Vibrio cholerae O1 Infection Induces Proinflammatory CD4+ T-Cell Responses in Blood and Intestinal Mucosa of Infected Humans


Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts; International Centre for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh; Department of Medicine, Harvard Medical School, Boston, Massachusetts; Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts; and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts

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Vibrio cholerae O1 is a noninvasive enteric pathogen and serves as a model for studies of mucosal immunity. Although symptomatic V. cholerae infection induces durable protection against subsequent disease, vaccination with oral killed whole-cell V. cholerae stimulates less long-lasting protection against cholera. In this study, we demonstrated that cholera induces an early proinflammatory cellular immune response that results in priming of Th1- and Th17-type cytokine responses to ex vivo antigenic stimulation and an increase in the ratio of Th1 to Th2 CD4+ T-cell responses. Comparable priming of Th1 and Th17 responses, with an increased ratio of Th1 to Th2 CD4+ T-cell responses, was not observed in subjects who received two doses of the oral cholera vaccine Dukoral (a whole-cell cholera toxin B subunit containing [WC-CTB] vaccine). These findings suggest that natural V. cholerae infection induces an early, proinflammatory cellular immune response, despite the apparent lack of clinical signs of inflammation. The failure of the WC-CTB vaccine to activate equivalent, CD4+ T-cell responses is a potential explanation for the shorter duration of protection following immunization with this vaccine. Additional studies are needed to determine whether these early T-cell-mediated events predict the subsequent duration of immunologic memory.

Vibrio cholerae causes 3 million to 5 million cases of diarrhea and over 100,000 deaths annually (2). Organisms colonize the epithelial surface of the small intestine and elaborate cholera toxin (CT), an essential virulence factor for all pandemic strains of V. cholerae (30). Because V. cholerae is a prototypical noninvasive pathogen, it serves as a model for the study of mucosal immunity (17).

Natural infection with V. cholerae provides greater than 90% protection against subsequent disease for at least 3 years in U.S. volunteer studies (the maximum period studied) and an average of 3 to 8 years on the basis of epidemiological studies in areas of endemicity (5, 19, 21). The mechanism(s) of protective immunity against cholera is not well understood but has been hypothesized to depend on anamnestic responses of memory B cells (16, 25, 35). Consistent with this, our group previously demonstrated that V. cholerae protein antigen-specific memory B cells remain detectable in the circulation for over a year following cholera, after circulating antibody levels have returned to baseline levels. In contrast, memory B-cell responses to lipopolysaccharide, a T-cell-independent antigen, were found to decrease more rapidly after infection (16). Such long-lasting memory B cells against V. cholerae protein antigens could play a role in mediating anamnestic responses and protection against subsequent infection.

In contrast, a currently licensed cholera vaccine, Dukoral (a whole-cell CT B subunit [WC-CTB] vaccine), consists of killed V. cholerae O1 supplemented with recombinant cholera toxin B subunit (rCTB) and provides over 60% protection. However, protection wanes by 3 years after vaccination, and the vaccine provides limited direct protection in children (6, 7). A second vaccine, licensed as Shanchol, is a bivalent preparation that includes a mixture of killed V. cholerae O1 as well as the O139 serogroup but does not include rCTB. The protective efficacy of this vaccine is greater than 65% over a 2- to 3-year observation period in India in all age groups, but long-term protection has not yet been characterized (33). Recently, we observed that memory-B-cell responses against V. cholerae antigens were substantially less following vaccination with Dukoral than following natural infection, despite initially similar antibody-secreting cell and plasma antibody responses (1). Because of its toxicity, both licensed cholera vaccines lack the ADP-ribosylating cholera toxin A subunit (29).

Because of the critical role of CD4+ T cells in follicular B-cell differentiation, we hypothesized that the induction of CD4+ T-cell responses might be critical to the subsequent development of memory-B-cell responses and for long-term

*p* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: jbharris@partners.org.

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‡ These authors made equal contributions.

# These authors share senior authorship and made equal contributions.

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protective immunity following cholera. We have previously demonstrated that the proliferation of circulating CD4 T cells in response to *ex vivo* stimulation with *V. cholerae* antigens peaks 7 days after cholera (3, 36), with the majority of these CD4 T cells expressing both memory (CD45RO CD45RA ) and gut-homing (α4β7) markers. Approximately 80% of these mucosal primed CD4 T cells areCCR7 negative, and 20% are CCR7 positive, consistent with effector and central-memory phenotypes, respectively (36).

To better understand the phenotype of these effector CD4 T-cell responses to *V. cholerae* infection and their role in subsequent memory development, we analyzed canonical lineage-specific cytokine responses of T cells to *ex vivo* stimulation with a *V. cholerae* membrane preparation (MP) after natural cholera and compared these to the responses seen following vaccination with the licensed WC-CTB vaccine.

**MATERIALS AND METHODS**

**Study design and subject enrollment.** Patients presenting to the hospital at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), with severe acute watery diarrhea with stool cultures positive for *V. cholerae* O1 were eligible for this study. Patients with significant comorbid conditions were excluded. Blood specimens were collected on the day after presentation (day 2) to allow for clinical stabilization and confirmation of a positive stool culture result. Adults from an unplanned urban area with demographic patterns similar to those observed in cholera patients and with no history of diarrhea during the previous 1 month were included as healthy controls. Additionally, healthy adult volunteers from a similar urban area were enrolled and vaccinated with two doses of the WC-CTB oral cholera vaccine on day 0 and day 14.

Because of the volume of blood required for the immunological assays, separate cohorts of patients, healthy controls, and vaccinees were enrolled for different components of the study. For the comparison of cytokine secretion in response to *V. cholerae* MP stimulation of whole blood, 7 patients and 5 vaccinees between the ages of 17 and 38 years were enrolled. The median age was 30 years in both groups. For the study of cytokine expression using intracellular cytokine staining/flow cytometry, 12 patients and 11 vaccinees between the ages of 16 and 50 years were enrolled. The median ages were 29 years in patients (mean, 29.9 ± 8.3 years) and vaccinees (28.6 ± 4.8 years) and 27 years in the control group (27.0 ± 5 years). A total of 14 cholera patients were enrolled in the endoscopy portion of the study for collection of duodenal pinch biopsy specimens. Specimens obtained from 5 healthy individuals with asymptomatic *Helicobacter pylori* infection enrolled in a separate and ongoing study were also utilized as controls. This cohort ranged in age from 22 to 45 years (median age, 30 years). All protocols of the study were approved by the Research and Ethical Review Committee of the ICDDR,B and the Institutional Review Board of Massachusetts General Hospital, and all participants provided written informed consent. The human experimentation guidelines of the U.S. Department of Health and Human Services were followed during the conduct of this research.

**Microbiologic examination of stools.** Stool from patients with a characteristic *V. cholerae* infection was positively stained for *V. cholerae* and enteric pathogens, including enterotoxigenic Escherichia coli, Salmonella, Shigella, and Campylobacter spp. A portion of the biopsy specimens was immediately fixed in 10% formalin (28). Another biopsy specimen was used for extraction of lamina propria lymphocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density centrifugation on Ficoll-Isopaque gradients (Pharmacia, Piscataway, NJ). PBMCs were resuspended at a concentration of 2 × 10e6 cells/ml in RPMI medium (Gibco, Carlsbad, CA) supplemented with 10% FCS (Gibco) and cultured in U-bottom tissue culture plates (Nunc, Denmark) in the presence of *V. cholerae* MP (10 μg/ml) or phorbol myristate acetate (PMA)-ionomycin at concentrations of 5 ng/ml and 1.0 μg/ml, respectively. Samples containing unstimulated cells were included as a negative control. Purified anti-CD28 (clone 28.2; BD Pharmingen) and anti-CD49d (clone 9F10; BD Pharmingen) at a concentration of 1.0 μg/ml were used for costimulation.

PBMCs and antigens were incubated for 2 h at 37°C in 5% CO2. After 2 h, brefeldin A (BFA; Sigma), (10 μg/ml) was added and cell cultures were incubated for an additional 4 h (15). Following the 6-h stimulation, cells were washed with phosphate-buffered saline (PBS) and 2% FCS. Washing pellets were stained for 1 h at 4°C with monoclonal antibodies and fluorescein isothiocyanate (FITC) (clone SK7) and anti-CD4-peridinin-chlorophyll-protein complex (SK3; Becton Dickinson Immunocytometry Systems [BD], San Jose, CA). Following surface staining, cells were washed and then incubated with fluorescence-activated cell sorter (FACS) lysing solution (BD Bioscience, San Jose, CA) for 10 min, washed, and then permeabilized with FACS permeabilizing solution (BD Bioscience, San Jose, CA) for 10 min at room temperature. Permeabilized cells were washed and then stained for 30 min at 4°C with fluorochrome-conjugated intracellular antibodies (i.e., anti-IFN-, anti-IL-13, anti-IL-10, and anti-IL-17) (BD Bioscience). Staining was performed by heating the sections in a microwave oven in sodium citrate buffer (pH 6.0). The sections were incubated with antibody against IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA), Horseradish peroxidase-conjugated mouse anti-rabbit antibody was used as the secondary reagent, and the sections were developed with diaminobenzidine as the substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted. QWin software (Leica Microsystems, Switzerland) with the Leica (DMLB) microscope was used for measuring the intensity of the IL-17-positive area. The area of the IL-17 staining was analyzed using an acquired computerized image analysis (ACIA) score. This metric has been described previously and is defined as the total positively stained area measured times the mean intensity of the positive area divided by the total area measured, including both negatively or positively stained tissues (31).

Another biopsy specimen was used for extraction of lamina propria lymphocytes (LPLs), as described previously (4). Biopsy specimens were washed in PBS and Hank’s balanced salts solution (HBSS; H4641; Sigma) and then incubated with EDTA (1 mM) and dithiothreitol (1 mM) with gentle vortexing. Supernatants were pooled and filtered through nylon mesh to obtain the intrathelial cell fraction. This fraction was washed, resuspended, and incubated in RPMI medium with 5 ml of collagenase and DNase (100 U/ml and 0.1 mg/ml, respectively; C0255 and D0255, respectively; Sigma) solution for 2 h at 37°C. This cell suspension was filtered through nylon mesh cloth, and the LPL fraction was resuspended in RPMI medium and placed in 24-well cell culture plates (BD...
Biosciences). Plates were incubated at 37°C in 5% CO₂ for 5 days. Culture supernatants were used to obtain the concentrations of cytokines (IL-6, IL-17, and IL-1β) using a Milliplex human cytokine/chemokine kit following the manufacturer’s recommended protocols (Millipore Corp., Billerica, MA). The concentration of IL-8 was measured using a human CXCL8/IL-8 DuoSet kit following the manufacturer’s recommended protocol (R&D Systems, Inc., Minneapolis, MN). Transforming growth factor β1 (TGF-β1) concentrations were measured using a Milliplex human TGF-β1 kit following the manufacturer’s recommended protocols (Millipore Corp).

**Statistical analyses.** To measure the statistical significance of the observed cytokine responses, the Mann-Whitney U test was used to evaluate comparisons of immune responses between groups of patients, vaccinees, and healthy controls. The Wilcoxon test was used to compare responses within groups at different time points. To evaluate trends in the immune responses after one and two doses of vaccine, a nonparametric trend test was used (9). A two-tailed P value of ≤0.05 was considered the cutoff for a significant difference.

**RESULTS**

Cytokine responses to *ex vivo* *V. cholerae* antigens in cell culture supernatants. To compare the cytokine responses to *V.
cholerae antigen stimulation of circulating T cells in patients with cholera and in recipients of the WC-CTB vaccine, we measured the concentrations of IFN-γ, IL-10, IL-13, and IL-17 in supernatants collected from cultures of whole blood, stimulated by the addition of V. cholerae MP, using methods described previously (34, 36). Cytokine levels were compared in patients and vaccinees in response to antigenic stimulation; unstimulated whole blood and PHA-stimulated whole blood were used as negative and positive controls, respectively.

As shown in Fig. 1, cholera patients demonstrated a significant increase in production of IFN-γ and IL-17 in response to ex vivo stimulation with V. cholerae MP. There was also a trend toward an increase in IL-13 in V. cholerae MP-primed cells (P = 0.06). There was no significant increase seen in IL-10. Similarly, there were no significant differences in cytokine levels in the unstimulated controls (see Fig. S2 in the supplemental material) or the PHA-stimulated controls at different time points, indicating that the priming of IFN-γ and IL-17 secretion in peripheral blood demonstrated on day 7 was V. cholerae antigen specific (see Fig. S1 in the supplemental material). The increased IFN-γ response to V. cholerae antigen stimulation remained detectable in the peripheral circulation through day 30 after infection. In contrast, subjects who received the WC-CTB vaccine did not develop a significant increase in IFN-γ, IL-13, or IL-17 production in response to ex vivo stimulation of whole blood with V. cholerae MP. There was a modest trend suggestive of increasing IL-10 responses after two doses of the cholera vaccine, but this did not reach statistical significance (P = 0.10).

Cytokine responses of specific V. cholerae antigen-stimulated CD4+ T-cell populations. Because the measurement of cytokine concentrations in cell culture supernatants does not distinguish the precise cellular origin of individual cytokines, we also performed flow cytometry-based intracellular cytokine staining (ICS) on PBMCs stimulated with V. cholerae MP to compare the proportions of individual CD4+ T cells expressing cytokines characteristic of Th1 (IFN-γ) and Th2 (IL-13) lineages.

Previous studies have focused on the ratio of Th1/Th2 responses as an important determinant of the mucosal immune response (12, 13, 26). As shown in Fig. 2C, infection with V. cholerae resulted in a shift toward a Th1 to Th2 response to V. cholerae MP stimulation, with a >2-fold increase in the ratio of IFN-γ-expressing/IL-13-expressing CD4+ T cells on day 7 following cholera compared to the ratios on day 2 (P = 0.008) as well compared to the ratios for healthy controls (P < 0.001). In contrast, no change in the ratio of CD4+ T cells expressing IFN-γ/IL-13 in response to stimulation with V. cholerae MP was seen at any time point after vaccination compared to the baseline measurement (P = 0.248 at day 7 and P = 0.203 on day 21). Furthermore, no change in the ratio of IFN-γ-expressing/IL-13-expressing cells was seen in patients or vaccinees in response to PHA stimulation, indicating that the increased Th1 and Th2 response observed in patients was specific to V. cholerae antigenic stimulation.

Confirmation of a mucosal IL-17 response in cholera patients. Cholera is a prototypical noninvasive noninflammatory diarrheal illness, and therefore, we had not anticipated a significant IL-17 response to V. cholerae antigen stimulation in peripheral blood of cholera patients. To confirm the induction of Th17 responses in mucosal tissues in cholera patients, we performed immunohistochemistry to examine IL-17 expression in duodenal biopsy specimens of patients recovering from cholera. As shown in Fig. 3, increased expression of IL-17 in situ was demonstrated in the lamina propria during acute cholera (day 2) compared to the expression on subsequent days and in control subjects, suggesting that infection with V. cholerae induces mucosal Th17 responses in lamina propria cells during the acute phase of illness that can subsequently be detected in the circulation by measuring responses of CD4+ T cells to V. cholerae antigenic stimulation.

We further recovered LPLs from duodenal biopsy specimens obtained immediately during acute illness (day 2), later in convalescence (day 30), and in healthy controls. LPL cell culture supernatants were used to measure cytokine expression, as cytokines secreted by the cultured LPLs on day 2 likely reflect the functional phenotype of these cells stimulated in vivo during V. cholerae O1 infection. As shown in Fig. 4, there was a trend toward an elevation of IL-17 in acute cholera (P = 0.07 compared to day 30, P = 0.09 compared to healthy controls), as well as increases in other proinflammatory cytokines, including IL-6 (P = 0.007 compared to day 30, P = 0.04 compared to healthy controls), which, along with TGF-β, is essential for the initial development of Th17 responses. Despite the lack of a clinically significant systemic inflammatory
response in cholera, IL-8 was also increased during acute illness \((P = 0.032\) compared to day 30). In contrast, TGF-β was expressed by LPLs at similar levels in acute- and convalescent-phase cholera samples.

**DISCUSSION**

Infection with *V. cholerae* provides long-term protection against subsequent disease (5, 19, 21). Although the mechanism(s) of protective immunity is not well understood, protection may be mediated by anamnestic immune responses to *V. cholerae* antigens (16, 25, 35). In support of this, memory-B-cell responses to *V. cholerae* protein antigens remain detectable for at least 1 year after infection, longer than circulating antibodies or circulating memory-B-cell responses to *V. cholerae* O1 lipopolysaccharide (16). Because helper T cells play a critical role in the development of long-lived memory B cells directed against protein antigens (8), we have hypothesized that the initial CD4\(^+\) T-cell response to *V. cholerae* infection may contribute to long-term protection against cholera.
Previously, we observed that patients recovering from cholera develop gut-homing central and effector memory CD4+ T-cell immune responses to *V. cholerae* protein antigens that peak in the circulation 7 days after cholera (3, 36). In this study, we characterized the functions of the cellular immune responses to *V. cholerae* O1 in cholera patients and vaccinees by measuring the lineage-specific CD4+ T-cell responses to *ex vivo* antigenic stimulation.

The paradigmatic cellular immune response to mucosal infection involves Th2-regulated IgA production; however, there are examples where Th1 or Th17 responses may be critical determinants of protective immunity against potentially invasive mucosal pathogens (12, 13, 23, 26). Because cholera is prototypical of a noninflammatory, toxin-mediated diarrhea (17), it was plausible that the disease would induce a CD4+ T-cell response skewed predominantly toward a noninflammatory Th2 response to *V. cholerae* antigens.

However, the findings of this study do not support this hypothesis. Instead, infection with *V. cholerae* primed Th1 and Th17 responses, with a shift toward Th1 to Th2 CD4+ T-cell responses. Despite the absence of overt pyogenic infection and gross histological changes in the gut during cholera, these responses are preceded by a mucosal innate immune response to infection that includes increases in IL-1β, IL-6, and IL-8 secretion by lamina propria lymphocytes. These results are consistent with increasing evidence that early responses to *V. cholerae* O1 infection include the upregulation of expression of proteins with presumed bacterial inhibitory properties, including lactoferrin and long palate, lung, and nasal epithelium clone 1 (IPLUNC1) (14, 28), and the migration of neutrophils to the lamina propria and epithelium during acute cholera (14, 28). It was previously shown that IL-1β expression is increased during acute cholera, a finding reproduced in this study. However, the finding that IL-6, a critical bridge between innate and adaptive immunity through the promotion of B-cell IgA class switch differentiation and T-cell differentiation (Th17 lineage), was increased in acute cholera is an extension of these findings with potential implications for the development of long-term immunologic memory.

Despite the similar antigenic composition of the WC-CTB vaccine and live *V. cholerae* O1 organisms encountered in natural infection, we observed significant differences in the CD4+ T-cell response in cholera patients and vaccinees. Compared to natural infection with *V. cholerae* O1, vaccination resulted in limited priming of cellular immune responses to *V. cholerae* antigens. In addition, while subjects recovering from cholera developed a shift toward increased Th1/Th2 CD4+ T-cell responses, a comparable shift in the Th1/Th2 ratio of T-cell cytokine production was not observed in subjects who received two doses of the vaccine.

The finding that natural cholera induces proinflammatory CD4+ T-cell responses greater than those seen in WC-CTB vaccine recipients is consistent with *in vitro* and murine models of the mechanisms of the adjuvant effects of CT holotoxin. In mice, the effectiveness of CT as an adjuvant is dependent on the presence of CD4+ T cells (18). The CT holotoxin induces a broad spectrum of CD4+ T-cell responses, including Th1, Th2, Th17, and regulatory T-cell responses, to coadministered antigens (20, 22). Recent evidence, also from a murine model, suggests that the induction of Th17 responses is essential for the mucosal adjuvant activity of CT holotoxin; specifically, the induction of an IL-17 response by CT is required to generate protective mucosal IgA antibody responses to coadministered antigens (11). Our clinical observations here similarly demonstrate that humans with natural cholera (and, hence, exposure to CT holotoxin) also develop *V. cholerae* antigen-specific mucosal IL-17 responses, despite the absence of overt pyogenic infection.

In contrast to the adjuvant effect of the CT holotoxin, the mucosal application of only CTB, the GM1 ganglioside-binding portion of the toxin, results in the differentiation of both adaptive and natural regulatory T cells that produce IL-10 and suppress Th1, Th2, and Th17 responses and promote tolerance to coadministered antigens (10, 22, 32). Thus, the presence of CTB in the WC-CTB vaccine may provide a possible mechanism by which vaccine-induced CD4+ T-cell responses to *V. cholerae* MP may be diminished or skewed toward the development of a Th2 T-cell phenotype. This may also explain why in initial clinical trials, the protective efficacy of the whole-cell-only vaccine (without CTB) against cholera was more persistent and ultimately afforded protection that exceeded the protection afforded by the WC-CTB vaccine by the third year of follow-up (62% for the whole-cell-only vaccine compared to 40% for the WC-CTB vaccine) (6, 7). Because the more recently licensed Shanchol vaccine does not include CTB, it may elicit a different profile of cellular immune responses to *V. cholerae* protein antigens than the WC-CTB vaccine and requires additional study.

In this study, we observed robust CD4+ T-cell immune responses in cholera patients that fell outside the predicted model for a noninvasive mucosal infection. However, in future studies, it will be critical to gauge the significance of these early CD4+ T-cell responses to *V. cholerae*. Because memory-B-cell responses to protein antigens persist longer than serum antibody responses, it may be useful to evaluate the association between early T-cell responses and the subsequent development and maintenance of long-term memory B cells. In addition, future studies are needed to examine the role of the CXCRI5-expressing follicular helper T cells, which likely play a particularly important role in regulating B-cell proliferation and class switching (24, 27). These studies may help further define the role of early T-cell responses in determining longlasting immunity to cholera.

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