Modulation of Cytotoxin Production by *Clostridium difficile* in the Intestinal Tracts of Gnotobiotic Mice Inoculated with Various Human Intestinal Bacteria

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Gnotobiotic mice died 2 days after inoculation of a cytotoxicogenic *Clostridium difficile* strain. Protection occurred when mice were previously inoculated with a strain of *Escherichia coli* or *Bifidobacterium bifidum*. Intestinal cytotoxin production was highly reduced in the surviving mice, whereas the *C. difficile* population level did not decrease to a great extent.

Pseudomembranous colitis has frequently been associated with *Clostridium difficile* (3, 5, 7). The pathogenic effect of such a bacterial strain is related to the production of two toxins: an enterotoxin and a cytotoxin (10, 11). The main experimental model is a conventional hamster treated with an antibiotic such as clindamycin (6). This treatment destroys the bacterial antagonistic effect and thus permits the growth of *C. difficile*. With this experimental model, an antagonism between toxicogenic *C. difficile* and nontoxicogenic *C. difficile* has been described (12). However, intestinal microflora cannot be fully controlled in such a model, so it is not possible to study bacterial interactions accurately. The purpose of this work was to investigate the role of various bacterial strains inoculated together with *C. difficile* on intestinal cytotoxin production and survival rate of gnotobiotic mice.

The cytotoxicity assay was performed in 96-well flat-bottom microtiter plates (Nunclon). Chinese hamster ovary cells (CHO-K1, Flow Laboratories, Inc.) were grown to confluency in F12 medium (Flow Laboratories) containing 10% newborn calf serum (GIBCO Laboratories). The cells were trypsinized (0.025% trypsin) and washed with 1 volume of F12 medium. Cells were diluted in F12 medium containing 10% newborn calf serum, 0.02 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate) buffer (Flow Laboratories), and antibiotics. Approximately 10^5 cells were distributed per well. Plates were covered with a press-sensitive film (Falcon 3073, Becton Dickinson) to prevent loss of CO_2 and then incubated at 37°C for 1 day. Samples of toxin preparation were titrated in threefold dilutions within the wells. After an overnight incubation at 37°C, plates were fixed with cold absolute ethanol. After drying, plates were stained with crystal violet (50 µl per well). Wells with approximately 100% round cells were easily recognized after washing with tap water. The cytotoxic titer was defined as the reciprocal of the highest dilution which rounds 100% of CHO-K1 cells. The gnotobiotic mice were reared in Trexler-type plastic film isolators fitted with a rapid transfer system (La Calhène) and were fed ad libitum a commercial diet (U.A.R.) sterilized by gamma irradiation. Cytotoxigenic *C. difficile* VPI 10463 was isolated from human tissue (2) and kindly provided by N. M. Sullivan. Strains of *Eubacterium ventriosum*, *Eubacterium aerofaciens*, *Clostridium ino-

cuum*, *Bacteroides vulgatus*, and *Bifidobacterium adolescents* were isolated by us from feces of two healthy adults and kindly identified by M. Sebald, Institut Pasteur, Paris. Strains of *Bifidobacterium bifidum*, *Escherichia coli*, and *Streptococcus faecalis* were isolated by us from the feces of three healthy neonates. All strains were cultured in brain heart infusion broth (Difco Laboratories). Fecal and cecal samples were introduced in an anaerobic chamber (1) immediately after collection. After homogenization, samples were serially diluted, and the strains were enumerated with the medium described by Aranki et al. (1). Incubation was for 48 h at 37°C. Morphology of colonies allowed a specific count of each strain. Bacterial strains isolated from feces of both neonates and adults were administered individually to axenic mice through the orogastric route (0.5 ml containing 10^6 CFU/ml). Bacterial implantation was controlled 4 days later by microscopic observation of a fecal 10^{-2} dilution. Mice were then challenged by the toxigenic *C. difficile* strain inoculated in the same way.

All of the strains inoculated before inoculation of *C. difficile* became established at a high level (10^9 to 10^10 bacteria per g) in dead mice as well as in sacrificed mice (Table 1). Control mice inoculated with *C. difficile* died within 2 days after infection. Large amounts of cytotoxin were found in the cecal contents, and *C. difficile* became established at the level of 10^{6.4} bacteria per g (Table 1 and Fig. 1B). All of the mice inoculated with *C. innocuum*, with *E. aerofaciens*, with *B. vulgatus*, or with *B. adolescents* and then challenged with *C. difficile* died within 3 days postinfection. The population level of *C. difficile* was significantly lower (P < 0.05) in dissociated than in monoassociated mice. However, this decrease was small (0.4 log_{10}) and did not significantly modify mortality rates and cytotoxin titers in both groups. Accordingly, in the following statistical comparisons, we considered the cytotoxin titer and *C. difficile* population level of dead dissociated mice as reference values. Previous inoculation of mice with *E. ventriosum* or *S. faecalis* induced a partial protection (about 50%) of the mice against *C. difficile* challenge (Table 1). In these two experiments, cytotoxin titers and population levels of *C. difficile* in ceca were not significantly different from those observed in previous groups of dissociated mice. In the following two experiments, mice previously inoculated with *E. coli* or *B. bifidum* were protected against challenge with *C. difficile* (Table 1). The experiment was repeated once. Mice were

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TABLE 1. Effect of various bacterial strains previously inoculated in axenic mice on mortality rate after *C. difficile* challenge. *C. difficile* cecal population level, and cytotoxin production
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<table>
<thead>
<tr>
<th>Strains inoculated before <em>C. difficile</em> (source)</th>
<th>Cytotoxin Titer (log_{10} ± SEM)</th>
<th><em>P</em></th>
<th>No. of bacteria per g of cecum (log_{10} ± SEM)</th>
<th>No. of dead mice/total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (neonate)</td>
<td>2.9 ± 0.1</td>
<td>&lt;0.001</td>
<td>7.3 ± 0.1</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td><em>B. bifidum</em> (neonate)</td>
<td>2.8 ± 0.6</td>
<td>&lt;0.001</td>
<td>7.7 ± 0.1</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td><em>S. faecalis</em> (neonate)</td>
<td>5.6 ± 0.3</td>
<td>NS</td>
<td>7.8 ± 0.2</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td><em>E. aerofaciens</em> (adult)</td>
<td>5.1 ± 0.2</td>
<td>NS</td>
<td>7.8 ± 0.06</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td><em>E. ventriosum</em> (adult)</td>
<td>5.6 ± 0.1</td>
<td>NS</td>
<td>7.9 ± 0.05</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td><em>C. innocua</em> (adult)</td>
<td>5.8 ± 0.1</td>
<td>NS</td>
<td>8.0 ± 0.2</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td><em>B. vulgatus</em> (adult)</td>
<td>5.7 ± 0.1</td>
<td>NS</td>
<td>8.1 ± 0.1</td>
<td>9.4 ± 0.2</td>
</tr>
<tr>
<td><em>B. adolescentis</em> (adult)</td>
<td>5.6 ± 0.1</td>
<td>NS</td>
<td>8.1 ± 0.05</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>None</td>
<td>5.8 ± 0.1</td>
<td>NS</td>
<td>8.4 ± 0.06</td>
<td>10/10</td>
</tr>
</tbody>
</table>

* Analysis of variance was made from results of the nine experiments (comparison of cytotoxin titers *F*<sub>1, 8</sub> = 24.3, *P* < 0.001; comparison of *C. difficile* population levels, *F*<sub>1, 8</sub> = 7.99, *P* < 0.001).

* Results of Student's *t* test performed after analysis of variance. Results of each experiment were compared with those of mice monoassociated with *C. difficile*, NS. Not significant.

* Healthy mice were sacrificed 30 days after inoculation of *C. difficile*.

* Healthy mice were sacrificed 8 days after inoculation of *C. difficile*.

sacrificed 2 days after *C. difficile* challenge and cecal contents were collected. The *C. difficile* population level in mice inoculated with *B. bifidum* was not significantly different from that of the reference mice, whereas it was significantly lower (*P* < 0.01) in mice inoculated with *E. coli* (Table 1). However, the antagonistic effect of *E. coli* against *C. difficile* was moderate since *C. difficile* was only three times less abundant in the cecum. By contrast, the cecal cytotoxin titers were 1,000 times lower in mice inoculated with *E. coli* or *B. bifidum* than in reference mice (*P* < 0.001) (Table 1). Kinetics of cytotoxin production were studied in feces of mice inoculated with *B. bifidum* and challenged 4 days later with *C. difficile* (Fig. 1A). *C. difficile* became established 1 day after challenge and remained at the same level during more than 1 month. Cytotoxin titers did not vary to a great extent throughout experiment but were far below cytotoxin titers of reference mice (Fig. 1B). No mice died during this experiment.

Our results show that some bacterial strains may prevent mortality due to *C. difficile*. However, this protection did not seem to be related to a strong antagonistic effect of these strains against *C. difficile*. On the contrary, there was a good correlation between survival of mice and cytotoxin titer in feces. Thus, a modulation of fecal cytotoxin production may occur without any strong bacterial antagonism and may persist for a long time in the intestinal tract to gnotobiotic mice. Enterotoxin titer was not measured in this study. However, it was shown that both toxins were pathogenic for hamsters and mice (4, 8, 9). The fact that mice inoculated with *E. coli* or *B. bifidum* survived the *C. difficile* challenge without diarrhea suggests that enterotoxin titers were also lower than those of dead mice. Protection against *C. difficile* challenge was observed with three strains isolated from neonates. They belonged to the main bacterial species found in fecal flora of healthy neonates. Further studies must be undertaken to determine whether other strains of *E. coli*, *B. bifidum*, and *S. faecalis* isolated from neonates could also protect gnotobiotic mice against pathogenic *C. difficile* strains. It may be assumed that such strains might provide a useful tool for preventing intestinal disorders induced by *C. difficile* toxigenic strains. However, the mechanism of modulation of cytotoxin production by these strains has to be elucidated.

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LITERATURE CITED


5. George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y.


