Effect of Environmental Stress on *Clostridium difficile* Toxin Levels During Continuous Cultivation

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A method for the continuous culture of *Clostridium difficile* has been described. It has been shown that subjecting continuous cultures of this microorganism to environmental stress results in increased levels of toxin in culture medium. Factors found to cause this release include alteration of the Eh from −360 to +100 mV or increasing the temperature from 37 to 45°C. The increased toxin levels were not associated with a change in viable cell density or the numbers of spores present. Additional studies have shown that subinhibitory concentrations of vancomycin and penicillin, but not clindamycin, also cause an increase in toxin levels during continuous culture. The increase in supernatant toxin levels occurs concomitant with a decrease in sonicated cell extract toxin levels. The data suggest that a number of factors can cause a release of toxin from *C. difficile* into the surrounding medium.

Recent reports (1, 4, 8) from a number of laboratories have documented the role of *Clostridium difficile* in antibiotic-associated colitis (AAC). It has been shown, for example, that *C. difficile* and a potent toxin produced by this microbe can be detected in stool from both patients and experimental animals with AAC. In addition, sterile culture filtrates of *C. difficile* containing high concentrations of toxin have been shown to produce the characteristic lesions of AAC in hamster ceca (3). Most of these studies have relied upon a cytotoxicity assay (1, 4, 8) to detect the toxin associated with *C. difficile*. More sensitive and specific immunochromatographic assays have not as yet been developed, in large part because the toxin of this microbe has not been obtained free from other contaminating proteins. Concomitant with the animal studies being done in this laboratory have been experiments designed to maximize in vitro toxin production for purification purposes. This study reports an in vitro system for production of *C. difficile* toxin and also reports the effects of a changing environment on culture toxin levels. The studies reported here are of additional interest since they may provide some insight into possible mechanisms of toxin production and release in vivo.

**MATERIALS AND METHODS**

**Microorganisms.** A single strain of *C. difficile* (Infectious Disease Laboratory HUC2-4) was used for all experiments. This strain was originally isolated from the stool of a patient with AAC and was characterized by established procedures (5). Strain HUC2-4 was chosen for study because of the consistently high toxin levels in vitro as determined by a cytotoxicity assay (2). The stock strain was grown for 48 h in prereduced chopped meat-glucose broth (Scott Laboratories, Fiskeville, R.I.) at 37°C and then aliquoted into small vials and frozen at −40°C until used. Before each experiment, a vial was thawed within an anaerobic chamber and plated onto a brucella base blood agar plate, and a single colony was subcultured to chopped meat-glucose for inoculating the fermentation vessel, after incubation.

**Continuous culture.** *C. difficile* was established in continuous culture using brain heart infusion broth supplemented with hemin and menadione as a nutrient medium. Cultures were grown in a C-30 Bioflo fermentor (New Brunswick Scientific Co., New Brunswick, N.J.), modified as described previously (10) to maintain anaerobic conditions. The fermentation vessel was fed via a peristaltic nutrient pump and was equipped with electrodes for monitoring Eh and pH. Electrodes were standardized against a quinhydrone reference solution (7) or phosphate buffer (pH 7.0), respectively, before each experiment. In addition to the Eh and pH, the culture was routinely monitored for optical density at 550 nm (OD550), viable cell density, spore count, toxin levels, and volatile fatty acid production. A portion of culture menstruum was removed for these assays via an aspiration sampling device and processed as described below.

Viable cell density and spore counts. After aspiration of a 10-ml aliquot, a sample was placed within the anaerobic chamber for determination of the viable cell density. Serial 10-fold dilutions of the culture were made using VPI dilution salts (5), and a 0.1-ml sample of each dilution was plated onto brucella blood base agar plates in duplicate. Plates were incubated within...
the anaerobic chamber for 48 h, and colonies were enumerated. Spore counts were determined by using identical procedures after heat shock at 80°C for 10 min. All counts were expressed as log<sub>10</sub> colony-forming units per milliliter.

OD measurements. The OD<sub>600</sub> was determined with a Coleman Junior II spectrophotometer standardized against sterile brain heart infusion broth supplemented with hemin and menadione.

Cytotoxicity assay. The presence of C. difficile toxin was quantitatively determined by using a modification of the cytotoxicity assay described previously (1). WI-38 cells (HEM Research Inc., Rockville, Md.) were used for all assays. Serial 10-fold dilutions of filter-sterilized specimens were made by using sterile phosphate-buffered saline (pH 7.4). A 0.2-ml aliquot of undiluted specimen and each subsequent dilution were placed into separate cell cultures containing 1.8 ml of medium 199 to give dilutions of 10<sup>-1</sup>, 10<sup>-2</sup>, etc. The cultures were incubated at 37°C and examined at 24 and 48 h for cytotoxic changes. The amount of toxin present in a given sample was expressed as the greatest dilution showing cytotoxic changes at 48 h.

Volatile fatty acid determinations. Quantitative estimates of the volatile fatty acid concentrations were made by extracting 0.5-ml aliquots with 1 ml of diethyl ether. Before extraction, 2-methylpentanoic acid was added as an internal standard to each sample at a concentration of 5 mM, and the pH was adjusted to 4.0 with 50% (vol/vol) H<sub>2</sub>SO<sub>4</sub> in water. The diethyl ether fraction was removed by aspiration, and 2-pl samples were injected onto a 4-m by 3-mm glass column packed with 6% Carbowax 20M triphosphoric acid (Supelco Inc., Bellefonte, Pa.). The column was maintained isothermally within a Shimadzu BA6AM chromatograph at a temperature of 170°C (Shimadzu Instruments, Columbia, Md.). Separation of volatile acid components was accomplished at a flow rate of 60 cm<sup>3</sup> of N<sub>2</sub> per min, and peaks were detected by a flame ionization detector. Fatty acids were identified by relative retention time versus the internal standard, and calculations of concentration were based on digital integration ratios of unknown acid versus internal standard using a Shimadzu 4AX digital integrator and a Hewlett-Packard 9830 programmable calculator.

Experimental design. After sterilization, the continuous culture apparatus and nutrient reservoir were purged with N<sub>2</sub> gas and allowed to stand for 48 h to check for sterility. The fermentation vessel was then inoculated and the culture was incubated at 37°C at an agitation rate of 200 rpm for 24 h. An initial sample was removed for viable cell density determinations, and a flow of sterile medium was introduced into the fermentation vessel at a rate of 0.5 ml/min, which corresponded to a D<sub>1</sub> = 0.085 h<sup>-1</sup>. The culture was monitored periodically as described above until a stable, continuous culture had become established. Alterations of the environment or addition of antimicrobials were made and the culture was monitored at 0, 2, 4, 6, 8, 24, 48, and 72 h, as described previously.

The Eh was adjusted from base-line values to +100 mV by the addition of potassium ferricyanide to the fermentation vessel dropwise so as not to exceed a concentration of 0.01 M in the vessel at any time. The pH was adjusted with either 1 N NaOH or 1 N H<sub>2</sub>SO<sub>4</sub>. The temperature was raised by using a variable thermostat and thermistor sensor within the fermentor and was also monitored with a mercury bulb thermometer. Antimicrobial agents were added to the fermentor vessel at concentrations shown to be subinhibitory by prior testing versus C. difficile HUC2-4. (The minimal inhibitory concentration of vancomycin was 0.5 µg/ml, that of penicillin was 0.5 µg/ml, and that of clindamycin was 2 µg/ml.) Vancomycin and penicillin were added at concentrations of 0.1 µg/ml, and clindamycin was added at a concentration of 0.5 µg/ml. For some experiments, inhibitory levels of antimicrobials were added to the fermentation vessel (vancomycin, 5 µg/ml, clindamycin, 50 µg/ml).

At each sample time, a 10-ml sample of culture was aspirated from the fermentation vessel and a 1-ml amount was filtered through a 0.45-µm filter for the cytotoxicity assay. Additional samples were placed into the anaerobic chamber for viable cell density determinations, OD<sub>600</sub> determinations, and spore density counts. A 1-ml sample was frozen at −40°C until used for assay of volatile fatty acids. For some experiments, a 4-ml amount was removed for comparison of cell-associated and supernatant cytotoxin. These samples were centrifuged at 10,000 × g for 20 min, and the supernatant was removed for assay. The cell pellet was washed in phosphate-buffered saline (pH 7.2) and repelleted by centrifugation at 10,000 × g. This procedure was repeated a total of three times. The final sample was resuspended in 4 ml of phosphate-buffered saline for sonication with a sonic dismembrator. A sample of the sonicated pellet was then tested for cytotoxicity as described previously.

RESULTS

Continuous culture of C. difficile. Within 24 h of inoculation, C. difficile population levels were greater than 10<sup>7</sup> colony-forming units per ml in the fermentor vessel. Stable populations of 10<sup>9</sup> colony-forming units per ml (± 1 standard error of the mean) were found within 72 h after starting a flow of sterile medium to the fermentation vessel at a D<sub>1</sub> = 0.085 h<sup>-1</sup>. Spore counts were 10<sup>9.8±0.14</sup> colony-forming units per ml, and the mean toxin titer was 10<sup>-2.5±0.09</sup>. The relatively low dilution rate was chosen to simulate the growth rate for bacteria in the large intestine and to stabilize the bacterial population within the fermentation vessel. Previous experiments had shown that a D<sub>1</sub> = 0.20 or greater resulted in population fluctuations which precluded accurate monitoring. The Eh of the culture menstruum was −361 mV ± 9.1, and the pH was 6.67 ± 0.2. The OD<sub>600</sub> remained at 0.330 ± 0.060 before environmental alterations. The major volatile fatty acids present were acetic, iso- butyric, butyric, isovaleric, and isocaproic acids, with acetic acid being present in greatest concentration at 20 mmol/liter.

Effect of alteration of Eh, pH, and temperature. Initial experiments were designed to...
determine whether alteration of the oxidation-reduction potential from -360 to +100 mV would affect either cell growth or toxin levels. It was found that this resulted in a 10- to 100-fold increase in toxin levels from base-line determinations (Table 1). The increase in toxin levels occurred within 4 h of the increase in Eh. Concomitantly, there was no change in viable cell density, spore counts, or optical density. After termination of potassium ferricyanide addition, the Eh rapidly returned to base-line levels.

Studies were also done which evaluated the effect of pH and temperature on C. difficile toxin levels during continuous culture. These studies indicate that alteration of the pH from 6.60 to 8.0 or 4.0 did not result in any increase in toxin levels (Table 1). Indeed, bacterial growth was inhibited and a slow decline in viable cell density and toxin levels within the fermentation vessel occurred at a rate compatible with a net bacteriostatic effect. Preliminary studies have shown that the cytotoxin is acid labile and alkaline labile, making it difficult to evaluate the role of pH in this system. On the other hand, raising the temperature from 37 to 45°C resulted in an increase in toxin levels within 4 h from 100- to 1,000-fold greater than those noted before alteration. The alteration of the temperature did not change the viable cell density or the number of spores. These data indicate that changes in the environment, such as Eh or temperature, resulted in increased levels of toxin during continuous culture of C. difficile.

Addition of antimicrobial agents to continuous cultures. Since our preliminary experiments had shown that culture toxin levels could be affected by environmental stress, it was of interest to determine whether antimicrobial stress would also cause an increase in toxin levels. The results of these experiments (Table 2) indicate that addition to the culture of vancomycin and penicillin at subinhibitory concentrations resulted in a 1,000-fold increase in toxin levels within 4 h. A representative experiment (Fig. 1) in which vancomycin was added to the continuous culture shows that the viable cell density did not change during the experiment despite an increase in OD550 from 0.250 to 0.760 over a 24-h period. The increased OD550 occurred concomitantly with morphological change in the organism, as determined by Gram stain, characterized by elongation and thickening of the bacterial cells. No increase in spore counts was observed during these experiments. Interestingly, the amounts of butyric acid present in culture filtrates increased from a mean of 4.5 to 14.1 mmol/liter, suggesting that metabolic as well as morphological alterations occurred when this antimicrobial was added. Exposure of continuous cultures to clindamycin at subinhibitory levels did not result in any demonstrable changes in toxin levels, viable cell density, OD550, or the numbers of spores present (Table 2).

Exposure of cultures to inhibitory levels of vancomycin or clindamycin resulted in a rapid decline in viable cell density and OD550, but no increase in toxin levels could be discerned.

Comparison of sonicated cell extract and culture supernatant toxin levels. Since the increased toxin levels noted in these experiments could reflect either a release of preformed toxin from the bacterial cell or synthesis of additional

| Table 2. Effect of antimicrobial agents on toxin levels during continuous culture |
|---|---|---|
| Factor | No. of observations | Concentration added (μg/ml) |
|  |  | Fold increase in toxin |
| Vancomycin | 4 | 0.1 | 100 to 1,000 |
| Penicillin | 3 | 0.1 | 100 to 1,000 |
| Clindamycin | 4 | 0.5 | 0 |

* Fold increase in toxin levels above preadjustment levels.

| Table 1. Effect of Eh, pH, and temperature on toxin levels during continuous culture |
|---|---|---|---|
| Factor | No. of observations | Base-line value | Adjust-ment to: | Fold increase in toxin* |
| Eh | 8 | -360 mV | +100 mV | 10 to 100 |
| pH | 3 | 6.60 | 8.0 | 0 |
| pH | 2 | 6.60 | 4.0 | 0 |
| Temp | 6 | 37°C | 45°C | 100 to 1,000 |

* Fold increase in toxin levels above preadjustment levels.

FIG. 1. Effect of subinhibitory concentrations of vancomycin on C. difficile toxin levels during continuous culture.
toxin, an effort was made to determine whether the toxin levels in the supernatant of these cultures were greater than or less than those found in sonicated cell extract obtained at the same time. The combined results of several experiments (Table 3) indicate that as toxin levels in culture supernatant increased during exposure of C. difficile to an adverse environment, the toxin levels in sonicated cell extract decreased proportionately. Before any alteration, sonicated cell extract and culture supernatant levels were comparable; however, within 8 h after the environment was altered, the supernatant levels of toxin increased by 100-fold, and the sonicated cell extract levels decreased by 100-fold. This decrease in sonicated cell extract toxin levels is not due to sonication alone, since sonication of cell-free supernatants did not result in any decrease in toxin levels. These data suggest that during exposure to certain adverse environments such as increased Eh and temperature, or subinhibitory levels of certain antimicrobial agents, C. difficile released toxin into the culture medium.

DISCUSSION

Continuous culture provides a unique means of manipulating conditions of bacterial growth. One may thereby simulate an in vivo situation in controlled fashion. By using this technology, the present studies have shown that the amount of C. difficile toxin present in continuous cultures is affected by changes in environmental conditions. Increased temperature, increased Eh, and subinhibitory levels of cell wall active antibiotics resulted in a marked elevation of toxin titers in the supernatant fluid. In the case of the antibiotics, this was shown to represent a net efflux of toxin from the intracellular to the extracellular compartments. Efforts to correlate changes in the population of viable C. difficile or of C. difficile spores with the increased levels of toxin were unsuccessful. Indeed, a comparison of spore counts and toxin levels during most experiments showed a correlation coefficient of 0.50. The importance of the toxin protein to C. difficile and to cellular function remains unknown. However, unlike certain clostridial toxins, it does not appear to be associated with sporulation.

One possible clue to the nature of storage and release of the toxin molecule resides in the environmental stresses which appear to cause its release from the cell. The Eh is thought to be an important factor in membrane transport, and it is known that potassium ferricyanide can interfere with active transport via proton motive gradient disruption (6, 9). It is possible that the toxin molecule is loosely associated with the cell membrane and that changes in the environment result in its release. These observations are supported indirectly by the effects of vancomycin and penicillin on toxin levels. Both antimicrobials disrupt the cell wall, and vancomycin interferes with cell membrane development. A protein loosely associated with the cell membrane might be released by cellular disruption of this nature. Although this is an appealing explanation, more data regarding toxin synthesis and release are required to rule out other possibilities.

Some of the environmental conditions manipulated in these experiments simulate intestinal changes in experimental animals treated with antimicrobials. For example, theecal Eh of hamsters increased from $-175$ to $+35$ mV after the administration of clindamycin to these animals (unpublished data). Changes in hamster cecal temperature during antimicrobial administration were minimal, however, with a mean increase from 37.5 to 39°C being measured. Nevertheless, the alterations of in vivo environment may prove to be important to the development of AAC, since the amount of toxin released from cells of C. difficile may be more important than the total number of viable organisms.

The observation that subinhibitory levels of antimicrobials, such as vancomycin and penicillin, could promote increased toxin levels is of particular interest. It has been shown that, despite a relative sensitivity of C. difficile to vancomycin, hamsters given this agent nevertheless succumb to C. difficile-induced disease (7). Unlike the situation with clindamycin, where the animals die during the course of drug administration, animals treated with vancomycin succumb from 5 to 7 days after cessation of antimicrobial therapy. It is conceivable that with vancomycin, C. difficile survives the relatively high cecal levels of the drug, and residual subinhibitory cecal levels may promote the development of AAC. The isolation of C. difficile strains sensitive to vancomycin from vancomycin-sensitive

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of observations</th>
<th>Baseline toxin</th>
<th>Fold increase in supernatant toxin</th>
<th>Fold decrease in sonicated cell extract toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>2</td>
<td>$10^{4}$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>$10^{4}$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1</td>
<td>$10^{4}$</td>
<td>1,000</td>
<td>100</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>$10^{4}$</td>
<td>1,000</td>
<td>100</td>
</tr>
</tbody>
</table>

* Reciprocal of the greatest dilution of material showing a cytotoxic response at 48 h.
cin-treated animals lends support to this theory. The relevance of these observations to human disease is unknown. However, it has been reported that a major portion of patients develop AAC after antimicrobials have been discontinued (11).

These studies report a method for studying C. difficile toxin production in vitro under controlled conditions simulating an in vivo environment. Further studies using this system may provide additional information regarding the nature of the lethal toxin produced by C. difficile.

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LITERATURE CITED