Serodiagnosis by Passive Hemagglutination Test and Verotoxin Enzyme-Linked Immunosorbent Assay of Toxin-Producing Escherichia coli Infections in Patients with Hemolytic-Uremic Syndrome

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Eight cases of hemolytic-uremic syndrome in which no pathogens were isolated were diagnosed serologically by a passive hemagglutination assay and a verotoxin (VT; Shiga-like toxin) enzyme-linked immunosorbent assay (ELISA). The passive hemagglutination assay employed formalinized sheep erythrocytes sensitized with soluble native antigen or heat-treated antigen (lipopolysaccharide [LPS]) from Escherichia coli O26, O111, O128, and O157 or flagellar antigen of nine different H serogroups of E. coli: H2, H7, H8, H10, H11, H12, H18, H19, and H25. All patients had antibodies against the native antigen and/or the LPS of E. coli O157, but positive agglutination with H7 was observed only in one patient. In the VT-ELISA with plates coated with purified VT 1 or VT 2, antibody against VT 2 was observed in the sera of five of six patients examined, but none of the patients possessed VT 1 antibody. These results indicate that the causative pathogen in these eight hemolytic-uremic syndrome cases is likely to be VT-producing E. coli O157. The passive hemagglutination assay described here is a very sensitive, simple, and rapid method. This assay is highly recommended for the serological diagnosis of VT-producing E. coli infections, particularly in patients infected by serogroup O157 strains. Furthermore, the VT-ELISA is useful in studying the role of VT in hemolytic-uremic syndrome.

Hemolytic-uremic syndrome (HUS) is manifested by the sudden onset of hemolytic anemia, thrombocytopenia, and acute renal failure about 10 days after the appearance of diarrhea and abdominal cramps. Although various bacterial and viral agents had been reported as the causative pathogens (15), Karmali et al. (9–11) reported that a verotoxin (VT; Shiga-like toxin)-producing E. coli (VTEC) strain, O157:H7, was detected frequently in the stools of patients with HUS. Since then, the correlation between HUS and VTEC has been well studied in America, Canada, and Europe (11, 12, 16, 17). In Japan, the incidence of VTEC infection has been increasing since an outbreak of VTEC O157:H7 infection at a kindergarten in 1990, but the correlation between HUS and VTEC has not been clearly demonstrated. The detection of VTEC in the stools of patients with HUS is the most definitive diagnostic criterion. It can sometimes be very difficult to detect VTEC, as the organism can be recovered from stools only for a short time after diarrhea. HUS appears about 1 week after the onset of diarrhea, at which time the pathogen usually has disappeared from the stool. Also, treatment with antibiotics has been started before HUS appears. On the other hand, HUS patients produce a high level of antibody response to the pathogen (13), and two studies (1, 3) have exploited this observation in the serodiagnosis of HUS. We have also reported the development of both a passive hemagglutination (PHA) assay using formalinized sheep erythrocytes (FSRBC) sensitized with soluble native antigen or heat-treated antigen (lipopolysaccharide [LPS]) of VTEC O157 and a VT enzyme-linked immunosorbent assay (ELISA) (21). In order to study the utility of these assays for the diagnosis of VTEC infection, we examined diarrheal patients in the outbreak which occurred at a kindergarten in 1990 and in three sporadic cases which occurred in 1991 and 1992. The PHA assay showed that 49 (74.2%) of 66 patients of the kindergarten outbreak and all of the sporadic case patients had antibodies to both or either the native antigen and the LPS of O157. In addition, anti-VT 2 antibody detected by the VT-ELISA was observed in the sera of 17 patients from the outbreak. In this study, we further evaluated the utility of these assays for eight HUS patients in whom no pathogens were detected.

MATERIALS AND METHODS

HUS patients. The eight HUS patients in this study were identified at six different hospitals from May 1990 to May 1993. All patients (A through H) are children 2 to 7 years old, as shown in Table 1. All patients presented to medical clinics at the onset of gastrointestinal symptoms and were subsequently hospitalized about 1 week after the onset. The most common symptoms included abdominal cramps, bloody or watery diarrhea, and fever. Vomiting was observed in three patients (A, B, and E). Bacterial examinations of fecal specimens from these patients were carried out at the hospitals with sorbitol IPA-bile salt agar (Kyokutou Seiyaku, Tokyo, Japan) and other media. VTEC or other pathogens were not isolated from these stools. One patient (D) was culture positive for E. coli O128, but the isolate did not produce VT. Hematological investigations were also carried out at the hospitals. Most of the patients suffered from hemolytic anemia, thrombocytopenia, and acute renal failure. They were diagnosed as having classical HUS. Their hemoglobin levels were 5.8 to 8.4 g/dl; haptoglobin levels, 6.8 to 21.8 mg/ml; platelet counts, 0.9 × 10^4 to 4.8 × 10^4 per mm; blood urea nitrogen levels, 39 to 132 mg/dl; and creatinine levels, 1.2 to 4.5 mg/dl.

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### TABLE 1. Antibody responses to antigens and VTs of E. coli in HUS patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Designation</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Wk after onset of diarrhea</th>
<th>PHA titer with FSRBC sensitized with:</th>
<th>Flagellar antigen</th>
<th>A405&lt;sup&gt;6&lt;/sup&gt; in ELISA coated with:</th>
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<sup>1</sup> Serum samples were diluted more than 100-fold for the PHA test and 1,000-fold for the ELISA.

<sup>2</sup> A value of 0.2 was determined to be the minimum indication of the presence of antibody.

<sup>3</sup> H2, H8, H10, H11, H12, H18, H19, and H25.

<sup>4</sup> Not done.

<sup>5</sup> Data are the reciprocals of the highest dilution which caused agglutination.

<sup>6</sup> VTEC O157:H7 was isolated, and the patient developed HUS (21). Serum was taken 5 days after the onset of diarrhea.

For each patient (B), all patients recovered after 2 or 3 months of treatment with aspirin, dipyridamole, heparin, or dialysis. In patient B, necrosis occurred in the colon region, and the colon was removed.

**Serum samples.** Serum samples were collected from the eight patients at the acute or convalescent phase. After examination of hematological parameters at the hospital laboratories, these serum samples were transferred to our laboratory and stored at −20°C until use.

Serum from 20 healthy adults and 10 healthy children were used as negative controls, and two of the HUS patients from the kindergarten outbreak (21) from whom fecal VTEC O157:H7 was isolated were used as positive controls.

**Bacterial strains.** For preparation of native antigen and LPS, four common VTEC O serogroups, strains TEC100 (VTEC O157:H7), TEC182 (VTEC O26:H11), TEC205 (VTEC O111:NM), and TEC347 (VTEC O128:H2), were used.

For preparation of flagellar antigen, strains TEC347 (O128:H2), TEC100 (O157:H7), TEC003 (O2:H8), TEC112 (O148:H10), TEC182 (O26:H11), TEC043 (O9:H12), TEC135 (O17:H18), TEC224 (OUT:H19), and TEC045 (O15:H25) were used.

**Purified VT 1 and VT 2.** VT 1 and VT 2 were purified from cultures of *Shigella dysenteriae* type 1 and VTEC O145:NM, respectively, by the following procedures. Each strain was inoculated into Trypticase soy broth (BBL) and incubated for 18 h with constant shaking. Sterile filtrate was mixed with solid ammonium sulfate up to a 70% saturation level. The precipitate was suspended in 0.05 M phosphate buffer (pH 6.0) and dialyzed against the same buffer. The dialyzed crude sample was loaded onto a DEAE-Sephacel column (Pharmacia) equilibrated with 0.05 M phosphate buffer (pH 6.0) and eluted with a linear gradient of 0 to 0.5 M NaCl in 0.05 M phosphate buffer, pH 6.0. Fractions showing activity in the Vero cell assay were pooled and dialyzed against 0.005 M phosphate buffer, pH 6.0. The dialyzed sample was applied onto a column of PBE (Pharmacia) equilibrated with 0.025 M imidazole·HCl, pH 7.4. Polybuffer 74 (Pharmacia)-HCl (pH 6.0) was used to elute VT 1 from the PBE column, and polybuffer-HCl (pH 3.6) was used to elute VT 2. VT 1 was eluted in a peak at pH 6.0, whereas VT 2 was eluted in a peak at pH 4.0. Toxic fractions were pooled, and solid ammonium sulfate was added to the pooled sample to 1.7 M. The mixture was applied to a fast protein liquid chromatography system (FPLC, Pharmacia) equipped with a phenyl-Sepharose CL-4B (Pharmacia) column which was equilibrated with 1.7 M (NH₄)₂SO₄-0.05 M phosphate buffer (pH 6.0), and the toxin was eluted with a linear decreasing salt gradient (1.7 to 0 M) in 0.05 M phosphate buffer, pH 6.0. Finally, the fractions from hydrophobic chromatography showing VT activity were directly applied to a column of TSK gel G 3000SW (Toso Co., Tokyo, Japan) equilibrated with 0.05 M phosphate buffer (pH 6.0) and fractionated in an FPLC system.

The purified toxin from cultures of each strain gave a single band on conventional polyacrylamide gel electrophoresis (5). In the Ouchterlony gel double-diffusion test between the purified toxin(s) and anti-Shiga toxin (22) or anti-VT 2 (23) serum (gifts from Y. Takeda, School of Medicine, Kyoto
University), the toxin from *S. dysenteriae* 1 formed a precipitin line with the anti-Shiga toxin serum but not with the anti-VT 2 serum. In contrast, the toxin from VTEC O145:NM formed a precipitin line with anti-VT 2 serum but not with the anti-Shiga toxin serum.

**Preparation of native antigen and LPS.** Strains grown in Trypticase soy broth (BBL) were cultured on nutrient agar plates at 37°C and incubated overnight. The organisms were harvested in saline and disrupted with a French press (Ohtake Co., Tokyo, Japan) at a pressure of 1,000 kg/cm². After centrifugation at 23,000 × g for 20 min, the supernatant fluid was filtered through a membrane (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). One filtrate was used for native antigen, and another filtrate heated at 121°C for 30 min in an autoclave and centrifuged at 1,500 × g for 20 min was used for heat-treated antigen (LPS).

**Flagellar antigens.** Nine kinds of flagellar antigens were partially purified from the culture grown in Trypticase soy broth by the method of Ibrahim et al. (8).

FSRBC sensitized with antigen(s). FSRBC (Japan Biotest Lab, Japan) were washed four times and resuspended at a concentration of 10% in saline. An equal volume of tannic acid (0.1 μg/ml; Japan Biotest Lab) was added, and the mixture was incubated in a 37°C water bath. After 30 min, cells were washed three times with cold saline and resuspended at a concentration of 10%. Antigens to be tested were added to the FSRBC at a concentration of 100 μg/ml. The mixture was incubated at 37°C for 30 min and left to stand at 4°C overnight; then the FSRBC were washed three times with saline to remove unbound antigen. The sensitized FSRBC were suspended at a concentration of 10% in saline containing 1% sodium azide and stored at 4°C until use. Control FSRBC were prepared in the same way except that the addition of antigens was omitted. Prior to use, the FSRBC stocks were diluted to a concentration of 1% in saline containing 0.1% bovine serum albumin (Sigma) and 0.01% Tween 80 (Sigma).

**PHA.** The PHA test was done in a microwell plate with V-shaped wells (Greiner), as described previously (21). The PHA titer of serum against each antigen was expressed as the maximum dilution that showed agglutination.

**Treatment of patient serum with 2ME.** To investigate the immunoglobulin class of the antibodies revealed by reactions in the PHA assay and VT-ELISA, positive sera of patients were treated with 2-mercaptoethanol (2ME) (6). Serum samples (0.1 ml) from patients were diluted 10-fold in saline, and an equal volume of 0.2 M 2ME (Wako Pure Chemical Industries, Tokyo, Japan) was added. After incubation at 37°C for 1 h, 2 ml of cold acetone was added to remove the 2ME and precipitate the serum protein. The mixture was centrifuged, and the acetone was removed with an aspirator. After the acetone treatment was repeated once, the pellet was resolved in 0.1 ml of saline and subjected to the PHA assay and VT-ELISA.

**VT-ELISA.** As described previously (21), the VT-ELISA was carried out according to the recommended procedure for ELISAmate (Kirkegaard & Perry Laboratories), using purified VT 1 and VT 2.

**RESULTS**

**Specificity of FSRBC sensitized with antigen(s).** The specificity of the FSRBC sensitized with antigen(s) was tested by the PHA assay, using specific antisera to the respective O or H antigens of *E. coli* (Denka Seiken, Tokyo, Japan). FSRBC sensitized with each of the native antigens or LPS of the four predominant VTEC O serogroups (O26, O111, O128, and O157) or the flagellar antigen of nine common H serogroups (H2, H7, H8, H10, H11, H12, H18, H19, and H25) agglutinated only with the homologous antisera. To assess the utility of the PHA assay, we measured the reactivity of 30 negative control serum samples to the various antigens. When twofold dilutions of the negative control sera in the range of 1:10 to 1:640 were subjected to the PHA assay, low PHA titers (1:10 to 1:80) against antigens O26, O157, H2, H11, and H25 were observed in some serum samples. As dilutions of 1:100 and greater did not result in positive reactions with any of the antigens tested, this dilution was used as the initial dilution of serum.

**Reaction of sera from HUS patients with native or LPS antigen and flagellins from *E. coli*.** The results of the PHA assay are summarized in Table 1. All serum samples from patients taken at the acute or convalescent phase reacted with the O157 native and LPS antigens, except sera from patients F and G taken 4 or 6 weeks after the onset of diarrhea; these reacted only with O157 native antigen. None of the sera reacted with antigens of the other VTEC serogroups, e.g., O26, O111, and O128. The PHA titers against O157 native antigen and LPS varied widely in individual patients. However, most of the patients had acquired significantly higher levels of antibodies against native or LPS antigens than against flagellins. The PHA titers tended to fall rapidly after the acute phase of the disease, but they were sometimes detectable 10 weeks after the onset of the disease, as in patients B and D. Furthermore, these antibodies were inactivated by treatment with 2ME.

**PHA testing for antibodies against nine common VTEC H antigens showed that only one patient, case B, had antibodies against H7 at the acute phase.** The PHA titer of serum samples taken at 1 and 2 weeks after the onset of illness was 1:900. After 4 weeks, the titer was below 1:100. These antibodies were also inactivated by treatment with 2ME.

**VT antibodies detected in patients’ sera by VT-ELISA.** In preliminary experiments, negative control sera diluted 100-fold gave an A₄₅₀ of 0.62 to 3.11 in wells coated with VT 1 or VT 2 and an A₄₅₀ of 0.24 to 0.35 in wells without antigens. Since the absorbance in both antigen-coated and uncoated wells with the 1,000-fold dilution of these sera was in the range of 0.01 to 0.04, this dilution was used in VT-ELISA.

On the basis of results of the VT-ELISA test of sera from patients in the outbreak caused by VTEC O157:H7 (21), an absorbance of 0.2 was established as the cutoff value to indicate the presence of anti-VT antibody. An absorbance of 0.11 to 0.19 was considered a suspicious positive reaction, and that below 0.1 was negative.

Six serum samples from the eight HUS patients were tested for anti-VT antibody. As shown in Table 1, ELISA readings for VT 2 showed that the absorbance of serum samples from patients ranged from 0.084 to 0.283. The sera from five patients in whom an absorbance of above 0.2 (cutoff value) was observed indicated the presence of anti-VT 2 antibody. Antibody against VT 2 was found mainly in serum samples taken within 3 weeks of the onset of illness. VT 2 antibody also tended to fall rapidly after the acute phase of the illness, although the serum sample from patient A taken 6 weeks after the onset of diarrhea still was positive for VT 2 antibody. These positive sera were also treated with 2ME, and nonspecific reactions showing an absorbance of >2.0 were observed in all samples tested. On the other hand, ELISA results for VT 1 showed absorbance of serum samples ranging from 0.075 to 0.186. None of the sera tested showed an absorbance higher than the cutoff value. However, a suspicious positive reaction was observed in two patients’ sera (patients E
and H) within 2 weeks after the onset of the illness. An absorbance of 0.141 for patient E and one of 0.186 or 0.163 for patient H were noted.

**DISCUSSION**

Investigations (19) of HUS in Japan carried out in 1991 showed that the incidence of HUS has been increasing since the occurrence of an outbreak of VTEC O157:H7 infection at a kindergarten in 1990. Approximately 100 HUS cases per year have been confirmed, and about 76% of these presented in typical fashion. Among these cases, gastrointestinal symptoms were the first signs of the disorder. In 43% of those cases, the causative pathogen was identified. E. coli was isolated from 76% of those stool samples in which any pathogen was identified. However, only 14% of these pathogens were VTEC. It is difficult to detect VTEC or any other pathogens in many cases of HUS. In the eight HUS cases in this study, no enteropathogens were detected in seven patients. Although E. coli O128 was isolated from one patient, this strain did not produce VT. Therefore, the development of other methods for the diagnosis of the causative pathogen in HUS is desirable.

In order to diagnose VTEC infections serologically, we previously developed a PHA assay for the detection of antibodies against native antigen, LPS, and the flagellar antigen of VTEC O157:H7, and a VT-ELISA to detect VT 1 and VT 2. Since the utility of these assays was studied with serum samples from diarrheal patients infected with VTEC O157:H7 (21), we further assessed the usefulness of these assays by examining eight HUS patients in whom the causative pathogen was not isolated. In addition, for the application of these assays to HUS, three predominant VTEC O serogroups (O26, O111, and O128) and eight common H serogroups of E. coli (H2, H8, H10, H11, H12, H18, H19, and H25) were added to the study, because VTEC strains belonging to various O or H serogroups apart from O157:H7 have been isolated from patients with HUS (10, 17).

The results showed that all patients had antibodies against the native antigen and/or the LPS of E. coli O157 in serum samples taken at the acute or convalescent stage of the illness, but none of the sera reacted with the O antigens of VTEC serogroups, such as O26, O111, and O128. In the PHA test for the nine common H antigens, only one patient (B) had antibodies against H7 in acute-phase serum samples. In the VT-ELISA, antibodies against VT 2 were observed in five of six patients examined.

With regard to serodiagnosis of HUS, Chart et al. (3, 4) developed a O157 LPS-ELISA, and recently Bitzan and Karch (1) reported an indirect hemagglutination assay that uses O157 LPS-coated sheep erythrocytes. In both assays, antibody against O157 LPS was observed in the sera of HUS patients. Those studies also recommended the detection of antibodies against native antigen or LPS of E. coli by means of ELISA, indirect hemagglutination or PHA for the diagnosis of VTEC infections. However, if VTEC or VT is not detectable. We also tried to detect antibody against flagellar antigens of VTEC. However, the results of the PHA test for antibodies against nine common VTEC H antigens showed that only one of eight patients had antibodies against H7 in acute-phase serum samples. In the remaining seven cases, antibody against H7 antigen could not be detected, although most of the VTEC O157 strains isolated from patients with hemorrhagic colitis or HUS are motile and possess the H7 antigen (12). In our previous work regarding the outbreak of VTEC O157:H7 (21), antibody against flagella antigen of H7 was not detected in 16 hospitalized patients or in 50 outpatients. Chart et al. (3) also could not detect antibodies against H7 in serum samples of 13 HUS patients in spite of their higher titers of antibody against O157 LPS. It is not clear whether the inability to detect the antibody against H7 is due to the methods used or to other factors. However, a possibility exists that some E. coli O157 strains in human infection possess cryptic H7 or nonimmunogenic H7. In either case, our PHA assay to detect antibody against flagella antigen may not be suitable for the serological diagnosis of VTEC infection.

In the serodiagnosis of VTEC infection with the PHA assay, there is a problem in that the LPS of E. coli O157 shares common antigenic epitopes with the LPS of certain serogroups of Brucella abortus (18), Yersinia enterococolitica (20), Salmonella spp. (20), and Vibrio cholerae non-O-1 strains (7). Antibodies directed against these organisms may give nonspecific reactions in the assay. Our data (unpublished data) showed high levels of cross-reaction between FSRBC sensitized with O157 native antigen or LPS and antibodies of B. abortus and Salmonella serogroup O30, but no cross-reaction was detected with Y. enterococolitica serotype O9 or V. cholerae non-O-1 serogroup Hakata. Since the symptoms of B. abortus infection are quite distinct from those of HC or HUS, misdiagnosis resulting from the cross-reaction in the serum seems unlikely. Similarly, Salmonella serogroup O30 is very rarely isolated from patients with diarrhea. On the other hand, nonspecific reactions with test sera in the PHA assay could be eliminated by the dilution of sera; therefore, the 100-fold dilution was considered appropriate for the PHA assay.

The PHA titers against O157 native and LPS antigens varied widely in individual patients. However, most of the patients acquired significantly high levels of antibody to both antigens in the early stages of infection. The PHA titers tended to fall rapidly after the acute phase of the disease, but they could sometimes be detected even 10 weeks after the onset of the illness. Similar trends in the antibody level of patients with HUS were observed in other studies (1, 3). Bitzan and Karch (1) indicated that this rapid fall in antibody titer to the E. coli O157 LPS may be due to the short half-life of immunoglobulin M (IgM). To study the immunoglobulin class related to positive reactions in the PHA assay and VT-ELISA, positive sera of HUS patients or those treated with 2ME because IgM in serum is segregated from the I9S to the 6.5S subunit by reduction through a sulfhydryl reagent such as 2ME (6). IgM antibody treated with 2ME becomes nonreactive. The treatment of the antibodies with 2ME in this study suggested that the immunoglobulin class of the antibodies detected in the PHA assay and VT-ELISA is IgM.

By using the VT-ELISA, an antibody response to VT 2 was observed in five of six patients' sera. This result indicates that VT-ELISA is useful as one method for the serodiagnosis of VTEC infection. Karmali et al. (11) reported that 4 of 15 cases of sporadic HUS and 2 patients with uncomplicated diarrhea who were siblings of HUS patients had neutralizing antibodies against the VT by Vero cell assay. VT-ELISA is highly sensitive and more convenient than the Vero cell assay to detect neutralizing antibody, but the usefulness of the VT-ELISA as a diagnostic tool may be limited to the general laboratory with access to pure VT. However, the VT-ELISA can be used to examine experimental verotoxemia in rabbits, monkeys, or other animals and to clarify the serological factors involved in HUS.

In the VT 1-ELISA, a suspicious positive reaction was observed in the serum samples from two patients. Although we used anti-human total immunoglobulin antiseraum as a secondary antibody, the use of fractionated immunoglobulin antiseraum may increase the specificity of the VT-ELISA.
In recent work reporting on the antibody against VT (2), a non-specific neutralizing effect of normal human sera against VT 2 and the non-IgG fraction was noted, suggesting the presence of a VT 2 inhibitor. In VT-ELISA, a nonspecific reaction was also observed in some serum samples from healthy individuals when the samples were diluted 100-fold. This reaction could be excluded by a higher dilution (1,000-fold) of serum. However, it is unclear whether these non-specific reactions are caused by some factor(s) in normal human sera. As one possibility, globotriosyl ceramide, one of the plasma-bound glycolipids, may be involved in these reactions. It has been consistently extracted from normal human plasma and recognized as a receptor of VT(2) (14).

The PHA assay described here is simple to perform and was sensitive enough to detect antibodies against VT-EC in patients with HUS. We highly recommend the PHA assay for the serological diagnosis of VT-EC infections caused mainly by serogroup O157. This assay is basically the same as that described by Bitzan and Karch (1), except for the use of FSRBC; however, the sensitized FSRBC are stable for 6 months at 5°C, the PHA can be performed immediately after the serum samples are obtained, and the results are available in 2 to 3 h. Furthermore, the VT-ELISA can be used to examine the VT in HUS and to study the role of VT in HUS.

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