

The role of *Escherichia coli* O 157 infections in the classical (enteropathic) haemolytic uraemic syndrome: Results of a Central European, multicentre study

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SUMMARY

To assess the importance of infection by Verotoxin (VT) producing *Escherichia coli* (VTEC) in children with HUS in Central Europe, stool and/or serum samples obtained from 147 patients from 28 paediatric centres were prospectively examined for the presence of VTEC and the kinetics of faecal VT titres (FVT), and for VT neutralization titres and antibodies against *E. coli* O 157 lipopolysaccharide, respectively. Ninety-two percent of the patients had classic (enteropathic) HUS (E⁺ HUS). Evidence of VTEC infection was obtained in 86% of them. VTEC/FVT were identified in 55/118 E⁺ cases (47%). A prominent feature was the frequent isolation of sorbitol-fermenting, VT2-producing *E. coli* O 157. H⁻. VT1 (C600/H19) was neutralized by 9%, and VT2 (C600/933W) by 99% of the initial serum samples from E⁺ patients, compared to 3% (VT1) and 100% (VT2) from age-related controls. Fourfold titre rises against VT1 and/or VT2 were observed in 13/70 (19%), and significantly elevated O 157 LPS IgM and/or IgA antibodies in 106/128 (83%) of the E⁺ patients. The ubiquitous VT2 neutralizing principle in the serum of HUS patients as of healthy controls warrants further investigations.

INTRODUCTION

The haemolytic uraemic syndrome (HUS) is a heterogenous group of vasculitic diseases characterized by microvascular angiopathy, haemolytic anaemia and renal impairment, occasionally involving additional organ systems [1, 2]. The classical (or epidemic) HUS is of growing paediatric and public health importance, accounting for the majority of acute renal failures in childhood. Despite earlier and better treatment, 14–31% of the patients still have a poor outcome or a severe course according to recent studies [3–5]. The pathogenesis of this form of HUS has been linked to enteric infections by verotoxin (VT) producing *Escherichia coli* (VTEC) [6]. At least three different verotoxins associated with human disease have been characterized. VT1, also termed Shiga-like toxin I (SLT-I) is genetically and serologically identical to Shiga toxin but differs from members of the more

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diverse VT2 group [7]. Prototype VT2 from *E. coli* strain C600/933W (SLT-II) [8] and VT2c (SLT-IIc) from *E. coli* strain E32511 (HSC) [9, 10] share identical A subunits but differ slightly in their B subunits [11] resulting in altered glycolipid receptor binding affinities [12, 13] and, consequently, in different Vero and HeLa cell toxicities [14–16]. VTEC strains producing various combinations of these toxins have been described; however, their respective contribution to the syndrome is not clear [8, 10, 11, 16, 17]. HUS is believed to develop after the absorption of significant amounts of VT from the gut into the circulation [17]. The diagnosis of VTEC infection in HUS as well as haemorrhagic colitis relies on the identification of VT-producing organisms and/or the demonstration of faecal VT [6, 17]. In view of the short excretion time of *E. coli* O 157 [18, 19], alternative diagnostic tools are needed. Fourfold rises of VT neutralization titres are considered by some authors as evidence of recent VTEC infections [6, 20, 21]. Recently, the use of enzyme-linked immunosorbent assays [22–24] and of an indirect haemagglutination assay [25] to detect antibodies against *E. coli* O 157 was reported. The evaluation of these diagnostic tools in different populations is therefore warranted.

In an open, voluntary study across Germany and adjacent parts of central Europe the relative frequency of VTEC involvement in childhood HUS was assessed using bacterial culture and cytotoxicity assays as well as the VT neutralization test and an Ig class-specific O 157 LPS ELISA. We further aimed to determine the contribution and limitations of these techniques to establish the diagnosis.

METHODS

Patients and samples

Stool and serum samples and clinical data were recruited according to a protocol that was circulated among members of the German-speaking Working Group of Paediatric Nephrology (Arbeitsgemeinschaft für Pädiatrische Nephrologie, APN) calling for submission of specimens from all children with acute HUS. HUS was defined by acute haemolytic anaemia with red cell fragmentation, and acute nephropathy; thrombocytopenia below 130/nl strongly supported the diagnosis [1, 2]. Between 1 August 1986 and 30 April 1991, 147 patients were enrolled from 28 paediatric centres throughout former East ($n = 8$) and West Germany ($n = 129$), from Eastern France (Alsace, $n = 1$), the northern part of Switzerland (Canton St Gallen, $n = 7$), and Austria (Vienna, $n = 2$). Stool samples were received from 129 of the 147, and serum samples from 144 patients. Most specimens were sent by express delivery; they were divided immediately after arrival and examined, or frozen at $-20\text{ }^{\circ}\text{C}$ until use. Twenty-two of the patients have been reported previously as part of a separate study [25]. Stool and serum samples from various controls, that were retained from routine laboratory work-up in most cases, were included as indicated.

Population figures for estimating the incidence of HUS were derived from the 1988 statistics for the Federal Republic of Germany and the former German Democratic Republic [26]. The patients' hometowns, identified by their postal codes, were classified according to the number of inhabitants [26].

Microbiological and cell culture techniques

Stool samples were examined for VT producing *E. coli* including O 157 and traditional enteropathic *E. coli* (EPEC), and for the presence of 'free' faecal VT (FVT). Briefly, 5–20 lactose-fermenting colonies were screened with EPEC O-group (Behringwerke AG, Marburg, Germany) and O 157 antisera by slide agglutination, and since mid-1988 also by the colony blot hybridization technique using synthetic oligonucleotide probes complementary to specific gene sequences of the VT1 A-subunit (probe 722) and the VT2 A-subunit (probe 849) [15]. After biochemical confirmation as *E. coli*, VT-positive colonies were serotyped as described [27]. Routine microbiological screening procedures for conventional pathogens were performed by local microbiological laboratories at the discretion of each centre. The presence of cytotoxin(s) was examined using Vero and HeLa S₃ cells. For the detection of FVT, stool samples were diluted with sterile physiological saline (1 g stool in 5 ml), thoroughly homogenized, and centrifuged; dilutions of the filter-sterilized (0.2 µm) supernatants were incubated with fresh suspensions of 1.2×10^4 cells/well of a microtitre plate (Nuncelox[®], Nunc, Roskilde, Denmark). Isolates were grown overnight in tryptic soy broth without additives (Oxoid, Wesel, Germany), extracted with 0.1 mg/ml polymyxin B (Sigma Chemicals, Deisenhofen, Germany), and tested for cytotoxicity. The verotoxin titres was defined as the reciprocal of the dilution causing 50% cell death per well (CD_{50%}). Identification as VT required demonstration of the characteristic cytotoxic effect and complete neutralization by anti-Shiga toxin and/or a reference anti-VT2 serum obtained by immunization of rabbits with VT2 from strain C600/933W.

Lipopolysaccharide preparation

Lipopolysaccharide (LPS) from *E. coli* O 157.H⁻ strain 493-1, isolated from a child with HUS, was separated by the hot phenol water extraction method (procedure I) of Westphal and Jann [28] and further purified using proteinase K (Sigma) followed by repeated phenol–water extractions and dialysis. Purified LPS was visualized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [29]. There were no protein contaminants detected by Coomassie blue staining of the SDS–PAGE gel or after blotting on nitrocellulose [29].

Serological assays

VT neutralization test. The patients' serum samples were investigated for their VT neutralizing activity using VT1 extracted from *E. coli* strain C600/H19 and VT2 from strains C600/933W [8] and 4220 (an O 157.H7 isolate from a child with HC). The latter strains hybridized only with the VT2 gene probe, and the toxins were completely neutralized by anti-VT2. In addition, control samples collected throughout the year from 40 children without recent enteric or renal disease, aged 4 months to 10 years (mean 3.7 years; median 2 years) were investigated. Geometrically diluted sera (50 µl) were incubated with equal volumes of either toxin (2 CD_{50%}) and assayed on HeLa cells. The residual cytotoxicity was assessed microscopically and quantitated by the dye absorption method of Gentry and Dalrymple [30]. The neutralizing capacity of each serum was calculated by

the least-squares regression analysis, and expressed as 50% neutralization titre (NT_{50%}).

O 157 LPS ELISA. Nunc-Immuno-Plate, MaxiSorb (Nunc) were coated with 0.5 µg LPS per well in 50 µl PBS and dried overnight at 37 °C. After extensive washing with PBS with 1% Tween-20 (Merck, Darmstadt, Germany), patient or control sera diluted in PBS containing 0.1% Tween and 2% fetal calf serum (Biochrom, Berlin, Germany) were added to the wells and incubated 2 h at 37 °C. Bound immunoglobulins were detected by alkaline-phosphatase-conjugated rabbit anti-human IgM and IgA diluted 1:1000 (Sigma), and *p*-phenyl phosphate as substrate (Behring). The reaction was stopped with 1 N NaOH after 30 min at 37 °C, and the A₄₀₅ was recorded against a blank well containing substrate solution and NaOH. Known negative and positive sera were run with each assay as controls. Results were confirmed by at least two separate assays. ELISA titration results were shown to be in good agreement with readings obtained with a single serum dilution (1:500). The specificity was demonstrated using heterologous *E. coli* O-group typing sera and LPS preparations. Selected serum samples were also subjected to immunoblotting using O 157 LPS as antigen as described [25]. ELISA control sera were obtained from 50 children without renal failure and without recent diarrhoea aged 0.5 to 15 years (mean 5.7 ± 4.5 years; median 3.0 years). The cut-off point to define a positive sample was calculated as the mean absorbancy plus 3 standard deviations of the control sera. The mean OD values (± 1 s.d.; dilution 1:500) were 0.130 ± 0.091 (IgM) and 0.007 ± 0.010 (IgA). OD values were multiplied by 1000 and expressed as ELISA units.

Clinical evaluation

Medical reports were reviewed for demographic information, prodromal illness, haematological and biochemical laboratory data, and clinical parameters using a standard questionnaire. HUS associated with intestinal prodromal symptoms such as diarrhoea, tenesmus or abdominal complications was referred to as classical, or enteropathic (E⁺) HUS, whereas the term 'atypical', or non-enteropathic (E⁻) HUS was used in the absence of primary enteric disease.

Statistical methods

Fisher's exact and *t* test were used as indicated.

RESULTS

The study population

One hundred and thirty-four of 145 patients (92%) with a known history had diarrhoea and other signs of enteropathy preceding the diagnosis of HUS (E⁺ cases). Eleven patients (8%) suffered from other forms of HUS (atypical, or E⁻ cases). Insufficient reports regarding the prodromal illness were obtained from the remaining two patients. The mean age at the onset of E⁺ HUS was 3.6 ± 3.2 years (range 3 months to 21.3 years, median 2.3 years), of E⁻ HUS 4.4 ± 4.1 years (1 month to 11.7 years, median 3.8 years) (difference not significant). Amongst the E⁺ patients, 76% were younger than 5 years, the male:female ratio was 1.15. Cases of E⁺ HUS were diagnosed throughout the year. For the complete years 1987-90,

Table 1. Characterization of verotoxin (VT) producing *E. coli* isolates from patients with classical HUS

VTEC isolate		No. of isolates	VT type
O-group	OH-serotype		
O 22	(O 22. H8)	1	VT1 (VT2)*
O 26	(O 26. H ⁻ , O 26. H11)	2	VT2
O 55	(O 55. H ⁻ , O 55. H6)	2	VT2
O 111	(O 111. H8)	1	VT2
O 157	(O 157. H ⁻)	6	VT2
	(O 157. H7)	3	VT2
ONT	(O 157. H?)†	2	n.d.‡
	(ONT. H ⁻)	1	VT2
	(ONT. H2)	1	VT1 (VT2)*

Note. Verotoxin (VT) was phenotypically characterized by complete neutralization of polymyxin B extracts with anti-VT1 (anti Shiga toxin) and/or anti-VT2 immune serum; the presence of VT1 and VT2 gene sequences was shown by hybridization with the oligonucleotides 722 and 849 (15).

* Differential neutralization studies of the polymyxin B extracts of the *E. coli* O 22. H8 and ONT. H2 isolates on HeLa and Vero cells suggest co-expression of VT1 and a VT2 variant toxin.

† H type not determined.

‡ n.d., not tested.

a monthly incidence above the average was seen from May to October (73.5%), with a peak in July and August, whereas 8/11 E⁻ patients presented between January and March ($P < 0.02$, Fisher's exact). A higher than expected number of patients lived in rural areas; 53.7% of all E⁺ HUS patients, but 40.3% of the general population were from settlements of fewer than 20000 inhabitants ($P < 0.05$, Fisher's exact). For the Northern part of Germany, served by the Hamburg University Children's Hospital, an incidence of approximately 1 case of E⁺ HUS per 100000 population below the age of 16 years and of 2.7 per 100000 below the age of 5 years was calculated.

Microbiological studies

Verotoxin-producing *E. coli* (VTEC) were identified in 19 out of 118 E⁺ patients from whom stool samples were obtained (16%). In two of them, VTEC had been isolated from siblings, rather than from the index patients. None of the nine E⁻ patients from whom stools were submitted yielded VTEC. Results of the primary stool culture were available from 75 patients: only 38 of 68 VTEC culture-negative E⁺ patients (56%), and 3 of 7 E⁻ patients (43%) grew coliform bacteria.

E. coli O 157 (non-motile and H7) was the most frequent serogroup involved (58%). The remaining strains belonged to at least five different serogroups (O 22, O 26, O 55, O 111, not typable) (Table 1). All but one VTEC isolate (ONT. H2) were sensitive to ampicillin, third-generation cephalosporins, aminoglycosides, tetracyclines and co-trimoxazole. Sorbitol was fermented by 4/4 *E. coli* O 157. H⁻ isolates tested. Using Vero cells, culture supernatants and polymyxin B extracts of the isolates were completely neutralized by anti-VT2 ($n = 15$) or by anti-VT1 and anti-Shiga-toxin ($n = 2$). Two isolates were not available for further characterization. VT production *in vitro* corresponded to the hybridization results

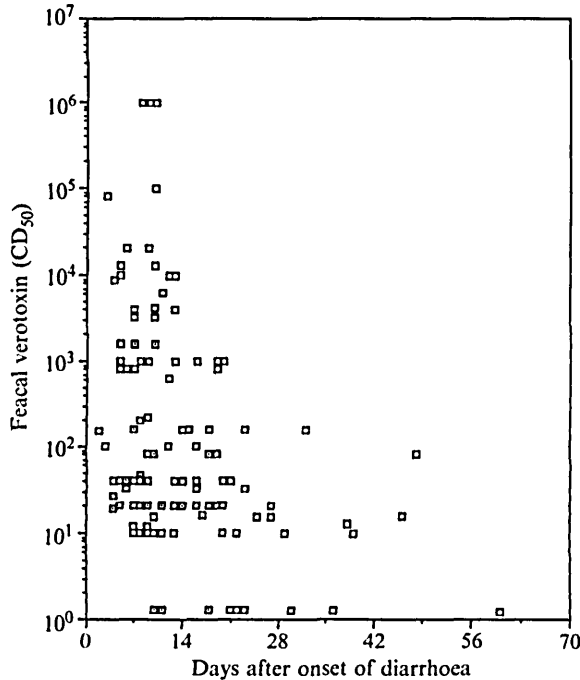


Fig. 1. Titration of stool filtrate cytotoxicity on Vero or HeLa cells (faecal verotoxin) found in 49 patients with classical HUS that was neutralized by anti-VT1 and/or anti-VT2 immune sera. Each dot represents a single stool sample collected at the indicated time after the onset of diarrhoea. All samples obtained from a given individual are included.

obtained with the VT1 and VT2 specific oligonucleotide probes. While two strains (*E. coli* O 22.H8 and ONT.H2) hybridized with both nucleotide probes, using HeLa cells, the extracted toxin was completely neutralized by anti-VT1 (Table 1). In addition, Vero cell toxicity of the stool filtrate of one of these patients was 10–100-fold greater than HeLa cell toxicity. From these data, the presence in both strains of VT1 and a VT2 variant was inferred.

Free (faecal) verotoxin. The presence of free (faecal) verotoxin (FVT) was demonstrated in the stool filtrates of 16 patients associated with VTEC strains, and of 36 additional patients from whom VTEC was not isolated. HeLa and/or Vero cell toxicity of the stool filtrates ranged from 15 to 10⁶ CD₅₀ (geometric mean 4.7×10^2 ; median 2×10^2 CD₅₀). The cytotoxic effect was neutralized by anti-VT1/anti-Shiga toxin in 2 cases, by anti-VT2 in 44 out of 52 cases (85%), and by a mixture of anti-VT1 and anti-VT2 in 6 cases (12%). None of the 9 E⁻ patients excreted neutralizable FVT.

Follow-up specimens for kinetic studies were available from 36 of the VTEC/FVT positive patients. FVT excretion varied from a few days to 6 weeks after the onset of diarrhoea (Fig. 1). Examples of individual FVT kinetics are shown in Fig. 2.

Other pathogens. *Salmonella* spp. were identified in the faeces of four patients with acute HUS; two of them yielded FVT (40 and 20 CD₅₀ respectively) that was completely neutralized by anti-VT2. In addition, rotavirus and cryptosporidia, and corona and respiratory syncytial virus (nasopharyngeal secretions) were

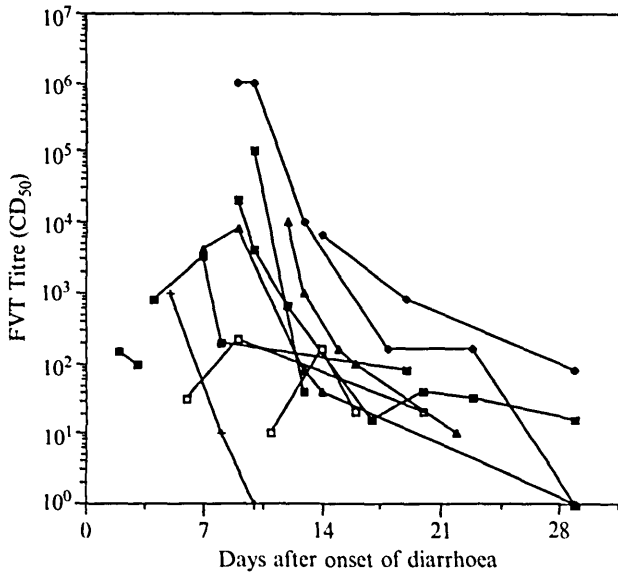


Fig. 2. Individual kinetics of faecal verotoxin titres (FVT) of selected patients with classical HUS. Each dot represents a single stool sample collected at the indicated time after the onset of diarrhoea.

identified. *Campylobacter jejuni* was isolated from a sibling suffering from diarrhoea, but not from the VTEC-positive index child. All but one of these patients had cultural and/or serological evidence of VTEC infection (Table 2). One *S. typhimurium* isolate was available for VT testing. The bacterial lysate and supernatant of this strain lacked Vero and HeLa cell toxicity. The polymyxin extract of the campylobacter strain displayed low levels of cytotoxicity that was not neutralized by anti-VT1 and/or anti-VT2 (Table 2).

Influence of the timing of stool collection. VTEC/FVT-positive stools were collected 8.0 ± 4.4 days (arithmetic mean, 1 s.d.) after the onset of diarrhoea, and 2.4 ± 2.5 days after the onset of HUS ($n = 51$), whereas VTEC/FVT-negative samples were collected 13.7 ± 10.8 days after the onset of diarrhoea and 7.6 ± 9.8 days after the onset of HUS respectively ($n = 53$). The difference in the timing

Table 2. Detection of potential pathogens other than VT producing *E. coli* in children with classical HUS

Pathogen	N	Evidence of VTEC infection
<i>Salmonella</i> spp*	4	4
Rotavirus	3	2
Respiratory viruses†	2	2
Cryptosporidium	1	1
<i>Campylobacter jejuni</i> ‡	1	1

Note. Evidence of infection by verotoxin-producing *E. coli* (VTEC) required isolation of the organism, and/or demonstration of faecal verotoxin and/or elevated anti-O 157 LPS antibodies.

* One *S. typhimurium* isolate was available for testing; its culture supernatant and lysate lacked HeLa and Vero cell toxicity.

† Corona and respiratory syncytial virus, respectively.

‡ Production of low levels of non-neutralizable cytotoxin on vero cells.

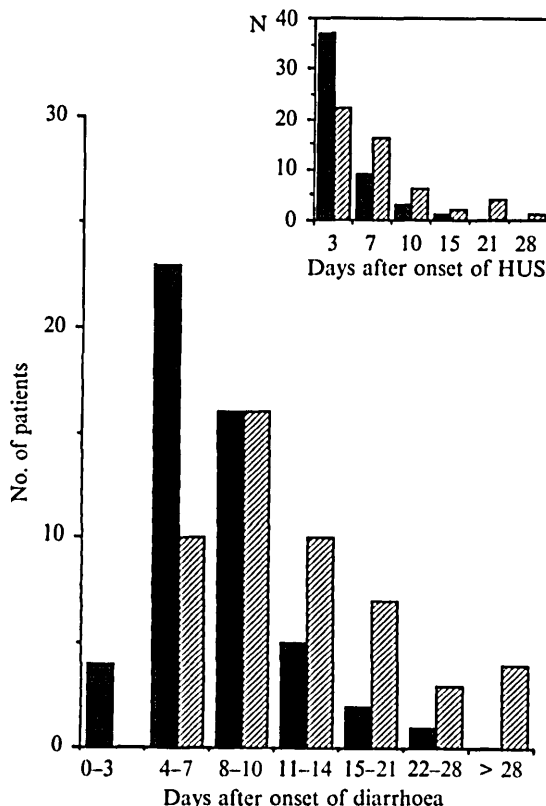


Fig. 3. Time dependence of the detection rate of verotoxin producing *E. coli* (VTEC) and/or faecal verotoxin (FVT) in patients with classical HUS related to the onset of diarrhoea and HUS, respectively. Solid bars: number of patients with VTEC/FVT positive samples; hatched bars: number of patients without demonstrable VTEC/FVT.

of VTEC/FVT-positive and VTEC/FVT-negative samples was significant ($P < 0.001$; *t* test). Thus, 27/37 (73%) of the stool samples collected within 1 week, but only 3/17 (18%) of the specimens collected later than 2 weeks after the onset of diarrhoea, were VT-positive ($P < 0.001$; Fisher's exact). The number of VT-positive stool samples in respect of the time of onset of diarrhoea and of HUS is shown in Fig. 3.

Serological studies

Verotoxin neutralization. Twelve of 131 serum samples obtained during the acute E^+ HUS neutralized VT1 at dilutions equal to or above 1:5 (median titre < 5; range < 5–80), whereas all but one initial serum neutralized VT2 (median 40; range < 5 to 320). Results obtained with VT2 from the wild-type isolate 4220 were very similar. VT1 and VT2 neutralization titres of the initial serum samples from E^+ patients did not differ significantly from the titres of the samples obtained from E^- patients and from control children of the same age group (Table 3). Paired sera taken 1–12 weeks apart were available from 70 patients. Seroconversion, or a fourfold change of the VT1 and/or VT2 neutralization titre was observed in 13/70 E^+ and 1/3 E^- patients (Table 3). In five patients, the specificity of the neutralization titre was at least partly consistent with the VT phenotype identified

Table 3. *Verotoxin neutralisation titres (NT₅₀) and E. coli O 157 LPS antibodies in patients with acute HUS and controls*

Assay	No. positive/no. examined (percent)			P*
	Classical HUS	Atypical HUS	Controls	
	Initial serum (neutralization titre ≥ 5)			
VT1	12/131 (9)	1/9 (11)	1/40 (3)	n.s.†
VT2	130/131 (99)	9/9 (100)	40/40 (100)	n.s.
	Seroconversion or fourfold rise of neutralization titres			
VT1	4/70 (6)	1/3 (33)	n.a.‡	n.s.
VT2	10/70 (14)	0/3 (0)	n.a.	n.s.
	Anti-O 157 LPS (IgA and/or IgM ELISA)			
	106/128 (83)	2/11 (18)§	0/40 (0)	< 0.001

* Fisher's exact test: enteropathic HUS *versus* atypical HUS and controls.
 † n.s., not significant.
 ‡ n.a., follow-up samples not available.
 § Only IgA (see text).
 || For the IgM as well as for the IgA ELISA.

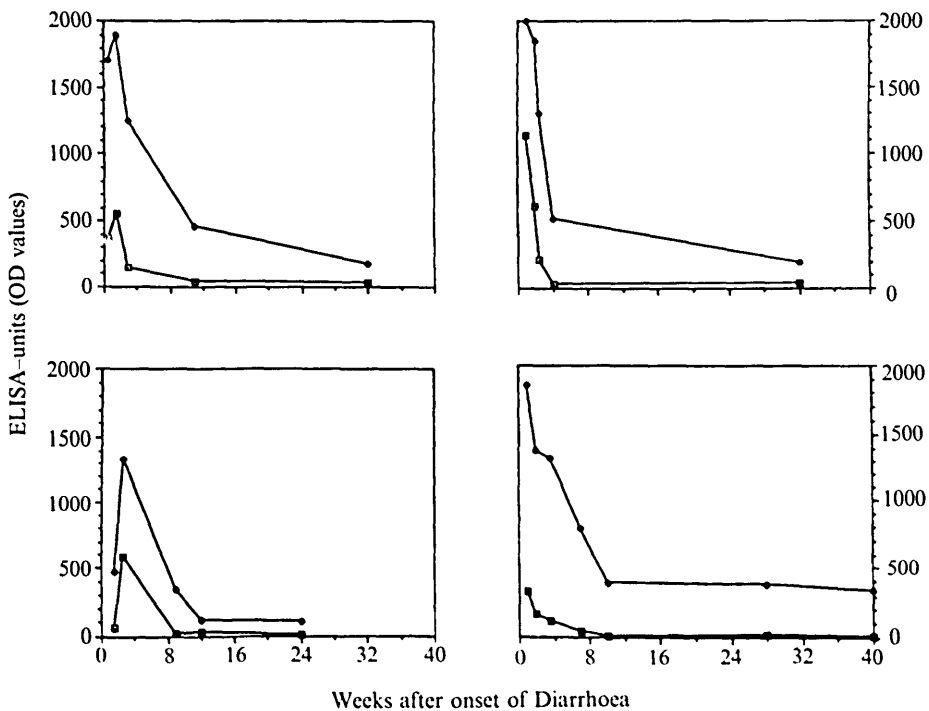


Fig. 4. Individual kinetics of O 157 LPS specific IgM and IgA antibodies in children with classical HUS. The cut-off level for positive ELISA readings was OD 0.400 (400 ELISA units) for the IgM assay (●---●), and OD 0.040 (40 ELISA units) for the IgA assay (□---□).

from the VT₂ isolate and/or from the faecal filtrate (most VT₂), whereas 25 VT₁-positive patients failed to exhibit significant titre rises.

E. coli O 157 LPS antibodies. Serum samples from 128 patients with classical, and from 11 patients with atypical HUS taken during the first week after

diagnosis of HUS were examined for antibodies to O 157 LPS by ELISA. Using a cut-off point of OD₄₀₅ 0.400 in the IgM assay and of 0.040 in the IgA assay, the sera from 100 E⁺ patients (83%) were positive (68 and 74% with the IgM and IgA assay, respectively). A second sample was available from 15 of the 28 E⁺ patients initially ELISA IgM and IgA negative; 6/15 follow-up sera were positive increasing the number of anti-O 157 LPS positive patients to 106 (83%). In contrast, elevated IgA readings (but not IgM) were obtained with the sera from two E⁻ patients. The difference between E⁺ and E⁻ patients was highly significant for either assay ($P < 0.001$, Fisher's Exact). Overall, a good correlation was observed between IgM and IgA values ($r = 0.75$, $x = 0.001$), and the ELISAs were in excellent agreement with O 157 LPS immunoblot results of selected sera (results not shown). Detailed analysis revealed positive ELISA readings in the sera from 9 of 10 patients with *E. coli* O 157 and from 4 of 7 patients with non-O 157 VTEC isolates. The magnitude of the IgM and IgA anti-O 157 LPS titres of VTEC/FVT culture-negative and culture-positive E⁺ patients did not differ significantly.

Follow-up studies showed that anti-O 157 LPS IgA and IgM declined to near cut-off levels 4 and 8 weeks, respectively, after the onset of diarrhoea. In some instances, elevated IgM titres were detected for as long as 7 months. Individual kinetics of LPS O 157 antibodies are shown in Fig. 4.

DISCUSSION

In this first comprehensive, prospective, multicentre study on microbiological and serological features of the paediatric HUS in central Europe, 92% of the cases were associated with enteric disease (E⁺ HUS). Based on one or more of the diagnostic criteria, i.e. (i) isolation of VT-producing organisms from the stool (VT production *in vitro* and/or presence of VT genes), (ii) demonstration of neutralizable faecal VT, and (iii) elevated serum IgM or IgA antibodies to O 157 LPS, 86% of the E⁺ cases had evidence of VTEC infection. The estimated annual incidence for the northern part of Germany of approximately one case of E⁺ HUS per 100 000 children younger than 16 years and of 2.7 per 100 000 children younger than 5 years is similar to figures reported elsewhere [3-5].

E. coli O 157 was the most frequent VTEC serogroup isolated. This corresponds to the high incidence of O 157 specific antibodies in VTEC/FVT-positive as well as culture-negative patients. The relative frequency of the non-motile O 157 strains is at variance with the predominance of O 157. H7 isolates reported from other areas [5, 17, 31, 32]. Most *E. coli* O 157. H⁻ strains isolated during this study as from other patients with HUS from Germany ferment sorbitol within 24 h [27, 33]. Both features are of diagnostic importance if selective media containing sorbitol or antflagellar antiserum are used [34, 35]. Many *E. coli* O 157 strains of North American origin implicated in HUS or HC reportedly produce VT1 and VT2, whereas a high proportion of European strains produce only VT2 [27, 31, 32]. This is reflected by a similar distribution of *in vivo* produced (faecal) VT as described in this study suggesting a major role of VT2 in the pathogenesis of HUS, whereas sole VT1 production seems to be found more frequently in VTEC strains isolated from watery or bloody diarrhoeas [27, 36, 37]. Production of a VT2 subtype (probably VT2e), together with VT1, was assumed in two *vt1* (772) and

vt2 (849) nucleotide probe-positive, non-O 157 *E. coli* isolates, based on differential HeLa and Vero cell toxicity and neutralization studies (Table 1). The $CD_{50\text{Vero}}$ of the stool filtrate of these patients exceeded the $CD_{50\text{HeLa}}$ by 1–2 orders of magnitude, illustrating the excellent correlation between the VT phenotype(s) found *in vitro* and *in vivo*.

A direct relationship was observed between the detection rate of VTEC/FVT and the time interval between the onset of diarrhoea and stool collection. Moreover, in sequential determinations of the faecal VT during the course of the disease, faecal cytotoxic activity was found to decrease rapidly in most instances. Few patients, however, excreted VT for remarkably long periods (up to 6 weeks) after the onset of diarrhoea. There was, however, no apparent correlation between the duration of toxin excretion and the clinical outcome.

Spread of *E. coli* O 157 within families and nursing facilities may occur [17, 38]. Here we have documented the presence of *E. coli* O 157 of the same O:H serovar and concordant faecal VT in family members of some of the index patients. Their symptoms varied from asymptomatic excretion to severe colitis. In an illustrative case the pathogen and high amounts of FVT (VT2) were identified in both (healthy) siblings and the mother, whereas only moderate amounts of the same VT type were excreted by the index patient who had been previously treated with antibiotics. In this and other instances where FVT-positive stools were obtained from family contacts, the toxin titre decreased as rapidly as in HUS patients indicating acute infection/colonization by the same pathogen rather than bacterial carriage. Early processing of the stools of siblings and parents might help to monitor the spread of VTEC infection and, eventually, to prevent secondary cases.

Various microbial pathogens have been incriminated as potential causes of the HUS [for review see 1, 39]. In this investigation, *Salmonella* spp., *Campylobacter jejuni*, and rotavirus, cryptosporidia, and respiratory viruses were identified during the acute disease. They are considered incidental findings, since all but one of these patients also had evidence of VTEC infection. However, their contributory role in the initiation of VTEC diarrhoea has not yet been addressed.

Rising titres of VT neutralizing antibodies have been described in patients with classical HUS [6, 20, 21]. In this study, fourfold titre increments against either toxin were rarely observed. Moreover, we showed that the sera from almost all patients as well as controls neutralized VT2 but rarely VT1, extending our previous observations [40]. Studies in our laboratory showed that the VT2 neutralizing activity of normal human serum was retained after the selective removal of the major immunoglobulin classes suggesting binding to other factor(s) than immunoglobulins (manuscript in preparation). We therefore conclude, that the diagnostic value of the neutralization test, at least in this population, is limited.

Recently, we reported the high incidence of anti-O 157 LPS antibodies in patients with acute HUS, detected by the indirect haemagglutination assay (IHA) and by transimmunoblots developed with anti-human IgM [25], confirming the diagnostic potential of O 157 LPS-based assays [22–24, 41]. Here we demonstrate that the majority of patients with classical HUS also produce a strong IgA response to this antigen. In most cases, the first sample collected shortly after

hospitalization was already positive with both tests. However, the decrease of O 157-specific IgA was more rapid compared to IgM. Lack of a measurable O 157 LPS antibody response in 17% of the E⁺ cases may have resulted from too early or late a blood sampling as demonstrated by our follow-up studies, and from infections by non-O 157 VTEC strains.

This prospective study comprising children with HUS from various distant locations over a period of 5 years demonstrates the clinical importance of VTEC O 157. H⁻ and H7 infection associated with HUS in Germany and central Europe. It was shown that a serological diagnosis is possible in the majority of cases by detection of acute-phase antibodies to O 157 LPS. In addition, the kinetics of FVT titres and of anti-O 157 acute-phase antibodies in natural infections were determined in a large patient population. It is not known why most patients do not mount a measurable VT antibody response. Further studies are needed to define the VT2 neutralizing principle in patients with HUS and in normal human serum, and their functional role in VTEC infections.

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