Immune responses to *Shigella dysenteriae* 1 and *Shigella flexneri* lipopolysaccharide and polysaccharide antigens in Bangladeshi patients with shigellosis

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**Summary**

The immune responses of adult Bangladeshi patients to diarrhoea caused by *Shigella dysenteriae* type 1 (n = 20) and *Shigella flexneri* (n = 12) were analysed and compared with that of 20 healthy individuals. Antigen-specific antibody secreting cells (ASC) were estimated by ELISPOT, the peripheral mononuclear cell response was determined by a lymphocyte proliferation assay, and serum and mucosal antibody responses were measured by ELISA. Purified lipopolysaccharides (LPS) and O-polysaccharides (PS) from *S. dysenteriae* type 1 or *S. flexneri* Y strains, respectively, were used as antigens. A significant increase (P<0.001) in the number of LPS-specific IgA-ASC was observed with a peak 6-8 days after the onset of disease followed by a rapid decline within 16 days. The mucosal IgA responses were similar. Serum IgA titres were highest 9-11 days after the onset of diarrhoea and significantly higher (P<0.001) than in the control group. The serum IgG levels in *S. dysenteriae* 1 group were almost twice (P=0.008) the level measured in the control group. The mean IgG titres in the *S. flexneri* group, however, were only slightly higher (P>0.05) than that seen in the control group during the acute phase (3-5 days after onset). A strong specific cellular immune response (P<0.001) to the homologous *S. dysenteriae* 1 and *S. flexneri* O PS antigens was observed in both groups 6-8 days after the onset of symptoms. The immune responses observed suggest that the patients had previously been exposed to shigella. The study demonstrates a good correlation between humoral and cellular immune responses which may play concomitant protective roles in the host defence against shigellosis. Informed consent was obtained from study patients and controls in accordance with the guidelines of the ethical review committee of International Centre for Diarrhoeal Diseases Research, Bangladesh.

**Key words:** Shigellosis, lipopolysaccharide, humoral and cellular immune responses

**Introduction**

Bacillary dysentery is a significant cause of morbidity and mortality in developing countries, and is a major cause of diarrhoea-related deaths among children in Bangladesh.4,5. *Shigellae* are able to penetrate and multiply within epithelial cells and lamina propria of the large intestine, which often lead to a strong inflammatory reaction with resultant ulceration and abscesses of the colon. The infection is usually self limiting and is restricted to the mucosal surface, but on rare occasions spreads beyond the lamina propria.

Shigellosis evokes both humoral and cell-mediated immune responses. Epidemiological surveys and studies in human volunteers and experimental animals have suggested that natural infection or vaccination confers species and serotype-specific immunity. The immune response was shown to be independent of serum antibody production. Antibodies elicited after shigellosis are directed against the shigella lipopolysaccharides
informed consent was obtained from each patient. Antimicrobial treatment (nalidixic acid and pivmecilliam) was started before the antibiotic sensitivity pattern of each bacterial isolate was obtained, when the severity of the disease so required. The patients were normally released from the hospital 4-5 days after admission (no traces of blood in faeces and not more than two soft stools/day) and they came for follow-up on days 7 and 11 from the time of presentation for medical attention.

Control group

The control group comprised twenty healthy adults aged between 20 and 50 years. The controls were selected from a population comprising people of lower socioeconomic status (shopkeepers, mechanics, daily labourers etc.) matching the patient population. The criteria for eligibility as control was that none of the individuals had any incidence of diarrhoea or enteric fever within the last 5 months. Written consent was obtained from each individual. The health of the subjects was assessed by their medical history, physical examination, and stool specimens were examined for bacterial pathogens and protozoa.

Study specimens and their preparation

Samples taken from the patients included peripheral blood mononuclear cells, serum and stool. Samples were taken from each patient on the day of admission (the onset of diarrhoea was approximately 2 to 4 days earlier) and 4, 7 and 11 days after admission. From each healthy control blood and stool samples were obtained only once. Mononuclear cells were separated by centrifugation of heparinized venous blood (~10 ml) on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Isolated cells were washed three times in MEM (Gibco, Grand Island, NY) and suspended in RPMI 1640 (Gibco) containing 10% (v/v) of heat inactivated foetal calf serum, 2 mM of L-glutamine (Gibco), 10 mM HEPES (Flow Laboratories, Herts WD3 IPO, England) and 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and the cell density adjusted to a concentration of 1 x 10⁶ cells ml⁻¹. Cell viability was checked by the trypan blue exclusion test. One part of the cell-suspension was used for the ELISPOT assay and the other part for the lymphocyte proliferation assay. Serum samples were collected by centrifuging the blood after clot formation and the serum was stored at −20°C. Two grams of each stool sample was mixed with phosphate buffered saline (PBS; pH 7.4: 1:5 dilution) containing 1 mg ml⁻¹ Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, USA), 1 mg ml⁻¹ phenylmethylsulfonyl fluoride (Sigma) 0.1 mM ml⁻¹ and 0.05% Tween 20. The mixture was vortexed and centrifuged at 2000 g at 4°C for 30 min. The supernatant was collected, filtered through a 0.45 μm pore size filter (Acrodisc, Gelman Sciences, MI, USA) and stored at −20°C in multiple aliquots until used.

Bacterial strains and preparation of antigens

S. dysenteriae type 1 strain B157a, S. flexneri type Y (SFL 111A) and Escherichia coli CVD 17-2 were grown in batch cultures as described and lipopolysaccharide (LPS) antigen extracted by hot phenol-water. O-antigenic polysaccharides (PS) devoid of lipid A were prepared from S. dysenteriae type 1 strain 114 Sd and S. flexneri type Y and E. coli CVD 17-2. Fatty acid analysis of isolated PS by gas liquid chromatography.

Materials and methods

Patient group

Adults with bloody, mucoid stool admitted to the diarrhoea treatment centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) were included in the study. A total of 40 patients (aged 18-58 y), with >25 faecal leukocytes and >20 red blood cells (RBC) per high power field (>400) and no protozoa in their stool were initially selected. Patients with stool culture positive for shigellae were enrolled in the study. The study was approved by the ICDDR,B ethical review committee, and written informed consent was obtained from each patient. Antimicrobial treatment (nalidixic acid and pivmecilli) was started before the antibiotic sensitivity pattern of each bacterial isolate was obtained, when the severity of the disease so required. The patients were normally released from the hospital 4-5 days after admission (no traces of blood in faeces and not more than two soft stools/day) and they came for follow-up on days 7 and 11 from the time of presentation for medical attention.

Control group

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did not show any detectable amount of fatty acids (less than 0.01% of each polysaccharide tested).

**Determination of antibody secreting cells (ASC) by ELISPT assay**

The number of ASC was determined by the modified ELISPT method. Ninety-six well plates with a nitrocellulose base (Millititter HA, Millipore Bedford, MA, USA) were coated overnight at 22°C with 100 μl of antigen or antibody. Affinity purified goat anti-human IgA, IgG and IgM (Sigma) at a dilution of 1:500 in PBS and purified LPS at a concentration of 20 μg ml⁻¹ in coating buffer (50 mM carbonate buffer pH 9.6) were used. Control wells were treated with coating buffer only. The microtitre plates were washed five times with PBS by suction into a Millititter vacuum filtration holder (Millipore). One hundred μl cell suspension (~10⁶ cells well⁻¹) was applied into each well and the plates incubated at 37°C overnight in a humidified atmosphere of 5% CO₂. Plates were washed five times with PBS. Subsequently biotin-goat-anti-human IgA, IgG and IgM (Sigma) diluted 1:500 in PBS were added and incubated at 22°C for 4 h. After five washes with PBS, avidin and peroxidase conjugated biotin (Vectastain, ABC Kit, Vector Laboratories, Inc., CA, USA) diluted 1:200 in PBS was added and the plates incubated for 1 h at room temperature. Finally after five manual washes the plates were stained using 3-amino-9-ethylcarbazole (Sigma) and H₂O₂ as substrate in 0.05 M sodium acetate buffer (pH 8.1). Dark brown spots, representing the isotypes or O-antigen specific IgA or IgG, were counted with the aid of a dissection microscope (Zeiss 47502, West Germany), and recorded as ASC per 10⁶ peripheral mononuclear cells.

**Determination of total immunoglobulin concentration**

Total IgA, IgG and IgM concentration of serum and faecal extracts from the patients with shigellosis were determined by using a turbidometric method. For this purpose Cryptas Bio centrifugal auto analyser (Roche, Japan) was used. The internal standard was human serum protein calibrator (Dakopatts A/S, Copenhagen, Denmark) and the antibodies used were rabbit anti-human IgA, IgG and IgM (Dakopatts).

**Determination of LPS-specific antibody titre by ELISA**

ELISA was performed in polystyrene microtitration plates (Nunc, Denmark) by LPS antigens. Wells of the plates were coated overnight with 100 μl of LPS (10 μg ml⁻¹ in 50 mM carbonate buffer, pH 9.6) at room temperature. Control wells were treated with coating buffer only. The plates were washed three times in washing buffer (0.15 M NaCl containing 0.05% (v/v) Tween 20). Serum samples diluted 1:1000 in incubation buffer (PBS containing 0.05% Tween 20) were added in duplicates to the wells and incubated for 22°C for 2 h. The plates were washed and alkaline phosphatase conjugated to rabbit anti-human IgA, IgG and IgM immunoglobulins (Dakopatts) were used at dilutions of 1:3000, 1:4000 and 1:2000, respectively, and incubated for 2 h at 22°C. After the final wash, 100 μl of p-nitrophenyl phosphate (1 mg ml⁻¹) in 1.0 M diethanolamine-HCl buffer, pH 9.8, was added as substrate. The optical densities (OD) were read after 100 min at 405 nm with a Titertek Multiscan photometer (Flow Laboratories, Irvine, Scotland). The results were expressed as relative titres: i.e. the OD values multiplied by the dilution factor (e.g. 1000). Net optical density was defined as the optical density of the antigen well minus the optical density of the corresponding background well.

Intestinal secretory IgA antibody to S. dysenteriae type 1 and S. flexneri O-antigen was measured by ELISA. Microtitre plates were coated as above with LPS from S. dysenteriae type 1 B157a, S. flexneri type Y and E. coli CVD 17-2. One hundred μl of faecal extracts (1:5 dilution) was applied to the plates and then incubated for 2 h at 37°C. After four washes, horseradish-peroxidase conjugated rabbit anti-human conjugates (Dakopatts) at 1:1000 dilution were applied and incubated for 2 h at 37°C. Subsequently, 100 μl of substrate solution containing 1.2-phenylenediamine dihydrochloride (OPD) in 0.1 M citrate buffer (pH 5.0) and 0.015% H₂O₂ was added. After colour development, the reaction was terminated with 25 μl 4N H₂SO₄. Optical density was read at 492 nm. Calculations were done as for serum samples.

**Antigen and mitogen stimulated lymphocyte replication responses (LR)**

One hundred μl of peripheral blood mononuclear cells (PBMs; 1 × 10⁶ cells ml⁻¹) were added to each well of a sterile 96-well U bottomed plate (Nunc) and 100 μl of either mitogen or antigen added to the wells (in triplicates). PBMs cultured in medium alone were used as controls. Lipid A free O-antigenic polysaccharides from S. dysenteriae type 1, S. flexneri Y and E. coli CVD at a concentration of 25 μg ml⁻¹ suspended in RPMI were used as antigens. Initial studies with PS indicated that the optimal stimulation of the lymphocytes occurred at a concentration of 25 μg ml⁻¹ (data not shown). Three mitogens were used for the stimulation assays: phytohaemagglutinin (PHA: Sigma; 1.25 μg ml⁻¹), Concanaval A (Con A; Sigma; 10 μg ml⁻¹) and Pokeweed mitogen (PWM; Sigma 10 μg ml⁻¹). Each mitogen was made up in complete culture medium as a stock solution, filtered, sterilized and stored at -20°C until used. Cultures were incubated at 37°C in 5% (v/v) CO₂ for 5 days. On the fifth day, 1 μCi [³H]thymidine (Amersham Sweden AB), was added to each well and the plates incubated overnight (16 h). Cells were harvested onto glass fibre filters using the Multimash cell harvester (Dynatech). The filters were air-dried and individual discs placed in 4 ml of scintillation fluid.
(SPM-14, Iso-Lab incorporation). The radioactivity was measured in a liquid scintillation counter (LKB Wallace, 1218 Rackbeta). The incorporation of [3H]thymidine was expressed as mean counts per minute (cpm) per triplicate culture. For each specimen, the net counts/minute was determined by subtracting the average counts/minute of a triplicate culture without antigen from the average counts/minute of a triplicate culture with antigen or mitogen. The mean net cpm values and standard deviation (SD) were determined for the control group.

**Statistical analysis**

Statistical comparisons were carried out with the Student's t test.

**Results**

**Clinical features**

Eight out of 40 patients had stool cultures with no growth of shigellae. Twenty patients had infection with *S. dysenteriae* type 1 and 12 were infected with *S. flexneri*. Antimicrobial therapy was given to all patients. Clinical resolution of diarrhoea occurred within 4 to 5 days of initiation of therapy. Stool cultures were usually negative for shigella after 2 to 3 days of antibiotic treatment.

**Analysis by ELISPOT assay**

**Total ASC**

The total numbers of cells secreting IgA, IgG and IgM as well as the number of LPS-specific ASC were determined by the ELISPOT assay. The maximum numbers of IgA-ASC (86 ± 17/10⁵ PMB, mean ± sn) and IgG-ASC (74 ± 17/10⁵ PMB) were observed in patients infected with *S. dysenteriae* 1 on days 6–8 after onset of infection (Figure 1a). The peak values were significantly higher (P < 0.03) than the acute phase values (samples taken on days 3–5). Patients showed a significantly greater ASC response compared to controls (P = 0.0001). The IgM-ASC were the highest (47 ± 7/10⁵ PMB) on days 6–8 and were significantly higher than the acute phase values (P = 0.01) as well as to the values seen in controls (P = 0.0001) even 12–16 days after infection.

In patients with *S. flexneri* infection (Figure 1b) the numbers of IgA-ASC (86 ± 11/10⁵ PMB) and IgG-ASC (75 ± 14/10⁵ PMB) peaked 6–8 days after diarrhoea started and were significantly higher (P = 0.0001) in comparison to those of controls. However, the peak response was not significant in comparison to the acute phase response (P > 0.05). The number of IgM-ASC in the patients were highest (55 ± 7/10⁵ PMB) 6–8 days after the onset of diarrhoea. Thereafter, it gradually declined but the magnitude of the response was still significantly higher (P = 0.0001) than in the controls on days 12–16.

**Specific ASC**

Peak responses occurred on days 6–8 after the onset of diarrhoea for most patients with *S. dysenteriae* type 1 infection. High numbers of *S. dysenteriae* type 1 LPS-specific IgA (13 ± 6/10⁵ PMB) or IgG (9 ± 4/10⁵ PMB) secreting cells were seen in the patients (Figure 2a); the responses represented approximately 15% and 12% of the total IgA or IgG producing cells, respectively. The strongest response was observed for IgA which was significantly higher when compared to the response of the control group (P = 0.0001) throughout the study period. The IgG-ASC response was highest on days 6–8 (P = 0.001) after which it declined rapidly to the control levels. The peak IgA and IgG-ASC responses on days 6–8 after the onset of disease were significantly higher than the respective acute phase responses (P = 0.004).

In *S. flexneri* infected patients (Figure 2b) the number of *S. flexneri* LPS-specific IgA (17 ± 6/10⁵ PMB) or IgG (11 ± 5/10⁵ PMB) producing cells were
around 18% and 14% of the total IgA-ASC or IgG-ASC, respectively. When compared with the IgA-ASC response in controls, the differences were highly significant \((P = 0.0001)\). The IgG-ASC response was short-lived; it peaked on days 6–8 \((P = 0.0001)\) and faded away within 11–16 days after diarrhoea \((P > 0.3)\). The peak IgA or IgG-ASC response was not significant when compared with the acute phase responses \((P > 0.05)\).

Low numbers of ASCs \((2–3/10^5\) PBM) against LPS from \(E.\) coli CVD 17-2 were seen in both groups of patients. Mainly IgA producing cells could be detected.

Measurement of anti-shigella antibody titres

Serum
In patients with \(S.\) dysenteriae type 1 infection (Figure 3a) the mean peak anti-LPS IgA titre occurred 9–11 days after the onset of diarrhoea \((324 \pm 108)\) and remained significantly higher \((P = 0.0001)\) in the serum up to 16 days in comparison to the IgA titres in controls. The relative IgG titres were highest \((726 \pm 232)\) in patients with \(S.\) dysenteriae type 1 infection at 9–11 days after onset of disease and remained elevated up to 16 days. The titres were significantly higher \((P = 0.008)\) when compared with the titres in healthy individuals. The differences in the IgM titres between the patients and the controls were also significant \((P = 0.002)\).

Anti-LPS IgA titres in \(S.\) flexneri patients were highest on days 9–11 after the onset of diarrhoea (Figure 3b) and remained significantly higher \((P = 0.0001)\) throughout the study period when compared to the IgA titres in the normal population. The relative IgG titres \((556 \pm 322)\) in \(S.\) flexneri infected patients were more or less constant throughout the study period. The IgM titres showed a pattern similar to that of IgG, although the values were much lower. There was no significant difference \((P > 0.05)\) in IgM responses between the patients with \(S.\) flexneri infection and the controls.

Faecal extracts
In \(S.\) dysenteriae 1 infection (Figure 4a) the peak titres were seen at days 6–8 after onset of diarrhoea \((2.89 \pm 2.09)\)
response they were still significant \((P = 0.01)\). s-IgA titres to the homologous \textit{S. dysenteriae} 1 LPS were highly significant \((P = 0.008)\) than those against heterologous \textit{S. flexneri} LPS.

The maximum s-IgA titres \((2.72 \pm 1.6)\) in the faecal extracts from patients with \textit{S. flexneri} infection (Figure 4b) were also seen 6–8 days after the onset of disease, although the titres were not significant when compared to the acute phase titres \((P > 0.05)\). The titres remained significantly higher \((P = 0.04)\) even in the 12–16 day samples. The difference between the antibody titres to \textit{S. flexneri} Y LPS and heterologous \textit{S. dysenteriae} 1 LPS in patients with \textit{S. flexneri} infection was not significant \((P = 0.06)\).

Figure 4. Mean faecal s-IgA anti \textit{S. dysenteriae} type 1 □ and \textit{S. flexneri} Y □ lipopolysaccharide responses in patients with either a, \textit{S. dysenteriae} type 1 \((n = 20)\) or b, \textit{S. flexneri} \((n = 12)\) infection at different time intervals after onset of dysentery and from healthy controls \((n = 20)\). Vertical bars represent \(\pm 1\) SD.

Total immunoglobulin concentration

Serum

In patients with \textit{S. dysenteriae} type 1 infection (Table 1), the mean IgA concentration was highest \((2.9 \text{ g l}^{-1})\) 6–8 days after the disease started. There was a mild decrease in IgA concentration to \(2.7 \text{ g l}^{-1}\) in samples obtained on days 11–16. The mean total \(\text{IgG}\) concentration in serum increased gradually from \(12.1 \text{ g l}^{-1}\) up to \(13.8 \text{ g l}^{-1}\) during the acute phase of infection. The mean IgM content of serum increased slightly from 1.05 to 1.2 \text{ g l}^{-1} during the study period \((-1.09 \text{ g l}^{-1})\).

The mean total IgA concentration in serum of patients with \textit{S. flexneri} infection was maximum \((2.7 \text{ g l}^{-1})\) on days 9–11 and decreased to \(2.5 \text{ g l}^{-1}\) on days 11–16. Mean levels of IgG were \(14.2 \text{ g l}^{-1}\) on days 3–6 which increased to \(15.3 \text{ g l}^{-1}\) at the end of the study period. The mean total IgM concentration was gradually increasing from \(1.1 \text{ g l}^{-1}\) (3-5 days) to \(1.3 \text{ g l}^{-1}\) 11–16 days after diarrhoea started.

Faecal extracts

The concentration of IgA in stool extracts of patients with \textit{S. dysenteriae} 1 (Table 1) was maximum \((0.42 \text{ mg ml}^{-1})\) on days 6–8 post-infection. Then there was a sharp decline in the IgA concentration reaching a level almost half \((0.22 \text{ mg ml}^{-1})\) of the peak value. Similarly the mucosal IgG concentration rose to a maximum of

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<th>Specimen and immunoglobulins</th>
<th>S. dys 1(^{a}) ((n = 20))</th>
<th>Patients infected with</th>
<th>S. flex(^{b}) ((n = 12))</th>
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<td>Days after onset of diarrhoea</td>
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<td>Stool ((\text{mg ml}^{-1}))</td>
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<td>IgG</td>
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Immunoglobulin concentration was measured as described in Materials and methods. S. dys 1\(^{a}\) - \textit{Shigella dysenteriae} type 1; S. flex\(^{b}\) - \textit{Shigella flexneri}.
Days after onset of diarrhoea

Control 3-5 6-8 9-11 12-16

Figure 5. Lymphocyte proliferation responses to O-antigenic polysaccharides (PS) isolated from S. dysenteriae type 1 and S. flexneri lipopolysaccharides in patients with either (a) S. dysenteriae type 1 (n = 20) or (b) S. flexneri (n = 12) infection at different times post-infection and in healthy controls (n = 20). Vertical bars represent ±1 SD.

1.61 mg ml⁻¹ on days 6-8 after illness started and rapidly decreased to 0.48 mg ml⁻¹ within 16 days. In patients with S. flexneri infection, concentration of IgA and IgG were the highest 6-8 days after diarrhoea (0.54 mg ml⁻¹ and 0.46 mg ml⁻¹ respectively) and decreased within 11-16 days to a concentration 10-13 times lower than the peak values.

Antigen specific lymphocyte replication assay (LR)

PHA, Con A and PWM were included as positive stimulation controls in the lymphocyte replication assay. The uptake of [³H]thymidine in unstimulated cultures of peripheral blood lymphocytes (PBM) from the patients or the controls were the same. The optimal stimulation to PS from S. dysenteriae type 1 (cpm 2244 ± 887) was found to occur between 6-8 days post-infection in the S. dysenteriae type 1 group (Figure 5a). Analysis by Student’s t-test showed that the difference in responses to PS between the patients and the control group on days 6-8 after onset of diarrhoea were highly significant (P = 0.0001). The response was much lower on days 11-16 after diarrhoea and was not significant (P = 0.9). The mean peak response in patients was significantly higher than the acute phase response (P = 0.001). The magnitude of lymphocyte proliferation response to homologous serotype shigella PS during the study period was notably higher (P = 0.0001-0.01) than that elicited by polysaccharides from heterologous bacteria.

Lymphocytes from patients with S. flexneri infection (Figure 5b) showed a strong response (cpm 3233 ± 1951) on days 6-8 after diarrhoea and the increases were significant (P = 0.001) when compared to the values in controls, but were not significant when compared to the acute phase values in patients (P > 0.05). The response, however, came down to the levels seen in controls (P = 0.66) after 11-16 days of infection. The lymphocyte proliferation response to homologous S. flexneri PS was significantly higher (P = 0.003-0.02) during the study period in comparison to that against heterologous S. dysenteriae 1 PS.

Discussion

Our study shows that there was a strong immunologic response in the patients with shigellosis. Analysis of the total ASC showed that the predominant response was IgA followed by IgG and IgM. Both the total IgA and IgG-ASC remained significantly elevated (P < 0.001) during the acute phase of the infection, reaching a peak around 6-8 days after onset of illness. This was consistent with the observation made in our own studies with a live oral S. flexneri vaccine (unpublished data), and in studies performed in experimentally-infected animals⁴ and human⁵. The duration of the response could not be studied for a longer period of time since the patients could not be followed up after 12-16 days. Increased numbers of LPS-specific ASC appeared in the peripheral circulation around 6-8 days after infection (Figure 2). The LPS-specific ASC in patients was significantly higher compared with responses seen in controls (P < 0.001). However, the ASC responses against the shigella LPS antigens abated after two weeks. The specific IgG or IgA responses were only 12-14% and 15-18% respectively, of the total number of immunoglobulin secreting cells. E. coli CVD 17-2 LPS was used as a negative control. E. coli CVD is an enteroradherent aggregative and a probable pathogen⁶ which has no relatedness to any of the known shigella O-antigens (unpublished data).

In most patients and five of the controls (25%) ASCs specific to the E. coli CVD LPS were found, although the numbers (2-3) were very low. The antibodies were mainly of the IgA subclass. Parallel results have been noted by others who justified the appearance of E. coli LPS-specific ASC as a result of polyclonal stimulation in patients with diarrhoea⁴. Parallel measurements of s-IgA in faecal extracts showed a pattern (Figure 4) similar to that obtained in the ELISPOT with peak responses against shigella LPS 6-8 days after the patients fell ill. The s-IgA
Results were in accordance with the findings of our vaccine studies in Vietnam (unpublished data) and those of Dinari et al. (1987) and Oberhalman et al. (1991). It is clear from our study that the mucosal antibody responses were more specific for the infecting organisms, and the response was limited to a short period of time. The intestinal secretory immune response did not correlate with the serum antibody responses in the patients, further supporting the concept that serum IgA immunoglobulin responses are not always mirror images of the s-IgA responses in secretions.

Analysis of the serum antibody responses against the shigella LPS showed statistically significant ($P < 0.001$) IgA increases in patients compared to the controls. In patients with S. dysenteriae type 1 infection, the relative IgG titre were significantly higher ($P = 0.008$) than the corresponding values in controls. However, the IgG titres in S. flexneri infected patients were barely higher than those in the controls ($P = 0.3$). The reason for this may be a result of a more severe infection caused by S. dysenteriae 1 than S. flexneri. We surmise that the lack of a rise in titres of LPS specific IgG in serum in patients with S. flexneri infection is a result of repeated frequent exposure to various S. flexneri serotypes, and therefore already high pre-existing titres. These data are in agreement with our studies from Vietnam and the studies of Oberhalman et al. (1991). Schulz et al. (1993), and Cohen et al. (1993). These responses in Bangladeshi patients were similar to those in primed Vietnamese patients but were unlike the unprimed Swedish patients. The initial elevated levels of serum IgG antibodies to homologous serotype LPS shows that anti-LPS-IgG titres are good indicators of previous shigella infections.

Patients infected with S. dysenteriae 1 or S. flexneri showed similar patterns of responses against specific shigella PS antigens in LR assays. The maximum stimulation was obtained during the acute phase of infection (6–8 days post-infection) and decreased rapidly, approaching the levels seen in controls within 2 weeks. The results further substantiate the proposition that shigellosis generates a systemic antigen-specific cell-mediated response, which apparently correlates with a reduction in clinical disease. Two of the persons in the control group showed a positive response to shigella PS, irrespective of previous known exposure. These control subjects might have experienced asymptomatic shigella infections in the recent past. Similar observations have been made in other studies of enteric infections where seven subclinical cases were found to occur for each clinical case of typhoid fever. These results are consistent with our previous data which shows that the controls had pre-existing elevated titres of IgG against LPS from shigella in serum. The LR response assays proved to be good measures of prior contact with shigella as has been demonstrated in several studies. However, the role these lymphocytes play in the host immune response to shigella infections is not clear. Chronic shigella infection has been reported in a patient with acquired immunodeficiency syndrome (AIDS) with impaired intestinal cell-mediated immunity. Moreover, CD4$^+$ cells in the gut lamina propria were shown to play a major role in host defence against cholera toxin-induced diarrhoea in mice. T-lymphocyte clones, responsive to S. flexneri, were generated from a patient with shigellosis. It should be emphasized that all patients were given oral antibiotics (nalidixic acid and pivmecillinam) as soon as admitted to the Clinical Research Centre of ICDDR.B. The possible influence of the antibiotic regimes on the immune responses is difficult to assess.

The functional role of the diffuse lymphoid compartment in the intestine in the host defence against shigellosis should be studied, to define better the cell-mediated immune mechanism. We surmise that in addition to the humoral and mucosal immune responses, cell-mediated immune responses are also important for curtailment of dysentery.

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References


5 Takeuchi A, Sprinz H, La Brec EH, Formal SB. Experimental acute colitis in the rhesus monkey following peroral infection with Shigella flexneri infection. Am J Pathol 1965; 52: 293–29


