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Examination of Diarrheagenicity of Cytolethal Distending Toxin: Suckling Mouse Response to the Products of the *cdtABC* Genes of *Shigella dysenteriae*

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Some strains of *Escherichia coli*, *Shigella* spp., and *Campylobacter* spp. that have been implicated in diarrheal disease produce cytolethal distending toxin (CDT). CDT induces unique morphological changes in Chinese hamster ovary cells, but its association with diarrheal disease is unclear. We studied the diarrheagenicity of CDT using the *cdt* genes that we originally cloned from *Shigella dysenteriae*. The *cdt* genes were subcloned into a high-copy-number plasmid in *E. coli* JM109 to achieve high-level CDT production into the culture supernatant. An isogenic CDT$^-$ derivative was constructed by deletion of the 0.9-kb sequence internal to the *cdt* genes. A suckling mouse model was established, in which the intragastrically administered culture supernatant of the CDT$^+$ *E. coli* strain induced excretion of loose and/or watery feces more often than did that of the CDT$^-$ strain in 24 h. The partially purified CDT preparation induced profuse watery diarrhea by 12 h in this model. High-level intestinal fluid accumulation in 4 h appeared to be related to the watery diarrhea. The results indicate that CDT is diarrheagenic to suckling mice and suggest that diarrheagenicity is dependent on CDT level. The preparations containing wild-type CDT induced tissue damage (necrosis and reparative hyperplasia) in the descending colon, whereas the tissues of the small intestines remained apparently intact. Association between the colonic damage and diarrhea is unclear and needs further investigation.

Various extracellular toxins produced by bacterial enteropathogens have been reported. Cytolethal distending toxin (CDT) was first described by Johnson and Lior in 1987 (9). The culture filtrates of a CDT-producing strain of *Escherichia coli* induced unique morphological changes in Chinese hamster ovary (CHO) cells; elongation of the cells was observed in 24 h, and then progressive cell distention resulting in cytolethal events was observed by 96 to 120 h (9). This unique biological activity allowed distinction of CDT from other extracellular toxins of *E. coli*, including heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), Verotoxin, hemolysin, and cytotoxic necrotizing factor (9, 11). CDT production from *E. coli* strains of many serotypes and from some strains belonging to *Shigella dysenteriae*, *Shigella boydii*, and various species of *Campylobacter* (1, 3, 8–11) was reported.

Molecular genetic studies of *E. coli* CDT were conducted with an O86:H34 strain by Scott and Kaper (20) and with an O128:H$^-$ strain by Pickett et al. (18). It was shown that in both strains, CDT was encoded by three consecutive genes (*cdtABC*) and the products of all three genes were required for the demonstration of toxin activity. However, the nucleotide sequences of the *cdtABC* genes cloned from the two strains showed only 33, 57, and 33% identity with *cdtA*, *cdtB*, and *cdtC* genes, respectively (17, 18, 20). Subsequently, the nucleotide sequences that were homologous to the *E. coli cdtA* gene reported by Scott and Kaper (20) were detected in strains belonging to *S. dysenteriae* and *Shigella sonnei* (17). We cloned the *cdt* homolog from one of the *S. dysenteriae* strains and analyzed the sequence (17); the sequences of *S. dysenteriae cdtABC* genes were 99% identical with those of *E. coli cdtABC* genes as determined by Scott and Kaper (20). Pickett et al. (19) reported the nucleotide sequence of the *cdtABC* genes of *Campylobacter jejuni* recently; the sequence had a low similarity to the *E. coli cdtABC* sequence that the same group reported previously (18).

The strains of *E. coli*, *Shigella* spp., and *Campylobacter* spp. that exhibited CDT activities were associated with diarrheal disease (1, 3, 8–11). However, experimental evidence demonstrating the diarrheagenicity of CDT in an in vivo model has not been obtained. Most animal assays employed so far for the examination of CDT-producing strains have been standard assays. These included the suckling mouse assay established for detection of *E. coli* ST (3, 10, 11, 15), rabbit ileal loop and permeability assays used for detection of *E. coli* LT or cholera toxin (CT) (3, 4, 9–11), and the Serény test for detection of invasiveness (11, 15). The results were either negative or considered negative, the latter due to confusion by the simultaneous production of LT or ST. However, some workers have observed interesting responses in the standard or semistandard animal assays. Bouzari et al. (4) reported the presence of inflammatory infiltrates in the submucosa and proliferation of epithelium in the rabbit ileum injected with either live cultures or culture supernatants of CDT-producing *E. coli* strains. Johnson and Lior (9) noted inflammatory responses in the rabbit permeability factor assay. The same workers also noticed hemorrhagic fluid and local inflammation in rat ileal loops, although the amount of fluid accumulation (FA) was not significant (10).

We wished to study diarrheagenicity of CDT. One possible approach to examine the specific effect of CDT would be to construct an isogenic CDT$^-$ strain from a clinical strain and compare the two strains in an appropriate animal model. However, if the parent strain also produces an enterotoxic factor(s) other than CDT, interpretation of the results may be difficult.

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We therefore employed a laboratory *E. coli* strain often used for gene cloning as the genetic background. The *cdtA*$^+$*B*$^+$*C*$^+$ genes cloned from *S. dysenteriae* were made to be expressed in the *E. coli* strain. An isogenic CDT$^-$ derivative was constructed by deletion of an internal sequence of the *cdt* genes for comparison with the parent strain. We chose a suckling mouse system as an animal model because of its value in simulating the actual course of diarrhea. However, we did not follow the standard protocol established for ST detection. The combination of the above approaches allowed us to demonstrate the diarrheagenicity of CDT and to obtain some insights into the action of CDT in this study.

MATERIALS AND METHODS

Construction of CDT$^+$ and isogenic CDT$^-$ *E. coli* strains. *E. coli* R1-1 was described previously (17). This strain harbors a recombinant plasmid, a pBR322 derivative bearing the 8.3-kb *Eco*RI-*Sal*I fragment cloned from the chromosome of *S. dysenteriae*. The recombinant plasmid was purified from R1-1. The 8.3-kb *Eco*RI-*Sal*I fragment containing the *S. dysenteriae cdtABC* genes was isolated from the plasmid and cloned into the *Eco*RI-*Sal*I sites of pUC119 (22), resulting in pKON1. Then, a 0.9-kb *Eco*RV fragment spanning more than 64.7% of the *cdtBC* sequence was removed from pKON1 by digestion with *Eco*RV followed by self-ligation. The resulting plasmid was named pKON2. Plasmids pKON1 and pKON2 were maintained in *E. coli* JM109 (23), and these strains were employed as CDT$^+$ and isogenic CDT$^-$ strains, respectively.

Toxin preparations. *E. coli* JM109(pKON1) and JM109(pKON2) were grown in LB medium (14) supplemented with ampicillin (200 μg/ml) with shaking (180 rpm) at 37°C overnight. Culture supernatants were obtained from the overnight cultures by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatants were filtered through a 0.22-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.). The preparations derived from JM109(pKON1) and JM109(pKON2) were named wild-type toxin (WT) and mutant toxin (MT), respectively.

CDT in the culture supernatant of JM109(pKON1) was partially purified as follows. Culture supernatant of *E. coli* JM109(pKON1) was obtained as described above. Ammonium sulfate was added to the culture supernatant to 60% saturation. The precipitate was dissolved in a phosphate buffer (50 mM phosphate, NaH_2PO_4-Na_2HPO_4 [pH 6.8]) and applied to a DEAE-Sepharose (Pharmacia-LKB Biotechnology, Inc., Piscataway, N.J.) column equilibrated with the phosphate buffer. The column was washed with the phosphate buffer and eluted with a 0 to 1 M sodium chloride gradient. Pooled fractions that showed CDT activity against CHO cells were applied to a Sephacryl S-200HR (Pharmacia-LKB Biotechnology, Inc.) column equilibrated with the phosphate buffer, and CDT was eluted with the phosphate buffer. Fractions that showed CDT activity against CHO cells were pooled. This pooled preparation was named partially purified toxin (PT).

CT and ST (STa) were purchased from Funakoshi Co., Ltd., Tokyo, Japan, and Sigma Chemical Co., St. Louis, Mo., respectively. These toxins were dissolved in the phosphate buffer at concentrations of 50 μg/ml and 50 ng/ml, respectively.

CHO assays. The CHO assay to detect CDT activity was performed as described previously (17). One unit of CDT activity was defined as the reciprocal of the dilution that caused lethality of 100% of the CHO cells.

Suckling mice and sample administration. Adult ICR mice were purchased from CLEAR Japan, Inc., Tokyo, Japan. Male and female mice were maintained at a 1:5 ratio to obtain suckling mice. Suckling mice weighing 1.8 to 2.5 g were removed from the mothers and starved for ca. 10 h at 25°C prior to sample administration. A 1/10-ml aliquot (total volume) of test sample in solution with Evans blue dye (0.04% vol/vol) was administered into the stomach of each suckling mouse via polyethylene intramedic gastric tubing (PE50; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan).

Diarrhea score assay. Each mouse that received a test sample was incubated on a white filter paper in a compartment at 25°C for 24 h. The mice excreting stained feces which were loose and/or watery in 24 h were judged diarrhea-positive animals. The diarrhea score was expressed as the percentage of the diarrhea-positive animals in each test group.

Kinetics of FA ratio. The FA ratio was determined by a modification of the method described previously (15). A group of five or more mice administered with test samples were incubated at 25°C for the specified period. FA was expressed as the ratio of the weight of stomach plus intestine to the remaining body weight. The FA ratio was determined for each animal, and the average and standard error was calculated for each test group. Average FA ratios were statistically compared by Student's *t* test.

Histological studies. Two animals were used for each examination. Small and large intestines were taken from the test animal and fixed in 10% formalin. The tissues were embedded in paraffin. Two 4-μm sections were prepared from portions 2 to 3 mm apart. The sections were stained with hematoxylin and eosin for morphological examination and by periodic acid-Shiff stain and alcian blue stain for visualizing mucin by standard methods and examined by light microscopy.

TABLE 1. CDT activities and diarrhea scores of various toxin preparations

Toxin preparation	CDT activity (U/ml)[a]	Diarrhea score (no. of positive animals/total no. of animals)[b]
WT[c]	1.0×10^4	67 (24/36)
PT[d]	8.4×10^5	100 (11/11)
MT[e]	<20	25 (7/28)
NEG-P[f]	<20	23 (3/13)
NEG-M[g]	<20	19 (3/16)
ST[h]	NT[i]	100 (10/10)
CT[j]	NT	100 (10/10)

[a] Determined by the CHO assay.
[b] The animals excreting stained loose and/or watery feces in 24 h after sample administration were judged positive.
[c] Culture supernatant of JM109(pKON1) containing WT.
[d] Obtained by ammonium sulfate precipitation followed by column chromatography.
[e] Culture supernatant of JM109(pKON2) containing MT.
[f] Culture supernatant of JM109(pUC119) employed as a negative control.
[g] Sterile LB medium employed as a negative control.
[h] ST of enterotoxigenic *E. coli*. A 0.1-ml preparation containing 5 ng of toxin was administered to each test animal.
[i] Not tested.
[j] A 0.1-ml preparation containing 5 μg of toxin was administered to each test animal.

RESULTS

CDT activity of toxin preparations. CDT activities of various toxin preparations are listed in Table 1. The MT was shown to be devoid of CDT activity. CDT activity of the WT preparation could be increased by partial purification. However, the increase in CDT activity was only 84-fold (PT). Part of the reason for the small increase is that a considerable amount of CDT was lost during partial purification because of toxin aggregation (16).

Diarrhea score assay. We first attempted to establish a suckling mouse model in which the difference in diarrheagenicity between the WT and MT preparations could be demonstrated. Visual inspection of the inoculated animals revealed that WT-inoculated mice excreted stained feces faster and more often than did MT-inoculated mice. We then defined the diarrhea score as described in Materials and Methods. This protocol was established based on the considerations described in the Discussion section. The culture supernatant of *E. coli* JM109 harboring the vector plasmid (NEG-P) and sterile culture medium (NEG-M) were employed as negative controls. ST and CT were used as positive controls. The results of the diarrhea score assay are summarized in Table 1. Some of the animals that received the negative-control samples exhibited a positive response for unknown reasons, thus giving low diarrhea scores. Animals given MT showed similarly low scores. Animals receiving WT showed much higher scores. When the toxin preparation with higher CDT activity (PT) was administered, the highest score was achieved. This score was equivalent to those obtained with the positive controls. Detailed time records for the diarrhea score-positive animals are given in Table 2. Most diarrhea scores were recorded during the late incubation period in the test groups that gave low total scores; MT-, NEG-P-, and NEG-M-inoculated animals gave scores of 21, 23, and 19, respectively during the incubation period from 12 to 24 h. Of the diarrhea-positive animals in the WT- and MT-inoculated groups, 70 and 32%, respectively, exhibited a positive

TABLE 2. Time course of diarrhea scores

Sample[a]	Diarrhea score during incubation period[b] of:				Total diarrhea score[a]
	0–3 h	3–12 h	12–18 h	18–24 h	
WT	0	8	39	20	67
PT	55	45	0	0	100
MT	0	4	4	17	25
NEG-P	0	0	15	8	23
NEG-M	0	0	0	19	19
ST	0	100	0	0	100
CT	0	100	0	0	100

[a] See Table 1 for explanation.
[b] Each incubation period was greater than the first value and less than or equal to the second value.

response by 18 h. All animals in the PT-, ST-, and CT-inoculated groups showed positive scores by 12 h. The pictures illustrating representative diarrhea patterns are shown in Fig. 1. Both loose and watery feces were excreted more frequently from PT- than WT-inoculated mice (Fig. 1B and C). PT-inoculated mice evidently excreted profuse watery diarrhea (Fig. 1C). These results indicate that CDT is diarrheagenic to suckling mice.

Kinetics of FA ratios. To obtain more evidence for the CDT-induced watery diarrhea, the kinetics of FA ratios after sample administration was examined. WT-induced FA values were slightly higher at all time points than those induced by MT (Fig. 2). Statistically significant differences, however, were detected at only 8 and 15 h ($0.02 < P < 0.05$ and $0.01 < P < 0.02$, respectively). FA values stimulated by MT were not statistically different from those stimulated by negative controls (Fig. 2, NEG-P and NEG-M), with one exceptional time point (NEG-P at 14 h [$0.01 < P < 0.02$]). The results indicate that CDT in the WT preparation stimulated a small amount of FA that culminated at around 15 h postinoculation.

FAs induced by WT, PT, CT, and ST were compared next (Fig. 3). PT-induced FA was very significantly ($P < 0.01$) and significantly ($0.02 < P < 0.05$) higher than WT-induced FA at 4 and 8 h postinoculation, respectively. The difference in FA was not significant at 15 and 24 h ($P > 0.2$). Thus, the watery diarrhea, which was more evident and observed earlier in the PT-inoculated group than in the WT-inoculated group (Fig. 1B and C and Table 2), appears to be correlated with FA. CT and ST exhibited high FA ratios at 15 h and 4 to 8 h, respectively,

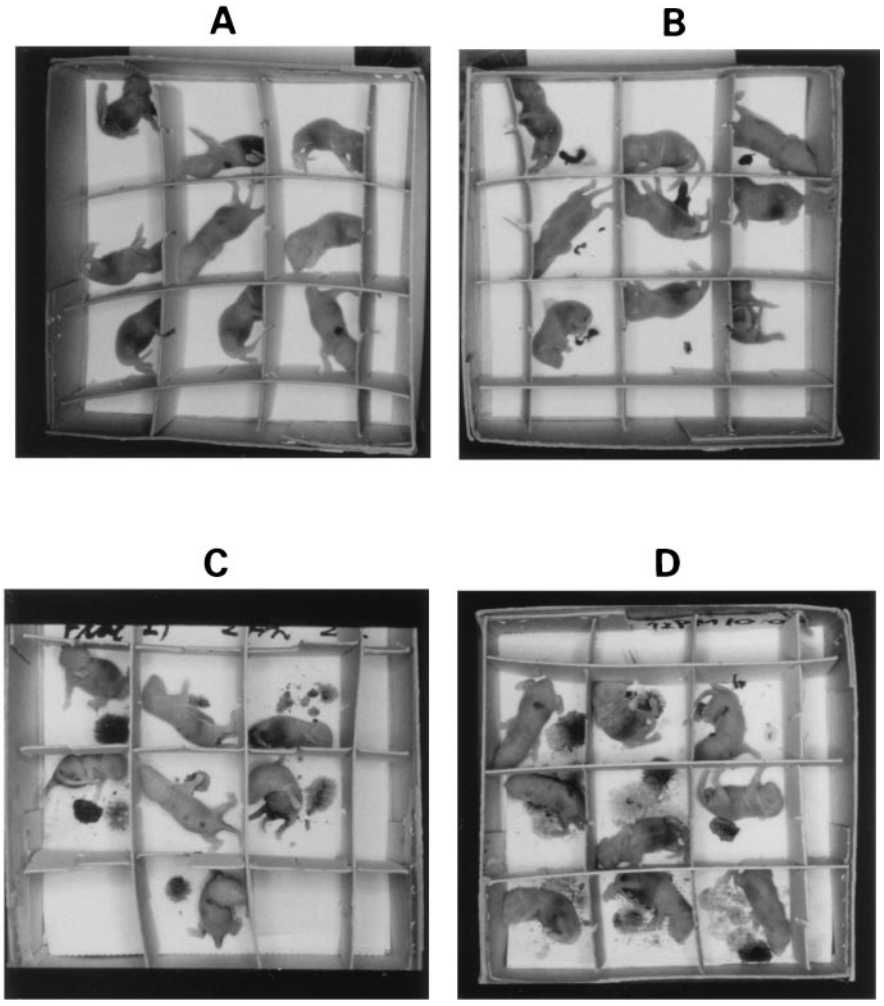

FIG. 1. Representative diarrhea patterns observed in diarrhea score assay. The photographs were taken after sample administration, at 24 h of incubation. Animals were given MT (A), WT (B), PT (C), and CT (D).

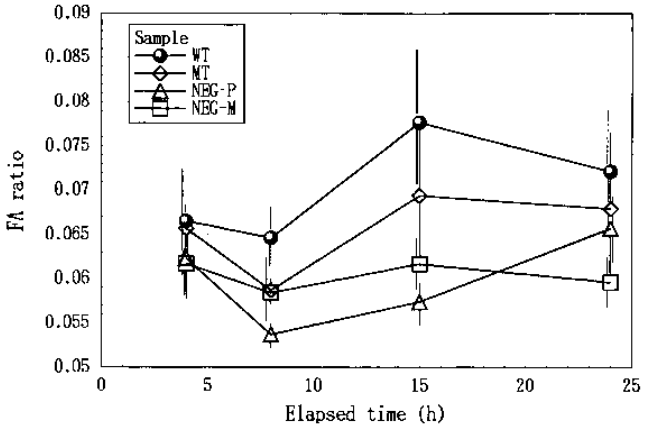

FIG. 2. Kinetics of FA ratios induced by WT, MT, NEG-P, and NEG-M. Five to 13 animals were used for each determination. Error bars indicate standard errors.

as reported previously by other workers (2, 5–7). The PT-induced early FA was kinetically similar to that of the ST-stimulated FA.

Histology. We examined whether CDT preparations administered to suckling mice induced any histological change in the intestinal tissues or not. Histologies were compared after sample administration, at 24 h of incubation.

When the tissues taken from various parts of the small intestines (upper and middle parts of jejunum and the middle ileum) were compared, no significant difference was detected among the animals that received sterile culture medium (NEG-M) and various toxin preparations (WT, MT, and PT). The representative histological findings of the tissue samples obtained from the middle jejunum are shown in Fig. 4. Neither destructive damage nor inflammatory response was noted in any of the samples. Examinations by periodic acid-Schiff stain and alcian blue stain revealed no remarkable difference in the distribution of the goblet cells in the tissue samples examined (data not shown).

On the other hand, CDT-induced histological changes were detected in the tissues of the descending colon (Fig. 5). The histology of the animals receiving MT did not differ from that of animals receiving NEG-M (Fig. 5A and B). On the other hand, WT elicited tissue damage; most of the mucosa was necrotic, and entire sheets of epithelial cells had sloughed off in some parts (Fig. 5C). Apparent reparative hyperplasia of epithelial cells was seen in the tissues of the animals that received PT (Fig. 5D).

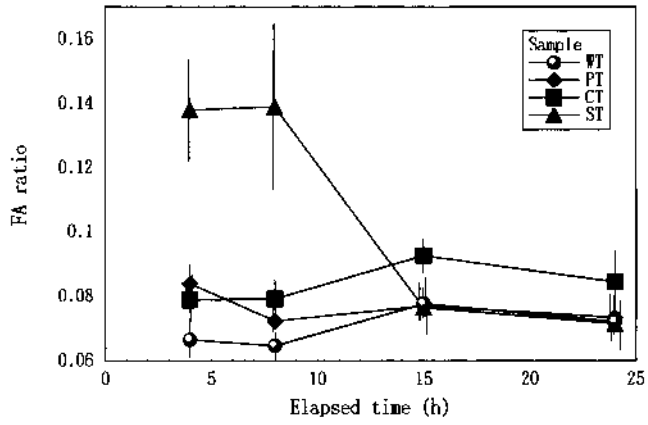

FIG. 3. Kinetics of FA ratios induced by WT, PT, CT, and ST. Five to 13 animals were used for each determination. Error bars indicate standard errors.

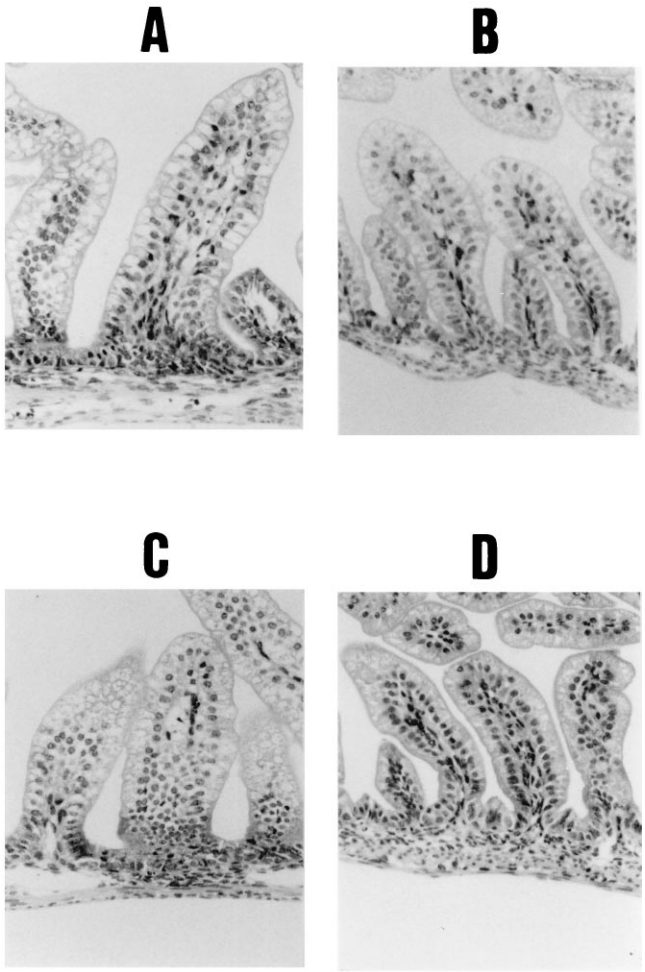

FIG. 4. Sections of the middle ileum stained with hematoxylin and eosin (magnification, ca. ×170). The tissues were obtained from animals that received NEG-M (A), MT (B), WT (C), and PT (D).

DISCUSSION

On the basis of the literature, standard animal assays established for known enterotoxins did not appear suitable for examination of CDT enterotoxicity. Therefore, we were interested in developing a model in which enterotoxicity of CDT could be examined. We employed suckling mice because they are economical and various types of response can be monitored in the whole animal. Isogenic pairs of CDT^+ and CDT^- strains were also useful for development of the model. We initially noted the difference in the secretion of stained feces when whole cultures of the isogenic strains were administered (data not shown). Subsequently, we found that the culture supernatants of the test strains could also be used to demonstrate the difference. We then established the diarrhea score assay to record the stained-feces response. There were two types of feces, loose and watery, that were excreted more often from the WT-inoculated than from the MT-inoculated mice. CDT induces various types of responses (elongation, disten-

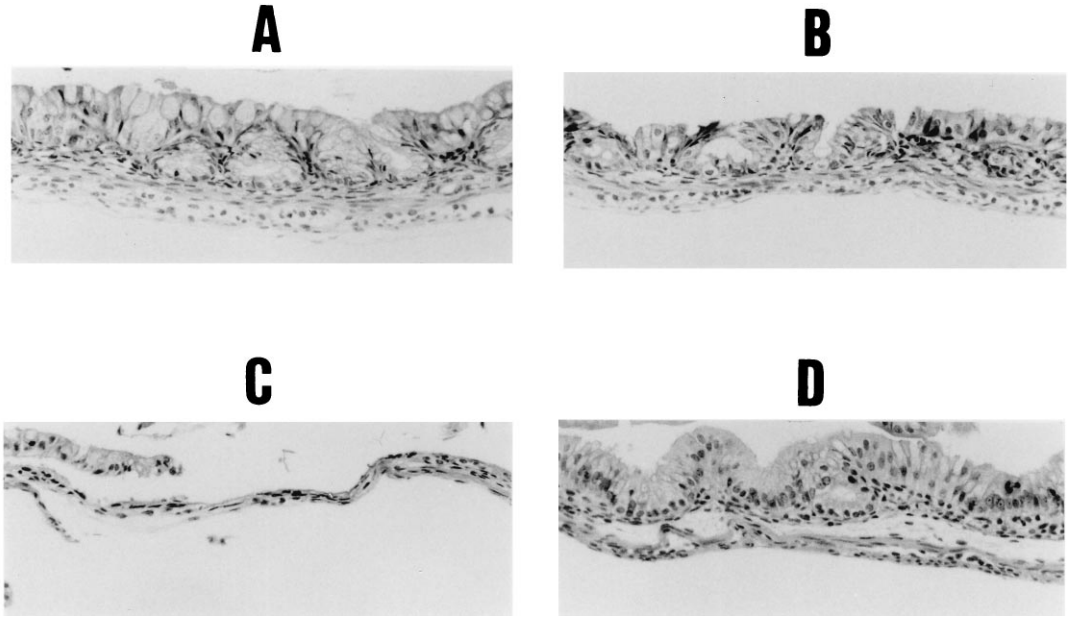

FIG. 5. Sections of the descending colon stained with hematoxylin and eosin (magnification, ca. ×170). The tissues were obtained from animals that received NEG-M (A), MT (B), WT (C), and PT (D).

tion, and lethality) in CHO cells, and the mechanisms responsible for these responses are not known at present. We therefore did not focus on watery diarrhea only and decided to use both loose and watery feces as indices of diarrhea. The WT preparation contained a relatively high-level CDT produced from the *cdt* genes on the high-copy-number plasmid. This preparation could induce the cytolethal response of CHO cells in 24 h, whereas the culture filtrate of *S. dysenteriae* containing a single copy of the *cdt* genes induced the same response at 96 to 120 h of incubation (16). We then decided to record the diarrhea score at 24 h postinoculation. Taking the above-mentioned factors into consideration, the suckling mouse protocol described in Materials and Methods was established after repeated preliminary experiments (data not shown). The 10-h starvation, in particular, was found essential to obtain reproducible results. The long observation period (24 h), however, seems to have contributed to relatively high background scores (Table 2, NEG-P, NEG-M, and MT).

The correlation between the level of CDT activity and the diarrhea score clearly indicated the diarrheagenicity of CDT (Table 1, MT, WT, and PT). This is supported by more detailed analyses of the diarrhea response. A comparison of the results obtained with WT- and PT-inoculated mice indicates that high-level CDT activity is responsible for profuse and frequent watery diarrhea (Fig. 1B and C), early diarrhea response (Table 2, WT and PT), and early FA response (Fig. 3, WT and PT). It seems reasonable therefore to conclude that watery diarrhea and early FA are associated and that these responses might be due to the CDT dosage. The effect of the CDT dosage at the level of cellular response could be inferred from the effect of the gene dosage described above. Evidence supporting this was obtained with WT and PT preparations; PT induced the cytolethal response of CHO cells earlier (ca. 12 h) than did WT (24 h) (16).

The early FA response is kinetically similar to the ST-induced response. ST-induced diarrhea in suckling mice is dose dependent, and this can be explained by the availability of free ST, the remnant not removed by binding to milk components, to low-affinity rather than high-affinity receptors (23). The low-affinity receptors appeared to mediate the secretory response (23). CDT-induced early FA and diarrhea might be dose dependent and are well reproduced in starved animals. These responses can be explained by the low affinity of the toxin to the yet unidentified receptors. A weak FA response culminating at around 15 h postinoculation was detected in WT-inoculated animals. It may be caused by the same mechanism as that induced by PT but may be delayed because of the lower CDT level. Further study to establish a purification method for CDT is needed. Purified CDT will facilitate the investigation of the dosage effect and the identification of CDT receptors. The results of these investigations may lend support for the above-described possibilities.

CDT induced tissue damage in the descending colon, whereas the tissues of the small intestines remained apparently intact. Other workers have reported that CDT-producing *E. coli* and *Campylobacter* spp. induced inflammatory responses in the small intestines (ilea) of rabbits (4) and rats (10), respectively. The discrepant results that we observed with suckling mouse small intestines may be due to differences in the sensitivities of the animals. Otherwise, the inflammatory responses observed by other workers may be due to factor(s) other than CDT produced simultaneously by the test strains. It is not clear whether the cytotoxic activity of CDT against the colonic tissue is associated with the diarrhea response. PT with high-level CDT activity induced more progressive tissue response (reparative hyperplasia) than did WT (necrosis). The frequencies of loose and watery feces were both dependent on the level of CDT activity. Both types of diarrhea could be related to the colonic damage. Alternatively, perhaps at least the early watery diarrhea is not associated with the cytotoxic activity of CDT. ST, LT, and CT stimulate the mucosal cells of the small intestine through enzymatic actions without tissue damage (12, 13, 21). LT induces elongation of CHO cells indistinguishable from that induced by CDT (9). In CHO cell assays of CDT, elongation is observed earlier than distention that leads to cytolethality. The cell elongation could be stimulated by a

mechanism distinct from that responsible for distention and cytolethality. If so, the mechanism associated with elongation could stimulate the small intestine and lead to watery diarrhea. Further studies on the identification and distribution of the CDT receptor(s) and analyses of the structure-function relationship of CDT by site-directed mutagenesis will help clarify the points mentioned above.

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