The Association Between Idiopathic Hemolytic Uremic Syndrome and Infection by Verotoxin-Producing Escherichia coli

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Forty pediatric patients with idiopathic hemolytic uremic syndrome (HUS) were investigated for evidence of infection by Verotoxin-producing Escherichia coli (VTec). Fecal VTec (belonging to at least six different O serogroups including O111, O145, and O157) or specifically neutralizable free-fecal Verotoxin (VT) or both were detected in 24 (60%) patients but were not detected in 40 matched controls. Ten of 15 of the former developed fourfold or greater rises in VT-neutralizing antibody titers, as did six other patients who were negative for both fecal VTec and VT. A total of 30 (75%) patients had evidence of VTec infection by one or more criteria. We concluded that a significant association exists between idiopathic HUS and infection by VTec. The detection of free-fecal VT was the most important procedure for the early diagnosis of this infection because, in our study, VTec were never isolated in the absence of fecal VT, whereas fecal VT was often present even when VTec were undetectable.

The hemolytic uremic syndrome (HUS) is characterized by a triad of features: acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia [1, 2]. Ever since its first description by Gasser et al. [1] in 1955, the syndrome has been reported in a variety of distinct clinical and epidemiological settings, and several different agents including drugs, chemicals, toxins, and microbes have been postulated as potential causes [2]. The prevailing view is that HUS is probably the end result of a number of different inciting events and pathogenic mechanisms [3].

The most common variety of the syndrome, the so-called idiopathic HUS of infancy and childhood [2], characteristically occurs a few days after the onset of an acute diarrheal "prodromal" illness. In a variant, referred to as "atypical HUS" [4], the prodrome is nondiarrheal and may consist of upper- or lower-respiratory-tract symptoms, fever, and vomiting. The other much less common types of HUS include a childhood form that is inherited [5], and adult forms that occur in association with pregnancy [6, 7], oral contraceptive use [8], malignant hypertension [9, 10], and various chronic illnesses [9, 11].

Idiopathic HUS is a leading cause of acute renal failure in childhood [12]. Although it occurs mostly in a sporadic fashion [12-14] a number of outbreaks of HUS have also been described [15-17] that indicate that the etiology may be of an infectious nature. The many microbes that have been implicated in this condition include bacteria such as Shigella dysenteriae type 1 (Shiga's bacillus) [18-21], Salmonella typhi [22], Campylobacter jejuni [23, 24], Yersinia pseudotuberculosis [25], Streptococcus pneumoniae [26], rickettsia-like organisms referred to as microtobiotics [27], and viruses such as coxsack-

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ievirus [28, 29], echovirus [30, 31], influenza [32], Epstein-Barr [33], and a Tacaribe-group virus (Por-
tillo) [12, 15]. The exact nature and frequency of the
association of these organisms with HUS requires
clarification.

In 1977 Konovalchuk et al. [34] described strains
of Escherichia coli that produced a cytotoxin that
was active on Vero cells (a line of African Green
Monkey kidney cells). This cytotoxin, referred to as
Verotoxin (VT), was found to be quite distinct from
the well-known heat-labile and heat-stable ent-
terotoxins of E. coli. The pathogenic significance
of VT-producing E. coli (VTEx) remains to be estab-
lished. In a preliminary communication [35], we
reported that VTEC, as well as free VT, were pre-
cent in the stools of some patients with HUS. These
findings stimulated a systematic and controlled study
using improved methodology, as well as additional
laboratory criteria, to establish the nature and the
extent of the relation between idiopathic HUS and
infection with VTEC. The results of these investiga-
tions are reported here.

Patients and Methods

Patients and specimens. A patient with ide-
opathic HUS was defined as a previously healthy
patient who developed the characteristic triad of
features (acute renal insufficiency, thrombocytopenia,
and microangiopathic hemolytic anemia) that
indicate HUS, following an acute diarrheal prodrom-
al illness, and who had no evidence of either the
inherited variety of HUS [5] or of disseminated intravascular coagulation (DIC) [36], as evidenced
by the prolongation of prothrombin and partial
thromboplastin times.

Forty-eight patients with a diagnosis of HUS
were referred to us between September 1980 and
September 1983. Thirty-three were managed at The
Hospital for Sick Children (Toronto) and 15 were
diagnosed and treated in other hospitals in Ontario
and Quebec.

Of the 48 patients, 40 with idiopathic HUS were
investigated in a controlled manner for evidence of
VTEC infection. Three patients with idiopathic
HUS were excluded because a stool sample was not
submitted, and five patients were investigated as a
separate group because they did not meet the
criteria for idiopathic HUS.

For each of the 40 patients with idiopathic HUS,
a control patient was selected who was matched for
age (usually within 20% of the age of the index pa-
tient), sex, and the same season of the year (within
three months of the onset of illness in the index
patient). For the first 17 patients with idiopathic
HUS, the controls were randomly chosen from
patients with Campylobacter enterocolitis whose
stools had been preserved at −70°C as part of a sepa-
rate study; they thus constituted a defined control
group with bloody diarrhea of known etiology.

For the next 23 patients, the controls were randomly
chosen in a prospective manner from otherwise
healthy patients who were admitted to our hospital
for elective orthopedic surgery and from healthy in-
fants at a local day care center. Two of the 40 pa-
ients with idiopathic HUS were siblings.

The specimens for investigation from the pa-

tients with HUS consisted of the first stool that was
submitted following admission; attempts were also
made to obtain acute and convalescent sera from
these patients. In the control group, only stools
were investigated.

Microbiological and serological investigations.
Stool samples from all patients were cultured for
Campylobacter, Salmonella, Shigella, Yersinia en-
terocolitica, and traditional enteropathogenic E. coli
serotypes by using established procedures; they were
examined by direct phase-contrast microscopy for
parasites and cellular exudates and by negative-
contrast electron microscopy for viruses.

Stools from all cases and controls were examined
for VTEC as follows. Twenty lactose-fermenting
colonies from a primary MacConkey agar culture
were subcultured onto 5% horse blood agar. Single
colonies from each subculture were inoculated into
Penassay® broth (antibiotic medium no. 3; Difco
Laboratories, Detroit) that was incubated overnight
at 37°C. The broth-culture supernatants were filtered
through 0.22 μm pore size membrane filters (Milli-
apore, Bedford, Mass.). These filtrates were tested for
cytotoxic activity in a tissue-culture assay system with
a Vero cell line (VERO 76; ATCC). Vero cells were
maintained in a tissue-culture medium (TCM) con-
sisting of minimal essential medium (Gibco
Laboratories, NY) with 10% fetal calf serum (Gibco);
the cells were grown as monolayers in 96-
well microtiter tissue-culture trays, and the latter
were used for cytotoxicity assays within 48 hr of prepara-
tion. Fifty microliters of serial two-fold dilutions of
the test filtrate were added to wells along one row
of the microtiter tray containing 200 μl of TCM per
well; the last well was used as a control and was in-
oculated with 50 μl of Penassay broth. The microti-
ter trays were incubated at 37°C in a 5% CO2 at-
mosphere and examined daily for up to three days for characteristic CPE. A filtrate of a reference VTEC strain (H.30; provided by J. Konowalchuk, Bureau of Microbial Hazards, Health and Welfare of Canada, Ottawa) was used as a positive control and was tested for cytotoxic activity with each new batch of Vero cells used. An ATCC strain of E. coli (ATCC 25922) was used as a negative control. All VTEC strains were confirmed as being E. coli by standard biochemical tests and were serotyped with antisera specific to the O, K, and H antigens.

In addition to the examination of culture filtrates for VT, each test strain of E. coli was also investigated for the presence of cell-bound (periplasmic) VT. Periplasmic cell extracts were obtained by polymyxin B sulfate treatment of bacterial pellets by using a method adapted from those described by Donohue-Rolfe and Keusch [37] and Griffin and Gemski [38] for releasing cell-bound Shiga toxin. Briefly, the method was performed as follows: the test E. coli culture was inoculated into 20 ml of Penassay broth that was incubated at 37 C for ~4–5 hr. The broth culture was then centrifuged at 10,000 g for 5 min, and the bacterial pellet was washed twice in sodium phosphate buffer (pH 7.2; 0.01 M). The pellet was resuspended in 1 ml of the buffer solution containing 0.1 mg/ml of polymyxin B sulfate (Burroughs-Wellcome, Quebec, Canada) and incubated for 30 min at 37 C. Finally, after recentrifuging and filtering this suspension, a bacteria-free supernatant was obtained. This was used for inoculating the Vero-cell assay system for the cytotoxicity test.

Free-fecal VT was investigated as follows: equal volumes of the fecal specimen and sodium phosphate buffer (pH 7.2; 0.01 M) were thoroughly mixed by using a vortex mixer and then centrifuged at 13,600 g for 5 min; a bacteria-free filtrate of the supernatant was then assayed for VT activity as described above.

The serological specificity of the fecal VT and the VT that was obtained directly from bacterial supernatants or cell extracts was determined by specific neutralization of the biologic activity in Vero cells by using a high-titered serum obtained from one of our HUS patients. This serum neutralized VT from VTEC reference strain H.30 to a dilution of 1:4,096. A negative-control serum was obtained from a patient who had no neutralizing antibody to H.30 VT. The test was performed as follows: equal volumes of the free-fecal VT or bacterial VT and the positive- or negative-control sera were preincubated at 37 C for 45 min. Aliquots of these mixtures were then assayed for VT as described. Neutralization was considered to be specific if the cytotoxic activity was absent after preincubation with the positive-control serum, but was still present after preincubation with the negative control.

The presence of VT-neutralizing activity in patients’ sera was investigated as follows (by using a modification of the method that we described earlier [35]): serial twofold dilutions of sera in TCM were preincubated with equal volumes of two, four, and eight 50% cytotoxicity doses (CD50) of a VT preparation. The last were prepared from a bacteria-free filtrate of an overnight Penassay broth culture of the reference VTEC strain, H.30. One CD50 was defined as the amount of VT activity required to produce a 50% CPE in the Vero-cell monolayer after three days of incubation at 37 C. After 1 hr of preincubation, 50-μl aliquots of the VT/serum mixtures were inoculated into the corresponding wells of a Vero-cell microtiter tray. Included in the assay were the appropriate toxin, serum, and cell controls. The microtiter trays were incubated at 37 C in a 5% CO2 incubator, and the end point was taken as the highest serum dilution causing inhibition of cytotoxic effect at three days. The end point was usually read in the rows inoculated with 2 CD50 units of VT, although occasionally it was easier to read in those rows containing the four or eight CD50 units of VT. Serological tests were only performed in cases when both acute and convalescent sera were available, and each serum pair was assayed on the same assay tray. The interpretation of rises in antibody titer between acute and convalescent sera was based on readings obtained from corresponding rows inoculated with the same toxin dose. The neutralization test was repeated on at least two occasions for each serum pair.

In addition to examining sera for VT-neutralizing antibodies, each serum-control well was also specifically examined for the presence of VT activity.

All strains of VTEC that were isolated from our patients with idiopathic HUS were examined by one of us (H. L.) for invasiveness by use of the Serény test [39], for heat-labile enterotoxins by use of the Chinese hamster ovary assay [40] and the Y-1 adrenal cell assay [41], and for heat-stable enterotoxins by use of the infant mouse test [42].

Results

Patients with idiopathic HUS. Clinical features. Forty patients were studied: 18 boys and 22 girls.
Their ages ranged from six to 226 months (mean, 45.2 ± 48.4; median, 26.5). The ages of the 40 matched controls ranged from five to 180 months (mean, 43.1 ± 49.1; median, 24). The onset of illness occurred in the first quarter of the year (January to March) for five patients, the second quarter (April to June) for 10, the third quarter (July to September) for 21, and the fourth quarter (October to December) for four.

The prodromal illness was characterized by diarrhea (40 patients), bloody stools (34 patients), abdominal pain (24 patients), vomiting (22 patients), fever (18 patients), and upper-respiratory-tract symptoms (8 patients). Abdominal pain was a consistent feature in older children, but this symptom could not be regularly assessed in infants less than two years of age. Vomiting, when present, was usually mild.

The mean interval between the onset of the prodromal illness and the diagnosis of HUS was 6.9 ± 3.3 days (range, two to 14 days; median, six days).

Clinical features present at the time of diagnosis of HUS included general malaise or lethargy (34 patients), anuria (13 patients), oliguria (11 patients), either disorientation or seizures or both (10 patients), peripheral edema (nine patients), temperature >38 C (seven patients), purpuric rash (six patients), prolonged coma (four patients), and moderate-to-severe dehydration (four patients). Four patients had ascites on admission or shortly thereafter. One 18-month-old patient died suddenly at home; a diagnosis of HUS was made at autopsy.

The white blood cell count of 38 patients on admission ranged from 6.6 to 44.1 × 10^9 cells per liter (mean, 20.2 ± 9.1; median, 19.0). The prothrombin and partial thromboplastin times were within normal range in each of 27 patients tested.

In general the illness ranged in severity from extremely mild to fulminating and fatal. Thirty-four patients (85%) made a full recovery, three (7.5%) had a residual neurological deficit, one (2.5%) had evidence of residual renal impairment, and two (5%) patients died.

**Microbiological and serological investigations.** The first stool sample taken from the 40 patients with idiopathic HUS for investigation was received at a mean of 10.6 ± 5.9 days (range, three to 28; median, eight) after the onset of the acute, diarrheal prodromal illness. Either VTEC or free VT or both were detected in 24 (60%) of these patients but in none of the matched controls (table 1).

Free-fecal VT was detected in all patients who were culture positive for VTEC (except for three in whom the test was not performed because of insufficient feces), as well as in 12 patients who were culture negative for VTEC (table 1). The fecal-VT titers ranged from two to 2,048 (geometric mean titer, 16; median, eight). There was no relation between the fecal-VT titer and the severity of the illness, but there was a tendency for the titers to be higher in stool samples received earlier in the course of the illness.

In patients from whom VTEC were recovered, the proportion of colonies that were VT positive ranged from 5%–100% when up to 20 E. coli colonies from the primary isolation medium were tested. In patients who were positive for fecal VT but were negative for VTEC, additional coliform colonies were investigated for VT production whenever they grew on the primary medium, but none of these were found to be VT positive.

The VT produced by all 12 VTEC isolates, as well as the VT in all positive fecal filtrates, was neutralized by the reference antiserum but not by the negative-control antiserum. VT activity was demonstrated in culture supernatants, as well as cell extracts, of all VTEC isolates. All other E. coli isolates (from cases and controls) that were negative for supernatant VT were also negative for cell-bound VT.

**Table 1. Evidence of Verotoxin-producing E. coli (VTEC) and free Verotoxin (VT) in stool samples of patients with idiopathic HUS and in matched controls.**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>VTEC and VT</th>
<th>VTEC only*</th>
<th>VT only†</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>(n = 40)</td>
<td>9</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Controls</td>
<td>(n = 40)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Stool samples not available for investigation of free VT. † VTEC were not detected despite testing 20 individual colonies from primary agar.

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and O(rough):nonmotile (one isolate). All 12 VTEC isolates were Serény negative, as well as negative for both heat-labile and heat-stable enterotoxins.

Acute and convalescent sera were available from 27 of the 40 patients. Acute sera were received a mean of 9.4 ± 4.0 days (range, four to 18; median, eight) after the onset of the acute diarreal illness, and convalescent sera were received a mean of 21.7 ± 8.6 days (range, 13–43; median, 18) after onset. Of the 24 patients who had either VTEC or VT in their stools, 15 had paired sera, and 10 of these showed a fourfold or greater rise in VT-neutralizing antibody titer. Of the 16 patients who did not have VTEC or VT in their stools, 12 had paired sera, and six of these showed a fourfold or greater rise in VT-neutralizing antibody titer. All serological tests were performed on at least two occasions, and the results were reproducible. In the present study, antigen (VT) and patients' sera were preincubated for 1 hr before inoculating them into Vero-cell monolayers. This approach produced more clearly defined end points than were seen with our previous method [35], in which sera and VT were added directly to Vero-cell monolayers without preincubation.

In 15 of the 16 patients who developed significant serological responses, the antibody titers in the acute sera ranged from four to 64 (geometric mean titer, 16; median, 16), and titers in the convalescent sera ranged from 32 to 256 (geometric mean titer, 64; median, 64). The remaining patient was unusual because her acute serum yielded a titer of 4,096 and the convalescent serum yielded a titer of 32,000.

Examination for the presence of VT activity in sera from 27 patients was negative in both the acute and convalescent sera.

When the results of the microbiological and serological procedures were analyzed, 24 (60%) of 40 patients showed evidence of VTEC infection by both microbiological and serological procedures; six (15%) showed evidence of infection by serological procedures only; and 30 (75%) showed evidence of infection either by microbiological or by serological procedures or both.

In three patients who had VTEC in their stool samples, other fecal pathogens were also present. These were rotavirus and E. coli O128:K72 (one patient), E. coli O128:K67 (one patient), and E. coli O128:K71 (one patient); the three entopathogenic E. coli serotypes were VT negative. No enteric bacte-
angiopathic hemolytic anemia [1, 2]. In idiopathic HUS these features are thought to develop secondary to endothelial damage and local intravascular coagulation [2, 11]. On the other hand, the same three features may also develop secondary to the activation of the coagulation pathways leading to disseminated intravascular coagulation (DIC) [36, 43, 44]. Thus even though features of HUS develop during DIC, evidence for the latter is not typically found in idiopathic HUS [11, 36]. DIC-associated HUS should therefore be distinguished from idiopathic HUS.

There have been reports of HUS occurring during outbreaks of *Shigella dysenteriae* type I (Shiga) dysentery in central America [19], Bangladesh [21], and India [20]. O’Brien et al. [45] have shown that Shiga toxin is closely related to the *E. coli* VT, but it remains to be established whether or not Shiga toxin is involved in the pathogenesis of Shiga-associated HUS. It is known that laboratory animals that die following the injection of Shiga toxin have lesions in several tissues that are compatible with ischemic injury [46, 47]. Cavanagh et al. [46] and Bridgewater et al. [47] proposed that Shiga toxin probably acts by damaging endothelial cells of small blood vessels, thus supporting the mechanism proposed for the pathogenesis of idiopathic HUS [2]. Smith et al. [48] have shown that rabbits inoculated with extracts of VTEC develop lesions similar to those that occur in rabbits given Shiga toxin.

The laboratory results from our patients closely resembled those described by others for idiopathic HUS [12, 14–17] but differ from those reported in cases of Shiga-associated HUS [21]. Koster et al. [21] showed that the latter was associated with circulating endotoxin, coagulopathy, and a leukemoid reaction (mean total white blood cell count, >80 × 10⁶ per liter). In contrast, coagulopathy and leukemoid reactions were not features in our patients. (Attempts to measure circulating endotoxin were not made.) Koster et al. [49] have also suggested differences at the histopathological level between Shiga-associated HUS and idiopathic HUS. These differences might be explained to some extent by the fact that shigelae are known to be invasive for epithelial cells [50] and are Serény positive [39], whereas VTEC are not.

Even though our findings indicate a close association between idiopathic HUS and VTEC, it should be noted that HUS is only one of several clinical manifestations that are associated with VTEC infection. We have observed two pairs of siblings with VTEC infection in which one sibling in each pair developed HUS while the other had only uncomplicated diarrhea [35]. Another condition that has been associated with VTEC is “hemorrhagic colitis.” Riley et al. [51] implicated strains of *E. coli* serotype O157:H7 in hemorrhagic colitis, and these isolates were subsequently shown to be VT producers [52, 53]. Three of the HUS cases in our study were also associated with serotype O157:H7. Hemorrhagic colitis [51] is clinically identical to the colitis of HUS [54], and radiological features of both conditions are typical of ischemic colitis [51, 55]. Thus the spectrum of clinical features associated with VTEC is wide, ranging from mild diarrhea to hemorrhagic colitis to idiopathic HUS.

Our study has established the groundwork for making a laboratory diagnosis of VTEC infection. Three different diagnostic criteria were used; namely, the detection of specifically neutralizable free-fecal VT, the culture of VTEC from stool samples, and the demonstration of significant rises in VT-neutralizing antibody titers in patient’s sera. Three-quarters of our patients had evidence of this infection by at least one of these criteria. In six (20%) patients the diagnosis was made only by serological means.

Of considerable practical diagnostic significance in our study was the finding that whenever VTEC were isolated in culture, the stool samples invariably contained free VT. In contrast, many patients had only free VT in their stools, and VTEC could not be detected even when 20 *E. coli* colonies from the primary isolation medium were examined. These observations indicate that the detection of free-fecal VT is the most important microbiological procedure in the early diagnosis of VTEC infection.

One possible explanation for the apparent absence of VTEC in stool samples that still contain free VT is that VTEC become greatly reduced in numbers (undetectable by the method used) shortly after the onset of diarrhea. This is supported by the work of Wells et al. [56] who, while investigating cases of hemorrhagic colitis, screened five colonies of *E. coli* from the primary isolation media for serotype O157:H7. They recovered this serotype from almost three-quarters of the patients whose stool samples were submitted within four days of the onset of symptoms but from none of those whose stool samples were sent seven days or more after onset. The
first stool sample for culture in our own study was received a mean of ~11 days after the onset of the acute diarrheal illness.

The cell culture–based methods that we have used for diagnosing VTEC infection are slow and labor intensive for routine diagnostic purposes. The development of genetic probes [57] and immuno- 
specific methods, however, should considerably simplify the diagnosis of this infection in the future.

The therapeutic implications of making a diagnosis of VTEC-associated HUS are not clear. There is no evidence to date that antibiotics influence the course of this disease. Whether or not immunization with “Verotoxinoid” has a role in altogether preventing the disease remains to be established.

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