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Lack of Evidence of Enterotoxin Involvement in Pathogenesis of Campylobacter Diarrhea

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CHAPTER 20

Lack of Evidence of Enterotoxin Involvement in Pathogenesis of *Campylobacter* Diarrhea

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INTRODUCTION

Several *Campylobacter* species are now recognized as important pathogens causing human diarrheal disease (7, 8), but specific virulence mechanisms are not yet well defined. *Campylobacter jejuni* and *C. coli* infection may result in classical dysentery with fever and the presence of blood and leukocytes in the stools, suggestive of an invasive process or cytotoxin production (3, 27, 37, 38). *Campylobacter* diarrhea may also be associated with episodes of loose or watery stools and the absence of fever, consistent with the effect of a choleralike enterotoxin (14, 33). Inflammatory diarrhea is the most common presentation in developed countries, whereas watery diarrhea is more often observed in developing countries (3, 6, 14). However, *Campylobacter* isolates from patients in both developed and developing countries have been reported to be enterotoxigenic (15, 19, 24).

Although toxin production is only one aspect of the total virulence potential of enteric pathogens, for many organisms, including *Vibrio cholerae* and *Clostridium difficile*, toxins play the major role in the pathogenesis of diarrhea (2). *Campylobacter* strains have been reported to produce an enterotoxin similar in structure, receptor, mechanism of action, and antigenic characteristics to cholera toxin (CT) and the heat-labile toxin (LT) of *Escherichia coli* (15, 19, 22, 23, 26, 33).

However, the following observations have raised questions about the biological role of *Campylobacter* enterotoxin: (i) there is no homology with *E. coli* or *V. cholerae* genes encoding enterotoxin production (29); (ii) several investigators have been unable to find toxin production by *Campylobacter* strains (17, 37); (iii) enterotoxin production, when present, is at low levels (20 to 2,000 times lower than for LT or CT) (19, 20); (iv) dehydration in *Campylobacter* enteritis is not a common clinical feature, in contrast to the secretory diarrhea caused by enterotoxigenic *E. coli* or *V. cholerae* (21); and (v) the typical *Campylobacter* clinical illness in developed countries is an inflammatory enteritis, but inflammation is not characteristically a response to enterotoxins.

To verify the role of enterotoxin in the pathogenicity of *C. jejuni* and *C. coli* diarrhea, useful approaches are to ascertain in vivo expression of the toxin or to demonstrate the presence of a host response to the toxin. If present, these findings would indicate that toxinogenesis has occurred in vivo. Either of these findings, if present, can be used as an indicator of the biological significance of toxin production.

In this chapter, we review previous data and present new observations on (i) whether patients from a developed and a developing country with sporadic cases of *Campylobacter* enteritis developed serologic responses to CT, as well as to

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Campylobacter common cellular antigens; and (ii) whether the isolated *Campylobacter* strains were toxigenic.

SURVEY OF PATIENTS WITH DIARRHEA IN THE UNITED STATES AND THAILAND

Populations Studied

For this survey, we sought to include persons with acute diarrheal illnesses in both developed and developing countries. An important part of our study design was the inclusion of appropriate controls. First, we used serum samples obtained between August 1982 and October 1984 from patients in Denver with sporadic acute inflammatory diarrheal illnesses (31). Entry criteria included an acute inflammatory enteritis, defined as abdominal pain, watery or loose stools, and either the presence of fecal leukocytes or a positive stool culture for a bacterial enteric pathogen, if fecal leukocytes were not present. Patients were classified into three groups as follows: 24 patients had confirmed *C. jejuni* or *C. coli* enteritis, 13 patients had enteritis due to other identified enteric pathogens, and 27 patients had enteritis of unknown cause. Serum samples also were obtained from July to December 1987 from children in Bangkok, Thailand, with acute diarrheal illnesses. Entry criteria included acute enteritis, defined as abdominal pain, watery or loose stools, and the isolation of *Campylobacter* or *Shigella* species (as a control group) as the sole pathogen. Children were classified into two groups as follows: 29 children (mean age, 13.5 months) had confirmed *C. jejuni* or *C. coli* enteritis, and 26 (mean age, 23.1 months) had *Shigella* enteritis. For both the U.S. and Thai patients, serum specimens were obtained during the initial visit to the outpatient department when the acute diarrheal illness was diagnosed (acute phase). For the Thai children, a second sample was obtained 14 days later. For all cases a convalescent-phase serum sample was obtained approximately 4 weeks after the onset of symptoms. Acute- and convalescent-phase serum samples collected from 12 Peace Corps volunteers infected with LT-producing *E. coli* (36), 12 single serum samples from healthy adults in Denver, and 100 serum samples from healthy children in Bangkok, Thailand, were used as controls in the assays for antibodies to enterotoxin. Serum samples had been stored at −20°C prior to testing.

Serologic Response to Enterotoxin

We first asked whether the infected persons and controls showed seroconversion to enterotoxin antigens. The ganglioside GM_1 enzyme-linked immunosorbent assay (ELISA) to detect anti-enterotoxin antibodies was performed essentially as described previously (31). In brief, polystyrene microtiter plates were coated with 0.15 μg of ganglioside GM_1, after which remaining binding sites were blocked with 0.1% gelatin in phosphate-buffered saline (PBS), with thimerosal and Tween 20 (PBS-T-T). The wells were then incubated with 0.1 ml of purified CT, the B subunit of CT, or LT (provided by R. A. Finkelstein) (1.0 μg/ml) in PBS-T-T containing 0.5% bovine gammaglobulin and 0.1% gelatin (PGG). After the plates were washed, a 1:100 dilution of the serum samples in PGG was added to duplicate wells and incubated. Peroxidase-conjugated goat antibody to human immunoglobulin G (IgG) diluted 1:2,000 was used, and

TABLE 1. Serum IgG response to CT in healthy controls and in patients in the United States with acute diarrhea[a]

Group	No. of persons studied	Mean ± SEM OD of serum in IgG ELISA	
		Acute phase	Convalescent phase
Healthy adults	12	0.12 ± 0.04	NA[b]
Infected with *E. coli* LT	12	0.14 ± 0.02	0.85 ± 0.13[c]
Infected with *Campylobacter* spp.	24	0.07 ± 0.10	0.07 ± 0.01
Not infected with *Campylobacter* spp.	13	0.12 ± 0.03	0.19 ± 0.09
No pathogen identified	27	0.12 ± 0.03	0.13 ± 0.03

[a] Adapted from reference 31.
[b] NA, not applicable.
[c] $P < 0.01$ by the paired t test.

antibody binding was measured as previously described (4).

Results of the ELISA determinations are presented in Table 1. These results were nearly identical to the results of the studies with the B subunit of CT or LT (data not shown). Acute-phase serum from all patients showed low optical density (OD) values. The positive control group (12 Peace Corps workers), known to be infected with *E. coli* LT, all seroconverted to the CT; OD values were significantly elevated in their convalescent-phase serum ($P < 0.01$). In contrast, none of the 24 U.S. patients with culture-confirmed *Campylobacter* infection showed seroconversion to CT. Identical results were noted in serum samples from 39 of the 40 other patients with diarrheal illnesses who were not infected with *Campylobacter* strains. The only seroconversion occurred in a *Shigella*-infected woman who had developed diarrhea in Mexico and who was probably coinfected with an LT-producing *E. coli* strain.

We then investigated the serologic response to enterotoxin in children with diarrhea and healthy control children in Thailand (Table 2). Of interest, the mean OD values of the acute-phase serum in the three groups of Thai children all were significantly higher than the value for the Peace Corps workers. The OD values of the convalescent-phase (28-day) serum samples from the *Shigella*-infected children were no higher than those of the acute-phase serum samples. None of the 23 *Campylobacter*-infected children seroconverted to CT, even though 12 (41.3%) children had watery diarrhea without fecal leukocytes or blood. Serum samples obtained 14 days after the onset of infection had OD values that were not significantly different from those of the acute- and convalescent (28-day)-phase serum samples.

Antibody Response to *C. jejuni* Surface Protein Antigens

The lack of serologic response to CT prompted us to investigate whether the patients from whom *Campylobacter* species were isolated had acute infection. To do this, we determined whether they showed serological responses to known cellular antigens. The antigen used in the *C. jejuni* surface protein ELISA was prepared as previously described (4). In brief, cells of three *C. jejuni* strains were extracted in glycine buffer (pH 2.2), and the supernatant was dialyzed against water, pooled, and used as the antigen at a final protein concentration of 5.0 μg/ml in 0.05 M carbonate buffer (pH 9.6). A 0.1-ml sample of this solution was added to each well of a flat-bottomed Immulon 2 plate. The screening-serum dilutions were 1:200 for IgM, 1:100 for IgG, and 1:50 for IgA determinations. Peroxidase conjugates of goat antibody to human IgG, IgA, and IgM were diluted 1:1,000. Assays were done in duplicate, results were read blindly, and reference sera were included in each ELISA run. For each patient, the OD value for each Ig class in the acute-phase sample was compared with that in the corresponding convalescent-phase sample. We defined seroconversion as occurring when a convalescent-phase serum sample showed a ≥100% increase from the OD value of the acute-phase serum sample. A threshold of 0.050 in the OD for the acute-phase sample was selected to eliminate low-level reactions that might meet the above criteria but were within the day-to-day variability of the assay. We investigated seroconversion to *C. jejuni* cellular antigens in children with diarrhea in Thailand (Table 3). Of the 29 children with *Campylobacter* infection, 22 (75.8%) showed seroconversion in at least one Ig class by day 14, and 19 (65.4%)

TABLE 2. Serum Ig response to CT in healthy controls and in patients with acute diarrhea in Thailand

Group	No. of persons studied	Mean ± SEM OD in IgG ELISA	
		Acute phase	Convalescent phase
Asymptomatic children	100	0.30 ± 0.05	NA[a]
Shigella-infected children	13	0.28 ± 0.07	0.25 ± 0.10
Campylobacter-infected children	23	0.34 ± 0.10	0.30 ± 0.05
Infected with *E. coli* LT[b]	12	0.11 ± 0.04	0.98 ± 0.10[c]

[a] NA, not applicable.
[b] This is the same group as shown in Table 1. Serum samples were reexamined simultaneously with the other serum samples shown in this table to provide a positive control group.
[c] $P = 0.006$ by the paired t test.

TABLE 3. Seroconversion to *C. jejuni* cell surface antigens in patients with sporadic diarrhea in Denver and Bangkok

Seroconversion by Ig class	No. of patients showing seroconversion					
	Campylobacter infected			Non-*Campylobacter* infected		
	Day 14, Bangkok (n = 29)	Day 28, Bangkok (n = 29)	Day 28, Denver (n = 24)	Day 14, Bangkok (n = 26)	Day 28, Bangkok (n = 26)	Day 28, Denver (n = 40)
IgA only	1	0	0	2	1	1
IgG only	2	4	4	0	0	4
IgM only	3	3	0	1	3	0
IgA and IgG	1	0	0	0	2	0
IgG and IgM	0	0	6	0	0	0
All three classes	9	10	10	0	1	2
Any class	22	19	21	3	7	7
None of the classes	7	10	3	23	19	33

showed seroconversion by day 28. Of 26 children who had acute *Shigella* diarrhea, only 3 (11.5%) seroconverted in any Ig class by day 14, but 7 (26.9%) seroconverted by day 28. This may have been due to intercurrent *Campylobacter* infection. In contrast to the *Campylobacter*-infected patients, only one (3.8%) patient showed seroconversion in all three Ig classes by day 28. Among the 24 U.S. patients with culture-confirmed *Campylobacter* infection, 21 (87.5%) seroconverted to the cellular antigens in at least one Ig class, 20 (83.3%) showed IgG seroconversion, 17 (70.8%) showed seroconversion in more than one Ig class, and 10 (42%) showed seroconversion in all three Ig classes (Table 3). For persons who had acute diarrhea due to enteropathogens other than *Campylobacter* species, 7 (17.5%) of 40 paired serum samples showed seroconversion to *C. jejuni* antigens in at least one Ig class, but only 2 (5%) patients with acute diarrhea from whom no enteropathogen was isolated showed seroconversion in all three Ig classes.

When the serologic results were analyzed on the basis of clinical information, no correlations were found for any group between the ELISA results and the presence of blood in feces, a history of fever, the presence of fecal leukocytes, or watery diarrhea. Moreover, no relationship was found between the ELISA results and the presence of a particular *Campylobacter* species.

Height of Antibody Response to *Campylobacter* Cellular Antigens

For the Denver population, for the acute-phase serum samples the distribution of levels of IgG and IgM *C. jejuni* antibody was not significantly different between patients with and without *Campylobacter* infection (Table 4); however, the IgA levels were higher in the patients with *Campylobacter* infection (P = 0.03), suggesting that the specific IgA level already had risen. Levels of IgA, IgG, and IgM to *C. jejuni* antigens among the *Campylobacter*-infected group were significantly higher in the convalescent-phase serum samples than in the acute-phase samples and were higher than in convalescent-phase serum samples from the non-*Campylobacter*-infected group.

Similar analysis for the Thai children showed that IgA and IgM antibody levels were not significantly different in the acute-phase serum samples taken from children with or without *Campylobacter* infection (Table 5). However, levels of IgG to *C. jejuni* antigens were higher in the *Shigella*-infected children than in the *Campylobacter*-infected group (P = 0.04). By 14 days, levels of serum IgA and IgM to *C. jejuni* antigens in the *Campylobacter*-infected group were significantly higher than in the *Shigella*-infected group. By 28 days, no differences in the levels of IgA and IgG were observed between children with or without culture-confirmed *Campylobacter* infection (Table 5), but IgM levels were significantly higher in the children with *Campylobacter* infection. OD values in the IgG and IgM assays of the *Campylobacter*-infected group were significantly higher in both of the postinfection samples than in the acute-phase samples (Table 5). However, IgA OD values in the 28-day samples were similar to the acute-phase values, indicating that the IgA immune response in those children was transient, whereas IgG values remained elevated.

TABLE 4. *C. jejuni* ELISA determination for serum samples of patients with acute diarrheal illnesses in Denver[a]

Group (no. of patients)	Mean OD ± SEM for: Acute-phase serum	Convalescent-phase serum (21–32 days)	*P*[b]
Campylobacter enteritis (24)			
IgA	0.29 ± 0.08	0.49 ± 0.10	0.024
IgG	0.34 ± 0.06	1.62 ± 0.31	0.0003
IgM	0.71 ± 0.15	2.02 ± 0.33	0.0005
No *Campylobacter* enteritis (40)			
IgA	0.16 ± 0.03	0.17 ± 0.03	NS[c]
IgG	0.33 ± 0.04	0.40 ± 0.05	NS
IgM	0.60 ± 0.07	0.85 ± 0.18	NS

[a] Adapted from reference 31.
[b] By paired *t* test.
[c] NS, not significant ($P > 0.05$).

Enterotoxin Production and Gene Probing in *Campylobacter* Isolates

Twenty-two *Campylobacter* isolates from the United States were incubated overnight at 37°C in an atmosphere of 85% N_2, 10% CO_2, and 5% O_2 in Casamino Acids-yeast extract (CAYE) broth supplemented with 1.0 μg of ferric chloride per ml, with shaking (100 rpm) in a water bath shaker, as previously described (31). Culture supernatants were examined in a GM_1 ganglioside ELISA to detect CT-like toxin (Table 6). CT at 25 ng/ml and uninoculated growth media were used as controls. All samples were added in 100-μl amounts in triplicate to a 96-well microtiter plate precoated with 1.5 μg of GM_1 ganglioside per ml, as previously described (34). After overnight incubation at room temperature, 0.1 ml of goat antibody to LT (provided by R. A. Finkelstein) diluted 1:500 was added to each well, and the plate was incubated overnight at 4°C. Peroxidase-conjugated goat antibody to human IgG was added and assayed as previously described (4). All the controls showed the expected reactions, but none of the *Campylobacter* isolates synthesized products recognized by antibodies to CT at any level approaching our threshold for positivity.

Because the lack of recognition of CT-like toxin by antibodies to CT in the culture supernatant did not rule out the possibility of CT-like toxin production in *Campylobacter* strains, we

TABLE 5. *C. jejuni* ELISA determination for serum samples of patients with sporadic diarrhea in Thailand

Group (no. of patients)	Mean OD ± SEM for: Acute-phase serum	Convalescent-phase serum (14 days)	Convalescent-phase serum (28 days)
Campylobacter enteritis (29)			
IgA	0.80 ± 0.29	1.14 ± 0.18	0.70 ± 0.11[a]
IgG	0.46 ± 0.06[b]	0.80 ± 0.10	0.81 ± 0.10
IgM	0.48 ± 0.12	1.40 ± 0.31[c]	0.77 ± 0.16
Shigella enteritis (26)			
IgA	0.76 ± 0.10	0.80 ± 0.15	0.69 ± 0.11
IgG	0.68 ± 0.11	0.67 ± 0.11	0.66 ± 0.10
IgM	0.31 ± 0.04	0.34 ± 0.06	0.45 ± 0.11

[a] Compared with day 14 ($P = 0.004$).
[b] Compared with day 14 ($P = 0.0002$) and day 28 ($P = 0.0001$).
[c] Compared with acute phase ($P = 0.005$) and day 28 ($P = 0.0005$).

TABLE 6. Production of *Campylobacter* cholera-like toxin by 22 *Campylobacter* isolates from patients with inflammatory diarrhea in Denver

Strain designation	ELISA antibodies to CT[a]	Enterotoxin titer in CHO cell assay
85-308	0.07	<1:2
85-309	0.03	<1:2
85-314	0.08	<1:2
85-316	0.08	<1:2
85-318	0.18	<1:2
85-322	0.24	<1:2
85-323	0.11	<1:2
85-328	0.10	<1:2
85-329	0.14	<1:2
85-331	0.12	<1:2
85-334	0.04	<1:2
85-337	0.12	<1:2
85-343	0.14	<1:2
85-352	0.02	<1:2
85-356	0.06	<1:2
85-360	0.17	<1:2
85-361	0.02	<1:2
85-365	0.10	<1:2
85-367	0.06	<1:2
85-370	0.12	<1:2
85-371	0.10	<1:2
85-374	0.02	<1:2
E. coli H 10407[b]	1.97	>1:256
Broth alone[c]	0	<1:2

[a] The ELISA results are the mean of triplicate determinations from at least two experiments. Results represent OD values. An enterotoxin titer of ≥0.3 is considered positive.
[b] *E. coli* H 10407 is a control strain known to be a high-level producer of LT.
[c] Broth represents uninoculated growth medium.

examined the cytotonic activity present in culture supernatant for these 22 *Campylobacter* strains in the Chinese hamster ovary (CHO) cell assay, performed by the technique described by Guerrant et al. (16). *Campylobacter* strains were grown in various media including brucella broth (1), brucella broth with the addition of amino acids (15), and CAYE (11), and the supernatants were harvested. Culture supernatants of *V. cholerae*, enterotoxigenic *E. coli*, and an *E. coli* strain known not to produce LT were used as controls. Criteria used to define a positive supernatant were as previously described (31). The positive control showed clear cytotonic activity (titer, >256) but none of the 22 *Campylobacter* culture supernatants tested showed any activity in the CHO cell assay (Table 6).

The 29 *Campylobacter* strains from children in Thailand were tested for the presence of the *E. coli* LT gene by using a hybridization assay with the LT DNA probe as previously described (13). A cloned DNA fragment described by Dallas (12) and an oligonucleotide described by Murray et al. (28) were both used. *E. coli* strains known to be LT producers or nonproducers were used as controls. Examination of the 29 *Campylobacter* isolates from Thailand under low-stringency conditions showed that none of the *Campylobacter* strains apparently possessed the gene encoding an LT- or CT-like toxin (Table 7).

SUMMARY

In the United States and other developed countries most cases of *Campylobacter* enteritis occur in the young adult population (18 to 45 years of age) and most cases are characterized by an inflammatory process with fever and fecal leukocytes (3, 6, 16). In contrast, in developing countries most cases of *Campylobacter* enteritis occur during the first 2 years of life (5) and symptomatic infection is rare in adults. In developing countries the spectrum of illness may include severe inflammatory illness, a mild secretory diarrhea, or an asymptomatic carrier state (5, 9, 14). Two explanations may be offered for the differences between the clinical and epidemiologic features of *Campylobacter* enteritis in developed and developing countries. First, *Campylobacter* strains in the United States and other developed countries may differ in virulence characteristics. Second, *Campylobacter* infection is far more common among children in developing countries than in children in developed countries, and repeated exposure to the organism may produce immunity.

We first examined whether *Campylobacter*-infected hosts from developed and developing countries seroconverted to CT, which is immunologically related to the *Campylobacter* enterotoxin (23, 26, 32). None of the 47 *Campylobacter*-infected patients studied showed seroconversion to CT. In contrast, Ruiz-Palacios et al. (33) in Mexico and Martin et al. (25) in the Central African Republic frequently found seroconversion to CT among children with *Campylobacter* infection. One explanation for these discrepant results is that *Campylobacter* strains in the United States may not be enterotoxigenic whereas those from developing countries are. However, in this study 23 (48.9%) of the 47 *Campylobacter*-infected patients were from Thailand, a developing country, and 25.5% of

TABLE 7. Hybridization assay with LT probe of *Campylobacter* strains isolated from children with diarrhea in Bangkok[a]

Bacterial type	No. of strains tested	No. of strains showing hybridization
E. coli LT+[b]	2	2
E. coli K-12 LT-	1	0
C. jejuni	25	0
Other *Campylobacter* species	4	0

[a] Hybridizations were conducted under low-stringency conditions as described previously (13).
[b] Control strain carrying LT-encoding plasmids.

these children did not have leukocytes or blood in their stools. Similar clinical characteristics have been reported in the Mexican and Central African Republic studies (25, 33). Coincident infection with LT-producing *E. coli* strains is common among children in developing countries (19, 36). Immunologic memory from prior LT-producing *E. coli* infections may have primed the response to *Campylobacter* enterotoxin in the other studies. In our study, acute-phase serum OD values to CT were two- to threefold higher in Thai children than in persons from developed countries, further indicating the high-level exposure of this group to enterotoxins.

In contrast, most of the *Campylobacter*-infected patients seroconverted to *C. jejuni* cell surface antigens. Overall, only 3 of 66 non-*Campylobacter*-infected persons seroconverted in all three Ig classes. However, two of those "non-*Campylobacter*-infected" persons probably had false-negative cultures, as occurs when enrichment or more sensitive culture methods are not used (30, 35). Another piece of evidence that those two patients may have been infected with *Campylobacter* species is that they also showed clear seroconversion to homologous and heterologous *Campylobacter* lipopolysaccharide, as described by Blaser and Perez-Perez (this volume). Among the *Campylobacter*-infected Thai children, the increase in IgA and IgM levels was transient, in contrast to a more prolonged response in the U.S. patients. All these results suggested that it is unlikely that *Campylobacter* strains isolated in developed countries differ in virulence from strains isolated in developing countries. Further evidence is the fact that travelers from developed to developing countries develop acute illness, as seen in developed countries (36).

Despite several reports that *Campylobacter* strains produce a CT-like toxin (22, 26, 33), we were unable to demonstrate production of toxin in the *Campylobacter* strains examined in this study by either ELISA or cell culture assay (16, 34). The lack of significant toxin production in the GM_1-binding assay was demonstrated by using three different antibody preparations that cross-react with *Campylobacter* CT-like toxin (23, 26). A parallel lack of enterotoxic activity was observed in the cell culture assay, despite our testing of several growth conditions and media to improve enterotoxin production, as suggested by other investigators (15, 18, 19, 26).

The discrepancies reported by different laboratories regarding the presence (10, 26, 33) or absence (17, 29, 31) of enterotoxin in *Campylobacter* strains may be related to the growth of the organism under in vitro conditions that are not optimal for toxin expression. Therefore, we investigated whether the 29 *Campylobacter* strains isolated in Thailand hybridized with the gene encoding *E. coli* LT or with an oligonucleotide probe for *E. coli* LT. The lack of hybridization at low stringency is consistent with the negative results from our other assays. In sum, we have found no evidence that supports a role for enterotoxin production in the pathogenesis of *Campylobacter* diarrhea in those areas of developed and developing countries.

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