Enumeration of Gut-Homing β7-Positive, Pathogen-Specific Antibody-Secreting Cells in Whole Blood from Enterotoxigenic *Escherichia coli* and *Vibrio cholerae*-Infected Patients, Determined Using an Enzyme-Linked Immunosorbent Spot Assay Technique

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*Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) are noninvasive mucosal pathogens that cause acute watery diarrhea in people in developing countries. Direct assessment of the mucosal immune responses to these pathogens is problematic. Surrogate markers of local mucosal responses in blood are increasingly being studied to determine the mucosal immune responses after infection. However, the volume of blood available in children and infants has limited this approach. We assessed whether an approach that first isolates β7-positive cells from a small volume of blood would allow measurement of the antigen-specific immune responses in patients with cholera and ETEC infection. β7 is a cell surface marker associated with mucosal homing. We isolated β7-expressing cells from blood on days 2, 7, and 30 and used an enzyme-linked immunosorbent spot (ELISPOT) assay to assess the gut-homing antibody-secreting cells (ASCs) specific to pathogen antigens. Patients with ETEC diarrhea showed a significant increase in toxin-specific gut-homing ASCs at day 7 compared to the levels at days 2 and 30 after onset of illness and to the levels in healthy controls. Similar elevations of responses to the ETEC colonization factors (CFs) CS6 and CFA/I were observed in patients infected with CS6- and CFA/I-positive ETEC strains. Antigen-specific gut-homing ASCs to the B subunit of cholera toxin and cholerab-specific lipopolysaccharides (LPS) were also observed on day 7 after the onset of cholera using this approach. This study demonstrates that a simple ELISPOT assay can be used to study the mucosal immunity to specific antigens using a cell-sorting protocol to isolate mucosal homing cells, facilitating measurement of mucosal responses in children following infection or vaccination.

Enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae O*1 are two major bacterial causes of severe acute watery diarrhea in developing countries (1). The two pathogens are noninvasive mucosa-associated organisms that colonize the small intestine, resulting in losses of fluid and electrolytes due to the action of enterotoxins. Acute infectious diarrhea is the second most common cause of death in children living in developing countries, surpassed only by acute respiratory diseases, and accounts for approximately 20% of all childhood deaths (2). Cholera toxin (CT) and lipopolysaccharides (LPS) are well-characterized antigens and virulence factors of *V. cholerae* O1. For ETEC, the heat-labile (LT) and heat-stable (ST) enterotoxins, as well as colonization factors (CFs), are important virulence factors (3–5). The CFA/I antigen has been recognized as an important colonization factor of ETEC and has frequently been detected in ETEC isolated from patients with diarrhea. The colonization factor CS6 has been shown to promote binding of ETEC to rabbit and human enterocytes and has frequently been present on ETEC isolated from humans with diarrhea (6, 7).

During natural infection and after vaccination, protection from noninvasive pathogens results from induction of a relevant antibody response. These antibodies are usually quantified in body fluids using immunological assays, solid-phase immunoassays, or functional tests (8). However, because of the anatomical compartmentalization of the adaptive immune system, measurement of antibody responses themselves in blood may not predict local immunogenicity against noninvasive mucosal infections for which protection is often mediated by local responses at mucosal surfaces. This anatomical compartmentalization also constitutes a major challenge when one is attempting to measure immune responses in other mucosal secretions, as responses induced at one given mucosal site, e.g., the small intestine, are not always reflected in secretions from distant mucosal tissues (9, 10). Therefore, the best approach for assessing local mucosal immune responses to infection is not necessarily obvious. None of the techniques developed so
far has gained general acceptance because of considerable variations in the content of mucosal secretions and practical difficulties in collecting these fluids or materials, especially in newborns and young infants. Furthermore, measurement of both the mucosal and systemic immune responses in neonates and young infants can be confounded by interference from maternal antibodies derived from breast milk or transudation of serum antibodies (IgG antibodies across the placenta). Specific antibody-secreting cells (ASCs) represent a subpopulation of immunoglobulin-secreting cells (ISCs) producing antibodies of known antigen specificity (e.g., against vaccine antigens). Previous work has shown that there is a transient migration of ASCs from intestinal tissue through the systemic circulation following mucosal stimulation (11). These cells are maturing and rehomogenizing to intestinal tissue and bear mucosal homing markers such as β7 (12), and their presence in the peripheral circulation is claimed to peak at days 6 to 8 following illness or vaccination (13). These cells can be directly evaluated, and immunoglobulins produced by these cells can be detected as secreted products in the supernatant of in vitro-cultured peripheral blood mononuclear cells (PBMCs) (14, 15). However, these and related assays usually require at least 3 to 5 ml of blood. As such, sensitive and specific procedures are needed to determine the responses in PBMCs that reflect prior mucosal infection or vaccination using the smaller volumes of blood that are available from young children and infants. To address this, we carried out a study that used a technique developed by Saletti et al. (13) to enrich gut-homing cells from blood using the gut-homing surface marker β7, and we validated this approach by collecting only 1 ml of blood and assessing the gut-associated antigen-specific immune responses in patients with cholera and ETEC infection in Bangladesh.

MATERIALS AND METHODS

Study groups and recruitment. We enrolled adult patients hospitalized at the Dhaka Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), who presented with severe acute watery diarrhea and gave informed consent. Patients whose stool specimens were positive for V. cholerae O1 and negative for other enteric pathogens (Shigella, Salmonella, and ETEC) were enrolled as cholera patients, while patients whose stool specimens were positive for ETEC but negative for V. cholerae O1 and other enteric pathogens were enrolled as ETEC-infected patients. Screening of stool specimens for other common enteric pathogens to exclude patients with coinfections was done as previously described (16, 17). Cholera patients recruited for the study were all infected with the V. cholerae O1 Ogawa serotype. The strains in the ETEC-infected patients recruited for the study were analyzed for expression of specific colonization factors, including the CFA/I group or CS6, as well as LT, LT/ST, or ST alone, by dot blot and PCR, respectively (18, 19). Healthy adult individuals, who had no history of diarrhea, fever, or antibiotic use in the 2 weeks prior to enrollment, were recruited as control participants and were matched by age and socioeconomic status to the V. cholerae/ETEC-infected participants. This study was approved by the research review and the ethical review committees of the icddr,b.

Subjects and sample collection. Heparinized venous blood samples were collected by venipuncture on days 2, 7, and 30 following the onset of hospitalization for V. cholerae-infected (n = 15) and ETEC-infected (n = 41; CFA/I group, n = 14; CS6, n = 8; CS7, n = 1; and CF negative, n = 18) adults and healthy controls (HC, n = 9). This study was conducted at the icddr,b in Bangladesh between May 2012 and June 2014.

Plasma separation. One milliliter of blood was collected in sodium heparin tubes. After centrifugation at 600 × g for 10 min, plasma was separated and collected in Eppendorf tubes and stored at −20°C for plasma antibody analysis.

Preparation of beads coated with anti-human β7. Sheep anti-rat IgG magnetic beads (Dynabeads; Invitrogen, Norway) were placed in a 9-ml culture tube (Pyrex, USA) containing 3 ml of cold fluorescence-activated cell sorter (FACS) buffer. For each blood specimen (1 ml), 25 μl of beads (107 beads) was used. The tube was placed in a magnet (Dynal MPC-6; Norway) for 2 min. The supernatant was aspirated without moving or detaching the tube from the magnet. The beads were washed with 3 ml of cold FACS buffer for 2 min, and this procedure was repeated twice. After removal of the tube from the magnet, beads were resuspended in FACS buffer in an initial volume of 25 μl/blood sample. Rat anti-human integrin β7 (BD Pharmingen, USA) was added, and the mixture was incubated for 30 min at 2 to 8°C with gentle tilting and rotation. Finally, the beads coated with anti-human β7 were added to mononuclear cells prepared as described below.

Cell preparation. After separation of plasma, the remaining blood was transferred into a Falcon tube (BD, USA) and mixed with 30 ml lysing solution (1 M NH4Cl solution) for lysis of red blood cells. The mixture was incubated for 7 min at room temperature and then centrifuged. The supernatant was removed (non-β7 cells), and the pellet was resuspended immediately to the initial blood volume (1 ml) in FACS buffer. The cells were washed twice and were resuspended in 1 ml FACS buffer and transferred to freshly prepared beads coated with anti-human β7 in a glass tube as described above. The cells and beads were incubated at 4°C for 45 min on a horizontal shaker. The tubes were then placed into the magnet, and the supernatant was aspirated. Cells were resuspended in RPMI complete medium (Gibco, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). After being counted, bead-separated mononuclear cells were plated onto the enzyme-linked immunosorbent spot assay (ELISPOT) plate for determination of the total immunoglobulin and the antigen-specific spots to ETEC and V. cholerae antigens. Depending on the retrieval of cells, 2,500 to 5,000 cells were used for determining the total immunoglobulin spots and 15,000 to 20,000 cells were used for detecting the antigen-specific spots; duplicate wells were used for the total immunoglobulin spots, while single wells were used for the antigen-specific spots. For the antigen-specific ASC enumerations, ELISPOT wells previously coated with specific antigens (i.e., LPS, cholera toxin B subunit [CTB], and CFA/I group colonization factor antigens) were prepared. Similarly, the total immunoglobulin-secreting cells (ISCs) irrespective of antigen specificity were enumerated in parallel wells coated with a mixture of affinity-purified goat antibodies to human IgM and light chains (17). The use of a horseradish peroxidase (HRP)-dependent two-color (3-amino-9-ethylcarbazole [AEC] and 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium [BCIP-NBT] substrate) ELISPOT procedure for detection has been previously described (13). The antigen-specific response was expressed as a percentage calculated as the number of antigen-specific gut-homing ASCs divided by the total gut-homing immunoglobulin-secreting cells of that same isotype; both the total ISC and the antigen-specific ISC numbers were adjusted to 106 prior to percent calculation.

Detection of CTB-specific antibodies in plasma. The recombinant choler toxin B subunit (rCTB)-specific IgA and IgG antibodies of patients infected with ETEC and V. cholerae were measured in plasma using a standardized enzyme-linked immunosorbent assay (ELISA) technique, as previously described (16, 20). As labile toxin B (LTB) has 80% nucleotide sequence homology and cross-reacts immunologically to CTB (21, 22), the plasma responses in the ETEC-infected patients were assessed using CTB as the antigen.

Statistical analysis. For the analyses of the antibody-secreting cells at different time points following infection, we used the Wilcoxon signed-rank test. We used the Mann Whitney U test to compare the
Gut-Homing Immune Responses

results between healthy participants and infected patients at different days after the onset of infection. The association between the ASC and plasma antibody responses was assessed using the Spearman correlation coefficient. We used GraphPad Prism 5.0 for statistical analyses and to generate the figures and considered a P value of <0.05 as significant.

RESULTS

Demographic characteristics of patients. In this study, we enrolled 15 adult cholera patients (median age, 30 years; 26.7% female) and 41 adult ETEC-infected patients (median age, 33.5 years; 46.3% female). In addition, 9 age-matched adult healthy controls (HC) were recruited. Of the patients infected with ETEC, 44%, 39%, and 17% were infected with LT-, LT/ST-, or ST-producing strains, respectively. The demographic, clinical, and microbiological characteristics of patients are presented in Table 1.

Total immunoglobulin responses. Total gut-homing immunoglobulin spots were enumerated per million gut-homing (β7⁺) cells at different days after diarrheal illness. Comparable levels of total gut-homing immunoglobulin-secreting cells (ISCs) were observed in cholera patients and ETEC-infected patients on different days (day 2, day 7, and day 30) after the onset of infection, and the levels in healthy controls were comparable to those on day 2 following infection (Fig. 1). In patients with cholera, we found a significant increase in total β7⁺ IgA ISCs at day 7 (Fig. 1A) compared to the numbers at day 2 (P = 0.005) and in healthy controls (P = 0.005), and the number of total β7⁺ IgA ISCs subsequently decreased at day 30 (P = 0.005). In the case of total gut-homing IgG ISCs, an increase was noted (Fig. 1B) at day 7 compared to the numbers at day 2 (P = 0.001) and in HC (P = 0.008), which then declined at day 30 (P = 0.0009). Similar to cholera patients, patients infected with ETEC also had significant increases in the numbers of total β7⁺ IgA-secreting cells at day 7 following infection (Fig. 1C) compared to the numbers at day 2 (P = 0.009) and in healthy controls (P = 0.008), and the numbers subsequently declined by day 30 (P = 0.0006) (Fig. 1C). The numbers of total β7⁺ IgG-secreting cells in patients infected with ETEC followed a pattern similar to that of total IgA ISCs (Fig. 1D).

CTB-specific responses. Antigen-specific immunospot assays were performed using CTB to assess the antigen-specific immune responses in both cholera and ETEC-infected patients. The CTB ELISPOT analyses showed increased percentages of CTB-specific gut-homing cells in patients with cholera, for both the IgA and IgG isotypes, at day 7 following infection compared to the levels at the other days following infection (days 2 and 30) and to the levels for healthy controls (Table 2). Both the IgA and IgG ASC responses to CTB in the ETEC-infected group, we found that the frequency of CTB-specific gut-homing IgA ASCs was higher at day 7 (Fig. 2C) than at day 2 (P = 0.0004) and in healthy controls (P = 0.006), and the numbers of CTB-specific gut-homing ASCs waned at day 30 compared to those at day 7 (P < 0.0001). For IgG responses, a similar pattern was observed (Fig. 2D), the percentage being elevated at day 7 compared to those at day 2 (P = 0.0007) and in healthy controls (P = 0.04) and decreasing at day 30 (P = 0.0003). The median percentages of β7⁺ IgG ASCs in both cholera patients (19.3%) and ETEC-infected patients (1.6%) were 4-fold higher than the median percentages of β7⁺ IgA ASCs (4.6% and 0.4%, respectively) at day 7 for the CTB-specific responses (Table 2). Both β7⁺ CTB-specific IgA ASC responses (median 4.6%) and the IgG ASC responses (median 19.3%) in patients with cholera were 12-fold higher than the CTB-specific responses in patients with ETEC infection (median values of 0.4% and 1.6%, respectively) (Table 2). In patients infected with ETEC strains positive only for heat-stable toxin (ST), the ELISPOT assay showed almost no response specific to CTB.

LPS-specific responses in cholera patients. We also assessed V. cholerae O1 LPS-specific responses in patients following cholera (Fig. 3). We demonstrated higher levels of IgA gut-homing ASCs on day 7 following infection than on day 2 (P < 0.0001) and in healthy controls (P < 0.0001), with responses in patients decreasing by day 30 (P < 0.0001) (Fig. 3A). Similarly, IgG gut-homing, LPS-specific ASCs were significantly elevated on day 7 (P < 0.0001) compared to the levels on day 2 and day 30 and in healthy controls (Fig. 3B).

CF-specific responses to ETEC infection. Colonization factors have a major role in ETEC infection, and immunity against colonization factors seems to play a critical role in mediating protection against ETEC infection (23, 24). For this reason, we also assessed immune responses using a CFA/I-specific ELISPOT assay for patients infected with ETEC strains expressing the CFA/I group colonization factor antigens and a CS6-
specific ELISPOT assay for patients infected with ETEC strains expressing the CS6 colonization factor alone or together with CS5. We found a significantly higher CF-specific response on day 7 than on day 2 or day 30 in patients infected with a CFA/I-positive ETEC strain and in healthy controls, respectively, for both CFA/I-specific IgA and IgG ASCs (Fig. 4A and B). We also detected significant $\beta 7^+$ immune responses to CS6 in patients infected with CS6-expressing ETEC strains. The frequencies of $\beta 7^+$ CS6-specific IgA and IgG ASCs were higher at day 7 than at day 30 and in healthy controls (Fig. 4C and D). We did not conduct the CS6-specific ELISPOT assay on day 2 as we had limited CS6 antigen and detection of this CF in stool isolates by the dot blot test required at least 2 days. Interestingly, patients with ETEC infections had stronger $\beta 7^+$ immune responses against the CS6 antigen (median of 4.6% of IgA ASCs and median of 11% for IgG responses on day 7) than against the CFA/I group (median values of 2.3% and 0.8% on day 7, respectively) (Table 2). No CFA/I-specific gut-homing ASC responses were observed in patients infected with CF-negative ETEC strains ($n = 18$).

**Association of gut-homing ASCs with antibody responses in plasma.** To determine the potential associations between gut-homing ASC responses and antibody responses in plasma, we compared CTB-specific IgA and IgG $\beta 7^+$ ASC responses on day 7 with the corresponding antibody responses in plasma on days 7 and 30 in patients infected with *V. cholerae* O1 ($n = 15$ at day 7 and $n = 14$ at day 30) and ETEC strains ($n = 29$) expressing LT or LT/ST. We found positive correlations between CTB-specific IgA and IgG $\beta 7^+$ ASC responses at day 7 and CTB-specific IgA and IgG antibody titers on days 7 and 30 in cholera patients (Spearman $r = 0.51$ to 0.8; $P = 0.0006$ to 0.05) (Fig. 5). Similarly, in patients infected with ETEC, CTB-specific gut-homing IgA and IgG ASCs at day 7 were positively correlated with CTB-specific IgA and IgG ELISA titers on days 7 and 30 (Spearman $r = 0.37$ to 0.62; $P = 0.05$ to 0.0003) (Fig. 6).

**DISCUSSION**

This study demonstrates that the immunomagnetic separation of $\beta 7^+$-positive cells from a small volume of whole blood followed by detection of antigen-specific ASCs by the ELISPOT assay can be utilized to determine gut-homing, antigen-specific ASC responses following diarrheal infections from two major causes, cholera and ETEC. These results support further application of this simple technique for studying B cell responses against different bacterial antigens and for host cell surface markers on B cells in young children and even in infants, fol-
The main goal of enumeration of gut-homing $\beta^+$ ASCs is to determine whether a participant responds to a specific antigen or vaccine and ideally whether the response measured is predictive of clinical efficacy; i.e., it constitutes a correlate of a specific antigen- or vaccine-induced immune protection. Although there is good correlation between plasma and ELISPOT data, no consensus exists as to whether a subject is a responder based on ASC ELISPOT data. In the past, most studies on human blood ASC responses have used an arbitrary cutoff to discriminate vaccine responders from nonresponders as well as from patients with natural infections (13). In addition, the unique benefit of measuring $\beta^+$ ASC responses is immunomagnetic enrichment of mucosally derived blood lymphocytes for the detection of human ASCs.

**TABLE 2** Numbers of total $\beta^+$ ISCs and percentages of antigen-specific $\beta^+$ ASCs in cholera and ETEC infection

<table>
<thead>
<tr>
<th>Antigen</th>
<th>IgA</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
<td>Day 2</td>
</tr>
<tr>
<td>Cholera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2,000</td>
<td>3,700</td>
</tr>
<tr>
<td>CTB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ETEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2,000</td>
<td>5,200</td>
</tr>
<tr>
<td>CTB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CFA/I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CS6</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* HC, healthy controls.

*b* Gut-homing ISC count (median, per million $\beta^+$ cells).

*c* Data are percentage (median) of total ISCs.

*d* ND, the assay was not done on that day.
(circulating plasmablasts) specific to any vaccine antigen, which also can be performed in resource-poor settings.

Antibody-secreting cells are an essential component of adaptive immunity and immunological memory (25). Detection of peak ASC responses at an early time point after booster immunization can be used to assess vaccine efficacy and subsequent immunological memory (26). ASCs induced by mucosal immunization mainly express α4β7-integrin (27). In mice lacking β7, the plasma cell numbers in the intestinal lamina propria are reduced 10- to 30-fold (28). Gut-homing ASCs in the blood shortly after infection are considered a marker of a subsequent mucosal response (29, 30); e.g., 80% of circulating ASCs in cholera patients have been shown to express α4β7 (31). Immune responses at the gut surface play a critical role in

![Fig 3](https://cvi.asm.org/)

**FIG 3** LPS-specific gut-homing antibody-secreting cell (ASC) responses in patients with cholera compared to those in healthy controls (HC). Percentages of LPS-specific IgA (A) and IgG (B) ASCs. Each symbol represents an individual data point for that patient, and the horizontal lines indicate the median responses for each study group. ***, P < 0.001.** *A* P value of <0.05 was considered statistically significant.

![Fig 4](https://cvi.asm.org/)

**FIG 4** CFA/I-specific (A, B) and CS6-specific (C, D) gut-homing antibody-secreting cell (ASC) responses in patients with ETEC infection compared with those in healthy controls (HC). Percentage of CFA/I-specific IgA (A) and IgG (B) ASCs and CS6-specific IgA (C) and IgG (D) ASCs. Each symbol represents an individual data point for that patient, and the horizontal lines indicate the median responses for each study group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. *A* P value of <0.05 was considered to be statistically significant.
mediating protection from cholera and ETEC infections, and previously infected patients may have an anamnestic immune response mediated by mucosal lymphocytes (24, 30, 32).

In this study, we confirmed that in cholera patients, total gut-homing IgA and IgG ASCs were elevated significantly on day 7 as expected. We also confirmed elevated gut-homing ASC responses on day 7 against lipopolysaccharide (LPS) and CTB, two important \textit{V. cholerae} antigens (14, 30, 33, 34). Duodenal ASCs specific for CTB are more numerous than those in the blood after oral immunization with B subunit whole-cell (B-WC) cholera vaccine (9) and killed whole-cell (WC) bivalent cholera vaccine (35), even though in natural infections, no significant differences are found between duodenal and circulating ASC responses (30). A significant correlation ($P = 0.005$; Spearman $r = 0.34$ to 0.67) has been observed between the CTB-specific IgA and IgG isotypes of antibodies in both cholera and ETEC infection at early and late convalescence (data not shown).

LPS-specific immune responses play an important role in the protective immunity to cholera, and LPS is an attractive target for vaccine design (30, 36–38). As for ASCs specific to CTB, duodenal ASCs specific for LPS are also more numerous than those in the circulation after oral immunization with killed whole-cell (WC) bivalent cholera vaccine (35), even though in natural infections, no significant differences are found between duodenal and circulating ASC responses (30). A significant correlation ($P = 0.005$; Spearman $r = 0.34$ to 0.67) has been observed between the CTB-specific IgA and IgG isotypes of antibodies in both cholera and ETEC infection at early and late convalescence (data not shown).

Although CTB is not an antigen of ETEC, CTB is homologous to LT, and cross-reactive immune responses against CTB of almost the same magnitudes as those against LT in patients with ETEC infection have been identified (21, 22). As for cholera, CTB-specific ASC responses have been shown to increase in blood after oral immunization with killed whole-cell ETEC vaccine containing toxoid antigen (40, 41) as well as in patients with ETEC diarrhea (31). In this study, we found, as expected, that CTB-specific $\beta^{+}$ IgA and IgG ASCs were increased at day 7 following ETEC infection, although the median percentage of CTB-specific gut-homing ASCs (for both IgA and IgG ASCs) was about 12-fold lower in patients infected with ETEC than those with cholera.

ETEC can stimulate immune responses to both homologous and cross-reacting colonization factors (CFs) (17). For instance, patients infected with CFA/I-expressing ETEC have been shown to respond with significant IgA antibody responses to the cross-reactive CS1, CS2, CS4, and CS14 colonization factors, in addition to the homologous CFA/I antigen (42, 43), and patients infected with CS14-expressing ETEC have also responded immunologically to CFA/I (44). Interestingly, this is true also for CF-specific ASC and memory B cell responses and development of highly avid IgA and IgG antibody responses (17). In this study, we found that patients infected with ETEC strains expressing the CFA/I group antigens developed CFA/I-specific $\beta^{+}$ IgA and IgG ASCs responses on day 7. Previous studies have also shown development of mucosal and systemic
immune responses against CS6 in humans infected with ETEC strains expressing CS6, with or without CS4 or CS5 (45), and oral immunization of mice with inactivated E. coli overexpressing CS6 induces substantial immune responses against this antigen (46). Patients infected with CS6-expressing ETEC develop not only CS6-specific IgG and IgA ASCs and antibody responses but also memory B cell responses and highly avid anti-CS6 IgA and IgG antibodies after onset of diarrhea (17). In this study, we confirmed that patients infected with ETEC strains expressing CS6 with or without CS5 also developed β7⁺ IgA and IgG ASC responses on day 7 after infection.

Antibody levels in the serum may correlate with the number of antigen-specific ASCs of that specificity and isotype (25). Uddin et al. reported significant correlations between circulating LPS-specific IgA ASCs on day 7 and IgA antibody levels to LPS in mucosal extracts; however, they found no correlation between ASCs and mucosal extract antibodies of the IgG isotype or for ASCs and antibody levels to CTB in mucosal extracts (30). One possibility for these results is that total ASCs, as opposed to gut-homing ASCs, were examined in this study. In this current study, we confirmed that β7⁺ ASCs of both the IgA and IgG isotopes specific to CTB significantly correlated with serum levels of IgA and IgG antibodies to CTB in patients with cholera and ETEC infections.

In the developing world, the volume of blood available for immunological studies, particularly in infants and children, is often a crucial limitation (47). The current approaches to determining circulating ASCs require a relatively large volume of blood (3 to 5 ml) (30) and a flow cytometry-based technique that is more expensive (39). Our current study suggests that these limitations can be overcome by enrichment of β7⁺ cells from as small a volume as 1 ml of blood, followed by an ELISPOT analysis. This should allow immunological assays of both B and T cells, possibly including cytokine production by specific T cells (48), to be conducted in individuals of all ages.

In summary, this study demonstrates that total as well as antigen-specific circulating gut-homing ASCs can be detected in a small blood volume and confirms that antigen-specific cells are elevated on day 7 following infection with both V. cholerae and ETEC. In addition, we found that the level of antigen-specific circulating ASCs on day 7 correlates with plasma antibody levels against CTB on day 7 and on day 30. The results suggest that oral vaccine studies might include determination of gut-homing ASCs to measure immune responses following vaccination, without requiring a substantial blood volume.

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