Immune response to verotoxin 1 and 2 in children with *Escherichia coli* O157:H7 hemorrhagic colitis and classic hemolytic uremic syndrome

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OBJECTIVES: To compare neutralizing antibody titres against verotoxin (VT)-1 and VT-2 between children with uncomplicated hemorrhagic colitis (HC) and those with classic hemolytic uremic syndrome (HUS). VT antibody titres were also compared in children with HC who received trimethoprim-sulfamethoxazole with those who did not.
DESIGN: Prospective study.
SETTING: Tertiary pediatric hospital.
POPULATION STUDIED: Children with HC (n=41) or classic HUS (n=12).
INTERVENTIONS: Serum antibodies against VT-1 and VT-2 were determined by quantitative neutralization.
MAIN RESULTS: Antibodies were detected in 40% (21 of 53) of serum samples for VT-1 and in 100% (53 of 53) of samples for VT-2. A positive immune response, defined as a fourfold increase in VT antibody titres or as a single titre of 1/64 or greater, was found in 0% (0 of 12) of patients with HUS compared with 7% (three of 41) of those with HC for VT-1 (P=0.4); and in 17% (two of 12) of patients with HUS compared with 22% (nine of 41) of those with HC for VT-2 (P=0.3). The rate of seroconversion against either VT-1 or VT-2 was comparable in treated and untreated patients with uncomplicated HC.
CONCLUSIONS: There was no evidence that neutralizing antibody levels against VT-1 or VT-2 in classic HUS or after antibiotic therapy are substantially different from those in patients with uncomplicated HC. *(Pour résumé, voir page 137)*

Key Words: *Children,* Escherichia coli *infections, Gastroenteritis, Hemolytic uremic syndrome, Immune sera*

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Réponse immunitaire à la vérotoxine 1 et 2 chez des enfants atteints de colite hémorragique à *Escherichia coli* 0157:H7 et de syndrome urémique et hémolytique classique

OBJECTIFS : Comparer les titres d'anticorps neutralisants contre la vérotoxine (VT-1 et VT-2) entre des enfants atteints de colite hémorragique non compliquée (CH) et des enfants atteints d'un syndrome hémolytique et urémique classique (SHU). Les titres d'anticorps anti-VT ont également été comparés chez des enfants atteints de CH qui recevaient du triméthoprim-sulfaméthoxazole et ceux qui n'en recevaient pas.
MODÈLE : Étude prospective.
CONTEXTE : Hôpital pédiatrique de soins tertiaires.
POPULATION ÉTUDIÉE : Enfants atteints de CH (n=41) ou de SHU classique (n=12).
INTERVENTIONS : Les anticorps sériques développés contre VT-1 et VT-2 ont été mesurés à l'aide d'une technique de neutralisation quantitative.
PRINCIPAUX RÉSULTATS : Les anticorps ont été décelés chez 40 % (21 sur 53) des échantillons sériques contre la VT-1 et chez 100 % (53 sur 53) des échantillons contre la VT-2. Une réaction immunitaire positive, définie comme une augmentation du quadruple des titres d'anticorps anti-VT ou comme un seul titre de 1/64 ou plus a été observée chez 0 % (0 sur 12) des patients atteints de SHU en comparaison avec 7 % (3 sur 41) des patients atteints de CH pour la VT-1 (P=0,4) et chez 17 % (2 sur 12) des patients atteints de SHU contre 22 % (9 sur 41) des patients atteints de CH pour la VT-2 (P=0,3). Les taux de séroconversion à l'égard de la VT-1 ou de la VT-2 étaient comparables chez les patients traités et non traités atteints de CH non compliquée.
CONCLUSIONS : Rien n'indique que les taux d'anticorps neutralisants dirigés contre la VT-1 ou la VT-2 dans la SHU classique ou après un traitement antibiotique ne soient substantiellement différents de ceux de patients qui présentent une CH non compliquée.

In North America, verotoxin-producing *Escherichia coli* serotype O157:H7 is the main etiological agent of both hemorrhagic colitis (HC) (1) and classic hemolytic uremic syndrome (HUS) (2,3). *E coli* O157:H7 produces two verotoxins (VT). VT-1 is almost identical to the toxin of *Shigella dysenteriae* serotype 1 except for one amino acid substitution, while VT-2 DNA is only 50 to 60% homologous (4). Multiple VT variants have also been reported (5). Both VT-1 and VT-2 have direct cytotoxic activity to vero cells (African green monkey kidney cells) (6). In HC, infection and ischemia may disrupt the intestinal mucosal barrier, possibly allowing translocation of VT and the initiation of renal endothelial cell damage leading to HUS. The cellular receptor for VT-1 and VT-2 is a glycosphingolipid globotriosyl ceramide, which has been identified on vero cells, human renal tissue (7) and human B lymphocytes (8).

Serum antibodies against various *E coli* O157:H7 antigens such as VT (3,9), outer membrane proteins (2), flagella (10) and lipopolysaccharide (2,11-13) have been shown in humans with HUS. Cytolytic antibodies against endothelial cells have also been described in patients with HUS (14). However, the significance of these findings is not well determined.

The incidence of HUS varies with age (4), and HUS pathophysiology may be related to the immune response of the host. We hypothesized that there is an increased immune response against VT in children with HUS. We compared neutralizing antibody levels in serum against VT-1 and VT-2 in children with classic HUS versus those with uncomplicated *E coli* O157:H7 HC. Second, in vitro studies have reported that trimethoprim-sulfamethoxazole (TMP-SMX) (15) and polymyxin B (16) may increase the amount of cytotoxin released by *E coli* O157:H7. We therefore set out to determine whether the magnitude of the immune response to VT-1 or VT-2 was modified by antibiotic therapy in children with proven *E coli* O157:H7 HC.

PATIENTS AND METHODS

Sainte-Justine Hospital is a tertiary care pediatric centre in Montreal. Informed consent was obtained from parents of all patients. The study was approved by the ethics committee of Sainte-Justine Hospital.

From June 1, 1989 to June 1, 1990 paired serum samples were collected from 12 children with full-blown classic HUS and from 41 children with culture-proven, uncomplicated *E coli* O157:H7 HC. Of these, 18 were randomized to receive TMP-SMX (4 mg/kg, twice daily for five days) and 23 to receive no antibiotic therapy. *E coli* O157:H7 HC was defined as the occurrence of an enteritis with bloody diarrhea and the identification of sorbitol-negative colonies on MacConkey sorbitol agar with an *E coli* biochemical profile and a positive slide agglutination (Difco Laboratories, Inc, Michigan) to serotype O157:H7. All strains of *E coli* O157:H7 were confirmed by the Laboratoire de Santé Publique du Québec. Classic HUS was defined as the occurrence of anemia with a hemoglobin value below the third percentile for age, thrombocytopenia (platelet count less than 100×10^9/L), presence of schistocytes on blood smear and acute renal failure with a creatinine value greater than the 90th percentile for age after a prodrome of enteritis. Atypical forms of HUS were excluded. Treated HC, untreated HC and HUS were established as independent diagnostic categories. Age, sex, hospitalization, time of onset of HC and time of serum collection were recorded for all patients.

Serum specimens: The acute phase serum was ob-

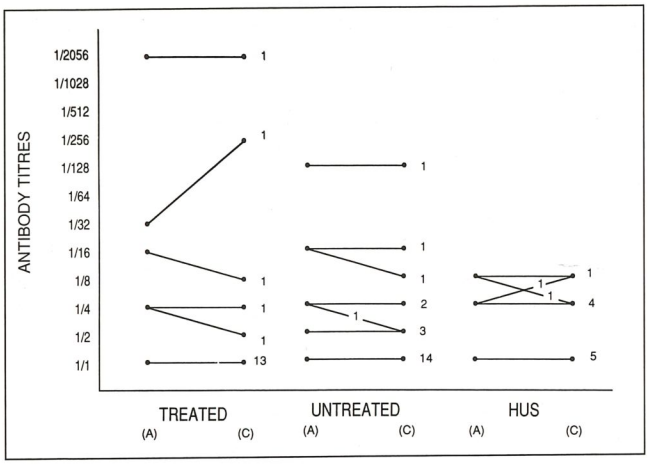

Figure 1) *Acute (A) and convalescent (C) neutralizing antibody levels in serum against verotoxin-1 in 41 patients with uncomplicated hemorrhagic colitis, among whom 18 were treated and 23 were untreated, and in 12 children with hemolytic uremic syndrome (HUS). Numbers to the right of C points indicate the number of patients with a specific antibody profile between acute and convalescent sera. The figure shows that most patients had low antibody titres against verotoxin-1, which were stable over time in all groups*

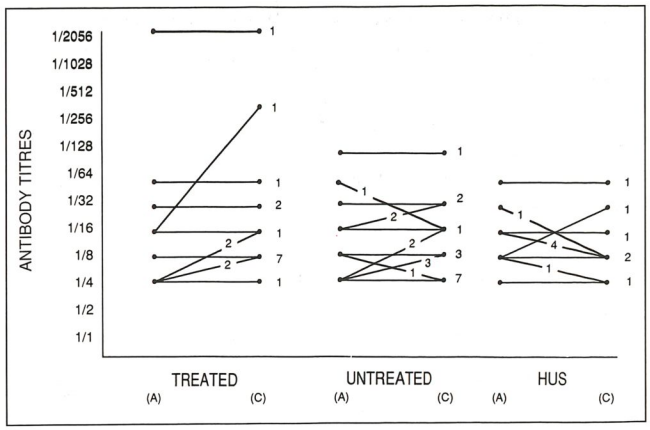

Figure 2) *Acute (A) and convalescent (C) neutralizing antibody levels in serum against verotoxin-2 in 41 patients with uncomplicated hemorrhagic colitis, among whom 18 were treated and 23 were untreated, and in 12 children with hemolytic uremic syndrome (HUS). Numbers to the right of C points indicate the number of patients with a specific antibody profile between acute and convalescent sera. The figure shows that, although a large number of patients had stable serum antibody levels, serum titre increased mainly in children with hemorrhagic colitis. Except for one patient, serum titres among children with HUS either were stable or decreased by one serum dilution*

tained at random from all patients with HC and from six patients who presented with HC but who subsequently developed HUS. The acute phase serum was taken at diagnosis in the remaining six children who presented with established HUS. The convalescent serum was obtained 10 days later from most patients. Sera were frozen at –80°C until analysis. All samples were tested for both VT-1 and VT-2 antibody by quantitative neutralization by a standardized method (17) modified as follows: 20 µL of each of VT-1 and VT-2 was added separately to 0.2 mL of vero cell monolayers to determine one unit of toxin; one unit of toxin activity was defined as the amount present in the highest dilution of a toxin preparation that caused 50% cell death (CD_{50}) after 48 to 72 h incubation. Toxin preparations were obtained from bacteria-free filtrates after overnight incubation with Evans Medium (18) of reference strains H19 for VT-1 and the authors' reference strain 90-2380 for VT-2. For neutralization, 30 µL of 2.5 units of each toxin was added separately to 30 µL of each serial dilution of patients' serum (1:2 to 1:4096). The toxin/antiserum mixtures were incubated at 37°C for 3 h then refrigerated at 4°C for 18 h; 20 µL aliquots of each toxin/antiserum mixture were then dispensed into corresponding wells of vero cell monolayers in microtitre trays. The microtitre trays were incubated at 37°C in a 5% carbon dioxide incubator and the end-point was the highest serum dilution causing inhibition of CD_{50} after 48 to 72 h of incubation. Included in the assay were appropriate toxins, serum and cell controls. Each serum pair (VT-1 and VT-2) was run on the same microtitre tray. To the authors' knowledge, there is no immunological cross-reaction between VT-1 and VT-2. A positive immune response was defined as a fourfold increase in VT antibody titres or as a single titre of 1/64 or greater (3).

Statistical analysis: The Fisher exact test was used to compare the numbers of patients with a positive serological response in the group with HUS versus the group with uncomplicated HC. The rate of seronconversion was also compared in treated versus untreated children with uncomplicated HC. Statistical significance was established at 0.05.

RESULTS

There were 53 patients (29 males and 24 females), of whom 41 (77%) were hospitalized. Median age was 62 months (range seven to 213) in patients with treated HC, 48 months (range three to 166) in those with untreated HC and 32 months (range 15 to 156) in those with HUS. Median time from onset of HC to acute serum sample collection was six days (range four to 11) in patients with treated HC, eight days (range three to 17) in patients with untreated HC and 6.5 days (range three to 16) in those with HUS. Median time between acute and convalescent serum collection was 11 days (range eight to 13) in patients with treated HC, 10 days (range seven to 21) in patients with untreated HC and 10 days (range three to 16) in those with HUS. Both age and time of specimen collection after onset of HC were comparable among groups. The time of randomization after onset of HC was similar in treated and untreated patients (mean ± SD): 7.4±5.0 and 7.2±2.7 days, respectively. Five patients with HUS required peritoneal dialysis and one was hemodialyzed.

Figure 1 shows acute and convalescent neutralizing antibody titres against VT-1 in children with uncompli-

cated HC (treated and untreated) and in those with HUS. Serum titres against VT-2 are presented in Figure 2. Antibodies were detected in 40% (21 of 53) of serum samples for VT-1 and in 100% (53 of 53) of samples for VT-2. A positive immune response against VT-1 was found in 0% (0 of 12) of patients with HUS compared with 7% (three of 41) of those with HC (P=0.4). Among the latter, two children were treated and one did not receive antibiotic therapy (P=0.3). A positive immune response against VT-2 was found in 17% (two of 12) of patients with HUS compared with 22% (nine of 41) of those with uncomplicated HC (P=0.3). Among the latter, 28% (five of 18) of treated patients and 17% (four of 23) of those who did not receive antibiotic therapy showed a positive immune response (P=0.2).

DISCUSSION

A fourfold increase between acute and convalescent serum titres is generally considered diagnostic of a recent infection. We also considered as positive any serum titre of 1/64 or greater because Karmali et al (3) showed the median convalescent serum titre to be 1/64 among 16 patients with HUS with seroconversion against VTEC. Time intervals of serum sample collection from patients with HUS and from those with HC and the time of sample collection after onset of HC were both similar to those reported by others (3,11). Dialysis could not have lowered antibody levels in the present study, except in the patient who was hemodialyzed.

The immune response to VT in children with *E coli* O157:H7 HC has not been completely described (6,19). Siddons and Chapman (20) reported comparable neutralizing antibody titres against VT-1 and VT-2 in patients with *E coli* O157:H7 HC and in healthy controls with a single serum sample. Chart et al (21), using ELISA, failed to differentiate between single serum specimens from patients with HUS and samples from apparently healthy controls; they also suggested that antibodies to VT-1 and VT-2 are of little value in the serodiagnosis of HUS caused by *E coli* O157:H7 (21). We detected antibodies against VT-2 more frequently than antibodies against VT-1; seroconversion against VT-2 also occurred more frequently than against VT-1. However, we cannot determine whether VT-2 is more immunogenic than VT-1 because we did not assess the excretion of free fecal VT-1 and VT-2 in vivo. It has been previously reported that strains of *E coli* O157:H7 may produce VT-2 more frequently than VT-1 (22,23).

The role of antibiotic therapy in *E coli* O157:H7 HC is unclear (24). Previous in vitro studies reported that TMP-SMX (15) and polymyxin B (16) may increase the release of VT produced by *E coli* O157:H7. If TMP-SMX does increase the release of VT in vivo, it was not reflected by an increased immune response to either VT-1 or VT-2 in our population. We recognize, however, that the power to detect such a difference between treatment and control groups was small (24). Contrary to our primary hypothesis, there is no evidence that levels of neutralizing antibody against VT-1 and VT-2 are different in classic HUS or after antibiotic therapy from those in patients with uncomplicated HC.

It was recently proposed that VT may be toxic to the immune system. Cohen et al (8) reported that the vast majority of shiga-like toxin-sensitive B lymphocytes are of the immunoglobulin (Ig) G and IgA committed subset, whereas most IgM and IgM/D producing cells are resistant. Abnormal subpopulations of T and B lymphocytes have also been described in HUS (25); however, the significance of these findings has not been determined. Lipopolysaccharide may be an important modulator of the inflammatory response of the host in HUS (26,27). More studies on the immune and the inflammatory response of the host are needed to help define the pathophysiology of classic HUS. Further analysis of variables in the clinical spectrum of *E coli* O157:H7 infections may identify risk factors for development of HUS.

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