Mucosal Immunologic Responses in Cholera Patients in Bangladesh

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Vibrio cholerae O1 causes dehydrating diarrhea with a high mortality rate if untreated. The infection also elicits long-term protective immunity. Since V. cholerae is noninvasive, mucosal immunity is likely important for protection. In this study, we compared humoral immune responses in the duodenal mucosa and blood of cholera patients at different time points after the onset of disease and compared them with those of healthy controls (HCs). Immune responses to lipopolysaccharide (LPS) and the recombinant cholera toxin B subunit (rCTB) were assessed by enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISPOT) assay. Significant increases in V. cholerae LPS-specific IgA and IgG antibody levels were seen in duodenal extracts on day 30, but the levels decreased to baseline by day 180; plasma V. cholerae LPS-specific IgA levels remained elevated longer. Levels of mucosal CTB antibodies also peaked on day 30, but the increase reached statistical significance only for IgG. A significant correlation was found between the CTB antibody-secreting cell (ASC) response in the circulatory system on day 7 and subsequent CTB-specific IgA levels in duodenal extracts on day 30 and the numbers of CTB-specific IgA ASCs in duodenal tissues on day 180. The proportion (0.07%) of mucosal V. cholerae LPS IgA ASCs peaked on day 30 and remained elevated through day 180 compared to that of HCs (P = 0.03). These results suggest that protective immunity against V. cholerae is not likely mediated by the constitutive secretion of antibodies at the mucosal surface; our results are consistent with those of other studies that suggest instead that anamnestic immune responses of mucosal lymphocytes may play a major role in protection against cholera.

Vibrio cholerae O1 is a noninvasive mucosal pathogen that colonizes the surface of the small intestine and elaborates cholera toxin (CT), an ADP-ribosylating toxin that causes secretory diarrhea. Although infection with V. cholerae induces protection against subsequent disease for several years (7, 10), the mechanisms of protective immunity are not understood (13). However, because V. cholerae is noninvasive, it has been hypothesized that protection is effected by antibodies at the mucosal surface.

The best-characterized marker of protective immunity to cholera is the plasma vibriocidal antibody, a complement-dependent bactericidal antibody that increases with age in areas in which cholera is endemic and is associated with protection from infection with V. cholerae. However, it is unlikely that vibriocidal antibodies directly mediate protection because there is no threshold vibriocidal-antibody titer at which protection against cholera is achieved (19) and studies with volunteers from areas where cholera is not endemic demonstrate that protective immunity to cholera persists even after vibriocidal-antibody titers have declined to undetectable levels (11).

Secretory IgA antibody (sIgA) is the predominant isotype on mucosal surfaces. The local IgA response is believed to play a major role in protective immunity from diarrhea caused by V. cholerae, since cholera is a gut-restricted, noninvasive mucosal infection. Cholera also induces both plasma IgG and IgA responses to V. cholerae antigens, but only levels of circulating V. cholerae-specific IgA antibodies are associated with protection (9). However, like plasma vibriocidal-antibody titers, plasma IgA responses remain elevated for only 6 to 12 months after cholera infection (8), while protective immunity after clinical cholera infection lasts substantially longer (7).

Because protective immunity after V. cholerae infection persists longer than detectable increases in serum antibodies, it has been hypothesized that protective immunity is generated by rapid anamnestic responses of memory B cells in the gut-associated lymphoid tissue (GALT) or in the blood. In support of this hypothesis, we found that circulating V. cholerae-specific memory B cells remain detectable for at least 1 year after cholera infection and persist longer than traditional measures of immunity to cholera (8). Furthermore, volunteers rechallenged with cholera toxin B (CTB) 15 months after initial exposure were able to respond with a rapid rise in detectable levels of sIgA in intestinal lavage in as little as 3 days after

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exposure (21), which is suggestive of an anamnestic response in mucusal tissues.

An alternative hypothesis is that the mechanism of protective immunity against *V. cholerae* infection is the ability of antibodies produced by antibody-secreting cells (ASCs) located in the lamina propria to maintain protection against *V. cholerae* upon reexposure. This hypothesis is consistent with the finding that the majority of *V. cholerae* antigen-specific ASCs, which are detected only transiently in the blood after cholera infection, express intestinal rehoming markers (17) and likely remain localized in mucosal tissues long after infection. For this reason, mucosal antibody levels may not be adequately measured by monitoring peripheral markers of immunity, such as the virobiocidal antibody, *V. cholerae*-specific-IgA, or other short-lived ASCs in the blood.

To evaluate these hypotheses regarding the nature of protective immunity to cholera, we directly measured mucosal immune responses in duodenal biopsy specimens from a cohort of 18 otherwise-healthy adults recovering from severe cholera infections. Specifically, we addressed the following questions: (i) whether the levels of *V. cholerae* antigen-specific ASCs and antibodies are increased for a longer period after cholera in the duodenal lamina propria than in the blood and (ii) whether there is a correlation between the levels of *V. cholerae* antigen-specific mucosal antibodies and ASCs in duodenal biopsy specimens and measurements of *V. cholerae* antigen-specific immune responses in the blood.

**MATERIALS AND METHODS**

**Study subjects.** Eighteen individuals admitted to the hospital of the International Centre for Diarrheal Diseases Research, Bangladesh (ICDDR,B), with acute cholera were enrolled in the study. The study was approved by the institutional review boards of the ICDDR,B and Massachusetts General Hospital, Boston, Massachusetts. Five healthy adults with asymptomatic *Helicobacter pylori* infection, evaluated as part of a separate study, were used as the healthy control group for comparison.

Duodenal biopsy specimens of around 1 mm² in diameter were obtained by standard forceps (Radial Jaw megabite; Boston Scientific) during acute infection (the second day of hospitalization after stabilization, termed day 2 in the study) and again on days 30, 180, and 360 after the onset of illness. For each blood sample, we measured the vibriocidal-antibody titer, the levels of IgG and IgA ASCs, and the proportions of circulating IgG and IgA ASCs specific to CTB and the homologous serotype of *V. cholerae* lipopolysaccharide (LPS). From the gut biopsy specimens, we measured the levels of IgG and IgA antibodies to CTB and LPS in duodenal extracts and the proportions of antigen-specific IgG and IgA ASCs among extracted lamina propria lymphocytes (LPLs), using an enzyme-linked immunospot (ELISPOT) procedure.

**Sample preparation.** Heparinized blood was diluted in phosphate-buffered saline. After the blood solution was centrifuged on a Ficoll-Isopaque instrument (LPLs), LPS IgG ASC responses were not measured because of the limited number of B cells. Wells were coated with KLH (Pierce Biotechnology, Rockford, IL) (2.5 μg/ml) for use as a negative control. For duodenal LPLs, LPS IgA ASC responses were not measured because of the limited number of cells obtained.

**Vibriocidal-antibody assay and CTB and LPS ELISAs in plasma.** The vibriocidal assay was performed as previously described, using guinea pig complement and the homologous serotype of *V. cholerae* O1 Ogawa (X-25049) or Inaba (T-19479) as the target organism (17). The vibriocidal titer was defined as the reciprocal of the highest plasma dilution that resulted in more than a 50% reduction in the optical density compared to that of the control wells without plasma. The CTB- and LPS-specific IgA and IgG responses in extracts from biopsy specimens (1) and plasma of patients and healthy controls were quantified using standardized ELISA protocols (8, 14, 17). Briefly, to quantify antibodies to CTB, ELISA plates were coated with ganglioside GM1 (0.3 nM/ml) followed by recombinant CTB (2.5 μg/ml) (gifts from A. M. Svennerholm, Gothenburg University). To quantify antibodies to LPS, ELISA plates were coated with the homologous serotype of *V. cholerae* LPS (2.5 μg/ml) (14). For each antigen, 100 μl either of saponin-treated extracts of biopsy specimens (duodenal extracts, 1:10 dilution) or of plasma (1:100 dilution) diluted in 0.1% bovine plasma albumin in phosphate-buffered saline was added per well. Horseradish peroxidase-conjugated secondary antibodies to human IgG or IgA (Jackson Laboratories, Bar Harbor, ME) were applied in separate wells. After a 90-min incubation at 37°C, the plates were washed and then developed with ortho-phenylenediamine (Sigma, St. Louis, MO) in 0.1 M sodium citrate buffer and 0.1% hydrogen peroxide. Plates were read kinetically at 450 nm for 5 min. The maximal rate of change in optical density was expressed as milli-absorbance units per minute, and ELISA units were normalized by calculating the ratio of the optical density of the test sample to that of a standard of pooled convalescent-phase sera from patients recovered from cholera run as a positive control on each plate.

**Statistical analyses.** Comparisons of immunologic responses were tested for significance using the Mann-Whitney U test. All reported *p* values are two tailed, with a cutoff *p* value of <0.05 considered the threshold for statistical significance.

**RESULTS**

**Study population.** Demographic, microbiologic, and clinical characteristics of the patients studied are presented in Table 1.
Eighteen patients (median age, 30 years; male, 17; female, 1) were enrolled in the study. Patients and controls were excluded from the study if they had a history of illness consistent with cholera in the previous year.

**Vibriocidal responses.** Consistent with those in previous studies, all patients in this cohort mounted strong vibriocidal responses and demonstrated seroconversion, represented by a 4-fold-or-greater rise in their vibriocidal titers (Fig. 1). The vibriocidal-antibody titer peaked on day 7 (geometric mean [GM] count, 2,281; 95% confidence interval [CI], 954.7 to 5,449; \( P < 0.001 \)) and remained significantly elevated through day 90 before declining to baseline levels at day 180 (GM, 95; 95% CI, 24 to 363; \( P < 0.1 \)).

**Antibody responses in plasma and in duodenal extracts.** In duodenal extracts, CTB-specific IgG and IgA antibody responses peaked at day 30, although the difference between day 2 and day 30 was statistically significant only for IgG antibodies (Fig. 2). In contrast, increases in the levels of CTB-specific IgA antibodies in plasma were seen through day 90 and in the levels of CTB-specific IgG antibodies in plasma until day 180, suggesting that CTB-specific antibodies remain elevated for a longer time in the plasma than in the duodenal mucosa. Levels of *V. cholerae* O1 LPS-specific IgG and IgA antibodies in the duodenal extracts were significantly elevated on day 30 compared to those for day 2 samples and controls. These levels declined to baseline by day 180, even though *V. cholerae* O1 LPS-specific IgA antibodies in plasma remained elevated through day 180. These results suggest that after cholera infection, preformed antibodies do not persist longer at the intestinal mucosal surface than in the blood.

**Peripheral and mucosal antibody-secreting cell responses.** Circulating *V. cholerae* antigen-specific ASC responses are short-lived after cholera infection (16). IgG and IgA ASC responses against *V. cholerae* LPS and CTB were all significantly increased on day 7 but declined to baseline levels by day 30 (Fig. 3). The proportions of mucosal CTB-specific IgA and IgG ASCs were slightly elevated on day 30 but did not differ significantly between study days or between patients and healthy controls. In contrast, the numbers of *V. cholerae* O1 LPS-specific IgA ASCs in LPLs peaked on day 30 and were found to be significantly higher in cholera patients than in healthy controls on days 2, 30, and 180, suggesting a relatively early response to LPS and the long-term persistence of LPS-specific IgA ASCs in the duodenal mucosa.

**Mucosal and systemic B cell responses.** The percentages of B cell, memory B cell, and gut-homing B cell populations in both duodenal LPLs and PBMCs from patients and healthy controls were quantified on different study days. We observed significantly higher gut-homing CD19+ cells (CD19+ β7+) at day 30 (\( P < 0.007 \)) and day 180 (\( P < 0.008 \)) in biopsy specimens from patients than in those from healthy controls (Fig. 4C), although there were no differences in the overall levels of CD19+ (Fig. 4A) and CD19+ CD27+ (Fig. 4B) B cells in the blood or duodenal mucosa of patients and those of healthy controls.

**Correlation between peripheral and duodenal markers of mucosal immunity to cholera.** Because the peak in circulating ASCs includes a large population of cells that are rehoming to the gut mucosa (16), we evaluated the correlation between the magnitude of the peak *V. cholerae* antigen-specific ASC response on day 7 and the subsequent levels of antigen-specific ASCs and IgA antibodies in gut mucosa (Table 2). We observed a significant correlation between the peak CTB ASC response on day 7 in the blood and the subsequent amount of CTB-specific IgA antibodies in duodenal tissue on day 30 and the number of CTB-specific IgA ASCs in duodenal tissue on day 180. However, there were no correlations observed between the peak in circulating LPS ASC responses on day 7 and subsequent mucosal LPS responses. Also of note, there was no significant correlation observed between simultaneous measurements of anti-
Peripheral markers of humoral immunity to cholera decrease to baseline levels before protective immunity against cholera ends. Possible explanations are that constitutive antibody secretion at the mucosal surface maintains protection upon reexposure and that increased levels of mucosal antibody secretion persist longer than detectable increases in circulating antibodies. However, in direct opposition, we found that the increases in circulating CTB- and LPS-specific antibodies actually persist for a longer time than the increases in the levels of these antibodies in duodenal tissue following cholera infection. This finding suggests that it is unlikely that protective immunity to cholera is mediated by constitutive antibody secretion at the mucosal surface.

In contrast, we did find that cholera patients had persistent increases in *V. cholerae* LPS-specific ASCs in duodenal tissue after cholera infection. *V. cholerae* LPS-specific IgA ASCs remained at significantly increased levels compared to those for healthy controls at least until day 180, long after circulating IgA ASCs returned to baseline levels. Although *V. cholerae* LPS-specific ASCs on day 180 represented only 0.07% of all lamina propria IgA ASCs, the lamina propria harbors 80% of all ASCs in humans (6), and it is possible that even a relatively small proportion of *V. cholerae* LPS-specific ASCs in the lamina propria may allow protective immunity against cholera.

Interestingly, the persistent increase in *V. cholerae* LPS-specific ASCs remained detectable despite the absence of detectable increases in IgA antibodies in mucosal tissues. This finding is of potential interest, since it has been recently demonstrated that IgA plasma cells may not secrete antibodies until induced to do so by the cessation of ongoing stimulation with the cytokine B cell-activating factor (5) or...
upon an encounter with an antigen (22). The lack of correlation between the proportion of LPS-specific ASCs in the lamina propria and tissue antibody levels seen in our study raises the possibility that some of the persistent *V. cholerae* LPS-specific ASCs detected by ELISPOT assay in the lamina propria may produce substantial quantities of antibodies only in response to certain stimuli on reexposure.

While such long-lasting *V. cholerae* LPS-specific mucosal ASCs may contribute to protective immunity, an alternative possibility is that memory B cells that are capable of both

FIG. 3. Peripheral blood and duodenal antigen-specific IgA and IgG ASC responses. Trends for anti-CTB IgA (A)-, anti-CTB IgG (B)-, anti-LPS IgA (C)-, and anti-LPS IgG (D)-specific ASC levels in patients and healthy controls on different study days are shown. Data are normalized to total IgA or IgG and expressed as percentages. *, statistically significant differences ($P \leq 0.05$).

FIG. 4. Peripheral blood and duodenal B cell responses in cholera patients on different days after the onset of infection compared to those in healthy controls. (A) Percentages of CD19$^+$ cells in total lymphocytes. (B and C) Percentages of CD19$^+$ CD27$^+$ (B) or CD19$^+$ $\beta7^+$ (C) cells among total CD19$^+$ cells. *, statistically significant difference ($P \leq 0.05$).
TABLE 2. Correlations between day 7 circulatory system IgA ASC responses and subsequent duodenal responses to specific antigens

| Antigen | First measurement (day) | Second measurement (day) | R⁰ | P valueb |
|---------|------------------------|--------------------------|----|---------|
| CTB     | Circulating IgA ASCs (7) | Duodenal ASCs (30) | 0.42 | 0.11 |
|         |                        | Antibodies in duodenal extract (30) | 0.78 | 0.008 |
|         |                        | Duodenal ASCs (180) | 0.77 | 0.04 |
| LPS     | Circulating IgA ASCs (7) | Duodenal ASCs (30) | 0.13 | 0.67 |
|         |                        | Antibodies in duodenal extract (30) | 0.16 | 0.77 |
|         |                        | Duodenal ASC (180) | 0.24 | 0.61 |

⁰ Spearman rank correlation coefficient showing the correlation between the first and second measurements.

b P values showing the significance between the first and second measurements.

proliferation and differentiation into plasma cells may mediate anamnestic responses that result in the amplification of humoral immunity on reexposure to an antigen. We have already found evidence that *V. cholerae* antigen-specific memory B cells persist in the blood much longer than antibodies and thus may contribute to long-term protection upon reexposure. However, we were not able to measure memory B cells in the duodenal biopsy specimens in this study because of the limited numbers of cells obtained.

In our previous study of memory B cell responses in blood after cholera infection (8), there were significant differences in the development of memory B cells into the T-cell-independent antigen LPS and the T-cell-dependent antigen CTB. We previously observed that peripheral IgG memory B cell responses to the protein antigen CTB persisted for a full year after cholera infection, but IgG memory B cell responses to LPS in blood waned more rapidly. Here, in mucosal tissues, we found the converse: *V. cholerae* LPS-specific IgA ASC responses in lamina propria persisted longer than IgA ASC responses to CTB in lamina propria. The lamina propria is a site of T-cell-independent antigen-induced IgA class switch recombination in the intestine, while T-cell-dependent IgA class switching is restricted to follicular lymphoid tissues (4). Our results suggest that LPS-specific IgA ASCs, derived from T-cell-independent IgA class switch recombination, may contribute to repopulating the lamina propria for a longer time than ASCs derived from follicular T-cell-dependent class switch recombination.

We found that the levels of circulating CT IgG and LPS IgG ASCs increased significantly at day 7 compared to those at the acute stage of infection and in healthy controls and then declined by day 30. However, we were not able to see any major differences in IgG ASC responses against CT in the gut. It has been shown previously that CTB IgG responses increased in the circulatory system after oral immunization with killed whole-cell enterotoxigenic *Escherichia coli* (ETEC) vaccine containing toxoid antigen (15, 24), as well as after natural cholera infection (16, 17) or ETEC diarrhea (16). Moreover, Quiding et al. (18) observed striking differences between duodenal and peripheral CTB ASC responses after oral immunization with B-subunit whole-cell (B-WC) cholera vaccine (18), with responses higher in the duodenum than in the circulatory system after vaccination. However, here we saw a different response following severe cholera infection. Further studies using intestinal immunocytes of the IgG antibody isotype (20) are needed.

The lack of correlation between concomitant measures of plasma *V. cholerae* antigen-specific IgA and ASCs and mucosal-tissue IgA and ASCs underscores the difficulty in identifying circulating markers of a protective immune response that is primarily dependent on anamnestic mucosal immune responses. Based on this study, the initial peak in CTB-specific ASCs is the best marker for a subsequent mucosal response to CTB. However, the T-cell-independent response to LPS appears to have different kinetics, and the initial ASC response in blood is not predictive of the ultimate magnitude of the mucosal anti-LPS response. The development of novel noninvasive assays for measuring anamnestic mucosal immune responses to antigenic challenge (somewhat analogous to the use of a tuberculin skin test) may ultimately provide better measurements of protective immunity against cholera and other mucosal infections than the measurement of circulating antibodies or ASC responses.

By flow cytometry, we have previously shown that the level of gut-homing B cells in the blood increases on day 7 compared to those in healthy controls and on day 2 of the illness (2). In this study, we found that the numbers of gut-homing CD19⁺ cells also increased significantly in the lamina propria late after infection, on days 30 and 180, compared to those on day 2 and in healthy controls. This finding suggests that gut-homing CD19⁺ cells may take some time to home back to the gut from the circulatory system. The baseline levels of CD19⁺ and gut-homing CD19⁺ cells in biopsy specimens were also relatively high in our healthy Bangladeshi controls, perhaps triggered by infection with other pathogens and the resulting recruitment of lymphocytes to this site (3). Further studies are needed to better understand the kinetics of antigen-specific B cell responses in the duodenal mucosa of *V. cholerae*-infected patients.

Taken together, our data are consistent with those of previous studies and provide additional evidence suggesting that protective immunity to cholera is not likely to be mediated by constitutive antibody secretion at the mucosal surface at the time of reinfection with *V. cholerae*. However, our data do not differentiate between the contributions to protective immunity of persistent *V. cholerae* LPS-specific IgA ASCs in the lamina propria or memory B cells capable of anamnestic immune responses upon reexposure. Additional studies are needed to address whether canonical memory B cells are present in mucosal tissue and whether these and/or circulating memory B cells contribute to protective immunity after cholera infection.

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