Effect of Various Diets on Toxin Production by Two Strains of *Clostridium difficile* in Gnotobiotic Mice

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When axenic mice fed a commercial diet were monoaassociated with two toxigenic strains of *Clostridium difficile*, 100% of them died 3 days after inoculation and both enterotoxin and cytotoxin were produced in their intestinal tract. However, when axenic mice were fed various semisynthetic diets before *C. difficile* challenge, some of them survived and their fecal cytotoxin and enterotoxin productions were highly reduced, whereas the *C. difficile* population level did not decrease to a great extent. Thus, gnotobiotic mice with *C. difficile* were a good model for the study of modulation by the dietary regimen of intestinal cytotoxin and enterotoxin production.

It has been well established that *Clostridium difficile* is responsible for pseudomembranous colitis (2, 7, 11), and it has been shown that its pathogenicity results from the production of two toxins, toxin A (an enterotoxin) and toxin B (a cytotoxin) (13, 22). Administration of a nontoxigenic strain of *C. difficile* and of a toxigenic one has been shown to exert a protective effect in hamsters and gnotobiotic rodents (3, 5, 23). In the latter case, this effect was accompanied by a modulation of cytotoxin production. Various intestinal bacteria of human origin were found to play the same role as a nontoxigenic *C. difficile* strain (4) in the protection of mice and modulation of toxin production. In vitro culture of toxin-producing strains of *C. difficile* showed that variations in the production of both toxins were affected by the nutritive substances of the culture medium (9).

The purpose of the present work was to determine whether dietary regimens affect toxin production in vivo. The conventional experimental model, i.e., the hamster, was not used here because the pseudomembranous colitis was induced by antibiotic therapy and the remaining flora was able to metabolize the diet. Several authors have reported that axenic rodents inoculated with different strains of *C. difficile* developed an experimental pseudomembranous colitis (6, 18, 24). Thus, gnotobiotic mice, in which the transformation of the diet in vivo is limited to an interaction between the host and the only strain present in their intestinal tract, were chosen as an experimental model.

**MATERIALS AND METHODS**

**Bacterial strains and bacterial counts.** Two toxigenic strains of *C. difficile* were used: strain VPI 10 463 (VPI), isolated from human tissue and kindly provided by T. D. Wilkins (1), and strain Mara, isolated by us from the feces of a human adult suffering from pseudomembranous colitis. These strains were cultured in semisolid medium B' (21). Fecal and cecal samples were diluted 10-fold, poured into tubes (8 × 400 mm; Touzart and Matignon, Vitry-sur-Seine, France), and immediately cooled to ensure prompt solidification (20). Incubation was for 48 h at 37°C.

**Enterotoxicity assay.** Enterotoxin was purified from a VPI culture poured in dialysis flasks surrounded by brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Incubation was for 4 days at 37°C in an anaerobic chamber. Crude enterotoxin was obtained after precipitation of culture broth with acetate buffer (20 mM, pH 5.5). Pure enterotoxin was prepared according to the method of Sullivan et al. (22). Purity was controlled by polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. Only one band, with a molecular weight of 40,000, was detectable by Coomassie blue staining.

Pure and crude enterotoxins were inactivated by mixing them with 1 volume of formaldehyde (Prolabo, Paris, France) for 1 week. Antiserum against crude enterotoxin was obtained from pigs immunized by the intramuscular route (10 mg per pig) with Freund complete adjuvant (Institut Pasteur Production, Ville d’Avray, France). Three challenges were done at 1-month intervals. Animals were killed 1 week after the last challenge, and blood was collected. Antiserum against pure enterotoxin was obtained from rabbits immunized with 0.5 mg of toxin per animal. The immunization schedule was the same as for pigs. Pig and rabbit antienterotoxin sera did not neutralize cytotoxin activity in tissue culture.

Enterotoxin amounts were determined in 96-well microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.) by using a double sandwich enzyme-linked immunosorbent assay (ELISA) technique derived from that of Lyerly et al. (15). The plates were activated with 100 μl of 1% glutaraldehyde (grade II; Sigma) in distilled water for 1 h at 4°C. After being washed with distilled water, 100 μl of antienterotoxin swine pooled immunoglobulins (5 ng of protein per ml) (bicarbonate buffer, 0.06 M, pH 9.6) was put in each well. The plates were incubated for 2.30 h at 37°C. Each well was washed with phosphate-buffered saline-0.05% Tween 20 (PBS-T); pH 7.0, containing 5% newborn calf serum (Industrie Biologique Francaise, Villeneuve la Garenne, France). Test samples (fecal or cecal suspensions) were added to the wells, serially diluted in PBS-T, and incubated overnight at 4°C. A purified enterotoxin dilution used as control was placed in one row of wells per plate. After being washed, 100 μl of antienterotoxin rabbit serum diluted 103-fold in PBS-T was added to each well. After incubation
for 1.30 h at 37°C and washing were done, 100 μl of goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma) diluted 1/2,000 in PBS-T was added to each well. After incubation for 2 h at 37°C and washings were done, the plates were incubated with 100 μl of substrate (p-nitrophenyl phosphate disodium hexahydrate; Sigma 104 phosphatase substrate) in diethanolamine buffer, pH 9.8, for 1 h at 37°C. The plates were read immediately with a spectrophotometer (Titertek Multiskan; Flow Laboratories) at 410 nm. For each plate, a calibration curve was obtained with pure enterotoxin. The limit of detection was about 0.2 ng of pure enterotoxin per ml. For each sample, optical densities at 410 nm in the linear part of the curve were transformed by using a calibration curve. The enterotoxin concentration corresponded to the average value of all estimations (expressed in log10 ng/g of feces). The limit of detection in feces was 5 ng/ml (0.7 log10 ng/ml) because dilutions are required before testing. Nontoxigenic C. difficile strains were negative in such tests.

**Cytotoxicity assay.** Cytotoxicity activity of C. difficile strains in cell lines of Chinese hamster ovary cells (CHO-K1) was assayed in 96-well microtiter plates (Nunclon; Nunc, Roskilde, Denmark) by the method previously described by Corthier et al. (4). The cytotoxic titer was defined as the log10 reciprocal value of the highest dilution at which 100% of cells are rounded.

**Cecal weight.** Mice were killed by cervical dislocation and weighed. Ceca were removed and weighed. Then, each cecum was dried for 1 week at 105°C to estimate its dry weight.

**Animals and diets.** C3H/He axenic adult mice were reared in a Trexler-type isolator fitted with a rapid transfer system (La Calhène, Velizy, France) and fed ad libitum various diets. Four regimens were used: a commercial diet RO1-40 (UAR, France) and on the basis of a simplified semisynthetic balanced. (SB) diet derived from “Animal Research Diets” (ICN Pharmaceuticals Inc., Irvine, Calif.), a protein-rich (HP) diet and a carbohydrate-rich (HC) diet were prepared (Table 1). The four diets were sterilized by gamma irradiation at 40 kGy.

The following experimental design was used. The control group was fed the commercial diet RO1-40, and other groups were fed the SB, the HP, and the HC diets. After a 15-day adaptation to the different diets, the four groups were challenged by the intragastric route with a 24-h culture of C. difficile containing approximately 108 viable cells. Only the control and SB diets were tested in mice challenged with strain Mara. Seven days after challenge, all SB, HP, and HC groups were fed the commercial diet RO1-40. Feces were individually collected from surviving mice and ceca were collected from dead mice for enumerating viable cells and detecting enterotoxin and cytotoxin. Preliminary experiments were made to ensure that the number of viable cells and the concentrations of both toxins were similar in ceca and feces of mice.

**Statistical analysis.** Variance analysis and Student’s t test were used for comparison of mean values of log counts, log cytotoxin titers, and log nanograms of enterotoxin per ml.

**RESULTS**

Reduction of cecum size. The SB diet reduced cecum weight in germfree and conventional mice as compared with control mice fed diet RO1-40. Both strains VPI and Mara reduced cecal size, compared with germfree mouse controls, whatever the diets. Under such conditions, cecum weights were similar to those of conventional mice (Table 2).

**Modulation of cytotoxin and enterotoxin production by C. difficile VPI in mice fed various diets.** All control mice fed the commercial diet RO1-40 were dead 3 days after inoculation with strain VPI. With the semisynthetic HP and HC diets, the mortality rates 5 days after inoculation were 30 and 10%, respectively, whereas all mice fed the SB diet survived (Fig. 1). In the three groups of mice fed the semisynthetic diets, the level of implantation was not significantly different from that of the control group (106 to 109 viable cells per g) except at 48 h, when a slight but significant difference was observed in mice fed the HC diet, compared with the control group (P < 0.05) (Fig. 2).

Two days after the C. difficile challenge, the cytotoxic titer was significantly lower (P < 0.01) in mice of the HP group than in mice of the HC and SB groups. This difference disappeared at day 7 of the experiment. Nevertheless, toxin production was 10- to 100-fold lower than that observed in control mice (P < 0.01).

Enterotoxin amounts were 102-fold lower in mice fed the three semisynthetic diets than in control mice (P < 0.001). A small production of enterotoxin was detected on day 7 of the experiment in some mice fed the HP diet (Table 3). After 7 days of the experiment, all surviving mice were again fed the commercial diet RO1-40. This change of feed did not affect the number of viable cells or the mortality rate. Whatever the initial diets, cytotoxin titers remained low and

![FIG. 1. Effect of diets on mortality of gnotobiotic mice monoassociated with C. difficile VPI.](http://iai.asm.org/)
stable during the whole experiment and enterotoxin was never detected.

Modulation of cytotoxin and enterotoxin production by \textit{C. difficile} Mara in mice fed various diets. As observed with strain VPI, all control mice that were fed the commercial diet RO$_3$-40 were dead 2 days after inoculation with strain Mara, whereas all mice fed the SB diet survived. The \textit{C. difficile} population slightly increased with time in mice fed the SB diet up to $10^7$ viable cells per g of feces (Fig. 3). Two days after challenge, there were no significant differences in the number of viable cells and cytotoxin titers between mice fed the SB diet and control mice. On day 7, the cytotoxin titers were significantly lower ($P < 0.001$) in the SB group than in the control group, although the numbers of viable cells were similar. Enterotoxin production was significantly lower on days 2 ($P < 0.05$) and 6 ($P < 0.02$), compared with control mice. There were no significant differences in the enterotoxin quantities between mice monoassociated with strain VPI (fed diet RO$_3$-40) and those monoassociated with strain Mara (whatever the diet) 2 days after challenge (Table 3).

After the change of diet on day 7, none of the surviving mice died and enterotoxin was never detected. Cytotoxin production increased gradually and was no longer significantly different from that of the control group on day 21 of the experiment, i.e., 15 days after the shift to diet RO$_3$-40.

### DISCUSSION

Both strains VPI and Mara, in the absence of competing bacteria, multiplied in the gastrointestinal tract of mice, produced high levels of enterotoxin and cytotoxin, and were lethal for gnotobiotic mice fed commercial diet RO$_3$-40. These findings are in agreement with the work of others, which suggests that simultaneous production of enterotoxin and cytotoxin is required to cause disease. Libby et al. (12) have reported that only children exhibiting a high cytotoxin and enterotoxin titer were sick. Lyerly et al. (14) showed that both toxins given intragastrically to conventional animals acted concomitantly; enterotoxin would disrupt the integrity of the intestinal mucosa and this would enable the cytotoxin to exert its pathogenicity.

Surprisingly, our results also showed that feeding can deeply affect the pathogenicity of \textit{C. difficile}. Mice monoassociated with strain VPI or Mara and fed semisynthetic diets survived. Previous studies (4, 5) demonstrated that some bacterial strains prevent death of gnotobiotic mice challenged with strain VPI and that a modulation of cytotoxin, and presumably enterotoxin, occurred, although the population level of strain VPI did not decrease to a great extent.
In this study, we have shown that various diets can also prevent mortality caused by *C. difficile*. This protection did not result from an antagonistic effect of these diets toward *C. difficile*. Indeed, the population levels of strain VPI and Mara were similar, whatever the diet. A marked reduction was observed in the cecal size of surviving and dead mice monoassociated with *C. difficile* VPI or Mara. This phenomenon has been reported in gnotobiotic rodents by several authors (6, 18) and did not seem to be involved in the protection. In mice fed the three semisynthetic diets, enterotoxin quantities fell very soon after challenge with strain VPI, whereas cytotoxin titers did not vary to a great extent. This could be caused either by the presence of components in the semisynthetic diets which could inhibit toxin production or by the absence of some components essential for toxin production.

Such a hypothesis does not alone account for this observation with strain Mara, since 1 week after challenge cytotoxin titers and enterotoxin production were still high in mice fed the SB diet, similar to dead mice monoassociated with strain VPI. Thus, it may be assumed that the SB diet could increase the host resistance to both toxins, so that high toxin production could be found in the intestine without leading to the death of mice. Enterocytes which form the intestinal barrier are in continuous turnover (8). The results of Perez-Schuel and Flores (19) suggest that in rats fed unbalanced diets a slowing-down of the cell turnover leads to prolonged activation of adenylate cyclase by cholera toxin. Moreover, Krivan et al. (10) have shown the existence of specific receptors of *C. difficile* enterotoxin on the surface of the intestinal brush border cells. Our SB diet may cause a slowing-down in the turnover of intestinal cells leading to a reduction in the number of neo-synthetized receptors able to bind enterotoxin. Because the integrity of the membrane was not damaged by the enterotoxin, the cytotoxin had no effect.

It might be assumed that the proteolytic activity changes according to diets and that the degradation of toxins varies in the different groups of animals despite a similar toxin production. In our opinion, this hypothesis is not valid, because only the level of toxin A was reduced. After the shift of the diet 1 week after the infection, levels of toxin remained low, although the protease activity was most likely similar to that of the control group.

It might be expected that in surviving mice the replacement of semisynthetic diets by the commercial diet would restore the same conditions as in the control group and then would permit the expression of pathogenicity of strains VPI and Mara. However, no case of mortality was observed. O’Hanley and Cantey (17) have shown that the immunoglobulin A antibody response occurred within 1 week in rabbits after experimental infection with invasive *Escherichia coli*. Moreau et al. (16) have demonstrated that bacterial urease production may be inhibited by an immune response of the mice. Furthermore, Czuprynski et al. (6) have detected an antitoxic antibody to the *C. difficile* (strain VPI) cytotoxin in sera from rats monoassociated for 21 days and have suggested that the toxin can interact with the immune system. Thus, it may be assumed that our mice were protected by antibodies after the change of diet. Another hypothesis could be made from the results of Corthier et al. (5), who showed that in gnotobiotic rodents a nontoxicogenic strain can modulate the cytotoxin production of strain VPI. It may be assumed that nontoxicogenic clones issued from our toxigenic strains might protect mice from them.

The experimental diets, although unbalanced in their protein and carbohydrate contents, were chosen to test the relative effect of protein and carbohydrate fractions on the modulation of toxin production. Whatever the diet, mice were protected. Therefore, this phenomenon does not seem to be related to the amount of protein and carbohydrate in the diets, but more probably to the nature of the components of these fractions, since the expression of pathogenicity was totally different according to diet. Our present studies aim at determining the components of diets which are involved in toxin production.

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**LITERATURE CITED**


