and the intestinal concentrations achieved were above the MIC. Methotrexate led to a marked change in the general state of the mice, with considerable weight loss (−20%) observed. This is probably due to the general systemic effects of methotrexate, although it had no effect on toxin production in C. difficile. For the mice treated with phenothiazines, the treatment was not found to slow the intestinal transit in these animals, as has been observed in humans, in whom these drugs often lead to a fecal stasis (6). It has been shown by Church and Fazio (7) and Talbot et al. (29) that fecal stasis resulting from age, surgery, disease of the digestive tract, or drugs is an important risk factor for PMC. Despite the slower intestinal transit induced by concomitant mecamylamine administration, phenothiazines had no effect on the production of toxins from C. difficile, which is the key pathogenic component in PMC.

In conclusion, both in vivo and in vitro experiments showed no changes in toxin production which could explain the development of PMC or postantibiotic diarrhea. As has been proposed by others (15, 20, 21), production of toxins A and B relating to PMC is most directly dependent upon the overgrowth of C. difficile in the intestine under clinical conditions. In the complex intestinal milieu characteristic of humans, many factors, such as the nature of the microbial flora before and after drug treatment (30), diet (18, 28), and the state of host defenses, undoubtedly play a role in C. difficile growth and resulting pathogenicity.

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