INFECTION AND IMMUNITY, July 1989, p. 2123–2127
0019-9567/89/072123-05$02.00/0

Vol. 57, No. 7

Relationship between Levels of *Clostridium difficile* Toxin A and Toxin B and Cecal Lesions in Gnotobiotic Mice

A. VERNET,[1] G. CORTHIER,[1]* F. DUBOS-RAMARÉ,[1] AND A. L. PARODI[2]

Laboratoire d'Ecologie Microbienne, Institut National de la Recherche Argronomique, Centre de Recherches de Jouy-en-Josas, 78350 Jouy-en-Josas,[1] and Chaire d'Histologie et d'Anatomie Pathologique, Ecole Vétérinaire d'Alfort, 94704 Maisons Alfort Cedex,[2] France

Received 27 August 1988/Accepted 31 March 1989

Various *Clostridium difficile* strains were studied with respect to their pathogenicity in monoassociated mice in relation to levels of toxin A and toxin B in vivo and in vitro. Two strains which were the most potent toxin producers in vitro induced mortality (100%); mice monoassociated with these strains were found to have high levels of both toxins in their ceca and an intense cecal epithelial ulceration together with a severe inflammatory process. No mortality was observed with the other strains. Strains which were moderately toxinogenic in vitro induced inflammation of the cecum but no ulceration, and no toxin A was found. Inflammation intensity was not related to toxin B levels. After 3 weeks, ceca returned to normal in spite of a chronic cytotoxin production. When compared with in vitro results, which showed a good correlation between the levels of the two toxins, toxin A amounts in vivo were found to be lowered relative to toxin B levels. The lack of detectable toxin A levels in animals infected with all but the two most highly toxinogenic strains prevented death. This work points out the importance of investigation of toxin A for the understanding of *C. difficile* pathogenicity.

Clostridium difficile is known as the major agent involved in the onset of human pseudomembranous colitis (1, 7, 9). Two toxins have been described, an enterotoxin (toxin A) and a cytotoxin (toxin B) (17). However, the respective roles of these toxins in the pathology following *C. difficile* infection have not been precisely established. The effects of *C. difficile* toxins given intragastrically to rodents were studied by Lyerly et al. (11), who observed that the pathologic activity of a culture filtrate was due to toxin A. High amounts of toxin B did not cause any significant response unless initially mixed with low amounts of toxin A (11).

Gnotobiotic rodents offer a well-defined model to study the pathogenicity of *C. difficile* because it is possible to study the interaction of *C. difficile* and its toxins with the host without any influence of other bacteria. In this model, animals infected by highly toxinogenic *C. difficile* strains develop a potentially lethal cecitis (3, 5, 13, 15, 18).

The purpose of this work was to investigate toxin production of various *C. difficile* strains (in vitro and in vivo) in regard to their pathological effect on gnotobiotic mice.

MATERIALS AND METHODS

Bacterial strains and counts. Eight *C. difficile* strains of different origins were used. Strain Si, originating from an asymptomatic child, was provided by A. Collignon, Faculté de Pharmacie, Châtenay-Malabry, France. Strain 786, a clinical isolate from an adult suffering postantibiotic colitis, was provided by M. Popoff, Institut Pasteur, Paris, France. Strain VPI 10463 (VPI) was isolated from an abscess and provided by N. M. Sullivan, Virginia Polytechnic Institute and State University, Blacksburg (5). Strains 88 and 660 had been isolated from neonates suffering from necrotizing colitis and were provided by O. Fontaine (6). Strain Mara was isolated by us from an adult suffering from pseudomembranous colitis (13). Strains R4 and M2 are clones derived from strains VPI and Mara, respectively (4).

* Corresponding author.

In vitro toxin production. The various *C. difficile* strains were grown within a dialysis bag in flasks containing autoclaved brain heart infusion broth (Difco Laboratories, Detroit, Mich.). The flasks were incubated for 4 days at 37°C in an anaerobic chamber. The dialysis bag contents were then collected for microbiological counts and toxin analyses. Assays were performed in triplicate with each strain.

In vivo experimental schedule. C3H/He axenic mice were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, Vélizy, France). They were fed ad libitum a commercial diet (RO3-40, UAR), sterilized by gamma irradiation.

Each group of mice (12 to 15 animals) was challenged with one *C. difficile* strain through the orogastric route with 0.5 ml of a 24-h culture (10^8 vegetative cells per ml). Two days after inoculation, vegetative cell numbers and production of toxin A and toxin B were assessed in the feces (or the ceca of dead mice). In surviving mice, fecal samples were collected and analyzed weekly for 3 weeks. For each *C. difficile* strain, three animals were killed on days 2 and 21 postinoculation and the ceca were excised and processed for histological examination.

Enumeration of *C. difficile* and investigation of toxins. The feces of living mice, the ceca of dead mice, or the dialysis bag contents were analyzed after appropriate dilution. Vegetative cells were enumerated in D medium (16) without safranin. Anaerobiosis was achieved by the use of tubes (8 by 400 mm). For toxin assays, samples were centrifuged (2,000 × *g* for 10 min) and the supernatant was recovered. A sample was kept at −20°C before toxin A determination; another fraction was kept at 4°C prior to cytotoxicity assay.

Toxin A quantitation was performed by the immunoenzymatic assay (enzyme-linked immunosorbent assay) derived from that of Lyerly et al. (12) as previously described by Mahé et al. (13). Samples collected in vivo were supplied with trypsin inhibitor (soybean type I-S from Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 1 mg/ml.

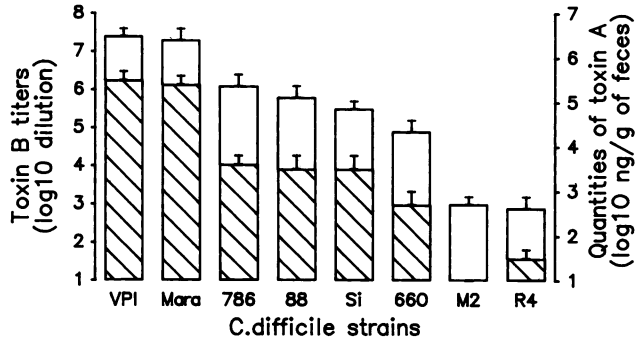

FIG. 1. Comparison of in vitro toxin A (hatched bar) and toxin B (open bar) production by different strains of *C. difficile*. The strains were grown for 4 days at 37°C in an anaerobic chamber.

Results were expressed as $\log_{10}$ nanograms per gram of feces or cecum.

The cytotoxicity assay was performed with Chinese hamster ovary cells (CHO-K1; Flow Laboratories) grown on 96-well microtiter plates (Nunclon) as described by Corthier et al. (3). The cytotoxin titer was defined as the $\log_{10}$ reciprocal value of the highest dilution rounding 100% of the cells.

Histopathology. Ceca were fixed in Bouin solution for 1 week and then kept in 70% ethanol. For microscopic preparation, they were embedded in paraffin, serially sectioned at 4-μm thickness, and stained with hematoxylin and eosin before microscopic observation. The symbols (−, +, ++, and +++) used in Fig. 3 correspond to the intensity of lesions estimated by the number of cells modified and/or the size of the area where the lesion occurred. The minus sign corresponds to the absence of lesions.

RESULTS

Toxin production in vitro. Whatever the strain, the numbers of viable vegetative cells after 4 days of culture were similar (around $10^{9.6}$ viable bacteria per ml of dialysis bag contents; data not shown). In regard to toxin production, three groups of strains were found (Fig. 1). The first group contains strains VPI and Mara, which were highly toxinogenic. The second (intermediate) group comprises strains 786, 88, Si, and 660, which were moderately toxinogenic. The third group includes the low-toxinogenic strains R4 and M2. It can be noticed that there is a good correlation between toxin A and toxin B levels ($r = 0.98$, $P < 0.01$).

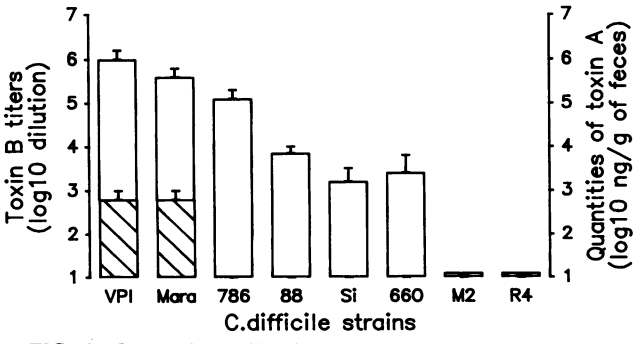

FIG. 2. Comparison of in vivo toxin A (hatched bar) and toxin B (open bar) production by different strains of *C. difficile*. Cecum samples were collected 2 days after inoculation of *C. difficile*.

TABLE 1. Kinetics of toxin B production in feces of mice monoassociated with various *C. difficile* strains

Day	Cytotoxic titer[a] with *C. difficile* strain:					
	786	88	Si	660	M2	R4
2	5.1 (0.3)	3.9 (0.3)	3.2 (0.2)	3.4 (0.4)	<1.5	<1.5
7	4.8 (0.1)	4.5 (0.1)	3.4 (0.3)	3.6 (0.2)	<1.5	<1.5
14	5.6 (0.1)	5 (0.2)	3.4 (0.1)	4 (0.2)	<1.5	<1.5
21	4.3 (0.3)	4.2 (0.3)	3.5 (0.2)	3.8 (0.2)	<1.5	<1.5

[a] The cytotoxic titer was defined as the $\log_{10}$ reciprocal value of the highest dilution rounding 100% of the cells. For each group, the number of *C. difficile* was constant ($10^{8.5}$/g of feces) during the entire experiment. Parentheses indicate standard error of the mean.

Toxin production and lethality in vivo. Axenic mice were monoassociated with the different strains. Microbiological analyses showed that whatever the type of strain, *C. difficile* established rapidly and in high numbers in the large intestine. On day 2 after inoculation, the mean level for all animals was $10^{8.3}$ viable bacteria per g of intestinal contents. However, only strains VPI and Mara were able to induce mortality. Mice harboring these strains developed a severe diarrhea, and 100% of them died within 2 days. No mortality was observed with the other strains.

The stability of toxin A in feces of axenic mice was assessed as follows. A known quantity of toxin A (3.5 $\log_{10}$ ng/ml) was mixed with feces diluted 1/3 and incubated for 1 h at 37°C. In such conditions, toxin A was not detectable (detection limit, 0.5 $\log_{10}$ ng/ml). Detection of toxin A was not statistically different from the control when trypsin inhibitor was added to the solution before incubation (3.4 ± 0.3 $\log_{10}$ ng/ml and 3.3 ± 0.3 $\log_{10}$ ng/ml, respectively). This is why trypsin inhibitor was systematically added to samples collected from mice. The toxin B titer was not affected by incubation with feces or cecum content: no change was observed after 1 day of incubation at 37°C in feces or cecum content diluted 1/4.

Toxins A and B were quantitated in the intestinal contents of the animals (Fig. 2). Moribund mice, namely, those

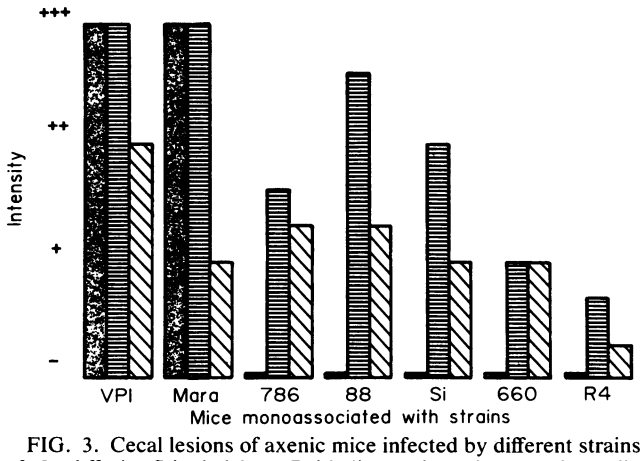

FIG. 3. Cecal lesions of axenic mice infected by different strains of *C. difficile*. Stippled bar, Epithelium: ulceration. Horizontally hatched bar, Submucosa: congestion, edema, and invasion with granulocytes. Diagonally hatched bar, Submucosa: invasion with monocytes. Symbols: −, no lesion; +, slight lesion; ++, moderate lesion; +++, severe lesion. Each point represents the mean value of three mouse examinations.

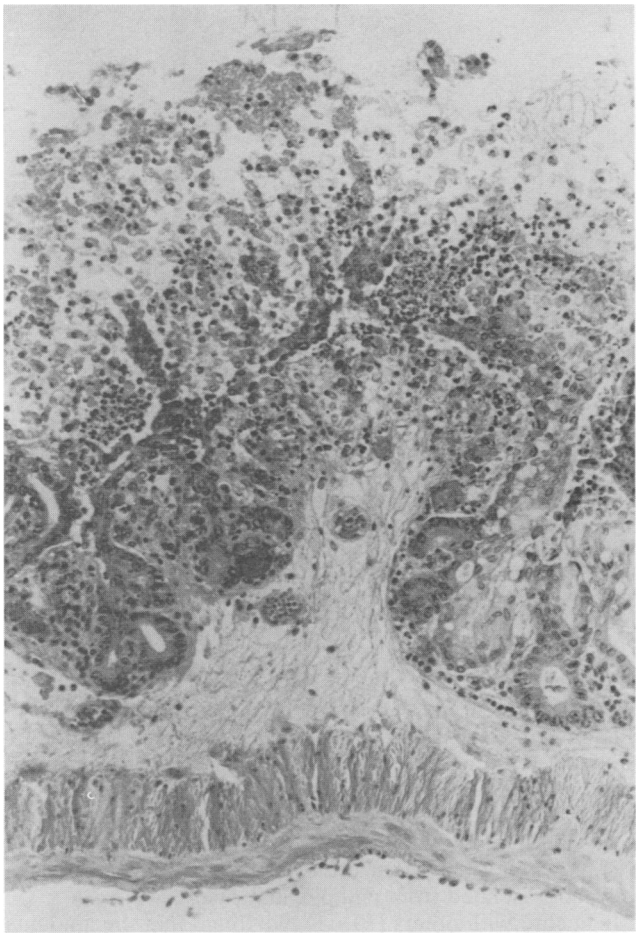

FIG. 4. Cecum of a moribund mouse 2 days after monoassociation with strain Mara (edema, congestion of submucosa, and disruption of epithelium). Magnification, ×140.

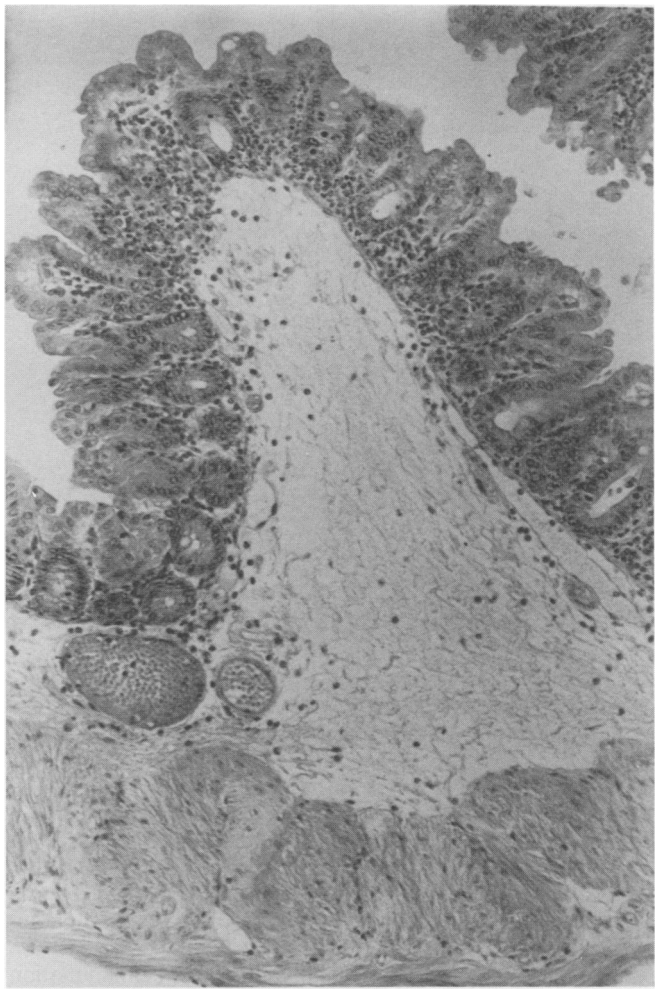

FIG. 5. Cecum of a mouse 2 days after monoassociation with strain 786 (edema and congestion of submucosa). Magnification, ×140.

monoassociated with strains VPI or Mara, were found to have high levels of both toxins in the cecum. No toxin A was detected in mice monoassociated with strains of the intermediate group, while toxin B titers still remained between 3.5 and 5. Of special interest was the observation that toxin B production in mice monoassociated with strain 786 (all of which remained healthy) was not statistically different from that found in moribund Mara-associated mice. Neither toxin A nor toxin B was detected when axenic mice were monoassociated with strain R4 or M2.

In monoassociated surviving mice, toxin B production did not decrease for at least 3 weeks (Table 1). Mice appeared healthy and no animals died despite relatively high toxin B levels in their feces.

When compared with in vitro results, in vivo toxin A levels were strongly reduced (VPI and Mara strains) or eliminated (remaining strains), while toxin B titers were only somewhat reduced.

Histopathology. Histopathological examination of the cecum was performed on mice monoassociated with each of the *C. difficile* strains. The ceca of moribund mice were characterized by a pronounced epithelial ulceration. Severe submucosal inflammation with congestion, edema, and granulocyte infiltration was noticed (Fig. 3 and 4). Mice harboring strains from the intermediate group had no epithelium ulceration in their ceca. Inflammatory edema of the submucosa occurred, but its intensity was not related to toxin B levels (Fig. 3 and 5). Mice monoassociated with the nontoxinogenic strain R4 were not affected or developed only a slight edema. Submucosal invasion by monocytes occurred with all *C. difficile* strains but was unrelated to pathology or toxin production.

Three weeks after inoculation, the ceca of surviving mice (inoculated with low-toxin-producer and nontoxinogenic strains) looked similar to those of control axenic mice (Fig. 6).

DISCUSSION

The usefulness of gnotobiotic rodents as experimental animals able to mimic human pseudomembranous colitis is now well established. In this model, no interaction with resident flora interferes with *C. difficile* establishment or toxin production. Animals develop nonhemorrhagic diarrhea, and the death rate can be very high (3, 18). An intense inflammation and typical pseudomembranes have been observed in moribund animals (5, 18), and this pathology was associated with the presence of high levels of toxin B (5) or of both toxins when toxin A was investigated (5).

In the present study we have quantified toxin A and toxin B levels in mice monoassociated with various toxinogenic

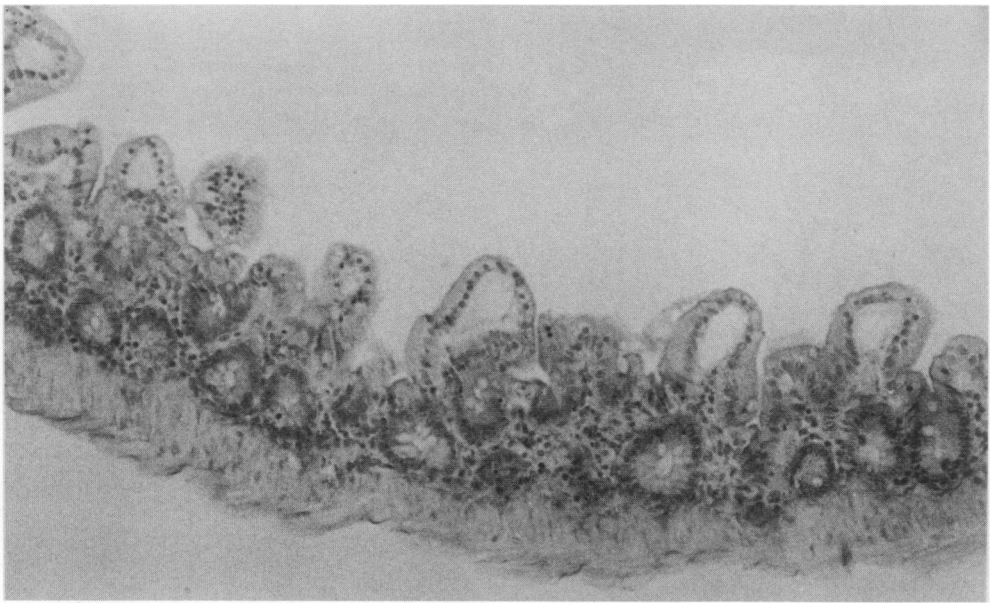

FIG. 6. Cecum of an axenic mouse. Magnification, ×140.

strains in regard to histopathological lesions. Toxin B was estimated by a biological effect which does not allow determination of toxin B concentration, whereas estimation of toxin A concentration was performed by enzyme-linked immunosorbent assay. Thus, the two toxin levels cannot be compared directly. Animal death was always associated with the presence of both toxins A and B and with a severe inflammation process characterized by epithelium ulceration and submucosal inflammation in the cecum. As no epithelium ulceration occurred in surviving mice, one can postulate that this lesion is characteristic of the lethal association with highly toxinogenic strains. Submucosal inflammation does not seem to be a critical lesion since it was observed in mice monoassociated with the nonlethal intermediate strains. Furthermore, the intensity of inflammation was not directly correlated to the level of toxin B.

Histopathological examination of the ceca from mice monoassociated with nonlethal strains revealed that the inflammatory process observed 2 days after inoculation had disappeared after 3 weeks, in spite of a chronic toxin B production. The same situation has been reported by others (5, 15) and is most likely due to a host immune response since antitoxin activity can be found in sera from animals or patients harboring *C. difficile* strains (5, 14).

Mice monoassociated with strains of *C. difficile* that produced intermediate amounts of toxins A and B in vitro survived even though stable, high levels of toxin B were observed in vivo (without detectable toxin A). This observation is in agreement with the work of Lyerly et al. using hamsters (11). These authors inoculated toxin B intragastrically in such a quantity that the titer observed in the cecum was similar to that found in our surviving mice. This inoculated toxin B did not affect hamster health significantly. On the other hand, it was shown in the same study that inoculated toxin A was lethal. In our model, it cannot be determined if death is due to toxin A alone or if both toxins are involved, since no strain produced toxin A without toxin B in vivo. Lyerly's experiments and our observations for gnotobiotic mice suggest that toxin B alone has a relatively minor pathological effect. However, the action of toxin B may depend on the tissue damage caused by toxin A (11).

Borriello et al. recently reported a lack of correlation between toxin A and B production in vitro and virulence to clindamycin-treated hamsters (2). Using the gnotobiotic mouse model, in which interactions between *C. difficile* and other microflora constituents are obviated, we found that the two strains that produced the highest amounts of the two toxins in vitro produced the most severe pathology in vivo. It is also noteworthy that the two most toxinogenic strains were both isolated from pathological cases.

In accord with others (10, 11), we found that the amounts of toxins A and B produced in vitro were correlated; i.e., strains that produced high levels of toxin B also produced high levels of toxin A. In vivo, however, we found toxin A levels to be sharply reduced compared to those of toxin B. It can be imagined that toxin A is produced but not detected. We have shown in this report that feces of axenic mice were able to reduce the sensitivity of immunological tests by a probable destruction of toxin A. This unexpected effect could be blocked by trypsin inhibitor, suggesting that a trypsinlike activity was involved. The proteolytic activity of the digestive tract had no effect on toxin B. Such enzymatic activity may occur in vivo before sample collection; toxin A would be degraded continuously in the intestinal tract, while toxin B would not be affected. Furthermore, toxin A may be fixed in vivo to brush border receptors (8). This is probably not true for toxin B: no receptor has been identified on brush borders (8). For all these reasons it is probable that toxin A detected in vivo was underestimated in regard to the amount of toxin A produced. This effect will not be observed in vitro. In dying mice, high levels of detectable toxin A may result from an overflow of degradation capacity or binding capacity of brush border receptors.

The important role of toxin A in the pathological process and the discrepancy between in vitro and in vivo production of toxin A point to the particular importance of toxin A determinations in studies of *C. difficile* pathogenicity.

ACKNOWLEDGMENTS

We thank P. Raibaud, R. Ducluzeau, and G. Elmer for help in improving the manuscript and P. Rapine for valuable technical assistance.

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