Toxin A of *Clostridium difficile* Is a Potent Cytotoxin

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*Clostridium difficile* is the cause of antibiotic-associated colitis in humans. The organism produces toxin A, which is generally known as the enterotoxin, and toxin B, which is known as the cytotoxin. Toxin A has been reported to have slight cytotoxic activity; in this study we show that cell lines (F9, OTF9-63, and P19) which express a carbohydrate to which toxin A binds are more sensitive to the toxin. These cell lines can be used as research tools for determining concentrations of biologically active toxin A and should also prove useful for studies of the mechanism of action of the toxin.

*Clostridium difficile*, which causes antibiotic-associated pseudomembranous colitis in humans, produces two toxins, A and B. Toxin B is one of the most potent cytotoxins known; only a pigcog is required to cause a cytotoxic effect on cultured cells (1, 11, 13, 14). Because of the high cytotoxic activity of this toxin, the neutralization of the cytotoxic activity by specific antisera is the "gold standard" for diagnosis of the disease. Interestingly, although toxin B is the toxin detected by the neutralization assay, it is not the toxin thought to be responsible for most of the symptoms of pseudomembranous colitis. Toxin A is thought to cause both the diarrhea and the destruction of the colonic mucosa which is characteristic of this disease (1, 10, 13, 14). Toxin B appears to have no effect on undisturbed colonic mucosa (1, 10, 13, 14).

Toxin A is not a classical enterotoxin in that the fluid response is not a clear liquid but rather a hemorrhagic exudate composed of serum components and cellular debris (10). There is some controversy over whether toxin A also is cytotoxic to cultured cells. Banno et al. (1) reported that toxin A had no cytotoxic activity; other laboratories reported the activity to the toxin to be 1,000- to 10,000-fold less than the activity of toxin B on the same cell lines (5, 11, 13, 14). Preparations of either toxin A or toxin B cause the same change in morphology of cultured cells, thus increasing the suspicion that the cytotoxic activity of the toxin A preparations might be due to contamination with toxin B. The main fact which would refute this concept is that specific antibodies to toxin B do not neutralize the cytotoxicity of toxin A (13). However, a direct demonstration that toxin A has cytotoxic activity independent of toxin B has not been presented.

In this paper, we show that some cell lines are much more sensitive to toxin A than those that have been tested previously. The morphological effect on these cultured cells appeared to be the same for each toxin, and these effects were neutralized by antibodies specific for the toxin tested.

**MATERIALS AND METHODS**

*C. difficile* VPI 10463 was grown in brain heart infusion dialysis flasks as previously described (13). Toxin A was purified by affinity chromatography using immobilized bovine thyroglobulin (9). Toxin B was purified by ion-exchange chromatography on DEAE-Sepharose CL-6B and immunoabsorption (11). The purity of the toxins was demonstrated by crossed immunoelectrophoresis and polycrylamide gel electrophoresis as previously described (13). Protein concentrations were estimated by the method of Bradford (2), with bovine gamma globulin as a standard.

Affinity-purified antibodies to toxin A were purified as previously described using homogeneous toxin A coupled to AffiGel 10 (Bio-Rad Laboratories, Richmond, Calif.) (12). Affinity-purified antibodies to toxin B were prepared similarly, using immobilized toxin B. The concentration of the affinity-purified antibodies was estimated using an *E*$_{280}$,1% of 14. For neutralization assays, the antibodies to the toxins were mixed 1:1 with serial dilutions of toxin, and the mixtures were incubated for 1 h on ice before addition to the culture wells (7).

The cytotoxic activities of toxins A and B on the mouse teratocarcinoma cell lines F9, OTF9-63, P19, and P19S1801A1 were determined by using the Chinese hamster ovary-K1 (CHO) cell line as a reference as previously described (7). The F9 cells were grown in Dulbecco modified Eagle medium containing 15% heat-inactivated fetal bovine serum on tissue culture plates coated with 0.1% gelatin. The OTF9-63, P19, and P19S1801A1 cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The CHO cells were grown in Ham F12 medium containing 2% fetal bovine serum. A cytotoxic unit was defined as the least amount of toxin that caused rounding of 90% of the cells after 18 h of exposure to the toxin.

**RESULTS**

Our initial hypothesis was that cell lines which expressed the trisaccharide to which toxin A binds would be more sensitive to the toxin than other cell lines. We chose to test three mouse teratocarcinoma cell lines which had been reported to express this carbohydrate structure on their membranes (4, 6). We compared the activity of toxin A on these cell lines to the cytotoxicity on CHO cells, which are not known to express this trisaccharide. Toxin A was 100-fold more active on these three cell lines than on CHO cells (Fig. 1).

We also tested the effect of toxin A on a subclone of the P19 cell line (P19S1801A1), which has been reported to express less of the trisaccharide than the other cell lines (6). This cell line was 16-fold less sensitive than the parent P19 cell line. The sensitivity of four other cell lines to toxin A has been reported previously by Donta et al. (5) (Table 1).
that were assayed for sensitivity the relative were 50 5 5 13 5 5 3 1.5 5
so level, action of which express thus indistinguishable to the cells were the most sensitive of these cell lines, but they are 10-fold less sensitive than the most sensitive cell lines reported on in this paper.
All of the cells we tested reacted to toxin A in the same manner as cells react to toxin B: they lost all of their distinctive morphology and became round, with very little attachment to the plastic. Cells treated with toxin A were thus indistinguishable on a morphological basis from the same cells treated with toxin B.

DISCUSSION
Our results support our initial hypothesis that cell lines which express the trisaccharide (4, 6) to which toxin A binds (3, 8) will be more sensitive to the toxin than other cell lines. The fact that a subclone of one of these cell lines which expresses less of this trisaccharide was less sensitive than the parent clone also strengthens this argument. This is not proof that this trisaccharide is a functional receptor for the toxin, but it is suggestive; considerably more work will have to be done to determine if this is the case, and we are now involved in this investigation.
The increase in cytotoxic activity of toxin A in these cell lines with no increase in the activity of toxin B demonstrates that toxin A definitely has a cytotoxic activity. The cytotoxic responses of cells to toxin A appear to be very similar if not identical to the responses to toxin B. The mechanism of action of these toxins has not been defined at the molecular level, so there is currently no way to determine whether these toxins have the same enzymatic mechanism of action. We believe that the enterotoxicity of toxin A could be the result of the same cytotoxic response that we report here. The enterotoxicity could result from disorganization of cells in the brush border, which would allow serum proteins and fluids to pass into the lumen.
The cell lines we tested should prove useful to investigators who desire to determine levels of biologically active toxin A. These cells are much more sensitive than the rabbit ileal loop test for enterotoxicity and are more sensitive than the enzyme-linked immunosorbent assay which our laboratory previously used to detect toxin A (Table 2). The cytotoxicity assay, when performed with any of these three cell lines, is the most sensitive test for the toxin so far reported. The cytotoxicity test for toxin B is also the most sensitive method available for detecting this toxin. The cytotoxicity test for toxin B is about 100-fold more sensitive than the toxin A cytotoxicity test when these more sensitive cell lines are used; thus, the cytotoxicity of toxin B would still mask the effect of toxin A unless specific antibody is used to neutralize toxin B.
The cytotoxicity assay for toxin B in feces is the standard to which other methods for diagnoses of C. difficile colitis are compared. Toxin B does not initiate the diarrhea and colitis and, in fact, has no effect on normal intestinal mucosa. Assaying for toxin B is useful because a positive test indicates that both toxins are present. We have never found a case of a strain producing toxin B without also producing toxin A, and in general about equal amounts of both toxins are produced in culture media. Because of the lack of sensitive tests for active toxin A, there have not been any studies of the levels of active toxin A versus levels of active toxin B in the feces of patients. The tissue culture cell lines we describe here could be used for such determinations. If these studies showed that the levels of active toxin A correlated better with the clinical course of the patient than did concentrations of toxin B, then assays for toxin A could be developed for routine use in clinical laboratories.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Organism or cell line</th>
<th>Minimum dose (ng) for positive reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterotoxicity</td>
<td>Rabbit</td>
<td>1,000</td>
<td>10</td>
</tr>
<tr>
<td>Lethality (LD_{50})</td>
<td>Mice</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>CHO</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>Indirect ELISA*</td>
<td>F9, OTF9-63, or P19</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>F9, OTF9-63, or P19</td>
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</tbody>
</table>

* LD_{50} 100% lethal dose.
* ELISA, Enzyme-linked immunosorbent assay.

TABLE 1. Activities of toxins A and B previously reported

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Minimal cytotoxic dose</th>
<th>Reference</th>
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<tbody>
<tr>
<td></td>
<td>Toxin A (ng/ml)</td>
<td>Toxin B (pg/ml)</td>
</tr>
<tr>
<td>Y1</td>
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<td>MHC</td>
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<td>5</td>
</tr>
<tr>
<td>WI-38</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

* Because of differences in the assays used in reference 5, activities of the toxins were normalized by using the CHO cell line as a reference to determine the relative sensitivities of the other cell lines. This table is limited to cell lines that were assayed for sensitivity to toxin A.


