JOURNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2272–2279 Vol. 39, No. 6
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.6.2272–2279.2001

Antibody Response to Shiga Toxins Stx2 and Stx1 in Children with Enteropathic Hemolytic-Uremic Syndrome

KERSTIN LUDWIG,[1]* MOHAMED A. KARMALI,[2]† VOLKAN SARKIM,[1] CHRISTOPH BOBROWSKI,[3] MARTIN PETRIC,[2] HELGE KARCH,[4] DIRK E. MÜLLER-WIEFEL,[1] AND ARBEITSGEMEINSCHAFT FÜR PÄDIATRISCHE NEPHROLOGIE‡

Klinik und Poliklinik für Kinder-und Jugendmedizin[1] and Medizinische Kernklinik und Poiklinik,[3] Universitäts-Krankenhaus Eppendorf, 20246 Hamburg, and Institut für Hygiene und Mikrobiologie der Universität Würzburg, 97080 Würzburg,[4] Germany, and Research Institute and Division of Microbiology, Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, and Department of Laboratory Medicine, University of Toronto, Toronto, Ontario, Canada M5G 1X8[2]

Received 31 October 2000/Returned for modification 20 December 2000/Accepted 22 February 2001

A Western blot (immunoblot) assay (WBA) for the detection of immunoglobulin G antibodies to Shiga toxins Stx2 and Stx1 in sera from 110 patients with enteropathic hemolytic-uremic syndrome (53 culture confirmed to have Shiga toxin-producing *Escherichia coli* [STEC] infection) and 110 age-matched controls was established by using a chemiluminescence detection system. Thirty-nine (74%) of the 53 culture-confirmed cases were infections with STEC serotype O157, and 14 (26%) were associated with infection by other STEC serotypes. The frequency of an anti-Stx2 response following infection by a Stx2-producing strain (34 of 48 cases; 71%) was higher than that of an anti-Stx1 response following Stx1-producing STEC infection (4 of 10). Furthermore, the frequency of an anti-Stx2 response in 110 control sera (10%) was significantly higher than the frequency of an anti-Stx1 response (1.8%) ($P = 0.0325$). For STEC O157 culture-confirmed cases WBA for toxin detection had a diagnostic sensitivity of 71% and a specificity of 90%. Because of its high specificity the assay might be a helpful tool for diagnosing suspected STEC infection when tests of stool samples or serological tests against various lipopolysaccharide antigens are negative. Furthermore, the prevalence of anti-Stx antibodies in healthy controls probably reflects the population immunity to systemic Stx-associated disease. It can thus serve as a basis for comparing immunity levels in different populations and for considering future Stx toxoid immunization strategies.

Infection by Shiga toxin-producing *Escherichia coli* (STEC), also referred to as verocytotoxin-producing *E. coli*, is associated with a clinical spectrum that includes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (20, 21, 35). The last ailment, characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, is the most serious complication of STEC infection (12, 22). The highest age-related incidence of STEC infection and of STEC-associated HUS is in young children (5, 12, 22, 37, 41). This suggests that STEC infection is associated with the absence of specific immunity, possibly to Shiga toxins (2, 23). The incidence of STEC-associated HUS is estimated to be about 2 to 3 cases per 100,000 children of less than 5 years of age (12), 1.4 cases per 100,000 children of less than 18 years of age (41) in North America, and 0.9 cases per 100,000 children of less than 16 years of age in Germany (5).

* Corresponding author. Mailing address: Universitäts-Krankenhaus Eppendorf, Klinik und Poliklinik für Kinder-und Jugendmedizin, Martinistr. 52, 20246 Hamburg, Germany. Phone: 49-40-428032702. Fax: 49-40-428035053. E-mail: kludwig@uke.uni-hamburg.de.

† Present address: Laboratory for Foodborne Zoonoses, Population and Public Health Branch, Health Canada, Stone Rd. West, Guelph, Ontario, Canada N1G 3W4.

‡ Members of this study group who participated (from five additional University Children's Hospitals in Germany) included the following: B. Hoppe, D. Michalk, and U. Querfeld, Cologne; J. H. H. Ehrich, G. Filler, and M. von Bredow, Charité Berlin; H. Ruder, Erlangen; U. John, and J. Misselwitz, Jena; and L. B. Zimmerhackl, Freiburg.

The detection of immunoglobulin M (IgM) and IgG antibodies to STEC lipopolysaccharide (LPS), primarily to *E. coli* O157 LPS but also to non-O157 LPS, has emerged as a useful and reliable diagnostic technique, especially when bacterial isolation fails (4, 5, 8, 11, 26).

Central to the genesis of HUS is the injurious action of systemic Shiga toxins on the endothelial cells lining the capillaries of the renal glomeruli and other tissues (22). Members of the Shiga toxin family consist of a similar subunit structure, with an A subunit (~32 kDa) with an enzymatically active A1 fragment (~27.5 kDa) that dissociates from a pentamer of B subunits (~7.5 kDa) during internalization and inactivates the 60S ribosomal subunit by removing one adenine from the 28S rRNA (6, 31). The three bacteriophage-encoded Shiga toxins produced by human STEC strains are Stx1 (type strain C600 [H19J]), Stx2 (type strain C600 [933W]), and Stx2c (type strains E32511 and B2F1 [7279]), which may be present alone or in combination (22, 24, 30, 42). Stx1 is virtually identical to Shiga toxin produced by *Shigella dysenteriae* type 1 but is serologically distinct from Stx2 (and Stx2c), with the toxins showing no cross-neutralization in tissue culture assay by homologous antisera (22, 30, 42). Stx2 is completely neutralized in vitro by antiserum to Stx2c, but Stx2c is only partially neutralized by anti-Stx2 antibodies (S. C. Head, M. A. Karmali, M. E. Roscoe, M. Petric, N. A. Strockbine, and I. K. Wachsmuth, Letter, Lancet **ii:**751, 1988).

To date, most of the studies investigating human antibody responses against Shiga toxins have been based on the detec-

tion of anti-Stx1 neutralizing antibody (NAb) in cell culture and anti-Stx1 IgG by enzyme-linked immunosorbent assay (ELISA) (5, 20, 22, 23). Using the latter, only about one-third of patients with STEC infection have been found to develop NAbs or IgG antibodies against Stx1 (23). Even though the frequency of anti-Stx1 antibodies in HUS is low, it is nevertheless significantly higher for HUS cases than for controls, indicating that the immune response correlates with STEC infection (23). The low frequency of anti-Stx1 responses by HUS patients was attributed to an inadequate antigenic stimulus by a toxin with a very high biological activity (23).

E. coli O157 is the most common STEC serogroup associated with human illness, and the toxin expressed most frequently by this serogroup is Stx2 (22, 43). However, information on the frequency of IgG antibody to Stx2 in cases and controls is very limited, largely due to technical difficulties in developing sensitive and specific assays. Several investigators have shown that virtually all sera from cases and controls are able to neutralize Stx2 (5, 21, 22). It was subsequently shown that the Stx2-neutralizing activity was not due to specific antibodies but rather to the nonspecific activity of high density lipoprotein in serum (7). Reymond et al. have shown that the Western blot assay (WBA) is significantly more sensitive and specific for detecting antibodies to Stx1 than either the NAb assay or ELISA (33).

The objective of this study was to develop a WBA to detect antibodies to Stx2 and to use the assay to investigate the relative frequencies of IgG antibodies to Stx1 and Stx2 in a large cohort of HUS cases and in age-matched controls.

(Part of this work appears in the doctoral thesis of Volkan Sarkim.)

MATERIALS AND METHODS

Patients and controls. One hundred ten patients with HUS (age range, 0.3 to 17 years; median, 3.1 years; mean, 4.0 ± 3.2 years [1 standard deviation {SD}]) and 110 age-matched controls (age range, 0.3 to 17 years; median, 3.0 years; mean, 4.1 ± 3.6 years [1 SD]) were investigated for antibodies against Stx2 and Stx1 by WBA. The HUS patients studied were those seen between June 1993 and September 1997 who had been investigated for STEC in stool specimens and for whom clinical data were available. Seventy-seven percent of the patients were younger than 5 years old. Ninety-one (83%) suffered from bloody diarrhea, while 19 (17%) had nonbloody diarrhea. Most patients lived in the northern part of Germany and were treated in the Children's Hospital, University of Hamburg. The remainder were treated in five additional pediatric centers throughout Germany (in Cologne, Berlin, Erlangen, Jena, and Freiburg). Fifty-one of the patients were male, and 59 were female (male/female ratio, 0.86).

Most of the children from the control group lived in the northern part of Germany. They had no history of diarrhea for the preceding 6 months and no clinical evidence of renal impairment, nor were they associated with an outbreak or sporadic case of HUS. Fifty-eight of the control patients were male, and 52 were female (ratio, 1.1). They were identified in different kindergartens and were undergoing a routine blood sampling before hepatitis B immunization or were from the out-patient endocrinology clinic and had exclusively small or tall stature and no other medical conditions. Informed consent was obtained from the parents of all children studied.

Sera used in the study. (i) Rabbit sera. To establish the specificity of the WBA, sera from three rabbits were used, one preimmunization and one each postimmunization with a toxoid of either Stx1 or Stx2, prepared by glutaraldehyde treatment (3). New Zealand White rabbits weighing approximately 1.8 to 2 kg were immunized subcutaneously with doses of 50 μg of either Stx1 or Stx2 toxoid mixed with equal volumes of Freund's complete adjuvant (Pierce Chemical Company, Rockford, Ill.) and boosted with 50 and 70 μg of Stx1 and Stx2 toxoid, respectively, mixed with Freund's incomplete adjuvant (Pierce Chemical Company) in three sequential weekly intervals (3, 34).

(ii) Control sera. Single serum samples from each control patient ($n = 110$) were investigated.

(iii) Patient sera. Two hundred ninety-eight acute-phase and follow-up sera from patients with HUS were investigated. Sera from patients with HUS who were culture positive for STEC ($n = 53$) and HUS patients without culture-confirmed STEC infections ($n = 57$) were investigated. The first sera ($n = 110$) from all HUS patients were collected 1 to 84 days after the onset of diarrhea (median, 8 days). Follow-up sera ($n = 188$; collected 5 to 401 days after the onset of diarrhea; median, 24 days) were available from 70 patients during their hospitalization, and depending on each patient's illness, after different periods of time after the acute stage of the illness, when the patients were seen in the out-patient nephrology clinic. Follow-up sera were not collected after a standard predetermined period. All serum samples were frozen at −20°C until use.

Isolation of STEC and characterization of STEC isolates by *stx* genotype. Stool samples were examined for STEC, including *E. coli* O157, and traditional enteropathogenic *E. coli*. STEC of serogroup O157 were isolated using immunomagnetic separation performed as described previously (18). Sorbitol-negative colonies from sorbitol-MacConkey agar plates were confirmed biochemically to be *E. coli* and were tested for the presence of O157 antigen using a latex slide agglutination test (28). To detect non-O157 STEC strains, bacterial growths from sorbitol-MacConkey agar were harvested into 1 ml of saline and screened for stx_1 and stx_2 gene sequences as described previously (19). To identify STEC strains in positive PCR samples colony blot hybridization with 100 to 200 well-separated colonies was performed using digoxigenin-labeled probes that were derived from strains C600 (H19J) and C600 (933W) using primer pairs KS7-KS8 (39) and GK3-GK4 (13, 19), respectively. The isolated STEC strains were serotyped as described previously (1). *stx* genotypes were determined using primer pairs KS7-KS8 (stx_1B) (39) and GK3-GK4 (stx_2B, $stx_{2c}B$) (13, 19). Differentiation of stx_2 and stx_{2c} genes was performed by restriction endonuclease analysis using *Hae*III and *Fok*I as described by Rüssmann et al. (38).

Shiga toxin preparation and purification. We have purified Stx2 from the *E. coli* strain R82(pJES) 120DH5α (kindly provided by J. E. Samuel, Department of Medical Microbiology and Immunology, College of Medicine, Texas A & M University, College Station, Texas), and Stx1 was purified from JB28, an *E. coli* TB1 strain transformed by recombinant plasmid pUC19B containing the stx_1 genes cloned from bacteriophage H19B (16, 34) (kindly provided by J. Brunton, University of Toronto, Toronto, Ontario, Canada) using sequential column chromatography (hydroxylapatite, chromatofocusing, and cibachron blue) as previously described (15). The Shiga toxin was resolved into its A and B subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 27-kDa band next to the A subunit is referred to as the A1 fragment (6, 30, 33). There were no protein contaminants detected by Coomassie blue staining of the SDS-PAGE gel before or after blotting onto nitrocellulose or by silver staining performed according to the method of Merril et al. (29). Figure 1 demonstrates the purity of the specific batch of toxins used in the present study.

WBA to detect IgG against Stx2 and Stx1. The WBA was adapted from the method of Towbin et al. (44). A standard concentration of Stx2 and Stx1 (25 μg) was run on an SDS-PAGE gel by using 9% stacking and 15% separating gels. The protein bands were transferred by Western blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, Calif.) for 1.5 h with a current of 0.14 to 0.20 A. Each membrane was cut into strips. One strip of each gel was stained with Coomassie blue to confirm the protein transfer. The strips were blocked for nonspecific binding for 1.5 h with Tris buffer (50 mM Tris, pH 7.4) containing 6% skim milk and 12% goat serum for Stx2 and 5% skim milk and 10% goat serum for Stx1. The blot was washed three times with Tris buffer at room temperature. Diluted serum samples (1:100 for human serum samples and preimmune rabbit serum samples and >1:100 to 1:10,000 for immune rabbit serum samples) in Tris-buffered saline (TBS) containing 1% skim milk and 4% goat serum for the Stx2 WBA and 1% skim milk and 2% goat serum for the Stx1 WBA were added to the blots and were incubated for 60 min at room temperature and then washed three times with TBS. A 1:10,000 dilution of goat anti-human IgG heavy-plus-light chains-peroxidase conjugate (Bio-Rad Laboratories) in TBS with 1% skim milk and 4 and 2% goat serum for Stx2 and Stx1, respectively, was added to the blots and allowed to react for 60 min at room temperature. The blots were then washed again three times in TBS. The antigen-antibody system was developed using a chemiluminescence detection system (ECL; Amersham, Little Chalfont, United Kingdom). The blots were exposed to a film (AR film; Kodak, Rochester, N.Y.) for 2 to 40 s and then developed. Each serum (patient sera as well as control sera) was tested at least twice. A serum was considered positive by showing an anti-Stx2 and/or anti-Stx1 IgG antibody response to at least one of the following: the A subunit, the A_1 fragment or the B subunit (see Fig. 2). Sera that showed a complete absence of binding were considered negative. The results of the WBA were read in a blinded fashion.

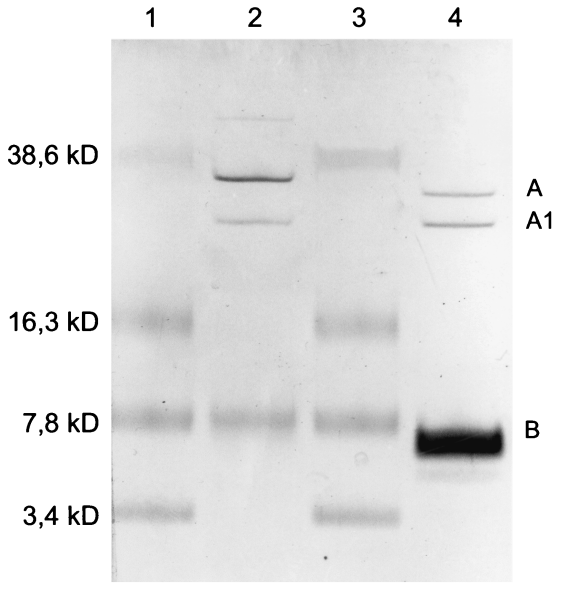

FIG. 1. SDS-PAGE demonstrating the purity of the specific batch of toxins used in the present study. Shown are the A subunit with the A1 fragment and the B subunit of Stx2 (lane 2) and Stx1 (lane 4) visualized after silver staining according to the method of Merril et al. (29). Lanes 1 and 3, low-molecular-weight protein standard (Rainbow unstained; Bio-Rad).

***E. coli* O157 and non-O157 LPS ELISA.** All 298 patients' serum samples as well as the 110 control serum specimens were tested for antibodies (IgM and IgG) against *E. coli* O157 LPS by ELISA as previously described (5).

Selected serum samples, especially those with a weak antibody response by ELISA, were also subjected to immunoblotting using O157 LPS as antigen and were tested for IgM and IgG antibodies (4). A sample was considered to be positive when there were detectable IgM and/or IgG antibodies in the O157 ELISA or immunoblot test.

Furthermore, serum samples without any antibody response (IgM or IgG) against O157 LPS by ELISA were tested for antibodies against the LPS of *E. coli* O26, O111, and O128, as described recently (26). A patient was considered to be positive for *E. coli* O26, O111, or O128 LPS when there were detectable IgM and/or IgG antibodies in the ELISA for one of the LPS.

Statistical methods. Chi-square values with Bonferroni or Yates correction, McNemar's test, the Kolmogoroff-Smirnoff test, and the Mann-Whitney U test were used as indicated. Statistical testing was performed using SPSS, version 10.0 (SPSS, Inc., Chicago, Ill.), on a standard personal computer.

RESULTS

Patient data. One hundred seven of 110 patients with HUS had diarrhea and other signs of enteropathy preceding the diagnosis of enteropathic HUS. The remaining three patients without diarrhea as a prodromal illness were considered to have enteropathic HUS because of demonstrable STEC in stool culture and concordant *E. coli* LPS antibodies (O157, one case; O26, two cases). Part of the data for two patients has been published previously (27; K. Ludwig, H. Ruder, H. D. Rott, and H. Karch, Letter, Lancet **347:**196–197, 1996).

Stool studies. STEC was isolated from 53 of 110 HUS cases (39 strains of serotype O157 and 14 strains of non-O157 serotypes) (Table 1). Stools from 57 HUS patients were negative for STEC by culture; however, for five of these cases *E. coli* strains without *stx* genes were isolated (four cases with *E. coli* O157 and one case with an untyped *E. coli* non-O157 strain).

TABLE 1. *stx* genotypes of STEC isolates from 53 HUS patients

STEC isolate	No. of patients	No. of patients with genotype			
		stx_2	stx_1	stx_2 and stx_1	stx_{2c}
O157	39	34[a]	1	4	
O26	5	3	2		
O69	1			1	
O103	1	1			
O111	3	1	1	1	
O165	1	1[b]			
Not typed	3	2			1
Total	53	42	4	6	1

[a] Two patients' stool samples also harbored the stx_{2c} genes.
[b] One patient's stool samples also harbored the stx_{2c} genes.

Frequency of antibodies against Stx2 and Stx1. (i) Rabbit sera. Rabbits immunized with a toxoid of either Stx1 or Stx2 developed a strong antibody response to the homologous toxin, and no cross-reactivity was seen with the heterologous Stx by WBA using rabbit sera at a dilution of ≥1:100. Dilutions of less than 1:100 showed weak cross-reactivity to the B subunit of the heterologous toxin. Sera from unimmunized rabbits and sera collected from rabbits before immunization remained negative for anti-Stx1 and anti-Stx2 IgG. Western immunoblots of rabbit sera using Stx1 are shown in Fig. 2 (lanes 9 and 10). Anti-Stx1 Western immunoblots with preimmune rabbit serum (Fig. 2, lane 9; nonreactive) and Stx1 immune rabbit serum (Fig. 2, lane 10; reactive to the A1 fragment of the A subunit and the B subunit) are shown. The A subunit was not detected after incubation with the Stx1 immune rabbit serum at the dilution of 1:5,000 used for this Western immunoblot (Fig. 2, lane 10), but it was visualized using Stx1 rabbit immune serum at dilu-

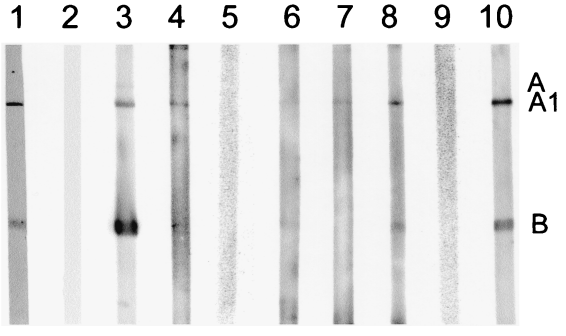

FIG. 2. Western immunoblot of human and rabbit sera against Stx1 as antigen (lanes 2 to 10). Lane 1 shows a strip of the PVDF membrane after blotting of Stx1, showing the A1 fragment and the B subunit visualized after Coomassie blue staining. Immunoblot strips from five human sera collected from HUS patients were used as standard control sera for the Stx1 WBA, two negative (lanes 2 and 5, nonreactive) and three positive (lane 3, reactive to the A subunit [weak], the A1 fragment, and the B subunit; lane 4, reactive to the A1 fragment; lane 6, reactive against both the A1 fragment and the B subunit [very weak]). Positive human sera from HUS patients (lane 7, reactive against the Stx1 A1 fragment [weak]; lane 8, reactive against the A1 fragment and the B subunit), preimmune rabbit serum (lane 9, nonreactive), and Stx1 immune rabbit serum at a dilution of 1:5,000 (lane 10, reactive against the Stx1 A1 fragment and the B subunit) are shown.

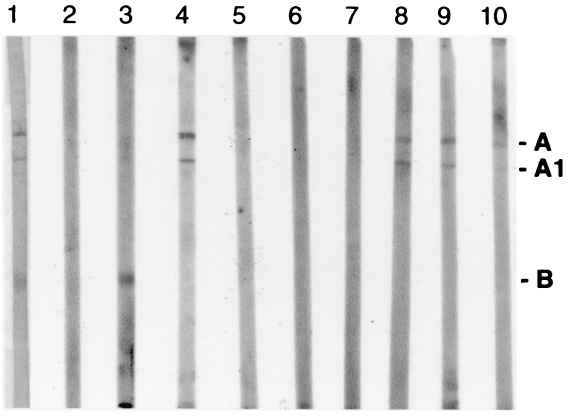

FIG. 3. Western immunoblot of sequential serum samples from a child with enteropathic HUS (lanes 6 to 10), the stained PVDF membrane (lane 1), and human control sera (lanes 2 to 5) using Stx2 as antigen. Lane 1, strip from the PVDF membrane after blotting and staining with Coomassie blue, showing the A subunit with the A1 fragment and the B subunit; lanes 2 to 5, human control sera. Two negative human control sera (lanes 2 and 5, nonreactive) and two positive human control sera (lane 3, reactive against the Stx2 B subunit; lane 4, reactive against the A subunit and the A1 fragment of Stx2) are shown. Western immunoblot strips of five sequential sera of a patient with enteropathic HUS are shown as follows: lanes 6 and 7, nonreactive 5 and 8 days, respectively, after the onset of diarrhea; lanes 8, 9, and 10, reactive 12, 17, and 38 days, respectively, after the onset of diarrhea, all showing an antibody response against the A subunit and the A1 fragment of Stx2.

tions of 1:100 up to 1:3,000 (data not shown), confirming the results of Reymond et al. (33).

(ii) Controls. In 110 sera from children without diarrhea and without kidney disease, no subject had both anti-Stx2 IgG and anti-Stx1 IgG. Eleven of 110 (10%) sera from control children showed anti-Stx2 IgG, whereas only 2 of 110 (1.8%) had antibodies against Stx1 and 97 exhibited no indications of either antibody (Fig. 3). The frequency of anti-Stx2 IgG in 110 control sera (10%) was significantly higher than the frequency of anti-Stx1 IgG (1.8%) ($P = 0.0325$; McNemar's test).

Antibodies against Stx2 and Stx1 in correlation to the *stx* genotype in HUS patients. The frequencies of anti-Stx1 and anti-Stx2 responses following STEC infection are shown in Table 2. In HUS patients with *E. coli* O157 and/or non-O157 isolates harboring the stx_2 gene alone or in combination with the stx_1 gene, 71% (34 of 48) showed measurable IgG antibodies against Stx2 alone ($n = 30$) or against both toxins ($n = 4$) (Table 2). Of four patients with *E. coli* isolates harboring the stx_1 genotype, two showed antibodies against Stx1 (Table 2). The sensitivity of the Stx2 WBA for an infection with stx_2 gene-expressing *E. coli* was 71%, and the specificity compared to controls was 90%; the positive and negative predictive values were calculated to be 76 and 87%, respectively. We did not have enough HUS patients infected by an *E. coli* isolate expressing the stx_1 genotype to determine the sensitivity of the Stx1 WBA, but the specificity compared to controls was shown to be 98%.

Antibodies against Stx2 and Stx1 in patient serum samples without *stx* genes in stool cultures. Thirty of 57 patients (53%) without STEC-confirmed HUS (for four cases *E. coli* O157 was isolated from stool culture and for one case *E. coli* non-O157 [untyped] was isolated, but no *stx* genotype was detected by PCR) showed detectable antibodies against Stx2 alone ($n = 28$) or against both toxins ($n = 2$). Antibodies against Stx1 were detected in six cases (10.5%).

TABLE 2. Detection of anti-Stx2 IgG and/or anti-Stx1 IgG in correlation to *stx* genes from STEC O157 and non-O157 isolates[a]

Isolate and antibody detected	No. of patients with genotype			
	stx_2	stx_1	stx_2 and stx_1	stx_{2c}
STEC O157	34[b]	1	4	0
Anti-Stx2 IgG	22	0	2	0
Anti-Stx1 IgG	0	0	0	0
Anti-Stx2 and anti-Stx1 IgG	1	0	2	0
STEC non-O157	8	3	2	1
Anti-Stx2 IgG	5	0	1	0
Anti-Stx1 IgG	0	1	0	0
Anti-Stx2 and anti-Stx1 IgG	1[c]	1	0	0
Anti-Stx IgG Total	29/42	2/4	5/6	0/1

[a] Cumulative results of anti-Stx2 and anti-Stx1 IgG in acute- and convalescent-phase sera are shown.
[b] Two patients without antibodies against Stx2 also harbored the stx_{2c} genotype in stools.
[c] This patient, showing anti-Stx2 and anti-Stx1 IgG in serum samples, harbored stx_2 plus stx_{2c} in stool specimens.

Dynamics and duration of antibody response against Stx2 and Stx1 in HUS patients. In total, 72 of 110 (65%) patient samples showed anti-Stx2 IgG and/or anti-Stx1 IgG in initial serum samples or during follow-up. Only anti-Stx2 IgG was detected in 58 cases, anti-Stx1 IgG alone was detected in 7 cases, and anti-Stx1 and anti-Stx2 IgG in combination were detected in an additional 7 cases. By McNemar's test, anti-Stx2 IgG was highly significantly more frequent than anti-Stx1 IgG in the patient population ($P < 0.001$).

For 38 patients (group I), no antibodies were detectable, either in initial serum samples collected 8.5 ± 5.2 days (mean ± 1 SD; median, 7.5 days) after the onset of diarrhea or in follow-up samples ($n = 55$) from 22 of these 38 patients, collected 56 ± 75 days (mean ± 1 SD; median, 21 days; range, 7 to 390 days) after the onset of diarrhea.

Of the 72 patients showing anti-Stx2 and/or anti-Stx1 IgG, 42 patients showed antibodies in the first available serum sample (anti-Stx2, $n = 36$; anti-Stx1, $n = 6$), collected 10.5 days (median) after the onset of diarrhea (group II). For 40 of these 42 patients, the time of the first serum sampling was 12.1 ± 7.2 days (mean ± 1 SD) after the onset of diarrhea (median, 10 days). However, for two patients the initial serum sampling was as late as 47 and 84 days after the onset of diarrhea. Both distributions, the 38 patients with negative serum samples (group I) and the patients of group II with antibody expression in the first serum sample after admission to the hospital, were not normal (Kolmogoroff-Smirnoff test). For both of these subgroups, a comparison of the time at which the initial samples were obtained yielded a significantly earlier time (median, 7.5 days) for the group lacking antibodies (median, 7.5 days versus 10.5 days; Mann-Whitney U test, $P = 0.0036$).

Additionally, seroconversion of anti-Stx IgG (anti-Stx2, 27 cases; anti-Stx2 and anti-Stx1, 2 cases; anti-Stx1, 1 case) was observed in an additional 30 patients 23 ± 31 days (mean ± 1 SD; median, 15 days) after the onset of diarrhea, while the last

of the initial negative serum samples were collected 8.8 ± 4.7 days (mean ± 1 SD; median, 7.5 days) after the beginning of diarrhea (group III).

Of all 72 HUS patients showing anti-Stx2 and/or anti-Stx1 IgG (groups II and III), sequential serum samples were available from 48 patients at variable intervals. However, the studies of follow-up sera were limited by the fact that the sera were not collected after a standard predetermined period, but it can be clearly stated that anti-Stx2 and/or -Stx1 IgG was detected in 6 of 26 investigated patients (23%) in the first week after the beginning of diarrhea, while in the second, third, and fourth weeks 67% (26 of 39), 84% (26 of 31), and 83% (20 of 24) showed anti-Stx2 and/or -Stx1 IgG. Thirty-one patients were investigated for longer than four weeks. Between weeks five and eight, 75% (21 of 28) showed anti-Stx2 and/or -Stx1 IgG, while 7 of 15 (47%) showed anti-Stx2 and/or -Stx1 IgG between weeks 9 and 12. In 8 of 18 (44%), anti-Stx2 and/or -Stx1 IgG was detected for longer than 3 months and up to over 11 months (335 days).

There were not enough samples showing anti-Stx1 IgG alone ($n = 7$) to compare the dynamics and duration of anti-Stx2 and anti-Stx1 IgG antibodies in the 72 patients. Furthermore, follow-up samples were only available from two of these seven patients. However, of special interest are samples from the seven patients showing anti-Stx2 and anti-Stx1 IgG in combination, including four cases with sequential samples; of these, three patients showed IgG antibodies first against one toxin, while in follow-up samples antibodies against both toxins were measurable.

A representative follow-up investigation of a patient is shown in Fig. 3 (lanes 6 to 10). The patient developed anti-Stx2 IgG in the third serum sample obtained 12 days after the onset of diarrhea (Fig. 3, lane 8).

It can be stated that there was no significant age difference between the children with anti-Stx1 or anti-Stx2 IgG (0.3 to 14.8 years; median, 3.1 years; mean, 4.13 years) and the HUS patients without any antibody response (0.4 to 17 years; median, 3.3 years; mean, 3.8 years).

Antibodies against the A and/or B subunit of Stx2 and Stx1 in 110 controls and 110 HUS patients. Serum samples reacting with no subunits or with the A and/or B subunit of Stx2 and/or Stx1 are shown in Fig. 4.

HUS patients develop antibodies against the A subunit of Stx2 and/or Stx1 significantly more often than controls do ($P = 0.0001$; chi-square test). The samples from HUS patients reacting with Stx2 and/or Stx1 showed antibodies only against the A subunit of Stx2 and Stx1 in 85% (55 HUS cases) and 71% (10 HUS cases) of the cases, respectively, including six patients reacting with both the Stx2 and Stx1 A subunits (Fig. 4). Five of 11 controls showing anti-Stx2 IgG had antibodies against the A subunit, and only one of two Stx1-positive control samples reacted with the A subunit of Stx1.

Antibodies against the B subunit of anti-Stx2 and/or anti-Stx1 were found more frequently in controls (7 of 13 controls versus 13 of 72 HUS patients; $P = 0.0145$; Yates-corrected chi-square test). Six of 11 positive control samples showed anti-Stx2 B subunit IgG, and 1 of 2 Stx1-positive controls had antibodies against the Stx1 B subunit. In HUS patients, antibodies against the B subunit of Stx2 and Stx1 were detected in 10 and 4 cases, respectively, including 5 and 2 cases, respectively, of reaction with both the A and B subunits and including 1 case of reaction with both the Stx2 and Stx1 B subunits (Fig. 4).

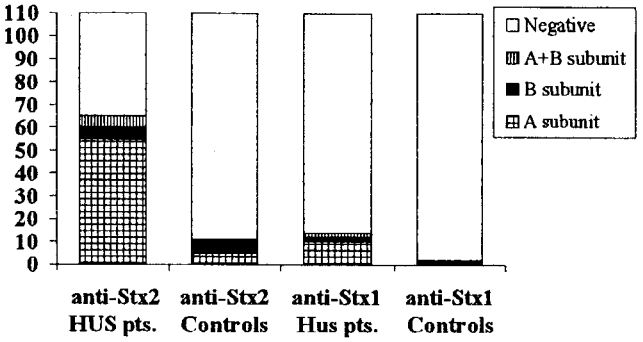

FIG. 4. Serum samples not reacting or reacting with the A and B subunits of Stx2 and Stx1 in patients (pts.) with enteropathic HUS ($n = 110$) and in age-matched controls ($n = 110$). In serum samples from 65 HUS patients anti-Stx2 IgG was detectable, including 7 patient samples with IgG antibodies against both toxins, 6 samples with IgG against both A subunits, and 1 sample with detectable IgG against the B subunit of both Stx2 and Stx1. Fourteen HUS patients showed measurable anti-Stx1 IgG in serum samples, including the seven patient samples with antibodies against both toxins. None of the controls had measurable antibodies against both Stx2 and Stx1.

Of 12 serum samples from HUS patients showing antibodies against the A subunit of Stx1, four had detectable IgG against both the A subunit and the A1 fragment (Fig. 2, lane 3; reactive to the A1 fragment and the A subunit [weak]), and eight were positive only for the A1 fragment (Fig. 2, lanes 4 and 6 [very weak], 7 [weak], and 8). Even though the A subunit of Stx1 was not visualized by Coomassie staining of the PVDF membrane (Fig. 2, lane 1), it was visualized after silver staining of the SDS-PAGE gel (Fig. 1, lane 4) and was detectable after incubation with human serum (Fig. 2, lane 3). Only 1 sample from 110 controls reacted with the A subunit of Stx1 without a reaction against the A1 fragment. The A subunit was not detected after incubation with the Stx1 immune rabbit serum with the dilution of 1:5,000 used for this WBA (Fig. 2, lane 10), but it was visualized using Stx1 rabbit immune serum at a dilution of 1:100 up to 1:3,000 (data not shown).

Samples from HUS patients reacting with the A subunit of Stx2 showed 88% ($n = 53$) reacting to the A subunit and the A1 fragment (Fig. 3, lanes 4 and 8 to 10), while 12% ($n = 7$) reacted with the A subunit only (data not shown). Five of six serum samples from controls positive for the A subunit of Stx2 were positive for the A subunit and the A1 fragment, while one reacted with the A subunit only. In contrast to Stx1, the A subunit of Stx2 was visualized by Coomassie staining of the PVDF membrane (Fig. 3, lane 1) and after silver staining of the SDS-PAGE gel (Fig. 1, lane 2). Furthermore, the Stx2 A subunit and the A1 fragment were detected after incubation with the Stx2 immune rabbit serum at every dilution tested (1:100 to 1:10,000) (data not shown).

Antibodies against *E. coli* O157 and *E. coli* non-O157 LPS using ELISA methodology. (i) Controls. Three out of 110 (2.7%) control children showed IgM or IgG antibodies against *E. coli* O157 LPS, two controls (1.8%) had elevated antibodies against *E. coli* O111 LPS, four out of 110 (3.6%) had measur-

able anti-O26 LPS IgM, and three (2.7%) showed antibodies against *E. coli* O128 LPS. Only one control showed IgM as well as IgG antibodies against *E. coli* O26 and *E. coli* O111 LPS and, additionally, had measurable anti-Stx2 B subunit IgG.

(ii) HUS patients. For STEC O157, O26, and O111 culture-positive patients ($n = 47$; Table 1), the LPS ELISA for the specific *E. coli* serogroups tested with ELISA detected antibodies in 94%. Forty-two of 43 (98%) *E. coli* O157 culture-positive patients (39 confirmed to have STEC infections; Tables 1 and 2) showed antibodies against *E. coli* O157 LPS. Four of five patients with *E. coli* O26 in stool culture and two of three with *E. coli* O111 in stools had detectable anti-O26 and anti-O111 LPS antibodies, respectively. Forty-eight of 52 patients without *E. coli* O157 or non-O157 culture-confirmed infection were confirmed as being infected with *E. coli* O157 on the basis of *E. coli* O157 LPS antibodies for 43 cases and on the basis of *E. coli* non-O157 LPS antibodies for 5 cases (O26, 4 cases; O128, one case). Four patients with negative stool cultures remained negative for *E. coli* O157, O26, O111, and O128 LPS antibodies. Immunoblot results compared favorably with the ELISA results for detecting anti-O157 LPS IgM and IgG antibodies.

DISCUSSION

In contrast to a growing body of data on antibody responses to Stx1, information on the frequencies of anti-Stx2 antibodies in cases and controls is very limited. This is because the development of assays for measuring anti-Stx2 responses has been difficult. This report indicates that Western blotting can be used successfully to detect antibodies to Stx2. While Takeda et al. reported in 1993 (T. Takeda, S. Dohi, T. Igarashi, T. Yamanaka, K. Yoshiya, and N. Kobayashi, Letter, J. Infect. **27:**339–341, 1993) a lack of detectable antibody titers in HUS children compared to healthy controls, we demonstrate serum IgG antibodies to Stx2 in 71% (34 of 48) of HUS patient samples expressing the stx_2 gene alone or the stx_2 and stx_1 genes in combination (Table 2). We found antibodies against Stx2 in 65 of 110 (59%) HUS patients investigated, including 7 patients with both anti-Stx2 and anti-Stx1 IgG. In comparison, only 11 of 110 (10%) control patients showed antibodies against Stx2. Only anti-Stx1 IgG was detected from seven HUS cases. In controls, two showed measurable anti-Stx1 IgG. Both anti-Stx2 IgG and anti-Stx1 IgG were found significantly more frequently in the HUS patients than in controls ($P < 10^{-20}$ and $P = 0.0002$, respectively, by chi-square statistics; even with the necessary Bonferroni correction, this is highly significant).

In 1990 Cohen et al. reported that B lymphoid cells are susceptible to Stx1 because they express Stx-binding glycolipids on their cell surfaces (10). By using in vitro B-cell activation studies they showed that the vast majority of Stx1-sensitive B cells belong to the IgG- and IgA-committed subset, whereas most IgM-producing cells are resistant to Stx. They concluded that this was the reason for the failure of long-term immunity to dysenteric disease (10). However, the high frequency of Stx1 NAbs in healthy farm residents found by Reymond et al. argues against a sustained immunosuppressive effect of Stx1 (32). Barrett et al. (2) discussed in 1991 a possible cytotoxicity of Stx2 to B lymphocytes and a hypothetical inhibition of the ability of humans to respond to Stx2 as a reason for the lack of response to Stx2-producing organisms. Because of the heavy- and light-chain reactivities of the conjugate used in this study, our WBA detected not only IgG but also, potentially, IgM and IgA. It is likely, however, that among the patients with HUS we are dealing in the acute phase and in short-term follow-up sera with IgM and IgG and in the control group we are dealing with IgG because all control persons were asymptomatic.

In 1993 Chart et al. discussed as a reason for a lack of Stx1 and Stx2 antibodies in 30 HUS patients as well as in 30 controls that the concentrations of Stx in patients may be too low to stimulate the immune defense system (9). Greatorex and Thorne tested sera from 27 *E. coli* O157:H7-associated HUS patients and 48 controls by ELISA and immunoblotting, but none of the samples were positive for anti-Stx2 IgG, IgM, or IgA (11). In contrast, in our study that includes 48 HUS patients infected by STEC expressing Stx2 or Stx2 and Stx1, the antibody response to Stx2 was quite common. The frequency of an anti-Stx2 response following infection with Stx2- or Stx2-plus-Stx1-producing strains (34 of 48; 71%) is higher than that of an anti-Stx1 response following Stx1- or Stx2-plus-Stx1-producing STEC infection (40%) (Table 2). Our results of the WBA for Stx1 showed 4 of 10 (40%; Table 2) HUS patients developing measurable anti-Stx1 IgG alone or in combination with anti-Stx2 IgG, with known stx_1 gene alone or in combination with the stx_2 gene in stool samples, confirming the results of Reymond et al. (33) and Greatorex and Thorne (11). Reymond et al. found a detectable antibody response against Stx1 in acute-and convalescent-phase sera in 10 of 19 (52.6%) patients with known STEC infections expressing either Stx1 alone or Stx1 plus Stx2 (33). Greatorex and Thorne could show that 9 of 27 (33%) HUS patients were positive for Stx1 IgG antibodies (11). The reasons for the frequency of antibodies to Stx2 being much higher than that of antibodies to Stx1 assume that Stx2-producing STEC are much more common in Germany than Stx1-producing strains. Therefore, a higher frequency of seropositivity to Stx2 (than to Stx1) in controls, as expected, was found, with the frequency of anti-Stx2 IgG in 110 control sera of 10% being significantly higher than the frequency of anti-Stx1 IgG (1.8%) ($P = 0.0325$; McNemar's test). However, the reason for the much higher frequency of anti-Stx2 responses in cases of Stx2-producing STEC infections compared to the frequency of anti-Stx1 responses in Stx1-producing STEC infections is not known. Because of the high endemicity of STEC expressing Stx2 in the population it is possible that many seronegative individuals already have had a primary exposure and that a positive response to Stx2 represents a booster response to a second exposure. This is strengthened by the fact that Stx1 and Stx2 have a high biological activity and a primary exposure may provide an insufficient antigenic challenge. Furthermore, it is supported by a case of HUS following bloody diarrhea without STEC in stool cultures. The patient developed a second episode of diarrhea in the fifth week after the onset of HUS which was nonbloody and followed by recurrent anemia with a lactate dehydrogenase increase and a haptoglobin decrease without affecting the renal values this time. The second diarrhea was followed by a new increase of O157 LPS IgA and IgM antibodies in serum samples, and stool cultures remained negative again. At this time (33 days after the onset of the first episode of diarrhea) the patient developed for the first time measurable anti-Stx2 IgG

antibodies in serum samples. However, as reported in the literature, there are at least five patients with a second repeated STEC O157-associated illness (36, 40; T. D. Piscione, M. A. Karmali, D. Stephens, R. Donckerwolcke, P. I. Tarr, E. Harvey, and G. S. Arbus, Abstr. 3rd Int. Symp. Workshop Shiga Toxin (Verocytotoxin)-Producing *E. coli* Infect., abstr. V213/I, 1997).

The duration of anti-Stx antibodies in follow-up samples from 18 patients investigated for over 3 months who developed anti-Stx2 and/or anti-Stx1 antibodies showed the disappearance of previously detectable anti-Stx IgG from 10 of 18 (56%) patients after the onset of diarrhea. However, from 40 patients only single serum samples and no follow-up serum samples were available; of these, 24 samples collected 11 days (median) after the onset of diarrhea had measurable anti-Stx2 and/or -Stx1 antibodies, while 16 samples without anti-Stx2 and/or -Stx1 IgG were collected 7 days (median) after the onset of diarrhea, consistent with the finding in 30 patients showing a seroconversion in follow-up samples collected 15 days (median) after the onset of diarrhea, while the first samples collected after 7.5 days (median) were negative. For group I (38 patients without any antibody response) and group II (42 patients with initially positive serum samples), a comparison of the time at which initial samples were obtained yielded a significantly earlier time (median, 7.5 days versus 10.5 days; $P = 0.0036$, Mann-Whitney U test) for the group lacking antibodies. These findings strengthen the need for investigating sequential serum samples.

The criterion for a positive sample in the Stx2 and Stx1 WBA used in this study was reactivity to either the A subunit, the A1 fragment, or the B subunit or a variable combination of the subunits. From HUS patients showing IgG antibodies against the Stx2 A subunit alone ($n = 55$) or against the A and B subunits ($n = 5$), 88% ($n = 53$) had antibodies against both the A subunit and the A1 fragment, and seven (12%) showed a reaction only against the A subunit. Of 12 HUS patient samples showing antibodies against the Stx1 A subunit ($n = 10$) or against the A and B subunits ($n = 2$), 4 had detectable antibodies against both the A subunit and the A1 fragment and 8 were positive only for the A1 fragment of the A subunit. Antibodies against the B subunit of Stx2 and/or Stx1 were found relatively more frequently in controls (7 of 13 controls versus 13 of 72 HUS cases; $P = 0.0145$, Yates-corrected chi-square test).

While in HUS cases showing anti-Stx2 IgG alone or anti-Stx2 plus -Stx1 IgG 10 of 65 (15%) patients developed antibodies against the B subunit of Stx2 (only B, $n = 5$; A and B, $n = 5$), 6 of 11 (54%) controls having anti-Stx2 IgG and 6 out of all 110 (5.4%) controls showed an antibody response against the B subunit of Stx2. These results confirm the study by Gunzer and Karch in which only one convalescent-phase serum from seven HUS patients showed a seroconversion against the stx_{2B} fusion protein, and 5.4% (14 of 260) of the controls had antibodies against the B subunit of Stx2 (14). Here we demonstrate that anti-Stx2 antibodies in serum samples of 30 HUS patients who had no detectable antibodies in the first samples showed a seroconversion after 15 days (median). The earliest anti-Stx2 IgG response was seen 3 days after the onset of diarrhea, and the latest was seen after 6 months.

The significance of reactivity to both subunits, including the A1 fragment of Stx2 and Stx1, as opposed to reactivity to either the A subunit, the A1 fragment, or the B subunit is not known and requires further studies.

It is unlikely that antibodies to Stx2 and Stx1 are cross-reacting, since we did not detect a similar amount of antibodies against Stx2 and Stx1 in controls (10 and 1.8%, respectively), and only 2 of 42 patient stool samples harboring the stx_2 gene showed anti-Stx2 and -Stx1 IgG in combination in serum and none showed anti-Stx1 IgG alone (Table 1). The weak cross-reactivity of rabbit sera at a dilution of less than 1:100, immunized with a toxoid of either Stx1 or Stx2, confirms the data of Bielaszewska et al. (3), who speculated that the toxoids may have led to the exposure of different epitopes. However, by using higher dilutions ($\geq$1:100) of rabbit sera no cross-reactivity was seen anymore, whereas serum samples from patients with HUS showed no cross-reactivity with the heterologous toxin by using dilutions of $<$1:100. The native toxins, in contrast to toxoids, may not result in the development of cross-reactive antibodies.

We did not have enough HUS patients expressing the stx_1 genotype to determine the sensitivity of the Stx1 WBA, but the specificity was shown to be 98%. The sensitivity of the Stx2 WBA was 71%, the specificity was 90%, and the positive and negative predictive values were calculated to be 76 and 87%, respectively. Furthermore, the observation that five HUS patients with *E. coli* isolates (O157, $n = 4$; not typed, $n = 1$) with no detectable *stx* genotype developed antibodies against Stx2 is of special interest because this may have been caused by a loss of the genotype upon subcultivation or the isolates may harbor a variant of the stx_2 gene (17). Therefore, the WBA for detecting Stx2 IgG antibodies is a valuable tool for serodiagnosis of HUS cases, especially those with no detectable genotype. In cases with no antibody response against a panel of various LPS antigens and for a growing number of *E. coli* serogroups responsible for HUS, the WBA is helpful for serodiagnosis. To date, it is not yet possible to determine whether seropositivity represents a primary infection or a secondary exposure. The relevance to diagnosis will vary according to the relative predominance of Stx1- or Stx2-producing strains in the population.

Laboratory animals immunized with Stx toxoids have been shown to be immune to systemic challenge by lethal doses of homologous or heterologous Stx (3). Given that no specific treatment is currently available for HUS, Stx toxoid immunization may be a practical option for limiting the morbidity and mortality associated with STEC infections in humans. However, knowledge about the nature and incidence of protective anti-Stx immunity in humans is still in its infancy. Current information on this subject is confined mostly to studies of the serum antibody response to Stx1.

The WBA for detecting anti-Stx2 IgG could provide comprehensive seroepidemiologic data on STEC infections as a basis for future Stx toxoid immunization studies as a potential practical option for limiting morbidity and mortality associated with STEC infections in humans, especially in children.

ACKNOWLEDGMENTS

K. L. was supported by a grant from "Stifterverband für die Deutsche Wissenschaft": "Sanitätsrat-Dr.-Emil-Alexander-Huebner-und-Gemahlin-Stiftung," Essen, Germany.

We thank Maike Westphal for technical assistance. The help of colleagues (M. J. Kemper, H. Altrogge, K. Timmermann, and M. Dietz) and the staff nurses of the pediatric nephrology unit in Hamburg in patient care and sample collection is gratefully acknowledged.

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