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Evaluation of cytotoxic activity in fecal filtrates from patients with *Campylobacter jejuni* or *Campylobacter coli* enteritis

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1. SUMMARY

We sought to determine the prevalence of cytotoxic activity in fecal filtrates from persons with *C. jejuni* or *C. coli* enteritis. Stool specimens were collected from 20 persons with *C. jejuni* or *C. coli* enteritis, 20 persons with acute diarrheal illnesses of other causes, and 9 healthy, asymptomatic persons. Fecal filtrates were then incubated with Chinese hamster ovary (CHO) or HeLa cells. The fecal filtrate from 1 of the 20 (5%) persons with *Campylobacter* enteritis was cytotoxic for HeLa cells at a titer of 1:40, and 10 (50%) were cytotoxic for CHO cells at maximum titers of 1:20. Cytotoxic activity for CHO cells at a median titer of 1:20 was also present in 40% of the fecal filtrates from persons with diarrhea due to causes other than *Campylobacter* enteritis, and in 33% of filtrates from healthy, asymptomatic persons. The observed low level of cytotoxicity in fecal filtrates from all patient groups studied likely resulted from non-specific factors, unrelated to the pathogenesis of *Campylobacter* enteritis.

2. INTRODUCTION

Although *Campylobacter jejuni* and *C. coli* are important causes of diarrheal illness worldwide, the pathophysiologic mechanisms whereby these organisms cause human disease remain poorly understood [1]. Cytotoxic activity has been demonstrated in culture supernatants of *C. jejuni* and *C. coli* grown in vitro [2-7], but the in vivo relevance of these observations is not clear. We therefore sought to detect cytotoxic activity in fecal filtrates from persons with *Campylobacter* enteritis. Fecal filtrates from persons with diarrhea due to other causes and from asymptomatic healthy persons were tested as controls.

3. MATERIALS AND METHODS

3.1. Patients studied

The following three groups of persons were studied: (1) 20 consecutively diagnosed patients with *C. jejuni* or *C. coli* enteritis; (2) 20 patients

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with acute enteritis. from whose stool cultures *C. jejuni* or *C. coli* were not isolated: and (3) 9 asymptomatic healthy persons. For each *C. jejuni* or *C. coli*-infected patient identified (group 1),the next patient to submit a stool specimen to the Microbiology Laboratory for culture was selected as a control (group 2). Upon preliminary identification of *Campylobacter* sp. by darkfield microscopy, stool specimens were promptly frozen at $-20°C$. and were stored at $-70°C$ for 1 to 10 months.

3.2. Determination of cytotoxicity

Stool specimens were suspended in phosphate-buffered saline (pH 7.2) at a ratio of 1 : 3, centrifuged at $3000 \times g$ for 30 min. and the supernatants passaged through a 0.22 μm filter. HeLa and Chinese hamster ovary (CHO) cells were cultured in modified Eagle's medium (Flow, McLean, VA) or F-12 medium (Gibco, Grand Island, NY), respectively, containing 10% fetal bovine serum. Serial two-fold dilutions of fecal filtrates were prepared using tissue culture media as diluents, and were mixed in final concentrations of 1 : 10 to 1 : 320 with freshly trypsinized cells in concentrations of 2×10^4 cells/ml. Dilutions of fecal filtrates that caused rounding of greater than 50% of cells within 24 h were defined as showing a cytotoxic effect. All samples were tested at least in duplicate. The protein concentrations of fecal filtrates were determined using a BCA protein reagent kit (Pierce, Rockford, IL). As controls for detection of cytotoxic activity, culture supernatants were prepared from *Shigella dysenteriae* type 1, strain 60R, non-01 *Vibrio cholerae*, strain 84-74, and non-toxin-producing *Escherichia coli*, strain 84-169.

3.3. Statistical methods

Proportions were compared by using a one-tailed Fisher exact test. Numerical values were compared by using a two-tailed Student's *t*-test.

4. RESULTS

4.1. Microbiology

Of the 20 patients with *Campylobacter* enteritis,

11 were infected with *C. jejuni*. 7 were infected with *C. coli*. and 2 were infected with strains that were no longer available for speciation. Fever. fecal erythrocytes, and fecal leukocytes were present in 55%, 85%, and 90%, respectively, of the patients with *Campylobacter* enteritis. Of the 20 symptomatic control patients, one was infected with *Cryptosporidium*, and one had *C. difficile* toxin activity detected in stool. No pathogen was identified in the remaining 18 patients by the routine diagnostic techniques employed.

4.2. Cytotoxicity of fecal filtrates and bacterial culture supernatants

Culture supernatants from three bacterial strains were tested as controls: supernatant from *S. dysenteriae* type 1 was cytotoxic for HeLa cells at a titer of 1 : 10, 240, supernatant from non-01 *V. cholerae* was cytotoxic for CHO cells at a titer of 1 : 2560, and supernatant from a non-toxigenic *E. coli* was not cytotoxic for CHO or HeLa cells. The fecal filtrate from 1 of the 20 (5%) patients with *Campylobacter* enteritis was cytotoxic for HeLa cells at a titer of 1 : 40, and 10 (50%) of these filtrates were cytotoxic for CHO cells at titers of 1 : 10 or 1 : 20 (Table 1). Elongation of CHO cells in response to the fecal filtrates was not observed. The fecal filtrates from *C. jejuni* and *C. coli*-infected persons did not differ significantly in rates of cytotoxicity. The presence of fever, fecal

Table 1

Cytotoxic activity in fecal filtrates from *C. jejuni* or *C. coli*-infected persons, persons with acute diarrheal illnesses of other causes, and asymptomatic, healthy persons

Population studied	n	Number (%) of fecal filtrates with cytotoxic activity for the indicated cell line [a]	
		HeLa	CHO
Campylobacter enteritis	20	1 (5)	10 (50)
Diarrhea of other causes		2 (10)	8 (40)
Healthy, asymptomatic	9	0 (0)	3 (33)

[a] Cytotoxic activity for HeLa and Chinese hamster ovary (CHO) cells was defined as rounding of greater than 50% of cells in a dilution of 1 : 10 or higher.

Table 2

Relationship between protein concentration and cytotoxin activity of fecal filtrates

Population studied	Characteristics of fecal filtrates (mean ± SEM)					
	Filtrates toxic for CHO cells		Filtrates toxic for HeLa cells		Filtrates not toxic for CHO or HeLa cells	All filtrates
	Protein [a]	Ratio [b]	Protein	Ratio	Protein	Protein
Campylobacter enteritis (n = 20)	7.8 ± 0.966	1.6 ± 0.201	2.0 [d]	20 [d]	3.5 ± 0.567	6.0 ± 0.812
Diarrhea of other causes (n = 19) [c]	4.1 ± 0.674	3.0 ± 0.495 [c]	6.2 ± 1.80	2.9 ± 1.62	3.2 ± 0.617	3.7 ± 0.492
Healthy, asymptomatic (n = 9)	2.4 ± 0.406	5.5 ± 0.754	NA [e]	NA [e]	3.1 ± 0.683	2.9 ± 0.470

[a] The protein concentration of filtrates is expressed in mg/ml.
[b] For each fecal fitrate with cytotoxic activity, the reciprocal titer of cytotoxin activity was divided by the protein concentration to obtain a cytotoxin-protein ratio.
[c] A patient with known C. difficile enteritis was excluded from this analysis.
[d] single case.
[e] No cases identified.

erythrocytes, or fecal leukocytes in these patients was not associated with the cytotoxicity of fecal filtrates. The cytotoxic activity in fecal filtrates also was not related to the interval between the onset of symptoms and the collection of fecal specimens or to the duration of storage of fecal filtrates at −70°C. Fecal filtrates from 2 of the 20 (10%) symptomatic control patients were cytotoxic for HeLa cells at titers of 1:10 or 1:20, and 7 (35%) were cytotoxic for CHO cells at titers of 1:10 or 1:20 (Table 1). In addition, the fecal filtrate from a patient with C. difficile enteritis was cytotoxic for CHO cells at a titer of 1:160. The fecal filtrates from 3 of the 9 (33%) healthy asymptomatic persons were cytotoxic for CHO cells at titers of 1:10 or 1:20; none were cytotoxic for HeLa cells. Thus, the prevalence of cytotoxic activity for CHO or HeLa cells in fecal filtrates from C. jejuni or C. coli-infected persons was not significantly different from the prevalence in fecal filtrates from persons with diarrhea of other causes (P = 0.5), or fecal filtrates from healthy, asymptomatic persons (P = 0.25) (Table 1).

4.4. Ratios of cytotoxin titer to protein concentration

We hypothesized that fecal filtrates with significant cytotoxin activity might contain disproportionately high ratios of cytotoxin activity to protein concentration. Therefore, a cytotoxin titer-protein concentration ratio was determined for each fecal filtrate by dividing the reciprocal titer of cytotoxin activity by the protein concentration of the specimen. The highest ratio (177) was detected in the fecal filtrate from a patient with C. difficile enteritis, an illness known to be toxin-mediated. Of the filtrates that were cytotoxic for CHO cells, those from patients with Campylobacter enteritis had a lower mean titer-protein ratio than those from the other two populations studied (Table 2). Therefore, using this method of standardization, we were unable to demonstrate significantly increased cytotoxin activity in fecal filtrates from patients with Campylobacter enteritis compared to controls.

5. DISCUSSION

Among non-immune populations, infections with C. jejuni or C. coli produce febrile illnesses in which diarrheal stools frequently contain leukocytes and blood [8]. Cytotoxins have been proposed as virulence determinants whereby C. jejuni and C. coli may cause inflammation. Culture supernatants from 32% to 100% of C. jejuni and C. coli strains isolated from humans are cytotoxic for cultured CHO, HeLa, or Vero cells [2–7], but the

significance of low titers of cytotoxin produced in vitro remains unclear. One possibility is that the in vitro conditions used for growth of the bacteria are suboptimal for toxin production, and that in vivo growth conditions are necessary for maximal production of toxin. Cytotoxin activity has previously been demonstrated in the feces of patients infected with *C. difficile* or enterohemorrhagic strains of *E. coli* [9,10]. We hypothesized that if cytotoxin were important in the pathogenesis of *Campylobacter* enteritis, cytotoxin activity might similarly be detectable in the stools of *Campylobacter*-infected patients.

In a Malaysian study, low-titer cytotoxic activity for HeLa cells was detected in the fecal filtrates from 5 of 19 (26%) *C. jejuni*-infected persons; however, filtrates from control subjects were not examined [5]. In this study, we have also detected cytotoxic activity in fecal filtrates from patients with *Campylobacter* enteritis, but the prevalences and titers of cytotoxic activity in fecal filtrates from patients with *Campylobacter* enteritis, persons with diarrhea of other causes, and healthy asymptomatic persons were not significantly different.

The lack of high-titer cytotoxic activity in fecal filtrates from patients with *Campylobacter* enteritis is consistent with the low levels of cytotoxicity observed in culture supernatants of *C. jejuni* of *C. coli* after in vitro growth [2–7]. The lack of consistently detectable cytotoxin production by these *Campylobacter* species in vitro or in vivo, and the lack of a consistent neutralizing antibody response to cytotoxins in infected persons [2,4] suggest that *Campylobacter* enteritis is not solely a cytotoxin-mediated disease. Alternatively, current testing methods may be inadequate to demonstrate the activity of cytotoxins produced by *C. jejuni* and *C. coli*.

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