Enteric infections induce a response of circulating pathogen-specific antibody-secreting cells (ASC). The expression of homing receptors (HRs) on these cells was studied in patients with diarrhea caused by *Vibrio cholerae* in Bangladesh, an area in which cholera is endemic. The gut HR, αβ2, was expressed by ~80% of the ASC, indicating mucosal homing of these cells. However, the peripheral lymph node HR, L-selectin, was also expressed by ~80% of the ASC specific to either cholera toxin or O antigen. In earlier findings after oral immunization in nonendemic areas, αβ7 has been expressed by ~100% and L-selectin by ~50% of the ASC. In comparison, the present data speak for a more systemic targeting of the immune response associated with long-lasting immunity in an endemic area. The results thus provide insight for the continued development and evaluation of vaccines.

Enteric infections associated with acute watery diarrhea caused by *Vibrio cholerae* result in substantial morbidity and mortality in children and adults every year in developing countries [1,2]. Even though the use of effective rehydration therapy has made a significant impact on mortality, it has little effect on morbidity. Since natural infection with this pathogen results in long-term protection [3], a natural approach for controlling cholera is the development of effective, affordable vaccines [4]. A thorough understanding of the immune responses that occur as a result of natural infection in an endemic area is essential.

*V. cholerae* is a noninvasive enteric pathogen. The immune response is initiated by antigen presentation, followed by migration of the stimulated antigen-specific B cells to regional lymph nodes to differentiate into specific antibody-secreting cells (ASC) [5]. These cells are distributed to appropriate tissues via the lymphatics and blood [5,6] in accordance with the concept of a common mucosal immune system [7].

The migration of the stimulated cells into their effector sites proceeds via specialized high endothelial venules [8–11]. The lymphocytes migrate into their target tissues guided by a combination of tissue-specific adhesion molecules, addressins, on high endothelial venules, and their ligands, the homing receptors (HRs), on lymphocytes, as well as other non–tissue-specific adhesion molecule combinations [8–11].

We have recently shown that patients infected with *V. cholerae* O1 or O139 exhibit a response of circulating ASC specific to cholera toxin (CT) and the O antigen (the lipopolysaccharide [LPS]), with a peak response ~7 days after the onset of diarrhea [12]. A similar antigen-specific response has been found in patients with enterotoxigenic *Escherichia coli* diarrhea (Qadri et al., unpublished data) and diarrhea caused by *Salmonella* species [13,14].

In the present study, we investigated the potential destination of the circulating antigen-specific ASC by studying their expression of HRs. In cholera patients, in whom the ASC responses were directed to both the LPS and to the CT components, we could examine separately the effect of the nature of the antigen (polysaccharide or protein) on the HR expression of the cells. The expression of homing-associated receptors was used to study the homing potentials of the pathogen-stimulated lymphocytes: αβ7 as the gut HR [15–17], L-selectin as the peripheral lymph node HR [18–21]. The stage of maturation of the ASC was evaluated by determining the expression of HLA-DR [22,23] and CD28 [24], expressed at the early and late stages of the B cell differentiation process, respectively. This information would provide insight into the mechanisms of the immune response to cholera known to result in protective immunity and would thus be valuable in the continued evaluation and development of new, effective vaccines.

**Materials and Methods**

**Study subjects and samples.** Eighteen patients with acute watery diarrhea and stool cultures yielding *V. cholerae* attending the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (located in Dhaka), were enrolled in the study. The patients were men of age 18–
50 years (median, 26). The control group consisted of 8 healthy volunteers (6 men and 2 women, aged 20–45 years [median, 27]) from Dhaka.

Ten milliliters of heparinized venous blood was collected from the patients at the acute stage after their rehydration in the hospital (day 2 or 3 after the onset of illness, designated d2 samples) for the determination of receptor expressions on all immunoglobulin-secreting cells (ISC). Twenty milliliters of blood for ASC and ISC determinations was also obtained at convalescence, 7–12 days after the onset of disease (designated d7 samples), when possible (see Results). From the healthy volunteers in the control group, 1 blood sample of 10 mL was obtained.

Stool samples of patients and controls were screened for *V. cholerae* O1 and O139 as described earlier [25]. Stool samples were also cultured to detect other enteric copathogens, including enterotoxigenic *E. coli* and *Salmonella*, *Shigella*, and *Campylobacter* species [26] and examined by direct microscopy to detect cyst and vegetative forms of parasites and ova of helminths.

**Separation of receptor-positive and -negative cell populations.**

The separation of peripheral blood mononuclear cells (PBMC) into receptor-positive and -negative populations has been described in detail earlier [14, 27, 28]. Briefly, PBMC were incubated with monoclonal antibodies to ααββ (ACT-1; Leukosite, Cambridge, MA), L-selectin (CD62L; Becton Dickinson, San Jose, CA), HLA-DR (Dako, Glostrup, Denmark), or CD28 (Becton Dickinson). The cells were washed and incubated with sheep anti-mouse immunoglobulin–coated magnetic beads (Dynal, Oslo). A magnet was placed outside the test tubes, and the receptor-positive cells attached to the beads were separated from the receptor-negative cells. The separated cell populations were used immediately for ASC or ISC determinations. The efficacy of the cell separation has been established in our previous studies [14, 27, 28].

**Determination of ASC.**

For the detection of *V. cholerae* O1 and O139 LPS–specific ASC, nitrocellulose-bottomed 96-well plates (Millititer HA; Millipore, Bedford, MA) were coated with purified LPS (25 μg/mL) [10] by procedures described recently [29]. For the detection of CT-specific ASC, the GM1-CT ELISPOT assay with recombinant B subunit of CT [30] was used as described earlier [29].

The freshly separated receptor-positive and -negative cell suspensions were incubated in the wells for 3 h at 37°C. Cells were removed by washing, and monoclonal mouse anti-human IgA1 and IgA2 (both from Nordic Immunological Laboratories, Tilburg, The Netherlands) or alkaline phosphatase–conjugated goat anti-human IgG (Sigma, St. Louis) or swine anti-human IgM (Orion Diagnostica, Helsinki) were added. The plates were incubated overnight at room temperature. Alkaline phosphatase–conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added to the plates with IgA-subclass antibodies and incubated for 2 h at 37°C. Thereafter, all plates were washed and chromogen substrate was added.

**Determination of all ISC.**

All ISC were quantified with ELISPOT as above for ASC, except that the plates were coated with rabbit anti-human IgA or IgM (both from Dako, Copenhagen) or goat anti-human IgG (Sigma).

**Statistics.**

The geometric means and SEs were calculated for ASC or ISC in each group. Arithmetic means (±SEs) were calculated for the percentages of receptor expression. Student’s *t* test was used for statistical comparisons. For the comparisons of cell numbers, log transformations were done. To assure statistical relevance of the results, only those based on ≥20 observed spots were used in the receptor analyses.

**Results**

**Clinical characteristics of the study groups.**

Of the 18 patients, 14 had severe and 4 had moderate dehydration on admission. They had a history of 4–36 h (median, 6.5) of watery diarrhea before hospitalization. Stool culture yielded *V. cholerae* O1 in 15 cases and *V. cholerae* O139 in 3 cases; no other bacterial pathogens were present. Stool microscopy revealed the presence of a few ova of *Ascaris lumbricoides* in 3 and hookworm in 3 patients. In the stool samples of the healthy controls, no bacterial pathogen was isolated: *A. lumbricoides* was detected in 1 and hookworm in 2 persons.

**ASC responses.**

Of the 18 recruited patients, 12 returned for follow-up visits on day 7. A specific ASC response was found in 11 of the 12.

In 9 patients, the number of LPS-specific ASC detected exceeded the inclusion limit of 20 detected spots against the *V. cholerae* LPS component (8/11 for O1 and 1/1 for O139) (figure 1). The responses were dominated by IgA in 4, IgG in 4, and IgM in 1 case. The IgA response to both LPS and CT was in all cases dominated by IgA1; IgA2 ASC represented on average 10% of total LPS-specific IgA ASC (range, 0–69%).

Of the 12 patients, 10 had ASC responses against CT exceeding the inclusion limit of 20 spots (figure 1). The response was dominated by IgG in all of the cases except 1, in which only a low IgM-dominated response was found. CT-specific IgM ASC responses were studied in 7 patients’ d7 samples. Five of these had IgM responses (range, 31–948 cells/10⁶ PBMC).
control group (52% ± 13%). A somewhat lower expression of L-selectin was found on ISC of the patients on day 2 (34% ± 7%; n = 15; P > .05 for both LPS and CT). On day 7, the expression of L-selectin on ISC was 54% ± 8% (n = 11). This increase was statistically not significant.

No difference was found in the expression of these HRs between the various isotypes (data not shown). The low number of patients with sufficient spots for IgA2 did not allow proper comparison between the two IgA subclasses (data not shown).

Expression of maturation markers. HLA-DR, a major histocompatibility complex II antigen expressed by differentiating B cells, was expressed by 69% ± 9% (n = 9) and 67% ± 12% (n = 7) of the LPS- and CT-specific ASC, respectively (figure 3A). In the control group, it was expressed on 43% ± 11% of the ISC (n = 8). It was expressed on 29% ± 5% (n = 15) and 50% ± 8% (n = 12) of patients’ ISC on days 2 and 7, respectively (P > .05). Day 7 ISC differed significantly from both LPS- and CT-specific ASC (P < .01 for LPS-ASC and P < .05 for CT-ASC).

CD28, a proposed plasma cell marker, was expressed on 42% ± 14% (n = 8) of the LPS-specific ASC and 36% ± 13% (n = 8) of the CT-specific ASC. In the control group, it was found on 37% ± 13% (n = 8) of the ISC. Of the patient ISC, 10% ± 7% (day 2; n = 15) and 26% ± 5% (day 7; n =

ISC. The numbers of all ISC (IgA1, IgA2, IgG, and IgM) of the patients were studied both in the acute phase of the disease (day 2) and in the convalescent stage (day 7). Compared with the values of the controls living in the same endemic area, the number of ISC were significantly higher on both days 2 and 7 (geometric mean, 792/10^6 PBMC for controls and 1669 and 2007/10^6 PBMC for d2 and d7 samples of patients, respectively; P < .05 for both). The difference in the number of ISC between d2 and d7 samples was not significant.

Expression of HRs. The gut HR αβ7, was expressed on 81% ± 6% (n = 9) of the LPS-specific ASC and 83% ± 7% (n = 10) of the CT-specific ASC of the patients (figure 2A). Both of these values were significantly higher than the expression of αβ7 found on ISC of healthy volunteers (42% ± 10%; n = 8; P < .01 for both comparisons).

On day 2, 30% ± 5% of the ISC of the patients (n = 15) expressed αβ7 (P < .001 compared with LPS-specific ASC and P < .01 compared with CT-specific ASC). It increased to 62% ± 7% on day 7 ISC (n = 11; P < .01); this was significantly lower than findings among the CT-specific ASC on the same day (P < .05).

L-selectin (CD62L), the peripheral lymph node HR, was expressed by 75% ± 7% (n = 9) and 76% ± 7% (n = 7) of LPS- and CT-specific ASC, respectively (figure 2B). These values did not differ significantly from those for ISC in the

Figure 2. Expression of αβ7 (A) and L-selectin (B) on all immunoglobulin-secreting cells (ISC) of healthy controls (dark bars), on ISC of patients on day 2 (open bars) and day 7 (shaded bars), on O1 or O139 lipopolysaccharide (LPS)-specific antibody-secreting cells (ASC; hatched bars), or on cholera toxin B subunit (CTB)-specific ASC (striped bars) in peripheral blood of patients with watery diarrhea caused by Vibrio cholerae. Nos. of patients and controls are indicated under each bar.

Figure 3. Expression of HLA-DR (A) and CD28 (B) on all immunoglobulin-secreting cells (ISC) of healthy controls (dark bars), on ISC of patients on day 2 (open bars) and day 7 (shaded bars), on O1 or O139 lipopolysaccharide (LPS)-specific antibody-secreting cells (ASC; hatched bars), or on cholera toxin B subunit (CTB)-specific ASC (striped bars) in peripheral blood of patients with watery diarrhea caused by Vibrio cholerae. Nos. of controls and patients are indicated under each bar.
Discussion

The present study investigated the targeting of the immune response in a severe enteric infection caused by *V. cholerae*, known to induce long-lasting immunity [3]. In a series of studies in Finland, we have determined the expression of HRs on pathogen-specific ASC and total ISC from patients with diarrhea caused by *Salmonella* species or *Campylobacter jejuni* [14] (Kantele et al., unpublished data). In addition, specific ASC after oral or parenteral vaccination with a *Salmonella* typhi Ty21a vaccine [18], after oral immunization with bivalent B subunit–O1/O139 whole cell cholera vaccine (Kantele et al., unpublished data), and after parenteral vaccination with tetanus or pneumococcal vaccines have been studied [14] (Kantele et al., unpublished data). In each case, the mucosal challenge in diarrhea or oral vaccination was found to induce an ASC response with a high proportion of cells expressing the gut HR αβ−, and a low proportion of cells expressing the peripheral lymph node HR L-selectin. Parenterally administered antigens, on the other hand, induced ASC responses with a lower proportion of cells expressing αβ− and a higher proportion of those expressing L-selectin. Similar findings have been reported after oral and parenteral vaccination of Swedish volunteers [31]. Consistent with these findings, we now found that the mucosal stimulation in cholera induced mostly (~80%) αβ−expressing ASC to both the LPS and the toxin components of the bacteria. Interestingly, however, and in contrast to the findings from Finland and Sweden, most (~80%) of the LPS- and CT B subunit–specific ASC expressed L-selectin, in spite of the mucosal route of antigen introduction.

When comparing the results of the present study with those obtained in studies conducted in Finland or Sweden, one should keep in mind that some fundamental differences exist between an endemic versus a nonendemic area. Differences in genetics and nutrition, in antigen load, and in gut cytokine environment (healthy vs. mild vs. severe state of disease) may exist between the groups of volunteers studied and also contribute to the differences in the observed immune responses. Moreover, in Finland and Sweden, the volunteers were mainly healthy persons receiving vaccines, whereas in Bangladesh the patients were severely ill and their intestinal epithelium damaged: In these patients, increased numbers of absorbed antigens could evoke more effectively systemic immune responses and thus account for the increased proportion of L-selectin–expressing cells. Finally, whereas in Finland and Sweden the encounter with the pathogen or vaccine strain represented a primary contact, in Bangladesh cholera is endemic, and previous exposures associated with symptomatic or asymptomatic infection are common [3, 25]. In our view, it seems plausible that the differences in the immune responses would be due to a secondary nature of the immune response elicited, and the targeting of the immune response in a secondary type of response would differ from that found after primary immunization. This line of thinking is strongly supported by our recent study in Finland, where results similar to these (high proportions of both αβ− and L-selectin–positive ASC) were found after an oral secondary immunization of volunteers primed either orally or parenterally with *S. typhi* Ty21a (Kantele et al., unpublished data). Moreover, in accordance with this is the finding that some memory B lymphocytes express both αβ− and L-selectin [32].

The fact that the proportion of L-selectin expressing cells among ASC increases on secondary mucosal contact with the antigen could be a result of several different mechanisms: a wider participation of nonmucosal tissues (e.g., peripheral lymph nodes or bone marrow) in the secondary immune response, recirculation of the L-selectin–expressing cells to the Peyer’s patches (see [33]) to amplify the immune response, different usage of integrins in primed persons dependent on selectin-mediated rolling of lymphocytes on secondary contact with the antigen, or a combination of the above. The lower proportion of αβ−expressing ASC detected in this study (83%) compared with that following oral primary vaccination (99%) or diarrhea induced by *Salmonella* species (100%; Kantele et al., unpublished data) may suggest an increased participation of nonmucosal tissues in the immune response in diarrhea in endemic areas.

Lymphocytes doubly positive for αβ− and L-selectin have recently been described in nonobese diabetic mice [34]. On the basis of our results, this could well be explained by a continuous exposure to the self-antigens and be comparable to secondary contact with the enteric pathogens in our studies.

When determining the expressions of HLA-DR and CD28, no obvious changes were observed in the patient ISC or ASC between the two time points studied. Similarly, no differences were found in the stage of maturation between the patient ISC or ASC and the control ISC. An unambiguous receptor expressed only by plasma cells has not been described as yet.

The IgA subclass analysis revealed a lower proportion of IgA2 ASC in patients with cholera in Bangladesh (10%) than in Finnish patients with *Salmonella*-induced diarrhea (33%; Kantele et al., unpublished data). Possible explanations include different sites of antigen stimulation (the upper vs. lower parts of the intestine) [35], the probable secondary nature of the infection, or genetic differences known to exist between races [36].

Hospitalized patients with cholera are severely dehydrated and under considerable physical and physiologic stress, which most likely affects their neuroendocrine homeostasis [37]. Hypovolemia can be thought to increase the possibility of cell-cell contacts and the probability of lymphocyte–high endothelial venule adhesion leading to extravasation of lymphocytes. The changes in corticosteroid production due to stress also alter the expression of various adhesion molecules of the cells [38]. The induction of shedding of L-selectin from granulocytes and monocytes has been reported on contact with various activators as well as in bacteremia or sepsis of newborns [19, 38, 39].

11) expressed CD28. The values for ISC, in both controls and patients, did not differ significantly from those of ASC.
addition, in vitro activation of T cells can lead to decreased binding to mesenteric lymph node high endothelial venules as observed in frozen section assays [40]. Also, both α4β7-integrins (α4β7; and αβ7) have been shown to be down-regulated on T cells on stimulation in vitro [41]. All of these data suggest that a severe diarrheal disease may have a significant effect on the homing potentials of all lymphocytes in the acute phase of the disease. Interestingly, a lower proportion of cells expressing HRs (L-selectin and αβ7) was found among patients’ ISC on days 2 and 7 compared with ISC of healthy controls. This could be due to down-regulation of these receptors on the ISC, an increase in the α4β7−L-selectin− cell population by cell division, or both. A similar decrease in the proportion of HR-expressing cells was not found in the Finnish patients with Salmonella diarrhea. Whether this low proportion of HR-expressing cells in the patients in Bangladesh is an effect of an exceptionally severe stage of illness (dehydration, heavy leakage through the intestinal epithelium) or some property of the enterotoxin remains to be studied.

In conclusion, enteric infections caused by V. cholerae O1 or O139 associated with acute watery diarrhea in areas in which cholera is endemic induces specific ASC, most of which express both the gut HR αβ7 and the peripheral lymph node HR L-selectin. This was true for both polysaccharide (LPS) and protein (CT) antigens. This expression pattern suggests a high potential of homing of these cells to both mucosal and systemic tissues. We propose that this is a consequence of the secondary nature of the challenge in an endemic area. Importantly, the study gives information on lymphocyte homing pattern in an immune response leading to a long-lasting immunity to an enteric pathogen. Also, in the acute phase of the disease, the targeting of peripheral blood ISC into tissues by their HRs was notably different from that observed at convalescence. The understanding of the targeting of the immune response should help in the future development and evaluation of effective vaccines against enteric infections.

References


