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Circulating Gut-Homing ($\alpha_4\beta_7^+$) 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 ($\alpha_4\beta_7^+$) 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 $\alpha_4\beta_7^+$ IgG ASC responders against Ipas was higher than that observed against PSSP1 or PSSP2, regardless of the *Shigella* serotype isolated from these patients. Thus, $\alpha_4\beta_7^+$ ASC responses to Ipas 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 specimens (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 inductive sites to the gut via the systemic circulation occurs soon after vaccination or infection (13–16). These homing lymphocytes include a contingent of antibody-secreting cells (ASCs) that are transiently circulating and whose frequency peaks in blood as early as 1 week after the onset of infection or after stimulation of the gut-associated lymphoid tissue (16–19). In these studies, mucosal immunity was determined by enzyme-linked immunosorbent spot (ELISPOT) assay using peripheral blood specimens collected about a week after antigen stimulation. ASCs express different sets of adhesion molecules in a tissue-specific manner, and the integrin $\alpha_4\beta_7$ mediates lymphocyte binding to specific mucosal adhesion molecules expressed in the gut (13, 14, 20).

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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 strains of *Shigella* organisms (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, Ipas 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 *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 & Beliaghata 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 blood 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* antiserum 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 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 antigens (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 antigens, the PCR-amplified fragments coding for IcsP and SigA amino acids, were cloned between the EcoRI and XhoI sites of pET21-d and pET24-d (Novagen, USA) using *S. flexneri* 2a 2457T as the template. The *Shigella* IcsP outer membrane protease removes the IcsA actin assembly protein from the bacterial surface and consequently modulates *Shigella* actin-based motility and cell-to-cell spread (38). The *sigA* gene encodes a protein with a predicted molecular mass of 139.6 kDa that has significant sequence similarity to several autotransporters from a variety of Gram-negative bacteria (39).

Detection of ASCs by ELISPOT assay. 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/10^3 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/λ antibodies (Southern Biotech, 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 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 (DynaMag-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[a]

No. of patients	Culture-based detection of:	Real-time PCR-based detection of:	C_T 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, EAEC	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	—

[a] Real-time PCR-based identification of pathogens was based on detection of specific genes as follows: for *V. cholerae*, the rRNA gene and O1*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.

rich for residual plasmablasts. The bead-based separation procedure resulted in depletion of 95% and 90% fluorescence-activated-cell-sorter (FACS)-detectable HLA-DR$^+$ CD19$^+$ cells and $\alpha_4\beta_7^+$ cells, respectively, in the negative fractions (16, 40). Furthermore, negatively sorted cells were reported to contain <1% of ELISPOT assay-detectable ISCs (41). Each well coated with *Shigella*-specific antigen received 50 μl of the suspension that contained either $\alpha_4\beta_7^+$ or $\alpha_4\beta_7^-$ cells. Each cell fraction was tested for numbers of ASCs specific to each *Shigella* protein antigen in an isotype (IgA or IgG)-specific manner. For this purpose, wells were developed with anti-human IgG horseradish peroxidase (HRP) conjugate (Southern Biotech, USA) along with anti-human IgA alkaline phosphatase (AP) conjugate (Southern Biotech, USA) with the use of substrates AEC (ImmunoBioScience, USA) and BCIP/NBT (Sigma, USA), respectively, as previously described in detail (16). Net numbers of spots in each well were obtained after subtracting the number of spots, if any, in PBS control wells. ASC numbers were expressed per 10^3 ISCs (determined in anti-κ/λ antibody-coated wells) of the corresponding antibody class (16). Specimens showing ≥5 spots/10^3 ISCs in the antigen wells were considered to represent positive responders.

Statistical analyses. The nonparametric unpaired *t* test was used for comparing ASC responses between shigellosis and non-shigellosis patients and for comparing ASC responses to different antigens (GraphPad Prism 6.01). To test for significance in terms of responders (≥5 spots/10^3 ISCs) to different Ipas by different antibody classes between shigellosis and non-shigellosis patients, we analyzed the data by chi-square test (GraphPad Prism 6.01).

RESULTS

Patient population and clinical features. Of the 89 patients enrolled in the study, just 37 returned for follow-up (see Table S1 in the supplemental material). These 37 volunteers had diarrhea 10 to 15 times a day at the time of admission to the hospital. Stool specimens were analyzed microbiologically for the presence of *Shigella* spp. and other bacterial enteropathogens. With the exception of one watery specimen, all of the *Shigella*-confirmed stool specimens were bloody and mucoidy. All volunteers were treated with antibiotics. Among the 37 volunteers, there were 28 men and 9 women. The duration of diarrheal illness ranged from ~5 to ~103 h, with a mean duration of 24 h.

Detection of bacterial enteropathogens. A total of 21 of 37 specimens collected from the study patients had *Shigella*-positive cultures by conventional microbiological assays (Table 1; see also Table S1 in the supplemental material). Serological characterization grouped these 21 *Shigella* spp. as follows: *S. flexneri* 3a, $n = 12$; *S. flexneri* 2a, $n = 5$; *S. sonnei*, $n = 2$; *S. flexneri* 4, $n = 1$; untypeable (UT) *S. flexneri*, $n = 1$ (Table 1). Among the 16 *Shigella*-negative specimens, bacterial enteropathogens were identified in 8 as follows: *V. fluvialis*, $n = 3$; *Campylobacter jejuni*, $n = 2$; *Pseudomonas*

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

ISC category	Cells positive for marker	Antibody class	Mean no. of IgA- or IgG-secreting cells ± SEM/ml of blood from diarrheal patients with indicated culture-confirmed disease status[a]		P value[b]
			Shigellosis ($n = 21$)	Nonshigellosis ($n = 16$)	
Gut homing	$\alpha_4\beta_7^+$	IgA	2,332 ± 635	2,182 ± 800	0.09
		IgG	1,951 ± 412	1,636 ± 768	0.004
Non-gut homing	HLA-DR$^+$ CD19$^+$ $\alpha_4\beta_7^-$	IgA	1,730 ± 352	913 ± 186	0.05
		IgG	448 ± 95	957 ± 212	0.02
Total	HLA-DR$^+$ CD19$^+$ $\alpha_4\beta_7^+$	IgA	4,061 ± 935	3,094 ± 900	0.07
		IgG	2,399 ± 438	2,593 ± 884	0.24

[a] Data are expressed as mean numbers (± standard errors of the means [SEM]) of IgA- or IgG-secreting cells (ISCs) expressing α4β7$^+$ and/or HLA-DR$^+$/CD19$^+$ per milliliter of blood.

[b] Data represent results of unpaired *t* tests.

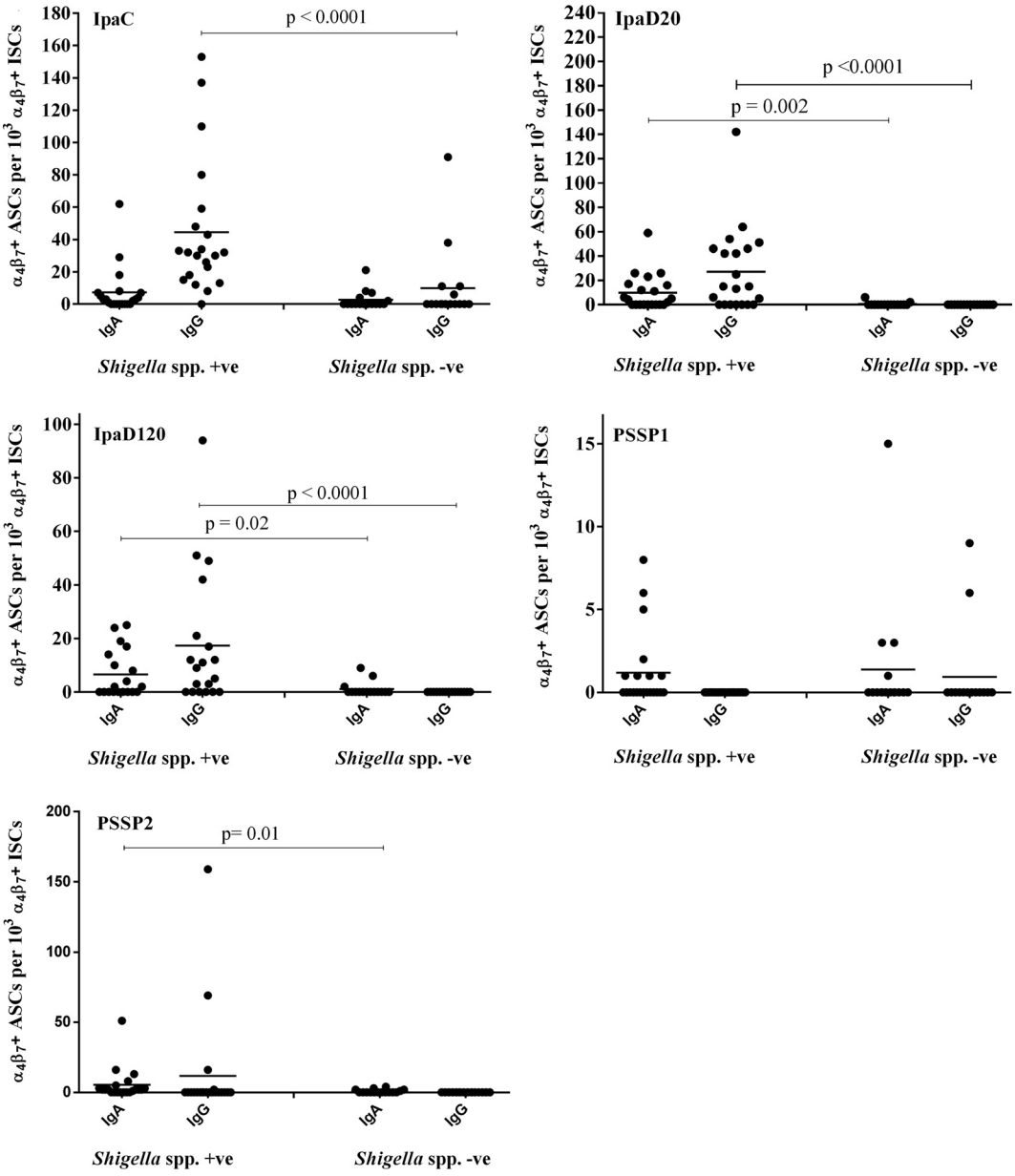

FIG 1 Gut-homing ASC ($\alpha_4\beta_7^+$) responses as determined by ELISPOT assays against different antigens among patients infected (+ve) or not infected (-ve) with *Shigella* spp. Responses were determined for the IgA and IgG antibody classes. Data were normalized for each antibody class and are expressed per 10^3 gut-homing ISCs. Nonparametric unpaired *t* tests were performed to determine statistical significance; horizontal bars represent mean values.

spp., $n = 1$; *V. cholerae*, $n = 1$; *V. parahaemolyticus*, $n = 1$ (Table 1). 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 ($\alpha_4\beta_7^+$) and non-gut-homing (HLA-DR$^+$ CD19$^+$ and $\alpha_4\beta_7^-$) fractions. The ELISPOT assay also enabled detection

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	No. of responders/total no. of diarrheal patients[a] (mean no. of antigen-specific gut-homing ASCs and range):			
	With shigellosis [*P* value][b]		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)

[a] Responders were defined as patients who yielded ≥5 spots per 10^3 gut-homing ISCs. Values in parentheses indicate mean numbers of antibody-secreting cells (ASCs) per 10^3 $\alpha_4\beta_7^+$ immunoglobulin-secreting cells (ISCs).
[b] *P* values represent results of chi-square (χ^2) tests.

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 10^3 gut-homing ISCs. 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 Ipa and PSSP1 antigens in the analysis of non-gut-homing ASCs (see Table S2 in the supplemental material). However, non-shigellosis patients showed significant differences in responses to PSSP2 antigens in the IgA ($P = 0.02$) class (see Table S2). Analysis of ELISPOT assay responders (≥5 spots per 10^3 gut-homing ISCs) 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-

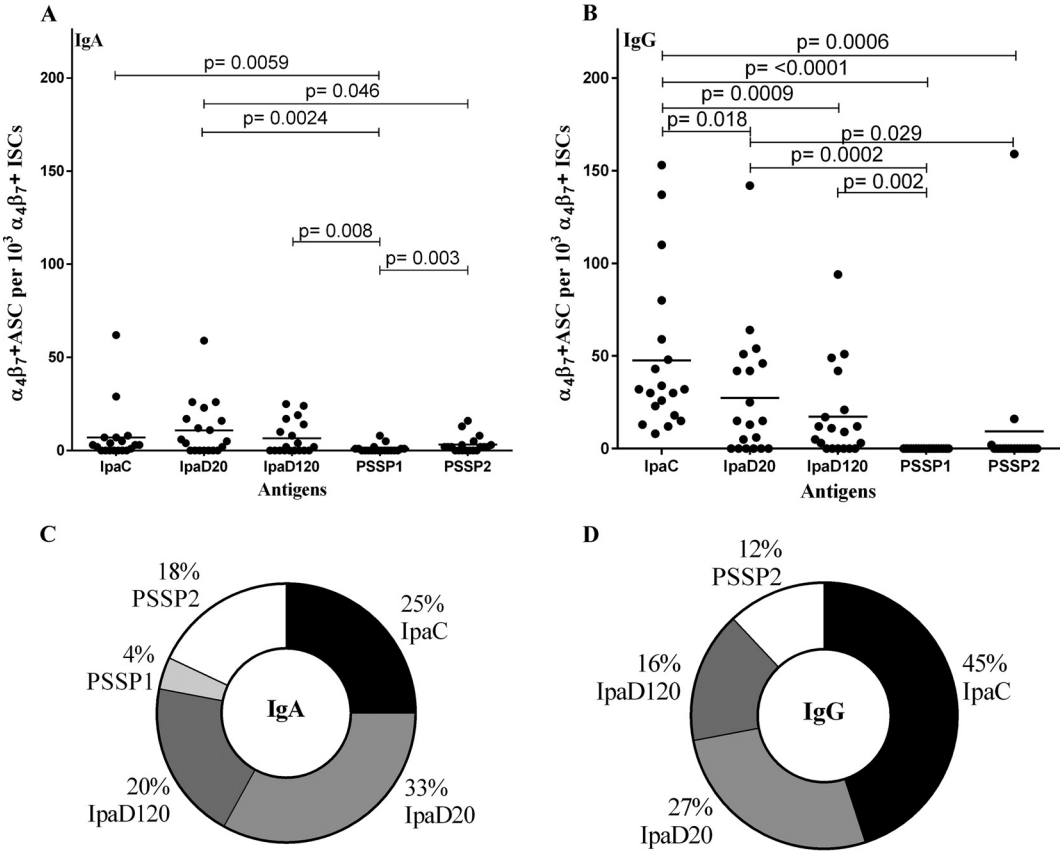

FIG 2 Gut-homing ASC ($\alpha_4\beta_7^+$) responses determined by ELISPOT assays in an antibody class-specific manner (A [IgA] and B [IgG]) and relative antibody class-specific proportions against different antigens (C [IgA] and D [IgG]) among shigellosis patients. Data were normalized for each antibody class and are expressed per 10^3 gut-homing ISCs. Nonparametric paired *t* tests were performed to determine statistical significance; horizontal bars represent mean values.

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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