Circulating Gut-Homing (α₄β₇⁺) Plasmablast Responses against Shigella Surface Protein Antigens among Hospitalized Patients with Diarrhea

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Developing countries are burdened with Shigella diarrhea. Understanding mucosal immune responses associated with natural Shigella infection is important to identify potential correlates of protection and, as such, to design effective vaccines. We performed a comparative analysis of circulating mucosal plasmablasts producing specific antibodies against highly conserved invasive plasmid antigens (IpaC, IpaD20, and IpaD120) and two recently identified surface protein antigens, pan-Shigella surface protein antigen 1 (PSSP1) and PSSP2, common to all virulent Shigella strains. We examined blood and stool specimens from 37 diarrheal patients admitted to the Infectious Diseases & Beliaghata General Hospital, Kolkata, India. The etiological agent of diarrhea was investigated in stool specimens by microbiological methods and real-time PCR. Gut-homing (α₄β₇⁺) antibody-secreting cells (ASCs) were isolated from patient blood by means of combined magnetic cell sorting and two-color enzyme-linked immunosorbent spot (ELISPOT) assay. Overall, 57% (21 of 37) and 65% (24 of 37) of the patients were positive for Shigella infection by microbiological and real-time PCR assays, respectively. The frequency of α₄β₇⁺ IgG ASC responders against Ipas was higher than that observed against PSSP1 or PSSP2, regardless of the Shigella serotype isolated from these patients. Thus, α₄β₇⁺ ASC responses to Ipa may be considered an indirect marker of Shigella infection. The apparent weakness of ASC responses to PSSP1 is consistent with the lack of cross-protection induced by natural Shigella infection. The finding that ASC responses to IpaD develop in patients with recent-onset shigellosis indicates that such responses may not be protective or may wane too rapidly and/or be of insufficient magnitude.

Shigellosis, a diarrheal illness, is caused by Shigella organisms. It begins with watery diarrhea and is followed by dysentery. Shigella is one of the five most important genera of pathogens that cause diarrhea globally (1). It is estimated that shigellosis causes more than 100 million episodes annually and that 90% occur in developing countries (2, 3). Studies show that the annual incidence rate may rise further due to identification of Shigella spp. in culture-negative diarrheal specimen (4). The emergence of multidrug-resistant Shigella spp. has also been reported (5, 6). Shigella spp. are considered category B bioterror agents by the U.S. Centers for Disease Control and Prevention (CDC) (7). Poor hygiene, limited access to safe drinking water, and malnutrition are among the many factors facilitating the spread and severity of Shigella diarrhea. Mortality due to shigellosis remains high amid effective treatments based on oral rehydration and antibiotics. The World Health Organization has made the development of a safe and effective Shigella vaccine a public health priority (8, 9). To date, development of an effective Shigella vaccine has remained elusive, although promising results from recent clinical trials have been reported (9, 10). Recent attempts have been made to correlate serum antibody responses with the presence of memory B cells against lipopolysaccharides (LPS) and IpaB antigens in human volunteers (11, 12). However, a major limitation for development of Shigella vaccine is the lack of knowledge regarding the nature and specificity of intestinal mucosal immune responses to Shigella antigens.

Local antibody formation and effector immune cells in the gut provide the first line of defense upon reexposure to Shigella infection (9). Migration of mature lymphocytes from mucosal induc-
Thus, detection of blood ASCs expressing $\alpha_4\beta_7$ may permit the identification of specific subsets of ASCs trafficking between the systemic circulation and the gut.

*Shigella* infection usually leads to production of *Shigella*-reactive antibodies in serum and intestinal secretions (21, 22). Protection against reinfection is thought to be mainly directed at the O antigen and hence is somewhat restricted to the infecting serotype or a cross-reactive serotype, a dominant concept in *Shigella* vaccine development efforts. Most of the previous studies have evaluated serotype-specific immune responses against *Shigella* spp. following natural infection or after immunization with vaccine candidates (23–26). More recent work suggests that, in addition to O antigen–specific responses, *Shigella* infection is followed by the production of local secretory IgA and serum IgG antibodies to bacterial virulence proteins (27). Studying the protein antigens that are common to all serotypes of *Shigella* became the obvious choice after the discovery of a large invasive plasmid in virulent *Shigella flexneri*; the plasmid was identified on the C terminus of *Shigella* infection (see Table S1 in the supplemental material). Subjects received oral vaccination with a protective antigen, termed pan-Shigella surface protein 1 (PSSP1), that has been identified on the C terminus of *Shigella* outer membrane protease IcsP and is conserved among all *Shigella* species and serotypes (35). Another, albeit less conserved, epitope called PSSP2 has also been identified as part of SigA, an autotransporter-like protease (36). In this study, we examined the frequency of circulating, gut-directed ASC responses to *Shigella* protein antigens in patients with recent-onset shigellosis in Kolkata, India, an area of high *Shigella* infection endemicity (1, 37).

**MATERIALS AND METHODS**

*Study group and clinical data.* The initial study population consisted of 89 adults admitted for acute diarrhea at the Infectious Diseases & Beliaghat General (ID&BG) Hospital, Kolkata, India. All subjects had 3 or more stool episodes per day with or without blood. All patients gave their informed consent per the study protocol, which was approved by the Institutional Ethical Committee. Enrolled subjects were followed for 1 month, and blood specimens were collected at different times. At the preliminary examination immediately after hospitalization, when the patient was enrolled into the study, 2-ml blood and stool specimens were collected. A second blood specimen (5 ml) was obtained 5 to 6 days after the onset of diarrhea. The 37 volunteers who returned for the second bleed collection were included for this study. Prior history of diarrheal illness was recorded for all enrolled subjects, as were stool specimen characteristics, age, sex, number of diarrheal episodes per day, and duration of illness (see Table S1 in the supplemental material). Subjects received oral rehydration therapy and antimicrobial treatment (chloramphenicol, norfloxacin, ofloxacin, tinidazole, or metronidazole).

*Microbiological analysis.* Stool specimens were analyzed by standard microbiological assays for detection of bacterial enteropathogens (37). In brief, diarrheal stool specimens were streaked on selective plates (thiosulfate-citrate-bile salts-sucrose, MacConkey agar, xylose lysine deoxycholate, Ryan medium, Hektoen enteric agar, and Columbia blood agar), and the colonies growing on the plates were tested biochemically for the presumptive identification of bacterial enteropathogens. *Shigella* spp. were further serotyped using a *Shigella* antisera kit (Denka Seiken, Japan). Confirmation of pathogroups of diarrheagenic *Escherichia coli* was made by multiplex PCR assays that included detection of enterotoxigenic *E. coli* (ETEC), typical and atypical enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC). Serogrouping of *Vibrio cholerae, V. parahaemolyticus, Shigella* spp., and *Salmonella* spp. was performed by the use of slide agglutination assays and commercially available antisera (Denka Seiken, Japan; Bio-Rad, France). Serotyping of *V. cholerae* O1 strains to Ogawa or Inaba was also performed.

*RT-PCR assay.* The 10 pairs of (either species-specific or virulence gene–specific) real-time PCR (RT-PCR) primers were used in this study for the detection of *V. cholerae*, *V. parahaemolyticus*, *Campylobacter* spp., *Shigella* spp., ETEC, EAEC, and EPEC (4). A 1-μl volume of DNA extracted from stool specimens by the use of a QIAamp DNA stool minikit (Qiagen, Germany) was used to detect enteropathogens in a 20-μl reaction volume containing Power SYBR green master mix (Applied Biosystems, USA) and 0.6 pmol/μl of PCR primers. Real-time PCR was performed in a 7900HT Fast RT-PCR machine (Applied Biosystems, USA) in which the tubes were initially heated to 50°C for 2 min followed by heating to 95°C for 10 min and were subjected to 35 cycles of PCR at 94°C for 20 s and 55°C for 20 s, with an extension step of 50 s at 72°C. Fluorescence signals were measured at the extension step of each cycle, and pathogen-specific threshold cycle ($C_T$) values were recorded. After PCR amplification, melting temperature ($T_m$) curve analysis was performed (4). For all assays, negative controls consisting of PCR-grade water were included.

*Description of antisera.* (PSSP1, PSSP2, IpaD20, IpaD120, and IpaC). Purified *Shigella* IpaD20, IpaD120, and IpaC were generously provided by Ariel Blocker, University of Bristol, United Kingdom. The PSSP antisera, the PCR-amplified fragments coding for IcsP and SigA amino acids, were cloned between the EcoRI and XhoI sites of pET21-d and used for immunization with vaccine candidates (28). Those invasion plasmid antigens (Ipas) comprises IpaA, IpaB, IpaC, and IpaD, against which substantial antibody levels have been detected in the sera of experimentally infected monkeys (29) and naturally infected children and adults (26, 30, 31). Furthermore, Ipa have been shown to be protective in animal studies (32–34). More recently, a novel and potentially cross-protective protein antigen, termed pan-*Shigella* surface protein 1 (PSSP1), has been identified on the C terminus of *Shigella* outer membrane protease IcsP and is conserved among all *Shigella* species and serotypes (35). Another, albeit less conserved, epitope called PSSP2 has also been identified as part of SigA, an autotransporter-like protease (36). In this study, we examined the frequency of circulating, gut-directed ASC responses to *Shigella* protein antigens in patients with recent-onset shigellosis in Kolkata, India, an area of high *Shigella* infection endemicity (1, 37).

Whole blood was used to detect circulating ASCs secreting antibodies (IgA and IgG) to different *Shigella*-specific antigens. These included migrating gut-homing ($\alpha_4\beta_7$) ASCs, non-gut-homing (HLA-DR $^+$ CD19$^+$ but $\alpha_4\beta_7$) ASCs, and total ASCs, whose levels were estimated by adding the gut-homing and non-gut-homing ASC numbers. Estimation of the levels of different types of cells was performed by single-step ELISPOT assay, and results were expressed as numbers of spots/10$^6$ immunoglobulin-secreting cells (ISCs) for each *Shigella* antigen. Assays were carried out in nitrocellulose-bottomed 96-well plates (Millipore, USA; catalog no. MSHAN4510) using a recently described whole-blood ELISPOT method (16). Wells were coated individually with five different types of purified *Shigella*-specific antigens (PSSP1, PSSP2, IpaD20, IpaD120, and IpaC) applied in 50-μl volumes at a final concentration of 5 μg/ml in phosphate-buffered saline (PBS). Additional wells coated with goat anti-human κ/A antibodies (Southern Bio- tech, USA) were used to enumerate the ISCs of each Ig isotype (16). Uncoated (exposed to PBS only) wells were used to detect nonspecific spots. Blood specimens (5 ml in an EDTA vial) collected ~5 to ~7 days after the onset of diarrhea (second bleed) were divided into five equal parts that were processed simultaneously. Red blood cells were lysed, and the remaining leukocytes were pelleted by centrifugation prior to immunomagnetic separation (16). Briefly, anti-mouse Ig-coated magnetic beads (Invitrogen, USA) were further coated with an anti-human $\alpha_4\beta_7$ mouse monoclonal antibodies (ACT-1; kind gift from E. C. Butcher, Stanford University School of Medicine, CA, USA) and used for capturing ASCs expressing $\alpha_4\beta_7$ (the gut-homing ASCs). This separation was done by using a magnetic separator (DynalMag-15 [catalog no.12301D]; Invitrogen). Cells bound to the beads and free beads were washed twice and concentrated magnetically. Negative cell fractions (i.e., those not expressing $\alpha_4\beta_7$) were further incubated with a mixture (1:1 bead/bead ratio) of beads coated with monoclonal antibodies to HLA-DR or to CD19 to en...
TABLE 1 Diarrheal patients with *Shigella* spp. and other pathogens detected by culture-based and real-time PCR methods

| No. of patients | Culture-based detection of: | Real-time PCR-based detection of: | \(C_r\) value or range |
|-----------------|-----------------------------|----------------------------------|------------------------|
| **With shigellosis** | | | |
| 1 | *S. flexneri* UT | *Shigella* spp. | 13 |
| 2 | *S. flexneri* 2a | *Shigella* spp. | 16–25 |
| 1 | *S. flexneri* 2a | *Shigella* spp., EPEC, EAEc | 17.1 |
| 5 | *S. flexneri* 3a | *Shigella* spp. | 16.4–22.96 |
| 1 | *S. flexneri* 3a | *Shigella* spp., EPEC | 20.8 |
| 1 | *S. flexneri* 3a | *Shigella* spp., EPEC | 22.19 |
| 1 | *S. sonnei* | *Shigella* spp. | 18.49 |
| 1 | *S. sonnei* | *Shigella* spp., ETEC | 14.48 |
| 2 | *S. flexneri* 2a | ND | — |
| 5 | *S. flexneri* 3a | ND | — |
| 1 | *S. flexneri* 4 | ND | — |
| **Without shigellosis** | | | |
| 1 | *V. fluvialis* | *V. parahaemolyticus*, EAEc | — |
| 1 | *V. fluvialis* | NKP | — |
| 1 | *V. fluvialis* | *Shigella* spp., EPEC | 26 |
| 1 | *V. cholerae* | *V. cholerae* | — |
| 1 | *V. parahaemolyticus* | *Shigella* spp. | 28 |
| 1 | *C. jejuni* | *Campylobacter* spp. | — |
| 1 | *C. jejuni* | *Shigella* spp., V. parahaemolyticus | 30 |
| 1 | *Pseudomonas* spp. | *Campylobacter* spp. | — |
| 1 | NKP | *V. cholerae*, ETEC | — |
| 1 | NKP | EPEC | — |
| 1 | NKP | EAEc | — |
| 1 | NKP | NKP | — |
| 4 | NKP | ND | — |

*Real-time PCR-based identification of pathogens was based on detection of specific genes as follows: for *V. cholerae*, the rRNA gene and O1v wb; for *V. parahaemolyticus*, the rRNA gene; for *Campylobacter* spp., the rRNA gene; for *Shigella* spp., ipaH; for ETEC, *lt* and *st*; for EPEC, *eaeA*; and for EAEc, aggR. ND, not done due to insufficient samples; NKP, no known pathogens; —, not applicable.

TABLE 2 Distribution of IgA- and IgG-secreting cells in diarrheal patients

| ISC category | Cells positive for marker | Antibody class | Shigellosis (n = 21) | Non-shigellosis (n = 16) | \(P\) value* |
|--------------|---------------------------|---------------|---------------------|--------------------------|-----------|
| Gut homing   | \(\alpha_\beta_7^+\) | IgA          | 2,332 ± 635         | 2,182 ± 800              | 0.09      |
|              |                           | IgG          | 1,951 ± 412         | 1,636 ± 768              | 0.004     |
| Non-gut homing | HLA-DR\(^+\) CD19\(^+\) | IgA          | 1,730 ± 352         | 913 ± 186                | 0.05      |
|              | \(\alpha_\beta_7^-\) | IgG          | 448 ± 95            | 957 ± 212                | 0.02      |
| Total        | HLA-DR\(^+\) CD19\(^+\) | IgA          | 4,061 ± 935         | 3,094 ± 900              | 0.07      |
|              | \(\alpha_\beta_7^-\) | IgG          | 2,399 ± 438         | 2,593 ± 884              | 0.24      |

*Data are expressed as mean numbers (± standard errors of the means [SEM]) of IgA- or IgG-secreting cells (ISCs) expressing \(\alpha_\beta_7^+\) and/or HLA-DR\(^+\)/CD19\(^+\) per milliliter of blood.

*Data represent results of unpaired t tests.
The most prevalent serotype was S. flexneri 3a followed by S. flexneri 2a and S. sonnei. The prevalence of S. flexneri 2a and 3a serotypes agrees with previously reported data from Kolkata, India (37). We reanalyzed 25 specimens by real-time PCR; 12 specimens had insufficient stool volume for real-time PCR analysis. Of the 25, 13 contained Shigella spp. by culture, and real-time PCR analysis also detected Shigella spp. In addition, real-time PCR analysis identified Shigella spp. in 3 specimens that were negative for shigellosis by the culture-based assay. Real-time PCR analysis also confirmed the culture-based findings for C. jejuni, V. cholerae, and V. parahaemolyticus as well as additional cases of V. cholerae, EAEC, ETEC, and EPEC infections (Table 1).

**B cell responses among shigellosis and non-shigellosis patients.** For immunological assessment, we used the ELISPOT assay with EDTA-treated blood. Due to insufficient blood specimens or technical failure (hemolysis), analyses could be performed for only 19 shigellosis and 15 non-shigellosis patients. Analysis priorities were IpaC, PSSPs, IpaD20, and IpaD120 antigens. A comparative analysis of ISCs (using spots developed in wells coated with goat anti-human κ/λ antibodies) was made between shigellosis and non-shigellosis patients. Data are summarized in Table 2. High degrees of variations of ISCs secreting different antibody classes (IgA and IgG) were noted for both gut-homing (HLA-DR⁺ CD19⁺) and non-gut-homing (HLA-DR⁺ CD19⁺) fractions. The ELISPOT assay also enabled detection...
of gut-homing ($\alpha_4\beta_7^+$) and non-gut-homing (HLA-DR$^+$ CD19$^+$ and $\alpha_4\beta_7^-$) ASCs that secreted antibodies against *Shigella* antigens for IgA and IgG antibody classes. Comparative analyses were also done for patients with or without culture-confirmed shigellosis. As shown in Fig. 1, no significant differences were observed for gut-homing ASCs against PSSP1 antigen between shigellosis and non-shigellosis patients per $10^5$ gut-homing ASCs. However, significantly higher responses were evident in the IgA class against PSSP2 antigen. For the IpaC antigen, significant differences were observed in the IgG class results, and for the IpaD20 and IpaD120 antigens, significant differences were observed in the results from both the IgA and IgG classes. There were no significant differences for shigellosis and non-shigellosis patients in responses to IpaC and PSSP1 antigens in the analysis of non-gut-homing ASCs (see Table S2 in the supplemental material). However, non-gut-homing patients showed significant differences in responses to PSSP2 antigens in the IgA ($P = 0.02$) class (see Table S2). Analysis of ELISPOT assay responders ($\geq 5$ spots per $10^5$ gut-homing ASCs) and gut-homing ASCs showed a significant trend in the levels of the class IgG responses against the antigens (IpaC $>$ IpaD20 $>$ IpaD120 $>$ PSSP) in shigellosis versus non-shigellosis patients (Table 3). Such a trend was also evident in gut-homing ASC responses for the IgG class against *Shigella* antigens (Fig. 2).

Correlations between culture-confirmed *Shigella* infection and antibody class-specific ASC responders were evaluated. For this, numbers of positive and negative responders among patients with or without culture-confirmed shigellosis were analyzed for sensitivity and specificity. Summarized data are presented in Table S3 in the supplemental material. Although the numbers of specimens were low, analysis showed trends with respect to sensitivity and specificity in detecting *Shigella* cases based on results of analysis of gut-homing ASC responders in the ELISPOT assay. Overall, values of both sensitivity and specificity of above 70% were obtained only with gut-homing ASCs secreting the IgG class of antibody against IpaC. ASC responders showed 100% specificity in detection of *Shigella* cases against other antigens (IpaD20, IpaD120, PSSP1, and PSSP2) in at least one of the two antibody classes; sensitivity remained about 50% or lower.

**DISCUSSION**

Gut tropism is required for the immune defense against several intestinal pathogens. Studies showed that levels of gut-homing ($\alpha_4\beta_7^+$) cells secreting antibodies to target antigens were higher following oral vaccination and were thought to confer protection against subsequent intestinal infections (27). We made a comparison between mucosal responses against different *Shigella* antigens among shigellosis and non-shigellosis patients. The observed high degree of variations of ISCs secreting different antibody classes (IgA and IgG) reflected variations in the immune status of the subjects. Polyclonal stimulation of ISCs was reported earlier in diarrheal patients and was mostly seen in the results from the IgA antibody class (42), which is similar to the results obtained in this study. Comparative analysis revealed that results from shigellosis patients did not show any significant differences in gut-homing and non-gut-homing ISCs secreting the IgA class of antibody. Significantly ($P < 0.0001$) higher levels of gut-homing ISCs than of non-gut-homing ISCs secreting the IgG class of antibody were evident in shigellosis patients, which perhaps augmented the significantly higher levels of gut-homing ASCs secreting the IgG class of antibodies, with a trend of IpaC $>$ IpaD20 $>$ IpaD120. These data are in congruence with the previously discussed higher systemic immunity to IpaC than to IpaD among shigellosis patients (29). Another important finding was the elicitation of ASCs against Ipas regardless of which of the *Shigella* serotypes had caused the diarrhea.

Recent preclinical studies showed that IpaD and PSSP1 antigens can induce cross-protection against experimental shigellosis caused by several species and serotypes (35, 43). We thus compared gut-homing ASC responses to IpaD20/IpaD120 and PSSP1 among shigellosis patients. Overall, ASC responses to IpaD20/IpaD120 were more frequent and of higher magnitude than the corresponding responses to PSSP1. Such responses were to a large extent contributed by IgG ASCs expressing $\alpha_4\beta_7^+$, an integrin playing a major role in directing the migration of mucosal plasmablasts to the intestinal mucosa (44). It is also worth noting that a substantial fraction of the gut-homing ASCs to IpaD20/IpaD120 were also of the IgA isotype. Since protection induced by natural infection is to a fair extent restricted to the infecting or closely related serotype and hence mainly directed to the capsular O oligosaccharide antigen, naturally induced immunity to IpaD may not develop to sufficiently high levels or may wane rapidly after infection. While this finding remains intriguing, since natural infection is thought to be serotype restricted and hence directed at the O antigen, it indicates that detection of protein-reactive ASCs in peripheral blood is a sensitive marker of active infection and may thus have diagnostic value.

This study also demonstrated that naturally induced ASC responses to PSSP1, an antigen recently identified as cross-protec-

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**TABLE 3** Frequency of gut-homing ($\alpha_4\beta_7^+$) antibody-secreting cells with responses to *Shigella* common protein antigens in diarrheal patients with and without shigellosis

| Antigen | With shigellosis | Without shigellosis |
|---------|----------------|--------------------|
|         | IgA            | IgG                | IgA            | IgG                |
| IpaC    | 7/21 (7, 0–62) [0.32] | 20/21 (45, 0–153) [<0.0001] | 3/16 (3, 0–21) | 5/16 (10, 0–91) |
| IpaD20  | 10/21 (10, 0–59) [0.008] | 13/21 (27, 0–142) [0.0001] | 1/15 (<1, 0–6) | 0/15 (0, 0) |
| IpaD120 | 7/19 (7, 0–25) [0.12] | 10/19 (17, 0–94) [0.0008] | 2/15 (1, 0–9) | 0/15 (0, 0) |
| PSSP1   | 3/21 (1, 0–6) [0.44] | 0/21 (0, 0) [0.1] | 1/16 (1, 0–15) | 2/16 (<1, 0–9) |
| PSSP2   | 4/21 (5, 0–51) [0.06] | 3/21 (12, 0–159) [0.11] | 0/16 (<1, 0–4) | 0/16 (0, 0) |

* Responders were defined as patients who yielded $\geq 5$ spots per $10^5$ gut-homing ISCs. Values in parentheses indicate mean numbers of antibody-secreting cells (ASCs) per $10^5$ $\alpha_4\beta_7^+$ immunoglobulin-secreting cells (ISCs).

* $P$ values represent results of chi-square ($x^2$) tests.

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tive in animal studies (35), were rather infrequent and generally much weaker than the corresponding ASC responses to IpaD (and, for that matter, IpaC). This observation may indicate that such mucosal antibody responses may not develop to a level conducive to immune protection or may wane too rapidly after *Shigella* infection to support protection against subsequent reinfection by *Shigella* bacteria of the same or different species and serotypes.

The numbers of positive responders to different *Shigella* antigens among patients with shigellosis differ from the numbers seen among patients without shigellosis. An evaluation (i.e., an analysis of the sensitivity and specificity) of the utility of the different antigens in detecting the recent onset of shigellosis has been attempted. Analysis showed 100% specificity for PSSP antigens in detecting the recent onset of shigellosis for the IgA isotype. However, very low sensitivity values were seen, which indicates that it is unsuitable for use as a good and reliable marker. Similar situations existed for the IpaD20/IpaD120 antigens, which showed detection specificity of over 80% in both the IgA and the IgG isotypes, with sensitivity of about 40%. In case of IpaC, the sensitivity and specificity values were higher than 70% for the IgG isotype and thereby showed its potential for use as a diagnostic marker, with positive and negative predictive values of 66.6% and 56.3% in detecting recent onset of shigellosis.

It is important to understand the nature of the immune responses that can be differentiated between humoral and local responses. Sera collected from the study subjects at hospital admission and ~5 to ~7 days after the onset of diarrhea were also evaluated by whole-cell enzyme-linked immunosorbent assay (ELISA) for analysis of different *Shigella* spp. (data not shown). No differences in serological reactivity were evident among sera collected at different time points after the onset of diarrhea. Sera from non-shigellosis patients and from shigellosis patients had comparable levels of reactivity. Data on humoral responses showed high levels of circulating antibodies in subjects residing in and around Kolkata, which can be considered indirect evidence of the endemicity of infections by *Shigella* spp. in the region.

Although natural infection with *Shigella* spp. may induce immunity to shigellosis, protection is species restricted and, by and large, O antigen serotype restricted and appears to be of short duration (42). It would thus appear that natural immunity to common protein antigens is not sufficient and/or does not last long enough to confer protection against shigellosis. Vaccine strategies based on overexpression of common protein antigens formulated with suitable adjuvants may thus be worth testing in the near future.

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**FIG 2** Gut-homing ASC (αβ⁺⁺⁺⁺) responses determined by ELISPOT assays in an antibody class-specific manner (A [IgA] and B [IgG]) and relative antibody class-specific proportions against different antigens (C [IgA] and D [IgG]) among shigellosis patients. Data were normalized for each antibody class and are expressed per 10⁵ gut-homing ISCs. Nonparametric paired *t* tests were performed to determine statistical significance; horizontal bars represent mean values.
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