Epidemiol. Infect. (1993), **110**, 1–7

A prospective study of exposure to verotoxin-producing *Escherichia coli* among Canadian children with haemolytic uraemic syndrome

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(*Accepted 10 August 1992*)

SUMMARY

Haemolytic uraemic syndrome (HUS) is a leading cause of acute renal failure in childhood. Although infection with *Escherichia coli* O 157.H7 has been associated with HUS in North America and Europe, only a limited number of studies have examined the role of other verotoxin-producing *E. coli* (VTEC) serotypes in this condition. To address this issue, we conducted a comprehensive, prospective microbiological study of patients treated for HUS at eight Canadian hospitals in the summer of 1990. Of the 34 consecutive patients with HUS enrolled over 4 months, *E. coli* O 157.H7 was isolated from the stools of 26, and other *E. coli* serotypes were isolated from four patients. In four subjects no pathogenic *E. coli* serotypes were identified on stool culture. Using oligonucleotide probes specific for VT-1 and VT-2, verotoxin genes were detected in the stool isolates of all patients with *E. coli* O 157.H7, and from two with other *E. coli* serotypes. Two other patients had at least a fourfold rise in anti-verotoxin antibodies. Strong evidence of exposure to a verotoxin was present in 30/34 (88%). Patients with *E. coli* O 157.H7 infection were more likely to develop an antibody response to VT-2 than to VT-1 (22/22 vs 12/22; $P = 0{\cdot}002$). These results further strengthen the association of HUS with verotoxin-producing *E. coli* in North America, and confirm that *E. coli* serotypes other than O 157.H7 are isolated in a small proportion of summertime HUS episodes.

INTRODUCTION

The haemolytic uraemic syndrome (HUS) is characterized by the abrupt development of haemolytic anaemia, thrombocytopenia, and renal injury [1]. It typically affects previously healthy children, with a peak incidence in those between 6 months and 5 years of age [2]. Over the last decade it has become clear that prior gastrointestinal infection with verotoxin-producing *E. coli* (VTEC), most commonly the O 157.H7 serotype, is strongly associated with the development of HUS in North America and Europe [3–6]. Recent studies in these

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regions, however, have reported isolation of *E. coli* O 157 . H7 in only 21–63% of patients [2, 4–7]. When the techniques for detection of VTEC are expanded to include tissue culture assays for free stool verotoxin, serum antibodies to verotoxin or to *E. coli* O 157 lipopolysaccharide, and DNA probes for verotoxin genes in stool, evidence of verotoxin exposure is identified in 75–100% of patients [7–10]. Clarifying the importance of VTEC serotypes other than O 157 . H7 would be important for the development of measures to prevent HUS. We therefore undertook a prospective, multicentre study of Canadian children with HUS using a clinical protocol that ensured early culturing of stools, and included comprehensive microbiological and serological methods for detecting evidence of verotoxin exposure.

METHODS

The study was conducted from 1 May 1990 to 31 August 1990 at the eight hospitals listed in the Appendix. Patients less than 15 years of age were included if they had been diagnosed by a paediatrician or paediatric nephrologist as having HUS [2, 11, 12] and if they satisfied the following laboratory criteria: (*a*) haemolytic anaemia of recent onset (haemoglobin concentration < 2 standard deviations from the mean for age, with schistocytes in the peripheral blood), and (*b*) creatinine concentration $>$ 97th percentile for age or urea > 7 mmol/l (20 mg/dl). Thrombocytopenia was regarded as supportive of the diagnosis.

Stool samples from all patients with HUS were submitted to the hospital microbiology laboratory within 24 h of admission, and were routinely inoculated onto a modified MacConkey agar containing 1% sorbitol instead of lactose. Colonies which failed to ferment sorbitol [13] were identified as *E. coli* by standard biochemical tests, and isolates were serogrouped by agglutination using O 157 antisera. All O 157 isolates from these patients were forwarded to the National Laboratory for Enteric Pathogens for confirmation, complete serotyping, and detection of verotoxin (VT) genes by the polymerase chain reaction (PCR) technique using oligonucleotide probes specific for VT-1 and VT-2 [14].

If no evidence of *E. coli* O 157 was identified at the hospital laboratory by the sorbitol–MacConkey screening, samples of stool were forwarded to the National Laboratory for Enteric Pathogens, and were cultured on selective agar media for the isolation of pathogenic *E. coli*. A minimum of 10 colonies were picked from the selective agar media and tested for the presence of verotoxigenic *E. coli* using a modification of the immunoblot method described by Perera and colleagues [15]. Colonies that were positive by the immunoblot method were selected for further investigation. Serotyping was performed with standard antisera to the O, K, and H antigens of *E. coli*.

Stool filtrates from all patients were assayed for the presence or absence of free faecal verotoxin using the cell culture technique described by Konowalchuk and colleagues [16]. To prepare these filtrates, equal volumes of faeces and phosphate-buffered saline (pH 7·2) were mixed and centrifuged at 15000 g for 5 min; the filtrate supernatants were then inoculated into Vero cells, as described by Karmali and colleagues [3].

Serum was obtained at the time of diagnosis, and again 2 months after discharge from hospital to minimize the small likelihood of detecting antibodies passively

acquired from red blood cell transfusions. The presence of serum-neutralizing antibodies to verotoxin was determined by the tissue culture assay method [16]. We considered exposure to verotoxin to be present if there was a positive stool culture for VTEC, free faecal verotoxin, evidence of verotoxin genes in stool isolates, or if there was an anti-verotoxin antibody titre of $\geqslant 4$ in the convalescent serum in those initially seronegative, a fourfold rise in the anti-verotoxin antibody titre in those initially seropositive, or a titre of $\geqslant 4$ in those with only one serum specimen. Comparison of the proportion of patients with antibody responses to VT-1 and VT-2 was performed using the McNemar test.

RESULTS

Patients

Thirty-four consecutive children with HUS (21 female, 13 male) were enrolled at the 8 hospitals over the 4 months of the study. The median age was 2·7 years (range, 1·1–14·6). The median highest recorded values for urea (27·4 mmol/l) and creatinine (345 μmol/l), and the median lowest recorded values for haemoglobin (58 g/l) and platelets ($34{\cdot}5 \times 10^9$/l) were similar to values from Canadian children with HUS from 1986 to 1988 [2]. Five patients were treated with haemodialysis, 10 with peritoneal dialysis, and 1 with both forms of dialysis. No patient was treated with intravenous immunoglobulin. The median length of stay in hospital was 11·5 days (range, 2–81). One patient died.

Microbiology

All 34 patients had diarrhoea; in 29 (85%) the diarrhoea was bloody. Stool cultures were obtained a median of 6 days after the onset of diarrhoea, and 94% were obtained within 10 days of the onset of illness. All those from whom no VTEC was isolated had stools cultured after the sixth day of gastrointestinal symptoms.

The results of stool cultures, assays for free faecal verotoxin, and analysis of bacterial isolates for VT genes are displayed in Table 1. *E. coli* O 157.H7 was isolated from 24 patients at the hospital microbiology laboratories, and from 1 further child on testing at the National Laboratory for Enteric Pathogens. Stool was not available in one patient whose sibling had HUS associated with *E. coli* O 157.H7 infection. Because this boy's acute and convalescent sera demonstrated fourfold changes in antibody titres to VT-1 and VT-2 (the same verotoxins present in his brother's stool), we have assumed that he was infected with an *E. coli* O 157.H7 with a similar verotoxin profile. Isolates were not available for verotoxin gene detection in two patients from whom *E. coli* O 157.H7 had been cultured at the referring institution.

Other *E. coli* serotypes were isolated from four children. Stool isolates showed evidence of verotoxin DNA by the PCR method in two of these subjects, but not from the *E. coli* O 6.H1 or O 18.K1.H7 isolates. *E. coli* O 6.H1 and O 18.K1.H7 have been isolated from patients with HUS in the past [17, 18]; the patients infected with these organisms had convalescent antibody titres to VT-2 of 1:4 and 1:8, respectively.

Of the 4 patients from whose stools no pathogenic *E. coli* was isolated, 2 had at least a fourfold rise in the antibody titre to VT-1, one of whom also had a fourfold

Table 1. *Verotoxin typing and free stool verotoxin in 34 children with HUS*

Isolate	N	Verotoxin typing of stool isolates				Free faecal verotoxin
		Neg	VT-1*	VT-2	VT-1 + VT-2	
E. coli O 157.H7	26†	0	0	2	22	5
E. coli O 6.H1	1	1	—	—	—	—
O 18.K1.H7	1	1	—	—	—	—
O 55.H7	1	—	—	1	—	1
O 111.NM	1	—	—	—	1	—
No growth	4	NA‡	NA	NA	NA	—
Total	34	2	0	3	23	5

* VT, verotoxin.

† In two patients with *E. coli* O 157.H7 infection, isolates were not available for verotoxin testing by PCR.

‡ NA, not applicable.

rise in titre to VT-2. The two remaining patients who did not have a fourfold rise had convalescent antibody titres to VT-2 of 4 and 8, respectively. The combined microbiological and serological data confirm that 30 of the 34 patients (88%) had definite evidence of exposure to VTEC. No patient had evidence of infection with *Campylobacter*, *Salmonella*, *Shigella*, or *Yersinia* species.

Acute and convalescent sera were available from 26 patients, while only 1 serum sample was available from the other 8. Twenty-two of the 26 *E. coli* O 157.H7 isolates had evidence of both VT-1 and VT-2 genes (2 had VT-2 only and 2 were not tested). Of the patients from whom these 22 isolates were obtained, significantly more developed antibody responses to VT-2 than to VT-1 (22/22 with antibodies to VT-2 vs 12/22 with antibodies to VT-1; $P = 0{\cdot}002$, McNemar test). This result was not due to inadequate ascertainment, as blood samples were available from both the acute and convalescent phases in all 10 patients with antibodies to VT-2 alone. Free stool verotoxins were present in 5 patients with *E. coli* O 157.H7 and in 1 with *E. coli* O 55.H7.

DISCUSSION

We conducted a detailed microbiological investigation of 34 consecutive patients with HUS who had been evaluated at 8 hospitals across Canada during the summer of 1990. The most striking result of this study is that 88% of children with HUS had definite evidence of exposure to verotoxin. The high rate of detection of verotoxin exposure can be attributed to the availability of routine screening of stools for *E. coli* O 157.H7 at the participating hospitals, the intensity of the search for verotoxin-producing *E. coli* in the stools of those who were negative for *E. coli* O 157 using the sorbitol–MacConkey screening method, and serological testing for acute and convalescent antibodies to verotoxin. Our study also demonstrates that verotoxigenic *E. coli* other than the O 157.H7 serotype are associated with some episodes of HUS, even during the time of peak prevalence of *E. coli* O 157.H7.

These results extend the findings of an earlier Canadian investigation [8] which

was conducted before the development of the polymerase chain reaction technique and before methods were available to detect VT-2. In that study, exposure to verotoxin was demonstrated in 30 of 40 (75%) children with HUS. Chart and colleagues [9] studied 60 British children with HUS, 14 (23%) of whom had evidence of exposure to VTEC as determined by bacteriological methods and by tissue culture methods to test for verotoxin. A further 35 had antibodies to the *E. coli* O 157 lipopolysaccharide, and 4 additional patients had evidence of other VTEC. While this study thereby identified exposure to VTEC in 48/60 (80%), it was not designed to detect serological evidence of verotoxin-producing organisms other than *E. coli* O 157.

Our results accord most closely with those from two recent prospective studies. The first identified faecal or serological evidence of Shiga-like toxin exposure in 48/51 (94%) Argentinian children with HUS enrolled over a 20-month period [19]. The second study enrolled 22 consecutive patients with HUS from Germany, 15 of whom had free faecal verotoxin or evidence of VTEC on stool culture; the remaining 7 subjects had significantly elevated titres of antibodies against the O 157 lipopolysaccharide [7]. Our investigation was conducted in the summer months; studies now underway will determine whether a similar pattern of exposure to verotoxin extends throughout the year, and will help determine whether exposure to specific verotoxins affects either the risk of developing HUS or the severity of the renal involvement.

Our patients with *E. coli* O 157.H7 infection were significantly more likely to develop an antibody response to VT-2 than to VT-1, despite being infected with organisms that had the genetic potential to produce both verotoxins. The reasons for this pattern are unclear, but the results suggest a more important role for either exposure or response to VT-2 in the pathogenesis of HUS. These findings are consistent with two earlier studies in which *E. coli* producing only VT-2 were more likely to be isolated from patients with HUS than from those with uncomplicated *E. coli* O 157.H7 gastroenteritis [20, 21].

Shiga-toxin and verotoxin are cytotoxic to endothelial cells [22, 23] and endothelial cell swelling is thought to be the initiating pathological event for the renal injury in HUS [24]. The results of the present study strengthen the epidemiological association of HUS with verotoxin exposure, and suggest that measures to prevent HUS must include diminishing the frequency of exposure to verotoxin or improving protection against its systemic effects.

APPENDIX

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ACKNOWLEDGEMENTS

The study was supported by a grant from the Research Institute of the Children's Hospital of Eastern Ontario, Ottawa, Canada. Support of the Canadian Pediatric Kidney Disease Reference Centre was provided by the Children's Hospital of Eastern Ontario Foundation, Ottawa, Canada. Dr Rowe was supported by a Career Scientist Award from the Ministry of Health of Ontario. We thank Ruth Moffatt and Florence Semple for their assistance and commitment to this project.

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