Detection of a Vacuolating Cytotoxin in Stools from Children with Diarrhea

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A cytotoxin inducing vacuolation in HEp-2 cells was detected in 19 (3.1%) of 618 stool specimens from children with diarrhea but in none of 135 from control children. Common enteric pathogens were found in only two (10.5%) of the 19 cytotoxin-positive stool specimens. The vacuoles induced by stool filtrates resembled those induced by the vacuolating toxin (VacA) of Helicobacter pylori. The vaculating toxin was heat-labile and protease-sensitive, and it had an apparent molecular weight of >100,000 but was not neutralized by an antiserum to H. pylori VacA. Although proper prospective case-control studies are needed to definitely assess the etiologic association between the new vaculating cytotoxin and diarrhea, the present study suggests that microorganisms of the gastrointestinal tract produce a Helicobacter-like vaculating toxin and may be responsible for cases of childhood diarrhea whose etiology is currently considered unknown.

The production of toxins is one of the most important pathogenic mechanisms by which many enteric pathogens may cause gastrointestinal diseases [1]. Some of these toxins induce morphological alterations in cell lines in culture [2], and these in vitro systems have been used to study their mode of action. Moreover, the direct detection of specifically neutralizable toxins in stool samples by means of cytotoxicity assays is a useful tool for rapid diagnosis of some intestinal infections as well as a valid diagnostic criterion when conventional microbiological procedures cannot detect the toxigenic organism in stool shortly after the onset of the disease. Finally, the presence of free toxin in stools demonstrates its in vivo production and points to its involvement in the genesis of illness.

Detection of free fecal toxins has contributed to clarification of the etiology of two important intestinal pathological conditions: pseudomembranous colitis [3] and hemolytic uremic syndrome [4]. Larson and co-workers [5] initially reported that stools from patients with pseudomembranous colitis contained a cytotoxic toxin and hypothesized that this was a bacterial toxin, although no specific microbe was implicated; subsequently, it was found that this toxin had the same physicochemical properties as a toxin present in culture filtrates of Clostridium difficile [6].

In 1977 Konowalchuk and colleagues [7] described strains of Escherichia coli that produced a cytotoxin (verotoxin [VT]) active on Vero cells. The detection of free VT in stools of patients with hemolytic uremic syndrome allowed Karmali and co-workers [8] to establish the etiologic role of the VT-producing E. coli in this syndrome.

Recently [9], we reported that stool filtrates from two of 63 children with diarrhea induced intracytoplasmic vacuolation in HEp-2 cell cultures resembling that produced by the Helicobacter pylori vaculating toxin (VacA), which is considered an important virulence determinant in the pathogenesis of the disease caused by this organism [10–13]. Since no other microbial product has been reported to induce similar morphological changes in cultured cells, we hypothesized that the toxic factor observed in stool filtrates could actually be the toxin of H. pylori [9].

We report here the results of a retrospective investigation of vaculating cytotoxic activity in a large sample of stool specimens collected during a study on the etiology of childhood diarrhea in Italy. We also attempt to characterize the cytotoxic factor detected in stool samples.

Materials and Methods

Stool specimens. The study was conducted on fecal specimens previously collected during a multicenter study on the etiology of childhood diarrhea in Italy (manuscript in preparation). The study involved six hospitals located in different cities of northern, central, and southern Italy. Six hundred and eighteen children with diarrhea (338 hospitalized patients and 280 outpatients) and 135 children who were free of enteric symptoms were observed from January through December 1992. Some of these patients have been described already [9]. Diarrhea was defined by ≥3 unformed stools in 24 hours. One child without diarrhea was enrolled for each five patients with...
diarrhea; these controls were selected from among children who were attending the same pediatric outpatient departments for treatment of minor ailments.

The age and sex distribution of the control group was similar to that of the group of patients with diarrhea. The children were younger than age 12 years; the mean age of both patients and controls was 31 months. Children who had been given antimicrobial agents for their current illness were not excluded. Clinical information was obtained by a review of the clinical charts and by a standardized questionnaire submitted to the children’s parents. In particular, the occurrence of fever (temperature of >37.5°C), vomiting, abdominal pain, and dehydration was ascertained.

**Stool examination.** Stool samples from children with and without diarrhea were collected on enrollment and examined for the presence of gross blood, leukocytes, enteric pathogens, and free cytotoxins. All the specimens were plated on MacConkey, salmonella-shigella, and thiosulfate-citrate-bile salts-sucrose agars, incubated at 37°C for 18 hours, and examined for the presence of *Salmonella* and *Shigella* species organisms, vibrios, and other enteropathogens by means of standard methods [14].

For detection of *Salmonella* species organisms, a portion of each sample was enriched in sodium selenite medium, incubated for 18 hours at 37°C, and then plated on salmonella-shigella agar and held at 37°C. *Campylobacter* species organisms were detected on charcoal-cefoperazone-deoxycholate agar plates after incubation at 42°C for 48 hours in a microaerophilic environment (Campy Pack; Oxoid, Basingstoke, UK). Putative *Campylobacter* colonies were submitted to gram staining and catalase and oxidase tests for confirmation of the identification [14].

*VT*-producing *E. coli* was identified by a Vero cell assay, as previously described [15]. Rotavirus and adenovirus antigens were detected by a latex agglutination test (Orion Diagnostica, Espoo, Finland). Intestinal parasites were identified by direct microscopic examination of stool. *Cryptosporidium* oocysts were also detected with a modified Ziehl-Neelsen stain [16]. Part of each stool specimen was frozen and maintained at −20°C until used for cytotoxicity assays.

**Detection of free cytotoxins and vacuolating activity in stools.** All stool samples from children with and without diarrhea were examined for their ability to induce cytopathic effects (CPEs), as previously described [17]. In brief, equal volumes of each fecal specimen and sodium phosphate buffer (pH 7.2; 0.01 M) were thoroughly mixed, centrifuged at 10,000g for 10 minutes, and filter-sterilized. Twofold serial dilutions of fecal filtrates were inoculated into Vero cell and HEp-2 cell monolayers grown in microtiter plates (0.02 mL of sample per well, containing 0.18 mL of cell culture medium). Cells were examined for CPE after incubation for 5, 24, and 48 hours at 37°C in an atmosphere of 5% CO₂. For samples showing CPE up to a 1:4 dilution, cytotoxicity neutralization assays were performed as previously described, with use of antisera to *C. difficile* cytotoxin, *Clostridium perfringens* enterotoxin, and *E. coli* VTs [17, 18].

Stool specimens were considered positive for vacuolating activity when an intracellular vacuolization was observed in at least 50% of the cells in the well. The cytotoxic titer was the highest final dilution of sample that yielded a positive response.

**Production of *H. pylori* VacA.** Crude *H. pylori* VacA was obtained by growing the cytotoxic strain CCUG 17874 in brucella broth (Oxoid) supplemented with fetal bovine serum (5% volume in volume) (Gibco Laboratories, Grand Island, NY) and Vitox 1% (Oxoid). Incubation was for 24 hours at 37°C with an atmosphere of 10% CO₂ in a rotary shaker set at 120 rpm. The culture was centrifuged, filter-sterilized, and concentrated by ultrafiltration on a YM-100 membrane (Amicon, Lexington, MA). The titer of VacA preparations was assessed on cell cultures as described above.

**Characterization of the stool vacuolating activity.** For evaluation of the uptake of neutral red by intracytoplasmic vacuoles, cells treated with either stool filtrates or crude *H. pylori* VacA were stained with 0.05% neutral red in cell culture medium for 4 minutes.

Heat stability was evaluated by heating fecal filtrates at 60°C and 70°C for 30 minutes. For assessment of enzyme sensitivity, samples were incubated with either trypsin (Sigma, St. Louis) or proteinase K (Sigma) (0.001% weight in volume) at 37°C for 60 minutes. A rough estimate of the molecular size of the toxic factor was obtained by ultrafiltration of stool filtrates on a YM-100 membrane (Amicon).

For the neutralization assay, stool filtrates and the VacA preparation were diluted up to a cytotoxic titer of 1:4. The above-described panel of antisera and an antisem showing neutralizing activity toward the *H. pylori* VacA (kindly provided by J. L. Telford, Siena, Italy) were used. VacA antisem was raised in rabbits with use of a partially purified preparation of the native toxin from *H. pylori* strain CCUG 17874 as antigen [19].

**Search for the organism producing the vacuolating toxin.** Isolation of *H. pylori* from stool samples showing vacuolating activity was attempted by means of the procedure reported by Thomas et al. [20]. The same stool samples were also streaked onto blood agar and incubated at 37°C in an aerobic and microaerophilic atmosphere for 48 hours. Colonies with different morphological appearances were picked up from each plate and grown in brucella broth supplemented with 5% fetal calf serum for 24 hours. Culture supernatant filtrates and bacterial extracts obtained by sonications were tested for vacuolation in HEp-2 cells.

**Search for fecal vacuolating activity in other groups of patients.** A group of children with diarrhea lasting for >10 days who were observed at a university pediatric clinic in Rome were prospectively studied from October through December 1993. Fecal samples from children who were found positive for vacuolating activity were collected at 5-day intervals during the illness and after the diarrhea subsided. Microbiological examination and the detection and titration of vacuolating activity were performed as above.
Table 1. Presence of free cytotoxins in stool specimens from 618 children with diarrhea and 135 asymptomatic children.

<table>
<thead>
<tr>
<th>Fecal cytotoxin</th>
<th>With diarrhea</th>
<th>Without diarrhea</th>
</tr>
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<tbody>
<tr>
<td>C. difficile cytotoxin</td>
<td>24 (3.9)</td>
<td>9 (6.7)</td>
</tr>
<tr>
<td>E. coli verocytotoxin</td>
<td>5 (0.8)</td>
<td>0</td>
</tr>
<tr>
<td>C. perfringens enterotoxin</td>
<td>5 (0.8)</td>
<td>0</td>
</tr>
<tr>
<td>Nonneutralizable cytotoxin*</td>
<td>6 (1.0)</td>
<td>3 (2.2)</td>
</tr>
<tr>
<td>Vacuolating cytotoxin</td>
<td>19 (3.1)*</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cytopathic effect not neutralized by the panel of antisera described in Materials and Methods.

Results

Vacuolating activity in the children’s feces. The results of cytotoxicity assays performed on filtrates of stool from children with and without diarrhea are shown in table 1. C. difficile cytotoxin and other factors inducing the death of cell monolayers but not neutralized by the available panel of antisera were more frequent in specimens from asymptomatic children than in those from the other subjects (cases), while C. perfringens enterotoxin and E. coli VT were found only in diarrheic stools. Vacuolating activity on HEp-2 cells was observed in 19 (3.1%) of 618 samples from cases but in none of the 135 samples from children without diarrhea (P = .03). Enteric pathogens including Salmonella species, Shigella species, Campylobacter species, Yersinia enterocolitica, VT-producing E. coli, rotavirus, adenovirus, Cryptosporidium species, and Giardia intestinalis were identified in 337 (56.3%) of the 599 children whose stool samples were negative for vacuolation but in only 2 (10.5%) of the 19 vacuolation-positive stools (P < .001). These two samples were found positive for rotavirus antigens and a VT-producing E. coli strain (O untypeable), respectively.

The mean age of patients whose stools were positive for vacuolating activity was 39 months (range, 7–96 months). The mean duration of diarrhea was 9.8 days (range, 1–20 days), but the symptoms were generally mild: 14 of the 19 children were outpatients and none required iv rehydration. Table 2 compares the clinical features of these patients with those of the other patients with diarrhea. Vomiting and abdominal pain, but not fever, were frequently observed in patients whose stools were positive for vacuolating activity. Most of the latter cases (14 of 19, or 74%) occurred in the cold season (between November and March), during which only 278 (45%) of the 618 total cases considered in the study were observed.

Characterization of the vacuolating activity. The vacuolation induced in HEp-2 cells by the 19 different stool filtrates was very similar and could not be serially passaged, a circumstance suggesting that the CPE was not due to a viral agent. To further exclude the presence of a vacuolating virus, aliquots of the fecal filtrates were centrifuged at 100,000g for 1 hour; the pellets were resuspended in PBS, and the supernatants were inoculated into HEp-2 cells. No vacuolating activity was observed in any pellet, while the supernatants were still positive at titers similar to those of the original fecal filtrates.

The CPE had a characteristic pattern, occurring diffusely and simultaneously throughout the cell monolayer within 6 hours and progressing to a maximum at 24 hours. The vacuoles strongly resembled those produced by H. pylori VacA. Figure 1a shows a representative CPE induced by a stool filtrate in HEp-2 cells, compared with that elicited by a culture filtrate of the cytotoxic strain of H. pylori CCUG 17874. The vacuoles induced either by VacA or by stool filtrates were avidly stained by neutral red (figure 1b).

In both cases vacuolation was a reversible phenomenon: vacuoles rapidly disappeared when the cell culture medium was replaced with fresh medium 6 hours after inoculation of the toxic samples. The fecal filtrates induced vacuolation in HEp-2 cells up to dilutions ranging from 1:4 to 1:128. In particular, CPE was observed up to a dilution of 1:4–1:8 in 14 samples and up to 1:16–1:128 in five.

When cytotoxicity assays were performed on Vero cells, this cell line proved to be far more susceptible to the effect of VacA than to the cytotoxin present in the stools. While the H. pylori CCUG 17874 culture filtrate induced vacuolation in

Table 2. Signs and symptoms in 618 children with diarrhea whose stools were positive or negative for vacuolating activity on HEp-2 cells.

<table>
<thead>
<tr>
<th>Sign or symptom</th>
<th>Positive for vacuolating activity (n = 19)</th>
<th>Negative for vacuolating activity (n = 599)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>6 (31.6)</td>
<td>376 (62.8)</td>
<td>.006</td>
</tr>
<tr>
<td>Vomiting</td>
<td>14 (73.7)</td>
<td>322 (53.7)</td>
<td>.08</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>12 (63.2)</td>
<td>213 (35.6)</td>
<td>.014</td>
</tr>
<tr>
<td>Severe dehydration</td>
<td>0</td>
<td>74 (12.4)</td>
<td>.1</td>
</tr>
<tr>
<td>Blood in stool</td>
<td>2 (10.5)</td>
<td>83 (13.9)</td>
<td>.8</td>
</tr>
<tr>
<td>Fecal leukocytes</td>
<td>3 (15.8)</td>
<td>203 (33.9)</td>
<td>.09</td>
</tr>
</tbody>
</table>
HEp-2 and Vero cells at comparable titers, only the three stool filtrates with the highest titer in HEp-2 cells induced vacuolation in <50% of Vero cell monolayers, even when the samples were tested undiluted.

A rabbit antiserum showing neutralizing activity toward VacA of *H. pylori* CCUG 17874 up to a dilution of 1:100 failed to neutralize the cytotoxicity of stool filtrates, even when diluted only 1:10. Vacuolation was inhibited neither by neutralizing antiserum to *E. coli* VT1 and VT2, *C. difficile* cytotoxin, and *C. perfringens* enterotoxin nor by convalescent sera from three patients whose feces specimens were positive for vacuolating activity.

In all 19 vacuolation-positive specimens, the vacuolating factor was completely inactivated by the heating of the stool filtrates at 70°C for 30 minutes and by protease treatment. It was retained by an ultrafiltration membrane with a pore size corresponding to a molecular weight cut-off of 100,000. It retained activity after storage at -20°C for 48 months and several freezing and thawing cycles.

**Search for the organism producing the vacuolating toxin.** The stool samples inducing vacuolation were cultured on specific and selective media for *H. pylori*, but no growth was obtained.

To verify if a bacterial species other than *H. pylori* was responsible for the vacuolating activity, filtrates of culture supernatants and of bacterial extracts obtained by sonication from different bacterial strains isolated from the positive stool samples were tested for vacuolation in HEp-2 cells. All were negative, and negative results were also obtained when supernatants of enrichment cultures of stools in brucella broth were tested.

**Search for fecal vacuolating activity in other groups of patients.** To study the persistence of the vacuolating cytotoxin in stools, we prospectively searched for new cases of vacuolating activity–associated diarrhea. On the basis of the characteristics of the patients found to be positive in the first retrospective survey, we decided to study a group of children brought to medical attention for diarrhea lasting for >10 days and occurring in the cold season (October–December 1993).

Two of the 15 patients examined in that period were found positive for vacuolating activity and were followed by means of repeated stool examination during the course of their illness. In both cases the vacuolating activity was detectable in stools during the episodes of diarrhea, while specimens collected after resolution of the intestinal symptoms were consistently negative in the cell culture assay (figure 2).

To verify if gastric colonization with cytotoxic *H. pylori* may result in shedding of active VacA in feces, stool samples from 20 patients who had gastritis associated with infection due to VacA-producing *H. pylori* but who did not have diarrhea or other intestinal disorders were examined by means of a cell culture assay. None of specimens showed vacuolating activity.

**Discussion**

Recently we reported that stool filtrates from two children with diarrhea induced intracytoplasmic vacuolation in HEp-2 cell cultures, strongly resembling that induced by VacA of *H. pylori* [9]. The results obtained in the present study by the analysis of a large number of stool specimens confirm that a vacuolating cytotoxin may be found in human stools and indicate that it is not *H. pylori* VacA. The presence of this novel cytotoxin appeared to be associated with diarrhea, since it was detected in 3.1% of stool specimens from symptomatic children but in none of those from healthy controls.
The absence of conventional enteric pathogens in all but two of the positive samples further suggests that the vacuolating cytotoxin was somehow involved in the genesis of diarrhea in those children. Moreover, the follow-up study of two cases of diarrhea associated with vacuolating activity showed that the cytotoxin could be repeatedly observed in stool while the diarrhea persisted, whereas it was no longer detectable in specimens collected after the diarrhea subsided.

Abdominal pain (often associated with vomiting) and absence of fever were significantly more frequent in patients with vacuolating activity–associated diarrhea than in the other children with diarrhea considered in our study. It is interesting that many cases occurred in the cold season, and in nine of them the duration of diarrhea was >1 week. Moreover, the vacuolating cytotoxin was found in the stool from as many as two children among the second group of 15 patients who were selected on the basis of these two features; this further suggests that the vacuolating activity–associated diarrhea might have a peculiar clinico-epidemiologic picture. This theory could be taken into account in the planning of future studies of selected patients to clarify the relationship between this new factor and diarrheal disease.

As far as the nature of the vacuolating activity is concerned, some of its features are compatible with those of the VacA of \textit{H. pylori}: the appearance and staining characteristics of the vacuoles, the heat lability and protease sensitivity of the factor, and the apparent molecular weight of >100,000 [11, 22, 23]. However, these similarities are quite nonspecific, and some other findings presented herein indicate that the vacuolating factor is not \textit{H. pylori} VacA: (1) an antiserum showing neutralizing activity toward VacA was not able to neutralize the vacuolating activity of stool filtrates; (2) Vero cells were more susceptible to VacA than to the vacuolating factor present in the stools; and (3) we could not isolate any \textit{H. pylori} isolate from the feces of our patients with the method used by Thomas et al. [20] to isolate this organism from the stools of heavily colonized subjects.

However, as for the Shiga-like toxin family [24], some degree of biological and immunologic heterogeneity among the vacuolating toxins produced by different strains of \textit{H. pylori} cannot be ruled out, since only a few strains have been studied and characterized so far [25]. Moreover, little is known about cytotoxin production by other members of the genus \textit{Helicobacter} that have been associated with intestinal disorders [26, 27].

We have no evidence regarding the site of the gastrointestinal tract in which the fecal vacuolating factor was developed and released. However, we observed that gastric colonization by cytotoxic strains of \textit{H. pylori} does not result in shedding of active toxin in stools. In fact, we did not observe any vacuolating activity in stool samples from 20 patients who had gastric disorders (but not diarrhea) and from whose gastric biopsic specimens VacA-producing \textit{H. pylori} strains had been isolated.

This finding suggests that the organism responsible for production of this cytoxin was colonizing the intestinal tract of our children with diarrhea. Many bacterial isolates obtained from the positive stool specimens were screened for cytotoxicity with negative results, but we cannot rule out the possibility that anaerobic organisms or unusual intestinal microbes requiring special culture conditions were the cause of vacuolating activity.

In conclusion, the results of this study suggest that microorganisms of the gastrointestinal tract that produce a vacuolating toxin may be responsible for cases of childhood diarrhea whose etiology is currently considered unknown. Proper prospective case-control studies are needed to definitely assess the etiologic association between the new vacuolating cytotoxin and diarrhea, and efforts will be made to isolate and identify the organism responsible for the production of this \textit{Helicobacter}-like toxin in vivo by its ability to produce the same toxin in vitro.
Acknowledgments

The authors are grateful to all the participants in the Study Group on Childhood Diarrhea of the Italian Association for Clinical Microbiology for their cooperation. They are also indebted to J. L. Telford for providing the VacA antiserum and to Ildo Benedetti and Fabio Minelli for their excellent technical assistance.

References