SEROLOGY

Antibodies to Shiga toxin in the serum of children with *Shigella*-associated haemolytic uraemic syndrome

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Antibodies to Shiga toxin (Stx) were measured in the sera of 49 children with *Shigella dysenteriae* serotype 1 infection, of whom 17 had haemolytic uraemic syndrome (HUS) and 32 had no complications (uncomplicated shigellosis, UCS). Children with HUS had lower levels of total IgG and IgM and lower IgM titres to Stx than those with UCS. The number of children with neutralising antibodies was similar in the two groups. Of the children with HUS, 11 had HUS on enrolment and six developed HUS subsequent to enrolment. Antibody titres in children who subsequently developed HUS were compared with those in children with UCS to assess whether differences in antibody titres occurred before the development of HUS. IgA titres to Stx were found to be higher in children who subsequently developed HUS than in those with UCS. However, logistic regression analysis revealed that titres of Stx antibodies in the serum were not significant risk factors for the development of HUS. Thus, although the levels of Stx antibodies were different in children with HUS, and higher IgA titres to Stx were identifiable in children who subsequently developed HUS compared with those with UCS, the relevance of these findings in the development of HUS remains to be elucidated.

Introduction

Haemolytic uraemic syndrome (HUS) is a triad of haemolytic anaemia, thrombocytopenia and acute renal failure that often complicates dysentery following infection with *Shigella dysenteriae* serotype 1 [1] or Shiga toxin-producing *Escherichia coli* (STEC) [2]. The mechanism(s) whereby these bacteria precipitate HUS is not understood, but Shiga toxin (Stx) produced by *S. dysenteriae* 1, and Stx1 and Stx2 (also known as SLT-I and SLT-II, respectively) produced by STEC appear to play a pivotal role. Stx and Stx1 are closely related and the base sequence of their genes differs by only three nucleotides [3]. It is not clear how these toxins induce HUS, but Stx and Stx2 can damage vascular endothelial cells *in vitro* [4, 5] either alone or in combination with cytokines and the bacterial lipopolysaccharide (LPS). More recently it has been

shown that injection of Stx2 in mice mimics the glomerular changes observed in HUS [6]. The importance of these toxins in the development of HUS has led to the hypothesis that antibodies to these toxins could prevent HUS. Serum antibodies to Stx1 and Stx2, neutralising as well as total immunoglobulin (Ig) isotypes, have been described in patients with STEC-associated HUS [7–10], but the titres were not as high or as consistent as those to the LPS [11]. Commercially available human intravenous immunoglobulin (IVIG) preparations contain neutralising antibodies to Stx and Stx1, but not Stx2 [12], and the use of IVIG in a trial of a limited number of children with HUS showed a beneficial effect [13]. Furthermore, human IVIG given to rabbits either before or within 1 h of Stx1 or Stx2 injection protected rabbits from developing diarrhoea due to Stx1 but not Stx2 [14]. Therefore, immunisation with toxoid for prevention of HUS in children infected with *S. dysenteriae* 1 or STEC [15] appears to be promising. However, the role of antibodies to Stx in protection against shigellosis and the development of HUS is unclear. Bangladeshi

patients with uncomplicated *S. dysenteriae* 1 infection elicit a strong antibody response to Stx with both IgA1 and IgG1 levels peaking at 9–11 days after the onset of diarrhoea [16]. Furthermore, both IgA and IgG responses to Stx are greater in patients with more severe illness, suggesting that these antibodies reflect disease severity rather than protection [17]. The present study describes Stx antibody levels (IgA, IgG, IgM and neutralising) in the sera of children with *S. dysenteriae* 1 infection with and without HUS, and also compares antibody titres between children with uncomplicated shigellosis (UCS) and those who subsequently developed HUS.

Materials and methods

Patient population

Initially, 49 children between 12 and 60 months of age attending the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, with complaints of dysentery (visible blood in stool, with or without fever) were enrolled. Stool and blood samples (5 ml, maximum) were collected from these children on enrolment. Freshly collected stools were examined microscopically for the presence of white (WBC) and red blood cells (RBC) and were cultured for enteropathogens [18]. Only children with culture-confirmed *S. dysenteriae* 1 were finally included in this study.

Laboratory investigations on blood included determination of haematocrit, total and differential leucocyte counts, platelet counts and the proportion of fragmented erythrocytes. Serum electrolytes and creatinine concentrations were measured when required. All children were treated with amdinocillin pivoxil or ciprofloxacin, while some received additional antibiotics for concomitant infections such as respiratory tract infections, septicaemia or middle ear infections. HUS was defined as serum creatinine levels $\geq 180/\mu\text{mol}$ of blood, signs of haemolysis, estimated either by haematocrit values falling by $\geq 10\%$ during hospitalisation or the presence of $\geq 0.5\%$ fragmented erythrocytes on peripheral blood smears, and platelet counts of $< 150\,000/\mu\text{l}$ of blood. Of all the children enrolled, 17 had HUS and 32 had no complications including HUS, acute renal failure, leukaemoid reaction or toxic megacolon (UCS). Of the children with HUS, eight had leukaemoid reaction ($\geq 40\,000$ WBC/ μl of blood, increased percentage of neutrophils and presence of immature neutrophils). Of the 17 children with HUS, 11 had HUS at the time of enrolment while six developed HUS subsequent to enrolment. The nutritional status of all children was determined by weight-for-age as a percentage of the National Centre for Health Statistics (NCHS) median. The study was approved by the Ethical Review Committee of ICDDR,B and only those children whose guardians gave informed, written consent were enrolled.

Collection and storage of serum and plasma samples

Serum was obtained from blood collected in sterile tubes and stored at -20°C until used. Plasma was collected from fresh blood separated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), in which plasma collected as a clear supernate above the band of mononuclear cells at the interface. It was filtered through a $0.22\text{-}\mu\text{m}$ pore size filter (Sartorius, Goettingen, Germany) and stored at -20°C until tested.

Determination of total immunoglobulins

Total IgA, IgG and IgM levels in plasma were measured by turbidimetry in a discrete analyser (COBAS-BIO; Roche) with monospecific antisera (Dakopatts, Glostrup, Denmark). Results were expressed as mg/ml of plasma.

Measurement of antibodies to Stx1 by enzyme-linked immunosorbent assay

Specific antibody titres to Stx1 were measured by an enzyme-linked immunosorbent assay (ELISA) as described previously [16]. Microtitration plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with $100\text{ }\mu\text{l}$ /well of purified Stx1 (a gift from Dr D. W. Acheson and Professor G. T. Keusch, New England Medical Center, Boston, MA, USA) [19] at $1.5\text{ }\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS)-Tween 20 and incubated overnight at 4°C . After blocking with PBS-Tween containing bovine serum albumin (BSA) 0.1% , serum samples, serially diluted in PBS-Tween containing BSA 0.1% , were added in duplicate to wells and incubated at 37°C for 2 h. After washing, horseradish peroxidase conjugated to rabbit anti-human IgA, IgM or IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1 in 1000 in PBS-Tween containing BSA 0.1% , was added and plates were incubated at 37°C for 1 h. The substrate, *o*-phenylenediamine dihydrochloride (OPD, Sigma) at $1\text{ mg}/\text{ml}$ in 0.1 M citrate buffer (pH 4.5)-hydrogen peroxide (Sigma) 30% , was added, and the reaction was stopped with 1 M H_2SO_4 after colour development. The OD was measured at 492 nm in a spectrophotometer (Titertek Multiskan Plus). Results were expressed as end-point titres which were determined as the interpolated dilution of the sample giving an OD_{492} of 0.2 above background. Background wells included those without samples. Calculations were done with a computer-based program (Multi; DataTree, Watthams, MA, USA).

Neutralisation assay

Antibody neutralisation titres were determined by the HeLa cell cytotoxicity assay [20]. Periplasmic extract (PE) was prepared from *S. dysenteriae* 1 strain 24583 (obtained from an adult patient with UCS) with polymyxin B [21] and used as a source of Shiga toxin. Briefly, HeLa cells at 2×10^4 cells/ml of minimal

essential medium (MEM) containing fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) 10% were grown as monolayers in flat-bottomed 96-well microtitration plates (Nunc) for 18–20 h at 37°C in a CO₂ 5% incubator. Serial two-fold dilutions of serum samples in MEM containing FBS 10%, from 1 in 4 to 1 in 64, were pre-incubated for 3 h with medium alone or PE diluted 1 in 8000 in MEM containing FBS 10% (which resulted in ~60% cytotoxicity of HeLa cells). A negative control of PE with medium and a positive control of PE with a neutralising anti-Stx monoclonal antibody (ICT7) [20] were always included in the assays. These were added in duplicate to HeLa cells and incubated for 18–20 h. Detached cells were removed and the remaining cells were fixed with formalin 5% in PBS, stained with crystal violet 0.13% in PBS containing ethanol 50% and formalin 2%, washed and dissolved with absolute ethanol. Optical densities were measured at 595 nm with a spectrophotometer (Titertek Multiskan Plus). Neutralisation of cytotoxicity by $\geq 20\%$ was considered as a positive response as described previously [12].

Statistical analyses

The significances of differences between two groups of children were ascertained by the *t* test (for continuous, parametric data) and the Mann-Whitney *U* test (for continuous, non-parametric data). The χ^2 test was used to compare proportions. Multiple regression analysis was used to assess the effect of nutritional status, duration of diarrhoea on enrolment, stool frequency in 24 h, sex and concomitant infections on Stx antibody titres. Logistic regression was used to determine whether serum Stx antibodies were risk factors for the development of HUS. Differences were considered to be significant when $p \leq 0.05$. Data were analysed with the Statistical Package for Social Sciences (version 6.0 for Windows, SPSS, Chicago, IL, USA).

Results

Patient population

The clinical characteristics of the children studied are shown in Table 1. Children in the two groups were comparable for age, sex, nutritional status, duration of diarrhoea on enrolment and the state of hydration (data not shown). Routine laboratory investigations (Table 1) showed higher numbers of WBC/ μ l of blood ($p < 0.001$) and higher percentages of polymorphonuclear cells ($p = 0.001$) and myelocytes ($p = 0.001$) in children with HUS when compared with those with UCS. These findings are not surprising, as 8 of 17 also had leukaemoid reaction, and are similar to previous reports where leukaemoid reaction accompanied most cases of HUS [1]. Compared with children with UCS, those with HUS had fewer platelets in blood ($p = 0.001$) and a greater percentage of fragmented erythrocytes ($p < 0.001$); these findings are consistent with the definition of HUS.

Table 1. Clinical characteristics and routine laboratory investigations of children on enrolment

Study group	Mean (SD) age (months)	Male, n (%)	Mean (SD) weight/age (% of the NCHS median)	Duration of diarrhoea (days)*	WBC $\times 10^3$ / μ l of blood	Mean (SD) percent PMNLs	Metamyelocytes (%)*	Myelocytes (%)*	Platelets $\times 10^3$ / μ l of blood*	Fragmented RBC (%)*
UCS	32.4 (14.7)	17 (53.1)	67.7 (11.0)	6.5 (5.0–8.8)	16.8 (13.9–19.5)	50.3 (19.2)	0.0 (0.0–1.0)	0.0 (0.0–0.0)	210.0 (181.3–237.5)	0.0 (0.0–0.0)
HUS	29.8 (9.7)	9 (52.9)	69.4 (10.2)	7.0 (5.0–8.0)	38.0 (21.9–46.4)	68.8 (12.9)	0.0 (0.0–3.0)	0.0 (0.0–1.5)	130.0 (72.5–206.3)	0.0 (0.0–1.1)
<i>p</i> value†	NS	NS	NS	NS	<0.001	0.001	NS	0.001	0.001	<0.001

NCHS, National Centre for Health Statistics; WBC, white blood cells; PMNL, polymorphonuclear cells; RBC, red blood cells; UCS, uncomplicated shigellosis; NS, not significant; HUS, haemolytic uraemic syndrome. * Values are median (25th–75th quartiles).

† The *t* test (for continuous, parametric data), Mann-Whitney *U* test (for continuous, non-parametric data) and the χ^2 statistic (for categorical data) were used.

Total immunoglobulins, titres of antibodies to Stx and neutralisation titres

Comparison of total IgA, IgG and IgM levels in plasma of children with HUS with those with UCS, showed that IgG and IgM levels were lower in children with HUS ($p < 0.001$ and $p = 0.036$, respectively, Table 2) while total IgA levels were similar. Serum IgM titres to Stx were lower in children with HUS ($p = 0.032$) than in those with UCS, while Stx IgG and IgA titres were similar in the two groups of children (Table 2). Because it is known that various clinical parameters may influence the immune response, a multiple regression analysis was performed to assess the effect of nutritional status, stool frequency in the 24 h before admission, duration of diarrhoea before enrolment, sex and concomitant infections on Stx antibody titres. None of these parameters had any effect on Stx antibody titres.

The numbers of children with serum neutralising antibodies to Stx were similar in the two groups of children (Table 2).

Comparison of children with UCS and those who subsequently developed HUS

Of the children with HUS, 11 had HUS on enrolment and six developed HUS after enrolment (median 3.5 days, range 1–6 days). Children who subsequently developed HUS and those with UCS were compared to assess whether changes in antibody levels were identifiable before the onset of HUS (Table 3). The two groups of children were similar in terms of sex, nutritional status (data not shown) and duration of diarrhoea before enrolment. Children who subsequently developed HUS had higher total WBC counts in blood ($p = 0.003$), and a higher percentage of polymorphonuclear cells ($p = 0.037$) and RBC fragments ($p = 0.021$) in blood than children with UCS (Table 3). Although these high levels were higher than normal, none of the children who subsequently developed HUS had a leukaemoid reaction at the time of enrolment. Of the total Ig levels, IgG was lower in children who subsequently developed HUS (median 4.2 mg/ml, 25th–75th quartiles 3.8–7.8) compared with those with UCS (median 8.0 mg/ml, 25th–75th quartiles 6.4–11.7; $p = 0.009$). Comparison of Stx antibody titres showed higher IgA titres in children who subsequently developed HUS ($p = 0.045$), while there were no differences in Stx IgG and IgM titres (Table 3). Logistic regression to assess whether Stx antibody titres were risk factors for the development of HUS showed no significance.

Discussion

The role of *Shigella*-specific antibodies in the development of HUS is unclear. Higher antibody titres to homologous LPS have been described in the plasma of

Table 2. Antibody titres in children with uncomplicated shigellosis and HUS

Study groups	Total immunoglobulin levels in plasma (mg/ml)*				Stx antibody titres in serum*			Number with serum neutralising antibodies to Stx (%)
	IgA	IgG	IgM		IgA	IgG	IgM	
Uncomplicated shigellosis	1.1 (0.9–1.3)	8.0 (6.4–11.7)	0.8 (0.7–0.9)		1698.2 (1004.5–3071.3)	3034.8 (1771.8–5354.7)	4247.9 (1506.8–5854.3)	7 (21.9)
Children with HUS	0.9 (0.7–1.1)	4.4 (2.8–7.4)	0.6 (0.4–0.9)		2347.0 (1402.0–3521.0)	2304.0 (2071.5–3693.5)	2031.0 (929.0–3206.0)	5 (29.4)
p value†	NS	<0.001	0.036		NS	NS	0.032	NS

* Values are median (25th–75th quartiles).

Stx, Shiga toxin; HUS, haemolytic uraemic syndrome; NS, not significant.

† The Mann-Whitney U test (for continuous, non-parametric data) and χ^2 statistic (for categorical data) were used.

Table 3. Comparison of features on enrolment of children with uncomplicated shigellosis and those who developed HUS subsequent to enrolment

Study groups	Duration of diarrhoea (days)*	WBC $\times 10^3/\mu\text{l}$ of blood*	Mean (SD) percent PMNLs	Fragmented RBC (%)*	Number (%) with serum neutralising antibodies to Stx	Stx antibody titres in serum*		
						IgA	IgG	IgM
UCS	6.5 (5.0–8.8)	16.8 (13.9–19.5)	50.3 (19.2)	0.0 (0.0–0.0)	7 (21.9)	1698.2 (1004.5–3071.3)	3034.8 (1771.8–5354.7)	4247.9 (1506.8–5854.3)
Subsequent HUS	5.0 (3.8–6.5)	25.3 (21.2–38.2)	69.5 (13.8)	0.0 (0.0–0.5)	2 (33.3)	3020.5 (2309.0–4369.0)	2702.5 (2230.0–3629.8)	2892.5 (641.5–3832.0)
p value†	NS	0.003	0.037	0.021	NS	0.045	NS	NS

Abbreviations: see Table 1.

*Values are median (25th–75th quartiles).

†The t test (for continuous, parametric data), Mann-Whitney U test (for continuous, non-parametric data) and the χ^2 statistic (for categorical data) were used.

children with complicated shigellosis (including HUS) than in children with uncomplicated shigellosis (UCS) [22]. Antibodies to Stx in *Shigella*-associated HUS have not been described. In adults with UCS, Stx antibody levels have been shown to correlate with disease severity [17]. Whether these findings suggest that more Stx is produced in children who are more severely ill with *S. dysenteriae* 1 infection is not known. In this study, Stx antibody titres were determined in the serum of children with *Shigella*-associated HUS and compared with those in children with UCS. Also, the Stx antibody titres were compared in children with UCS and those who subsequently developed HUS to assess whether changes in Stx antibody titres were associated with the development of HUS.

Studies on adults with UCS have shown that total IgG levels are lower in sera but higher in the stools of patients with shigellosis compared with healthy controls, suggesting that low levels in serum are secondary to increased loss in the stool [16]. Although IgA was also lost in the stool, in the same patients, serum IgA levels were not lowered [16]. This is possibly because locally, in the rectal mucosa, there is increased production of IgA [23]. In the present study, children with HUS had similar plasma levels of IgA, but lower levels of IgG and IgM than children with UCS. The lower IgG and IgM levels in plasma could be caused by increased loss in the stool, and the unaltered levels of plasma IgA may reflect increased production locally, as it has been shown that there are more IgA positive cells in the rectal mucosa in patients with greater inflammation [23]. Unfortunately, antibody levels in the stool were not measured in this study. Another possibility is direct inhibition of antibody secreting cells by Stx, as Stx has been shown to inhibit stem cells producing IgG- and IgA-secreting cells, but not IgM-secreting cells [24]. However, the finding of lowered IgG and IgM levels and similar IgA levels in children with HUS and UCS in the present study contradicts this suggestion. Also, differences in antibody levels in these two groups of children caused by the influence of Stx on production of antibodies suggest that the production of Stx is different in children with HUS and those with UCS. A preliminary and limited determination of Stx concentrations in stools from these children showed no differences between the two groups of children (unpublished observations).

Comparison of Stx antibody titres in the serum of children with UCS and HUS showed lower titres of Stx IgM in children with HUS, while the Stx IgG and IgA titres were similar in the two groups. Similar results have been reported from children with *E. coli* O157:H7-associated HUS [11]. The reason for this selective decrease in Stx IgM titres is not clear. One possibility is that children with HUS are experiencing a secondary infection. Moreover, it is not known

whether IgM Stx antibodies have a role in protection against *Shigella*-associated HUS.

To assess whether antibodies in the serum are risk factors for the development of HUS, children with UCS were compared with those who developed HUS subsequent to enrolment. The latter had lower total IgG levels, which could be due to greater loss in the stool or to inhibition by Stx. Higher IgA Stx titres may be a reflection of a more severe illness in children with HUS compared with those with UCS, or secondary to stimulation by cytokines such as IL6, which is known to be elevated in the sera of children with HUS [25]. However, as logistic regression (carried out to assess whether Stx antibodies in the serum influenced the development of HUS), showed no significant association, the significance of different Stx antibody titres in the serum of children with HUS remains unclear.

In summary, differences in Stx antibodies occur in children with *Shigella*-associated HUS and some differences are observed before the development of HUS. However, the role of these antibodies in HUS remains to be elucidated.

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