MAJOR ARTICLE

Adaptive and Inflammatory Immune Responses in Patients Infected with Strains of *Vibrio parahaemolyticus*

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In patients with diarrhea caused by *Vibrio parahaemolyticus,* antibody-secreting cell responses to thermostable direct hemolysin (TDH), lipopolysaccharide (LPS), and whole-cell bacteria were seen. TDH- and LPS-specific responses were seen in serum samples, and immunoglobulin A antibody responses were observed in stool. Levels of C-reactive protein and nitric oxide metabolites increased in the systemic circulation at the onset of illness. Tumor necrosis factor–α and lactoferrin levels were high during the acute stage in mucosal secretions and in plasma, whereas interleukin-1β levels were high only in mucosal secretions. Duodenal and rectal biopsy specimens obtained at the onset of illness showed an acute inflammatory response. The lamina propria showed edema, congestion of blood vessels, and hemorrhage, with an increase in levels of polymorphonuclear neutrophils and macrophages. Strains belonging to different serotypes exhibited varying resistance to killing by serum; the O8:K21 strain was most sensitive. Infection with *V. parahaemolyticus* results in B cell responses and an acute inflammatory response that is self-limiting.

Vibrio parahaemolyticus strains are becoming an increasing cause of concern as causative agents of acute gastroenteritis in a number of locations, including India [1], Bangladesh [2, 3], Japan [4], Taiwan [5], and the United States [6]. The serogroups O3:K6, O4:K68, and O1:KUT are believed to have acquired pandemic potential [2, 7, 8]. *V. parahaemolyticus* is responsible mainly for gastroenteritis, although wound infections and septicemia also may be caused by this organism. *V. parahaemolyticus* has been known to cause diarrhea in travelers and army personnel [9], and it also has been a cause of concern among immunocompromised individuals, including those with leukemia, liver disease, and human immunodeficiency virus infection and AIDS [10, 11]. Symptoms of *V. parahaemolyticus* infection usually include acute, self-limiting diarrhea (caused by consumption of contaminated food) and, in some cases, septicemia. Only strains of *V. parahaemolyticus* with the *tdh* gene are capable of causing gastroenteritis [12].

A number of serogroups among the >72 recognized O:K serotypes of *V. parahaemolyticus* can cause the disease. The most important virulence factor identified to date is thermostable direct hemolysin (TDH) [13]. TDH-related hemolysin also has been identified as a virulence factor [13, 14]. Enteroinvasiveness of the bacteria has been reported in a rabbit model, in which the organism invaded, colonized, and produced inflamma-

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Informed consent was obtained from patients and control subjects. This study was approved by the ethical review committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B; Dhaka).

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tion in the small intestine [15]. However, very little is known about immunological and inflammatory responses in patients who have natural infection with the pathogen. In the present study, we have attempted, to our knowledge for the first time, to study the mucosal and systemic immune responses in patients infected with *V. parahaemolyticus* and the involvement of different inflammatory components. To examine antigen-specific mucosal immune responses, we studied the circulating antibody-secreting cell (ASC) responses, which can be considered a proxy measure of the mucosal immune response in the gut [16], and we used stool extract preparations to investigate intestinal antibody responses [17]. In addition, we studied systemic immune responses to TDH and lipopolysaccharide (LPS) in serum. Inflammatory responses were evaluated by investigating the levels of the bactericidal protein lactoferrin, oxidant-mediated defense factor (nitric oxide; $NO^{\cdot}$), C-reactive protein (CRP), and the cytokines tumor necrosis factor (TNF)–α and interleukin (IL)–1β. Responses were studied during the acute stage of the disease (after the onset of illness) and at different phases during convalescence. Duodenal and rectal biopsy specimens from patients were also examined to discern changes in gross histopathology and the contribution of the inflammatory cells to the pathogenesis of the disease. Responses in patients were compared with those seen in healthy volunteers for all study parameters.

SUBJECT, MATERIALS, AND METHODS

Study subjects and clinical evaluation. Twenty-six adults and 2 children infected with *V. parahaemolyticus* were enrolled in the study. They were recruited from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) hospital in Dhaka between July 2000 and October 2001. The ICDDR,B has a 2% surveillance system, in which stool samples from every 50th patient attending the hospital are screened for enteric pathogens. During the study period, *V. parahaemolyticus* was detected in samples from 30 patients. We see >100,000 diarrhoeal patients at our hospital every year, and, extrapolating from these data, it can be assumed that many of those patients are infected with *V. parahaemolyticus*. For our study, we screened stool samples from patients who were admitted to the hospital and who may or may not have been included in the 2% surveillance system. Patients who had characteristic "meat wash" (reddish and watery) or rice-water diarrhea and met our study criteria were evaluated. Only those whose stool samples tested positive for *V. parahaemolyticus* and who consented to participate in the study were enrolled. Patients whose stool samples tested positive (by methods described elsewhere [18]) for other common bacterial pathogens were excluded. The degree of dehydration in the patients ("severe" to "some") was assessed by a physician, according to the Denver system [19]. In addition, 20 men and 10 women (age range, 18–45 years) with no history of diarrhea during the previous 3 months, whose stool samples tested negative for enteric pathogens, and who were of socioeconomic backgrounds similar to those of the patients were randomly recruited from in and around Dhaka city. Stool and plasma samples collected from 10 patients with *V. cholerae* O1 [20] and 10 with *S. dysenteriae* type 1 infection [21] from July 1999 through September 2001 were also tested for inflammatory responses, for comparison with those from patients infected with *V. parahaemolyticus*.

Bacteriological examination of stool samples and stool occult blood testing. Watery stools with a characteristic "meat wash" or rice-water appearance were cultured on thiosulphate citrate bile-salt sucrose (TCBS) agar (Eiken). Patients with stool samples from which greenish mucoid colonies were isolated were recruited for the study. Suspected *V. parahaemolyticus* colonies were presumptively identified by a battery of biochemical tests [8] and serotyped by the slide agglutination test, using commercial antisera against the O and K antigens (Denka Seiken). Stools were cultured to detect other enteric pathogens, including *V. cholerae* [22], enterotoxigenic *Escherichia coli* [23], and *Salmonella, Shigella,* and *Campylobacter* species [18] and were examined by direct microscopy for cyst and vegetative forms of parasites and ova of helminths. The stools of the healthy control subjects were similarly screened. Stool occult blood was assayed using the modified guaiac acid procedure [24].

Detection of virulence genes with polymerase chain reaction (PCR). PCR assays were performed to test for the presence of the species-specific *toxR* gene, as well as the 2 virulence genes *tdh* and *trh* [2]. A group-specific PCR was carried out to ascertain whether a particular isolate belonged to the pandemic genotype. PCR for the filamentous phage f237 (open-reading frame [ORF] 8), which is another marker for the pandemic genotype [8], was also carried out, using procedures described elsewhere [2].

Sample collection. Samples of venous blood and stool were collected from patients after the patients had been rehydrated. This occurred on the second day of hospitalization (day 2), ~2 days after the onset of diarrhea (day 0); for the purposes of the study, this was considered to be the acute stage of the illness. Samples were also collected 5, 12, and 28 days later, during convalescence (i.e., days 7, 14, and 30 after the onset of the disease). Samples collected on the day of admission (day 1) were used to assess changes in stool from patients. For control specimens, a single sample of blood and another of stool were collected from healthy subjects. Peripheral blood mononuclear cells (PBMCs) were isolated from blood collected in heparinized vials (Vacutainer System; Becton Dickinson) by gradient centrifugation on Ficoll-Isopaque (Pharmacia). Plasma collected from the top of the Ficoll-Isopaque gradient was stored in aliquots at −20°C until ELISAs were performed. Plasma and

serum separated from blood collected in EDTA-coated sterile vials and in vials that did not contain any additive, respectively, were aliquoted and stored at −70°C for sensitive assays. Stool samples obtained from patients and healthy control subjects on different study days were frozen immediately at −70°C. Stool extracts were prepared by mixing stool (1 g of stool in 4 mL of buffer) with PBS containing EDTA (0.05 *M*), protease inhibitors, soybean trypsin inhibitor (100 μg/mL), and phenylmethylsufonyl fluoride (10 m*M*) [22]. One milliliter of stool extract was equal to 0.25 g of stool. Stool extracts were frozen in aliquots at −70°C.

Biopsy specimens were collected from adult patients from the second part of the duodenum by a standard endoscopic procedure, using local anesthetic and biopsy forceps (Megabite endoscopic forceps; Microvasive). The rectal biopsy samples were taken 10–12 cm from the anus, using a sigmoidoscope (Olympus).

Antigens. *V. parahaemolyticus* LPS was extracted by the hot phenol/water method [25] and purified as described elsewhere [26]. Whole-cell (WC) bacterial antigens were prepared using live *V. parahaemolyticus* strains grown on TCBS agar, using a procedure described elsewhere [22]. Clinical *V. parahaemolyticus* strains of serotypes in serogroups O1 (strain 003, serotype O1:K25), O3 (strain 001, serotype O3:K6), O4 (strain 029, serotype O4:K55), O5 (strain 008, serotype O5:KUT), and O8 (strain 024, serotype O8:K21) were used to prepare antigens for the immunological assays. TDH was purified from *V. parahaemolyticus* 4750, a clinical strain isolated in Japan, by a method described elsewhere [27].

Detection of ASCs in blood. The Ficoll-Isopaque–separated mononuclear cells (MNCs) were assayed for TDH-, LPS-, and WC antigen–specific ASCs by a 2-color ELISPOT technique [16]. Individual wells of poly L-lysine (Sigma)–treated, nitrocellulose-bottomed 96-well plates (Millititer HA; Millipore) were coated with 0.1 mL of WC antigen (5×10^{10} cfu/mL) and LPS (25 μg/mL), layered with 0.5% glutaraldehyde, and stored at −20°C until use [22]. Wells were coated with purified TDH (0.1 mL; 5 μg/mL) and incubated overnight at 4°C. Samples from patients infected with O1 ($n = 8$) and O3 ($n = 12$) *V. parahaemolyticus* were tested with WC and LPS antigens from both homologous strains (i.e., testing of samples from O1- and O3-infected patients with antigens from O1 and O3 strains, respectively) and heterologous strains (i.e., testing of O1- and O3-infected patients with O3 and O1 strains, respectively), as well as TDH. Numbers of cells secreting antibodies of the IgA, IgM, and IgG isotypes were determined. ASC responses to all antigens were studied using PBMCs collected from healthy control subjects at a single time point.

Detection of TDH- and homologous LPS-specific antibodies in serum and stool. Serum samples collected from patients at the acute and convalescent stages of infection were tested at a dilution of 1:200 with homologous LPS (2 μg/mL) and TDH (1.0 μg/mL) by ELISA, using procedures described elsewhere [17]. Optical density (OD) was measured kinetically at 450 nm for 5 min and expressed as mOD per minute [28]. Stool extracts prepared from samples from patients and healthy control subjects were tested for response to homologous LPS and TDH, and responses were measured kinetically and expressed as the specific titer per microgram of total IgA.

Detection of soluble mediators and cytokines. For determination of CRP concentrations in serum (in mg/dL), a fluorescence polarization immunoassay procedure was carried out using the Abbott TDx analyzer (Abbot Laboratories). The lactoferrin content of plasma and stool was measured using a commercial EIA kit (Oxis International), and units were expressed as nanograms per milliliter of total protein in stool extracts or nanograms per milliliter of plasma. The final products of $NO^{\cdot}$ in vivo are nitrate (NO_3^-) and nitrite (NO_2^-). The total nitrate and nitrite (designated "NO_3^-/ NO_2^-") content of plasma was measured photometrically using a commercial kit (Cayman Chemical) [21]. NO_2^-/NO_3^- was expressed as a micromolar concentration in plasma. ELISA kits with amplified-sensitivity reagents were used to assess the levels of IL-1β and TNF-α (reagents from Pharmingen and Genzyme, respectively) in plasma and stool extracts.

Resistance to killing in* V. parahaemolyticus *strains isolated from patients. To test the susceptibility of *V. parahaemolyticus* to human serum, culturing for 2 h of bacteria grown in Luria broth containing 3% sodium chloride was used. Pooled human serum prepared from 10 healthy volunteers was used for the serum bactericidal assays. Bacteria were added to physiological saline to a concentration of $\sim 1 \times 10^5$ cfu/mL, this solution was added to serum at a level of 10%–95%, and tests for susceptibility to killing were carried out, using procedures described elsewhere [29]. The viability of bacteria was determined immediately before and after incubation for 2 h. Control aliquots were mixed with serum that had been heated to 56°C for 30 min. Neat and diluted samples in physiological saline were plated on TCBS agar in triplicate and incubated overnight. Each experiment was repeated at least 3 times, and the bacteria count was expressed as the mean of 3 determinations. Killing of bacteria was calculated as the percentage of viable bacteria, in comparison with control vials containing heat-inactivated serum.

Histopathologic examination. Mucosal punch biopsy specimens were collected from adult male patients from the duodenum and from the rectum during the acute stage (day 2; $n = 17$), early convalescence (day 7; $n = 17$), and late convalescence (day 30; $n = 10$). Duodenal and rectal biopsy specimens obtained from 10 healthy adult males were treated as control samples. Formalin-fixed, paraffin-embedded tissues from the adult patients were sectioned at 3 μm and stained

with hematoxylin-eosin. An enzyme-histochemical staining procedure was used to detect and quantitate mast cells and neutrophils by measuring chloroacetate esterase activity using pararosanilin dye [30]. Coded sections from each specimen were examined by a histopathologist who was unaware of the culture report and clinical profile of the patient. For evaluation of the biopsy specimens, histopathological features described elsewhere [31] were used.

Statistical analysis. The Wilcoxon signed-rank test and the Mann-Whitney *U* test were used, where applicable, for statistical analyses. $P \leq .05$ was considered to be statistically significant. Analyses were carried out using SigmaStat (Jandel Scientific). Data were expressed as median value and interquartile range or as geometric mean (GM) and range (GM − SEM to GM + SEM).

RESULTS

Clinical history of study subjects. Of the patients recruited in the study, 26 were adults (median age, 29 years), and only 2 were children (ages 10 and 11 years). The ratio of men to women was 25:3. Of the 28 *V. parahaemolyticus*–infected patients, 17% suffered from severe dehydration and 45% from moderate dehydration. No signs of dehydration were recorded for the remaining patients. Rehydration with intravenous fluid was needed by 59% of patients (median fluid requirement, 2 L/patient), and all patients received oral rehydration therapy. The median stool frequency after admission was 18 stools/day, and the median frequency of vomiting was 6 episodes/day at the initial stage (day 1). The patients were treated with doxycycline (75%), ciprofloxacin (3%), or erythromycin (7%) or were not given any antibiotic therapy (15%).

Of the 28 patients recruited in the study, 42% were infected with strains of the O3:K6 serotype and 21% with strains of the O1:K25 serotype, both of which belonged to the pandemic genotype. A mixture of other, nonpandemic serotypes (O1:K56, O8:K21, O5:KUT, O3:K29, O4:K37, and O4:K55) was also observed. All isolates were shown to have *tdh* and *toxR* by PCR. Of these isolates, 18 strains were shown to be positive by group-specific PCR and to have ORF-8. *V. parahaemolyticus* was the only bacterial pathogen isolated from the patients included in this study. Microscopic examination of stool revealed the presence of few ova of *Ascaris lumbricoides* in 2 patients and both *Giardia* and *Entamoeba histolytica* in 1 patient. No bacterial pathogens were isolated from stool from healthy control subjects. However, *Giardia* was isolated from 1 healthy adult, and *A. lumbricoides* was isolated from 2 healthy adults.

Occult blood and leukocytes in stool. Examination of stool collected during the acute stage showed occult blood of moderate to severe grade (2+ to 4+) in 68% of patients, occult blood of mild grade (1+) in 8%, and no occult blood in the remainder. In the majority of patients, no leukocytes or red blood cells were found in stool.

Leukocytes in blood. Higher levels of leukocytes in blood were seen in patients at the acute stage of the disease than in healthy control subjects (table 1). In adults, the levels remained high for up to 7 days after the onset of diarrhea but decreased to levels seen in healthy control subjects by day 30 after onset (data not shown). Total lymphocyte counts were lower in patients at the acute stage of illness than in control subjects. Percentages of polymorphonuclear neutrophils (PMNs) in blood were high in patients at the acute stage of infection, compared with convalescence ($P = .011$ to $P < .001$) or with healthy control subjects ($P = .027$).

Mediators in serum and stool. Levels of CRP were higher in patient serum samples collected during the acute stage of the infection than in samples collected during convalescence ($P \leq .001$) or in samples from healthy control subjects ($P \leq .001$) (table 2). Approximately 80% of the patients infected with *V. parahaemolyticus* had a CRP level >7 mg/dL in serum during the acute stage. The median level was 8.3 mg/dL, which was higher than that among patients with *V. cholerae* O1 infection (4.1 mg/dL; $P \leq .045$) and lower than that among patients with shigellosis (12.0 mg/dL; $P = .04$). High levels of lactoferrin were seen in plasma and stool samples collected from patients during the acute stage, compared with samples collected during convalescence and samples from healthy control subjects ($P \leq .001$). This increase was greater than that seen in patients with *S. dysenteriae* type 1 infection ($P \leq .001$) but similar to that seen in patients with cholera (*P* was not significant). Levels of the proinflammatory cytokine TNF-α were high in both plasma and stool from patients at the acute stage of *V. parahaemolyticus* infection, which was similar to the response seen in patients with shigellosis. TNF-α levels were significantly higher in stool samples (but not in plasma) from patients with shigellosis (median, 2280 pg/mL; $P \leq .001$) than in samples from patients infected with *V. parahaemolyticus* infection (median, 33.1 pg/mL; table 2) at the acute stage. Levels of IL-1β were high in stool samples from *V. parahaemolyticus*–infected patients at the acute stage of infection ($P = .001$), compared with convalescence and with stool from healthy control subjects ($P = .002$). In patients infected with *S. dysenteriae* type 1, the levels of IL-1β were even more highly elevated (median, 13,000 pg/mL; $P \leq .001$) than in patients with *V. parahaemolyticus* (median, 310.9 pg/mL) at the acute stage. No increase in IL-1β levels was seen in plasma samples from patients with *V. parahaemolyticus* infection or from patients with shigellosis. Levels of the inflammatory cytokines TNF-α and IL-1β were below the limit of detection in stool and plasma samples from patients with cholera.

Histopathological examination of tissue sections. Hematoxylin-eosin–stained sections from duodenal and rectal bi-

Table 1. Sociodemographic characteristics of patients infected with *Vibrio parahaemolyticus* and healthy control subjects and clinical features at the acute stage (onset) of disease.

Variable[a]	Patients (n = 28)	Control subjects (n = 30)
Age, median years (range)	30.0 (21.0–40.0)	25.0 (23.0–38.0)
Duration of illness before hospitalization, median h (range)	6.0 (4.0–24.0)	NA
Dehydration, no. (%) of patients		
Severe	5 (17.2)	NA
Some	13 (45.0)	NA
None	10 (37.8)	NA
Temperature >37.8°C, no. (%) of patients	1 (4.5)	NA
Blood leukocyte count, median cells × 10^2/mm^3 (IQR)	113.0 (100.0–150.0)[b]	90 (85–92)
Blood lymphocyte count, median % (IQR)	20.0 (16.3–28.8)[b]	35 (33–38)
Blood polymorphonuclear neutrophil count, median % (IQR)	72.0 (62.5–76.0)[b]	55 (50–57)
Occult blood in stool, no. (%) of patients		
4+ to 2+	17 (68)	NA
1+	2 (8)	NA
0	5 (20)	NA
Results of microscopic examination of stool, no. (%) of patients		
>50 Leukocytes/high-power field	2 (6.9)	0
Red blood cells		
0	11 (40.7)	NA
1–10	9 (33.3)	NA
11–20	6 (22.2)	NA
>50	1 (3.7)	NA

NOTE. IQR, interquartile range; NA, not applicable.

[a] Most parameters were studied at day 2 after onset of illness; tests on stool were carried out on day 1 (i.e., on admission in the hospital).

[b] $P \leq .05$ (statistically significant), compared with healthy control subjects.

opsy specimens obtained from *V. parahaemolyticus*–infected patients during the acute stage of infection showed an acute inflammatory response (figure 1*A* and 1*C*). The surface epithelium showed epithelial degeneration and denudation in both the duodenum and rectum. PMN infiltration of villi and crypt cells was seen in most cases. The lamina propria showed prominent congestion of villus and pericryptal blood vessels and hemorrhage, especially of the proximal villi. Quantification of PMNs in pararosanilin-stained sections demonstrated that the numbers of PMNs were higher in duodenal specimens obtained during the acute stage (GM, 5.58 cells/mm^2) than in these locations in healthy individuals (GM, 0.28 cells/mm^2; P = .002). Biopsy specimens obtained during early convalescence (day 7 after onset) showed fewer changes in the villus epithelium but more-prominent intraepithelial lymphocytes. Scattered PMNs were seen in the lamina propria, and marginated blood vessels with swollen endothelial cells were observed. Evidence of eosinophil degranulation was seen toward the crypt base. Although epithelial damage was not seen during late convalescence (figure 1*B*), PMNs persisted in the lamina propria (GM, 3.0 cells/mm^2).

At the rectal site, the mucosa showed changes similar to those seen in the duodenum during the acute stage of infection (figure 1*C*). The surface epithelium showed evidence of damage, irregularly placed nuclei, vacuolization, detachment, and denudation. The lamina propria showed mild edema, congestion of blood vessels, and hemorrhage involving the full width of the mucosa; endothelial swelling; and PMN margination. The numbers of PMNs were higher during the acute stage in rectal sections (GM, 7.36 cells/mm^2) than in these locations in healthy individuals (GM, 1.2 cells/mm^2; P = .002), and these cells were localized in the lamina propria region of the villus and crypt, rather than in the epithelium (P = .006). The epithelial changes were smaller during early convalescence and subsided by day 30 after onset of infection (figure 1*D*). The PMN response and vascular changes, although smaller, persisted until late convalescence.

ASC responses. *V. parahaemolyticus*–infected patients had IgA, IgG, and IgM ASC responses to TDH at the acute stage of the disease that remained high until early convalescence (day 7 after onset). No statistically significant difference was seen in the response during the acute stage and during early convalescence in any antibody isotype (figure 2). Numbers of ASCs

Table 2. Levels of mediators of immune response in blood and stool of *Vibrio parahaemolyticus*–infected patients with diarrhea and healthy control subjects.

Mediator	Level in blood sample			Level in stool sample		
	Acute stage	Convalescent stage	Control	Acute stage	Convalescent stage	Control
CRP, median mg/dL (IQR)	8.9 (4.1–12.0)[a,b]	0.6 (0.49–0.79)	<0.5	ND	ND	ND
Lactoferrin, median ng/mL (IQR)	296.4 (232.4–354.4)[a,b]	193.6 (138.0–208.8)[a,b]	123.8 (65.1–156)	65.3 (43.7–97.6)[b]	50.4 (24.3–112.9)	31.5 (13–48.0)
$NO^{\cdot}$ metabolites, median NO_2^-/NO_3^- (IQR)	244.4 (188–276)[b]	161.0 (128–248)[b]	70 (64–160)	ND	ND	ND
TNF-α, median pg/mL (IQR)	65.8 (43–86)[a,b]	38.1 (13.5–51.4)[b]	11.0 (8.3–14.5)	33.1 (20.4–60.7)[a,b]	11.3 (9.1–18.2)[b]	3.2 (1.60–5.5)
Interleukin-1β, median pg/mL (IQR)	23.0 (5.2–29.2)	16.4 (9.2–29.0)	16.8 (7.2–25.6)	310.9 (60.4–340)[a,b]	0.55 (0.22–11.5)	0.20 (0.05–1.2)

NOTE. Acute-stage samples were obtained on day 2; convalescent-stage samples were obtained on day 3. Samples from healthy control subjects were obtained on day 0. CRP, C-reactive protein; IQR, interquartile range; ND, not done; $NO^{\cdot}$, nitric oxide; NO_2^-/NO_3^-, total nitrite and nitrate content; TNF, tumor necrosis factor.

[a] $P \leqslant .05$ (statistically significant), compared with convalescent-stage samples.

[b] $P \leqslant .05$ (statistically significant), compared with control samples.

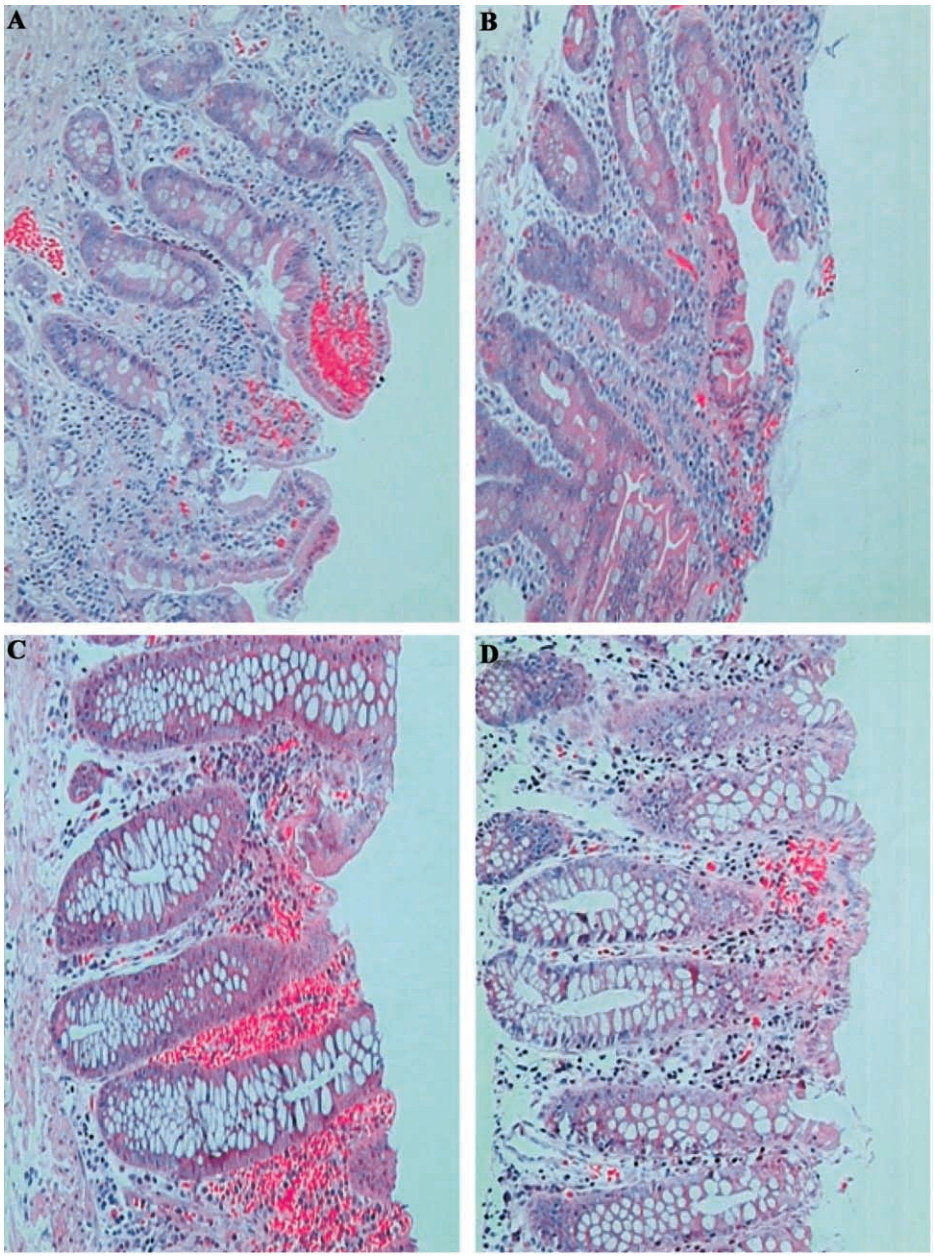

Figure 1. Acute inflammation was seen in paraffin-embedded hematoxylin-eosin–stained sections of duodenal *(A* and *B)* and rectal *(C* and *D)* biopsy specimens. The duodenal and rectal sections obtained during the acute stage, at day 2 *(A* and *C)* after onset of diarrhea, and during convalescence, at day 30 *(B* and *D),* are shown for patients with acute watery diarrhea caused by infection with *Vibrio parahaemolyticus.* (Original magnification, ×400.)

were significantly higher in patients than in healthy control subjects. As has been reported elsewhere, the ASC response decreased, and IgA, IgG, and IgM ASCs could not detected by day 14 after onset of the disease [16, 22]. The ASC response was measured on day 14 only in 6 patients in whom it was poor or not present (GM, 2.1 ASCs/10^7 MNCs).

The ASC response to homologous and heterologous antigen types in patients infected with *V. parahaemolyticus* strains in the O1 ($n = 8$) and O3 ($n = 12$) serogroups was studied using both WC bacterial antigen and LPS (table 3). In both groups of patients, a response was seen not only to homologous WC and LPS antigens but also to heterologous antigens, and the response was predominantly in the IgA and the IgM isotypes and was weak in the IgG isotype. The kinetics of the response, however, differed for patients infected with strains in the O1 serogroup and patients infected with strains in the O3 serogroup. *V. parahaemolyticus* serogroup O1 stimulated the maximum IgA ASC response to WC or LPS antigen at the acute stage, rather than at day 7, whereas patients infected with strains in the O3 serogroup showed a stronger response later, at day

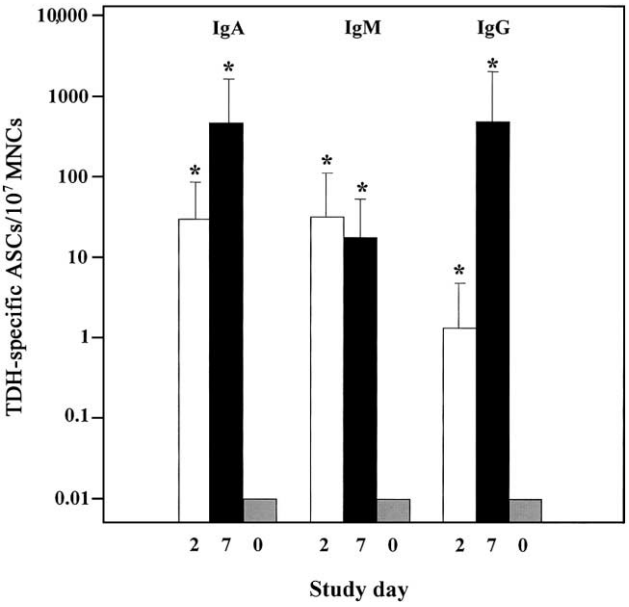

Figure 2. Antibody-secreting cell (ASC) responses to thermostable direct hemolysin (TDH) in study subjects. TDH-specific IgA, IgM, and IgG responses in peripheral blood are shown in patients during the acute stage (day 2; *white bars*) and convalescence (day 7; *black bars*) and in healthy control subjects at a single time point (day 0; *gray bars*). Bars show geometric means; lines show ranges (+1 SEM). *$P \leq .05$ (statistically significant), compared with healthy control subjects.

7 after onset. A similar pattern was seen in the responses to heterologous WC and LPS antigens in these 2 groups of patients. A significant difference was seen in the response during the acute stage and that during early convalescence (table 3). Healthy control subjects showed a poor ASC response to the O1 and O3 LPS antigens, one that was significantly lower than the response seen in patients ($P \leq .04$).

Serum antibody responses to TDH and LPS. *V. parahaemolyticus*–infected patients had IgA antibody responses to TDH at day 7 of onset of illness (GM, 38.4; $P = .002$) that remained high until late convalescence (GM, 41.3; $P = .002$). A similar IgG response was seen, although the levels were increased later, at ~14 days after onset of illness. A much weaker IgM response to TDH was seen. The levels of TDH-specific IgA, IgM, and IgG in patients at all stages of infection were significantly higher than those seen in the healthy control subjects ($P \leq .001$).

The response to homologous LPS was studied in patients infected with *V. parahaemolyticus* serotypes O1 ($n = 8$), O3 ($n = 13$), O4 ($n = 2$), O5 ($n = 3$), and O8 ($n = 2$). The response to homologous LPS, when data from these patients were analyzed collectively, was significant by day 7 in the IgA (GM, 9.2 mOD/min; $P = .001$; 4.3-fold increase), IgM (GM, 66.4 mOD/min; $P = .01$; 2.3-fold increase), and IgG (GM, 26.6 mOD/min, $P = .015$; 2.4-fold increase) isotypes, and levels of all 3 immunoglobulin isotypes remained higher than they were during the acute stage, in most cases, until day 14 after onset ($P = .02$ to $P = .001$). The IgG response to LPS-specific antibodies remained high through 30 after onset. Levels of antibodies to O1, O3, O4, O5, and O8 *V. parahaemolyticus* LPS were much lower in healthy control subjects than in *V. parahaemolyticus*–infected patients between days 7 and 14 after onset ($P \leq .05$).

Antigen-specific IgA antibody response in stool. *V. parahaemolyticus*–infected patients had IgA responses to TDH and homologous LPS in stool (table 4). Approximately 68% of the patients showed a ≥3-fold increase in TDH-specific response by day 7–14. A response to homologous LPS antigen was also observed in ~56% of the patients at approximately the same time points. Antibody levels in healthy control subjects were significantly lower than those seen in patients; during convalescence, levels to TDH were, on average, 12–15-fold lower, and levels to O3 LPS antigen were 8–10-fold lower.

Killing of strains of* V. parahaemolyticus *by serum. To relate the virulence of the strains to the immune response generated after natural infection, we tested the resistance to killing by serum of 28 strains of 8 O:K serotypes that were isolated from patients in this study. Strains of the O1:K25 and O1:K56 serotypes were most resistant and were not killed, even in a solution containing 95% human serum, whereas others, such as strains of the O8:K21 and O3:K6 serotypes, were very susceptible and could be lysed even in a solution containing 20%–30% serum. In all cases, different isolates, if they belonged to the same O:K serotype, demonstrated similar susceptibility to killing by serum.

Relationship between resistance to serum and immune response. The cumulative immune response in the 8 patients who were infected with the serum-resistant strains (i.e., O1:K25 and O1:K56 serotypes) was compared with that in the 17 patients who were infected with strains that were more sensitive (i.e., O8:K21, O5:KUT, and O3:K6 serotypes). It appeared that the more-virulent, serum-resistant strains were those that induced an earlier ASC response to LPS, at day 2 after onset of infection. The IgA response to homologous LPS in serum during early convalescence was also significantly higher in patients infected with O1:K25 and O1:K56 serotypes (7-fold higher; $P = .036$) than in patients infected with other serotypes. Thus, infection with the serum-resistant phenotype of *V. parahaemolyticus* strains appeared to evoke an earlier and stronger response to LPS. No other difference was found between the 2 groups of patients, even in the clinical characteristics of the infection, including dehydration status and the disease severity.

DISCUSSION

This is the first report, to our knowledge, of a detailed and systematic study of the adaptive and inflammatory immune

Table 3. Antibody-secreting cell (ASC) responses to whole-cell bacteria and lipopolysaccharide in 8 patients infected with *Vibrio parahaemolyticus* O1, 12 patients infected with *V. parahaemolyticus* O3, and 30 healthy control subjects.

Antigen type, subject group, antigen serogroup	IgA response, GM ASCs/10^7 MNCs (IQR)			IgM response, GM ASCs/10^7 MNCs (IQR)			IgM response, GM ASCs/10^7 MNCs (IQR)		
	Day 0	Day 2	Day 7	Day 0	Day 2	Day 7	Day 0	Day 2	Day 7
Whole cell									
Patients									
Homologous									
O1	—	94 (54.3–153)	19.95 (9.2–43.2)	—	34.1 (9.7–119.7)	16.5 (7.2–37.9)	—	3.0 (1–9)	7.6 (3.2 –17.8)
O3	—	8 (4–16.1)	83 (61–112)	—	8.14 (9.1–16.1)	152.1 (94–247)	—	2 (1.2–2.3)	10.1 (6.3–16.3)
Heterologous									
O1	—	117.2 (73–189.2)	14 (6.5–29)	—	45.6 (12.5–166)	6.1 (2.6–14.4)	—	2.3 (1–5.5)	0
O3	—	9.3 (4.1–21.1)	43 (33.3–55.5)	—	7.8 (3.1–19.8)	38.9 (16.9–89.3)	—	1.94 (1.3–3.0)	1.9 (1.3–3)
Control subjects									
O1	9.0 (1.0–21.0)	—	—	5.0 (1.0–15.9)	—	—	1.0 (1.0–1.0)	—	—
O3	6.5 (1.0–18.2)	—	—	3.0 (1.0–5.7)	—	—	1.5 (1.0–14.5)	—	—
Lipopolysaccharide									
Patients									
Homologous									
O1	—	47.1 (30.1–74)	40.3 (30.1–154.9)	—	83.2 (68.9–100.5)	137 (83.6–225)	—	0	13.4 (5.5–32.5)
O3	—	7.4 (3.3–17)	43.7 (23.2–82.2)	—	30.1 (5.4 –32)	86.7 (39.8–189)	—	3.4 (1.8–6.3)	3.6 (2.1–6.1)
Heterologous									
O1	—	71 (53–95.3)	4.2 (2.2–8.1)	—	105.7 (76.4–146)	9.3 (3.4–25.6)	—	4.5 (2–10.6)	1.7 (1–2.7)
O3	—	6.8 (3.5–13.1)	36.1 (25.5–51)	—	8.1 (3.3–20)	74 (46.5–116.7)	—	0	2.7 (1.6–4.3)
Control subjects									
O1	4.2 (1.0–12.0)	—	—	6.0 (3.9–11.0)	—	—	6.9 (1.0–21.0)	—	—
O3	6.0 (2.0–16.5)	—	—	8.0 (5.0–15.9)	—	—	3.0 (1.0–21.1)	—	—

NOTE. Homologous responses were the responses of patients infected with *V. parahaemolyticus* O1 or O3 to O1 or O3 antigen, respectively; heterologous responses were the responses of patients infected with *V. parahaemolyticus* O1 or O3 to O3 or O1 antigen, respectively. Responses in healthy control subjects were studied at a single time point on day 0. GM, geometric mean; IQR, interquartile range; MNCs, mononuclear cells.

Table 4. Antigen-specific IgA antibody responses in stool samples from patients infected with *Vibrio parahaemolyticus*.

Parameter	Response to thermostable direct hemolysin				Response to lipopolysaccharide			
		Convalescent stage				Convalescent stage		
	Acute stage	Day 7	Day 14	Day 30	Acute stage	Day 7	Day 14	Day 30
GM IgA (range)[a]	2.6 (2.1–3.1)[b]	3.8 (3.0–4.9)[b]	5.8 (4.5–7.3)[b,c]	3.4 (2.7–4.4)[b]	2.7 (2.2–3.3)	3.6 (2.8–4.5)[b]	3.3 (2.7–3.9)[b]	2.2 (1.9–2.6)[b]
Subjects with response,[d] % (*n*/*N*)	NA	37 (10/27)	64 (16/25)	42 (8/19)	NA	48 (13/27)	40 (10/25)	26 (5/19)
Increase, *x*-fold[e]	NA	3.0	3.6	3.0	NA	3.0	3.5	3.7

NOTE. Acute-stage samples were obtained on day 2. GM IgA for 24 healthy control subjects was 0.07 (0.01–0.20) for thermostable direct hemolysin and 0.3 (0.03–0.5) for lipopolysaccharide. GM, geometric mean; NA, not applicable; *n*/*N*, no. of subjects with response/no. tested.

[a] Range is GM − SEM to GM + SEM.

[b] $P = .05$ to $P < .001$, compared with healthy control subjects (Wilcoxon rank sum test or Mann-Whitney *U* test).

[c] $P = .04$ to $P = .02$, compared with convalescent-stage samples (Wilcoxon rank sum test or Mann-Whitney *U* test).

[d] Subjects with an increase of ⩾2-fold in IgA from the acute-stage measurement were considered to have demonstrated a response.

[e] Change from GM measured on day 2; calculated for subjects with responses only.

responses in patients with diarrhea who were infected with *V. parahaemolyticus*. Our patients had a short history of stay at the ICDDR,B hospital, similar to that observed for patients with acute watery diarrhea who were infected with *V. cholerae* O1 or O139 or enterotoxigenic *E. coli* [20, 22], and the duration of the illness was shorter than that of patients with shigellosis [32]. There were differences between the inflammatory responses in patients in the present study and those in patients with other forms of acute watery or invasive diarrhea. The inflammatory response at the acute stage in the gut and in the circulation in most cases was less severe than that observed in patients with shigellosis [33] but more severe than that seen in patients with cholera.

Markers of enteric inflammation, such as stool occult blood, have been found to be a specific indicator of invasive disease [34], as well as of diarrhea caused by enteroaggregative *E. coli* [35]. We have observed occult blood in the stool of a significant proportion of patients with *V. parahaemolyticus* infection, although leukocyte and red blood cell counts were not high. This distinguishes *V. parahaemolyticus* infection from *Shigella*-associated diarrhea, in which there is an increase of all 3 components in the stool [33]. A possible reason for the lack of increase in leukocyte and red blood cell levels may be lysis of red blood cells and damage to leukocytes by hemolytic and cytotoxic activities of TDH [36]; this is different from the action of *Shigella* enterotoxins.

V. parahaemolyticus is known to cause systemic infections in the human host [11], and it is capable of a more than superficial colonization and can penetrate the lamina propria of the small intestine in the rabbit ileal loop [37]. Levels of the majority of inflammatory mediators in the patients in the present study increased in stool and/or in blood. This suggests that, in natural infection, virulence antigens other than TDH (e.g., LPS and capsular polysaccharide antigens) may induce stimulation of these inflammatory cells and proinflammatory factors. Levels of the proinflammatory cytokines TNF-α and IL-1β were high at the onset of infection in our patients. These cytokines may be produced by PMNs, macrophages, and other inflammatory cells in the mucosal arena [38]. There was an increase in the number of PMNs in blood and a decrease in the number of lymphocytes. This has been documented in other acute bacterial infections [39]. Stool testing and histopathological evaluation showed an increase in the number of macrophages at duodenal and rectal sites. Increased production of lactoferrin in local and systemic circulation suggests that PMNs are activated as a result of the inflammatory response. This can also be linked to the increase in the number of PMNs in the systemic circulation and local sites in the duodenum and rectum. Epithelial cells are also a source of IL-1β. This cytokine also could have been released from the damaged epithelial tissues into the intestinal lumen. The increase in the levels of lactoferrin and $NO^{\cdot}$ metabolites in specimens from patients is also indicative of the strong inflammatory response induced in natural infection. The increase of $NO^{\cdot}$ metabolites suggests that up-regulation of the oxidative killing mechanism is increased, possibly by phagocytes, including macrophages and intestinal epithelial cells; the intestinal epithelium is a major site of $NO^{\cdot}$ production. Studies in animals have shown a histocytic response, which suggests that macrophages play a role in the response to *V. parahaemolyticus* infection [15], and, in the present study, we found an increase in the number of macrophages in both the duodenum and the rectum.

The results of our study show that *V. parahaemolyticus*–associated diarrhea in humans leads to inflammatory responses that have features similar to those seen in disease caused by *Shigella* species [21, 33] and that appear to be more severe than those seen in disease caused by *V. cholerae* O1 or O139 [20]. We have not found that levels of the 2 proinflammatory cytokines increase in patients with cholera (data not shown), whereas an increase has been documented in patients with shigellosis. A difference in the response to the 2 forms of bloody diarrhea is that the healing process is quicker and levels of

inflammatory cytokines decrease more rapidly in TDH-associated bacterial infections than in shigellosis. The mediators may remain elevated for as long as 15–45 days after onset of illness in shigellosis. Levels of the acute-stage protein, CRP, increased much more in patients with *V. parahaemolyticus* infection than in patients with cholera [20] and to a degree comparable to that seen in patients with shigellosis [33]. The upregulation of $NO^{\cdot}$ metabolites and lactoferrin in patients infected with *V. parahaemolyticus,* however, appeared to be comparable to that seen in the patients with cholera [20].

The inflammatory immune response in *V. parahaemolyticus*–induced diarrhea appears to reflect the self-limiting pattern of the clinical disease. The acute inflammatory response in the circulation and the local secretions at the mucosal surface subsided and reverted to preinfection conditions very soon, by approximately day 7 after the onset of infection. This is in keeping with the action of TDH in IEC-6 cells [40], in which it has been shown that, once the toxin is removed from the environment, the cells rapidly recover their original morphology.

Strains of both the pandemic and nonpandemic types were isolated from our patients. We were not able to discern any difference between the responses of patients infected by pandemic strains ($n = 18$) and patients infected by nonpandemic strains ($n = 10$) in clinical characteristics, B cell responses, or inflammatory responses. This suggests that the attributes of the pandemic strains, other than virulence in the host, may be responsible for the worldwide spread of infection with these strains.

The strains of *V. parahaemolyticus* isolated from our patients showed varying susceptibility to the bactericidal effects of serum. Those belonging to the O1:K25 and O1:K56 serotypes, which were more resistant, induced a relatively earlier B cell response. We speculate that the early immune response to these pathogens could be the result of their increased ability to evade killing by the host, which allows them to colonize and persist in higher numbers, resulting in a earlier humoral immunological response.

Because *V. parahaemolyticus* causes secretory diarrhea, the small intestine has been considered to be the site of action in the human host. Small-intestine damage was found during autopsy of 4 persons who died of *V. parahaemolyticus*–induced food poisoning [41]. In the present study, however, we show that both small-intestine and large-intestine tissues are affected by the pathogen. We observed epithelial damage, denudation, and hemorrhage at both sites. Our results suggest that both locations of the gut are affected by colonization with the pathogen. Gastroenteritis caused by *V. parahaemolyticus* results in strong systemic and mucosal B cell responses to TDH and LPS. An IgA ASC response indicates mucosal activation. Both the antigens also induced an increase in the presence of IgM ASCs, which suggests that this was a primary response to the antigen. In addition, TDH also induced strong IgG responses, similar the responses to cholera toxin reported elsewhere [22]. The IgG response is also indicative of a systemic component, which is a possibility; *V. parahaemolyticus* results in denudation and damage of the gut epithelium, and antigens can reach the lamina propria. It is possible that TDH can be taken up by Peyer's patches and transported within macrophages to the mesenteric lymph nodes and spleen to induce a systemic response; this mechanism has also been proposed for cholera toxin [42]. In addition, TDH may bind to cells via the GT1b ganglioside receptor [43, 44], which is believed to be a toxin-binding epitope on T cell surfaces [45] and to induce an innate immunity. Thus, both the mucosal and systemic compartments of the immune systems are activated.

We do not know whether natural infection due to *V. parahaemolyticus* results in an O:K serotype–specific or a TDH-specific protective immune response. This is the first study to document the immune response after natural infection, and more work, especially on mucosal and cellular responses and on immune modulators, needs to be carried out before the detailed mechanism of immune response in the host can be understood.

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