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Serum Antibodies to *Escherichia coli* Serotype O157:H7 in Patients with Hemolytic Uremic Syndrome

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Sera from 13 patients with hemolytic uremic syndrome (HUS) and 8 healthy control subjects were examined for antibodies specific for bacterial antigens of *Eschericia coli* serotype O157:H7. Bacterial components, including outer membrane proteins (OMPs), lipopolysaccharide (LPS), and flagella, were reacted with sera by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting and by enzyme-linked immunosorbent assay. All 13 serum samples from HUS patients contained high-titered antibodies of the immunoglobulin M class against O157 LPS and some OMPs. These same sera reacted weakly with some of the major OMPs, but not the LPS, of non-O157 strains of *E. coli*. Sera from patients did not contain antibodies to non-O157 LPS or H7 flagella. The possibility of using *E. coli* serotype O157 LPS in an enzyme-linked immunosorbent assay for the routine diagnostic testing of sera from HUS patients for evidence of O157:H7 infection is discussed.

Hemorrhagic colitis (HC) is a form of gastroenteritis characterized by abdominal cramps and bloody diarrhea which in most cases is self-limiting (22, 23) but may develop into idiopathic hemolytic uremic syndrome (HUS), resulting in microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (17, 34). A variety of agents have been implicated as a possible cause of HUS. Strains of *Escherichia coli* producing Vero cytotoxin (VT) have been isolated from the stools of patients with HC and HUS. The most common *E. coli* serotype is O157:H7 (11, 12, 14, 24), although VT-producing *E. coli* strains belonging to serogroups O26, O104, O153, and O163 have also been isolated from patients with HUS (12, 24).

VT is cytotoxic for African green monkey kidney cells and is distinct from *E. coli* heat-labile enterotoxin and heat-stable enterotoxin (15). Two types of VT have been recognized, termed VT1 and VT2 (25), and the genes encoding both of these toxins are bacteriophage encoded (26, 28). Gene probes have been prepared for both VT1 and VT2 and have been used for both the detection and characterization of VT-producing *E. coli* (24, 27, 36, 37).

VT-producing *E. coli* strains were first suspected as being a cause of HC when VT-producing strains of *E. coli* serotype O157:H7 were isolated from an outbreak of HC associated with consumption of undercooked hamburgers (22, 23). Since then, several outbreaks of HC caused by *E. coli* serotype O157:H7 have been reported in North America (22) and more recently in the United Kingdom (29). Karmali et al. (14) were the first to demonstrate VT in fecal extracts, providing evidence that VT was produced during pathogenesis.

The detection of fecal VT-producing *E. coli* or VT or both has proved essential in the diagnosis and epidemiology of cases of HC and HUS. Unfortunately, the bacteria that cause the disease and the toxins they produce are only detectable in fecal specimens for a limited time following the onset of disease (22–24). Human infections caused by *E. coli* serotype O157:H7 have been shown to result in a host immune response as evident by the raising of antibodies to VT (13) and agglutinating antibodies to *E. coli* serotype O157:H7 and seroconversion in patients with HUS (20). In the present study, the immune response of patients with HUS was examined, with particular emphasis on determining key antigens that could conceivably be used in immunological tests to determine a role of *E. coli* serotype O157:H7 in cases of HC and HUS in which VT-producing *E. coli* and VT were not detected.

MATERIALS AND METHODS

Bacteria. *E. coli* serogroup O157 strains E34500, E32511, E30480, and E30228 were isolated from patients with HUS and HC (27). With the exception of the nonmotile strain E32511, strains had flagellar antigens of type 7. Strains E20513 (O111:H2), E19004 (O119:H6), E2348/69 (O127:H6), E24582 (O142:H6), and E28956 (O26:H−) belong to enteropathogenic *E. coli* serogroups. *Yersinia enterocolitica* E41497 (serogroup O9) was also used. Bacteria were stored on Dorset egg slopes and are held in the culture collection of the Division of Enteric Pathogens. *Brucella abortus* 10503 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London. This strain was stored as a lyophilized preparation and grown on blood agar prior to use.

Bacteria were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) alone or in Trypticase soy broth containing the iron-chelating agent Desferal (1 mg/ml; CIBA-GEIGY Corp., Summit, N.J.) (3). Iron-restricted growth was confirmed by testing culture supernatants for the bacterial siderophore enterobactin (1, 3, 19). For the preparation of outer membranes (OMs) and lipopolysaccharide (LPS), bacteria were grown in 200 ml of Trypticase soy broth (37°C, 16 h, 120 rpm).

Sera. Thirteen serum specimens obtained from patients with HUS from whom strains of *E. coli* serotype O157:H7 had been isolated were received by the Division of Enteric Pathogens (Table 1). Control sera were from two adult males, four adult females, and two infants. Clinical details were incomplete, but any known prodrome of diarrhea or bloody diarrhea has been shown in Table 1.

OMs. OMs were prepared from sonicated (150 W, 3 min,

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TABLE 1. Details of patients with HUS

Patient no.	Location[a]	Age of patient	Serum sample (days)[b]	Titer of antibody to LPS[c]	*E. coli* serotype O157:H7	
					Strain no.	VT produced
003	Wolverhampton/?	18 mo	60	100	E29962	2
011	London/Peterborough	6 yr	?[d]	1,000	E30979	2
040	Great Yarmouth/?	10 yr	75[d]	2,000	E36419	2
041	Ipswich/?	9 yr	7[d,e]	4,000	E35413	2
048	Cardiff/Carmarthen	17 mo	14[d]	3,160	E36307	1 and 2
050	Burton-on-Trent/Repton	18 mo	6	15,850	E36303	2
052	London/?	11 yr	?[d]	4,000	E36320	2
155	Glasgow/?	63 yr	12[d,e]	20	E39118	1 and 2
244	Leeds/Huddersfield	14 mo	9[d,e]	250	E42031[f]	2
282	Stoke/Stoke	18 mo	7[d,e]	20,000	E42568	2
310	Liverpool/Southport	2 yr	3[d]	12,600	E43635	1 and 2
327	Leeds/Wakefield	2 yr	5[d,e]	2,000	E43393	2
340	Lincoln/Newark	22 mo	7[d]	12,600	E43415	2

[a] Sending laboratory/home address.
[b] Approximate number of days following onset of disease before blood sample was taken.
[c] Titer of serum antibody reaction with O157 LPS by ELISA.
[d] Diarrhea when known.
[e] Blood in stool when known.
[f] Nonmotile.

O°C) bacteria (4) by selectively solubilizing the cytoplasmic membrane with Sarkosyl (BDH, Poole, England) (6). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg of OM protein (OMP) was applied per lane.

SDS-PAGE. Bacterial proteins and LPS were separated by SDS-PAGE (16) as described previously (3), using a constant current (50 mA) for exactly 3.5 h. Profiles were stained with either Coomassie blue (8) or silver (32, 38) or used for immunoblotting.

Immunoblotting. OM and LPS profiles were transferred onto nitrocellulose sheets and reacted with antisera (30 μl per lane) as described previously (8). Profiles immobilized on nitrocellulose were separated by cutting with pinking shears to ensure accurate realignment following reaction with antisera. Antibody-antigen complexes were detected with ^{125}I-iodinated immunoglobulin (8) raised against human immunoglobulin G (IgG) (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and IgM (Sigma Chemical Co., St. Louis, Mo.). Each lane was reacted with approximately 5 μg of immunoglobulin containing 10^6 cpm of activity, and reactions were detected by autoradiography. Membrane-bound LPS profiles obtained by proteinase K digestion of whole bacteria were blocked in hemoglobin solution (8) containing the serine protease inhibitor phenylmethylsulfonyl fluoride (10 mM) (Sigma) to prevent residual amounts of proteinase K from digesting the added immunoglobulins.

LPS. LPS for SDS-PAGE and immunoblotting was prepared from strain E32511 by proteinase K digestion (10) and stained with silver (32) or used for immunoblotting. For SDS-PAGE, the LPS from 100 μg (wet weight) of bacteria was applied per lane. LPS for enzyme-linked immunosorbent assays (ELISAs) was prepared from strain E32511 by the hot-phenol procedure of Westphal and Jann (35). SDS-PAGE profiles of isolated LPS did not contain any contaminating proteins as detected by silver staining (38).

Flagella. Flagella were partially purified from strain E30480 by heat extraction (60°C, 30 min). Following incubation, bacteria were sedimented by centrifugation (5,000 × *g*, 30 min, 4°C) and flagella were pelleted by ultracentrifugation (100,000 × *g*, 1 h, 4°C). The nonmotile strain E32511 was used as a flagellum-negative control.

ELISA. ELISA plates were coated with LPS from strain E32511 or flagellar preparations from strain E30480 and E32511 (1 μg in 100 μl of coating buffer [1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ per liter, pH 9.6]). After being washed in phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Tween 20 (PBS-Tween), unfilled protein-binding sites were blocked by adding 200 μl of 1% (wt/vol) bovine serum albumin in PBS per well (30 min, 37°C). Sera diluted in PBS-Tween were added to ELISA plates (100 μl per well) prior to incubation at room temperature for 2 h. After the plates were washed with PBS-Tween, 100 μl of alkaline phosphatase-conjugated goat anti-human total immunoglobulin antiserum (diluted 1:350 in PBS-Tween; Sigma) was added to the wells and the plates were incubated at room temperature for 2 h. Following washing as above, *p*-nitrophenol phosphate (1 mg/ml; Sigma) in carbonate buffer (pH 9.8) was added to the plates (200 μl per well) and the plates were incubated at room temperature for 100 min in the dark. The reaction was stopped by the addition of 25 μl of 1 M NaOH, and the intensity of color was determined by reading the A_{405}.

RESULTS

Reaction of sera with OMs. It is known that normal human sera contain antibodies to *E. coli* antigens, including the iron-regulated OMPs (IROMPs) and the major OMPs (8, 9). The IROMPS are expressed when bacteria are grown under iron restriction (8, 9, 19) and are known to be expressed by pathogenic bacteria in vivo during infection (7). In the present study, bacteria were grown under iron restriction in an attempt to mimic the bacterial phenotype encountered by the host during infection with *E. coli* serotype O157:H7. Bacteria grown in Trypticase soy broth containing Desferal produced the siderophore enterobactin, indicating that bacteria were indeed growing under iron restriction. The OMP profile of *E. coli* E30228 (Fig. 1, lane 1) grown under iron restriction showed the two major OMPs, OMP C (40 kilodaltons) and OMP A (32 kilodaltons), and three IROMPs of 81, 78, and 74 kilodaltons. The IROMPs were not expressed under iron-replete growth (Fig. 1, lane 2).

Sera from 13 patients with HUS and 8 controls were reacted by immunoblotting with replicate OM profiles from strain E30228 grown under iron restriction. With an anti-

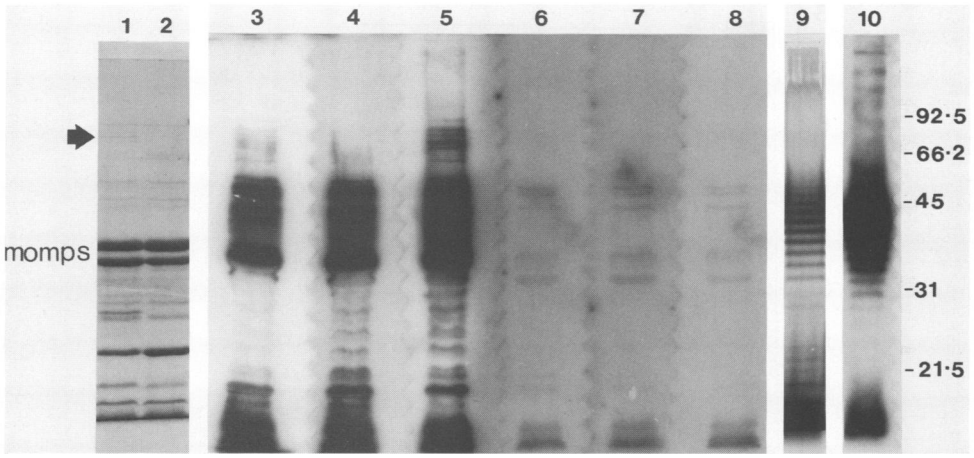

FIG. 1. SDS-PAGE OMP profiles of *E. coli* serotype O157 grown under iron restriction contained the major outer membrane proteins (momps). New OMPs (arrow, lane 1) were absent from profiles of bacteria grown under conditions of iron repletion (lane 2). Replicate profiles of lane 1 reacted strongly with sera from HUS patients (lanes 3 to 5) but only slightly with control sera (lanes 6 to 8). Purified *E. coli* serotype O157 LPS (lane 9) reacted strongly with sera from HUS patients (lane 10). A 30-μg sample of OMP was applied per lane. The LPSs from 100 μg of proteinase K-digested bacteria were used for each lane. Molecular size standards are given in kilodaltons.

human IgG second antibody, sera from HUS patients and control sera reacted weakly with OMP A and other minor OMPs (data not shown). With an anti-human IgM second antibody, all 13 HUS serum samples gave a strong reaction with the OM profile. Only one serum sample gave a clear reaction with the IROMPs (Fig. 1, lane 5); however, all the HUS sera gave a strong reaction with what appeared to be high-molecular-weight LPS and some OMPs (representative profiles are shown in Fig. 1, lanes 3 to 5). This type of reaction was not detected with eight control serum samples (representative profiles are shown in Fig. 1, lanes 6 to 8). The possible reaction of HUS serum antibodies with LPS was further examined. Figure 1, lane 9, shows a silver-stained SDS-PAGE profile of purified O157:H7 LPS from strain E32511, with the polysaccharide chains separating to give a characteristic ladder pattern. Reacting HUS sera with replicate profiles of purified LPS showed that the major antigen detected in OM profiles was indeed LPS (Fig. 1, lane 10).

To assess the specificity of HUS serum antibodies to O157:H7 antigens, we also reacted sera from patients with the OM profiles of five strains of *E. coli* belonging to other serotypes (O111:H2, O119:H6, O127:H6, O142:H6, and O26:H−). Sera reacted weakly with OMP A of the five control strains, but no reaction with the LPS of these strains was detected. The results in Fig. 2 show the reaction of two HUS serum samples with OM profiles of *E. coli* E20513 (O111:H2) (lanes 1 and 5), E19004 (O119:H6) (lanes 2 and 6), E2348/69 (O127:H6) (lanes 3 and 7), and E24582 (O142:H6) (lanes 4 and 8).

Reaction of sera with flagella. The antibody response of sera from patients to the H antigen was also examined. Flagella prepared from strain E30480 migrated on SDS-PAGE gels as a band of 60 kilodaltons. This protein was absent from extracts made from the nonmotile strain E32511 (data not shown). The sera from HUS patients did not react with the H7 antigen (data not shown).

LPS ELISA. The immunoblot reaction with O157 LPS of the 13 serum samples from HUS patients was quantified with purified LPS in an ELISA alongside 8 control serum samples. The serum dilution giving an A_{405} value of 0.5 was calculated for each serum. The titers obtained with sera from HUS patients (mean, 5,968 ± 6,811) were significantly higher ($P = 0.001$) than those of control sera (mean, 326 ± 399). Since HUS sera contained significantly higher levels of antibodies to O157 LPS than control sera, all sera were diluted (100 times) in PBS-Tween prior to reaction with LPS-coated ELISA plates. Of 13 serum samples from HUS patients, 8 gave optical density values in excess of 2.0; in contrast, 5 of 8 control serum samples yielded values below 0.5 (Fig. 3).

Reaction of sera with non-*E. coli*. Serological cross-reactions between the O-antigens of *E. coli* serotype O157, *B. abortus*, and *Y. enterocolitica* (O9) have been reported with serum from HUS patients (20) and hyperimmune rabbit serum (5, 30). When SDS-PAGE LPS profiles were reacted with HUS sera, antibodies were found to react with the LPS of *B. abortus* (Fig. 2, lane 9). However, the LPS of *Y. enterocolitica* was not recognized by HUS sera, and exposing immunoblots to X-ray film for 15 days (instead of the usual 2 days) detected a reaction with a single band comigrating with the dye line, but a reaction against LPS was not observed (Fig. 2, lane 10). The silver-stained O157 LPS

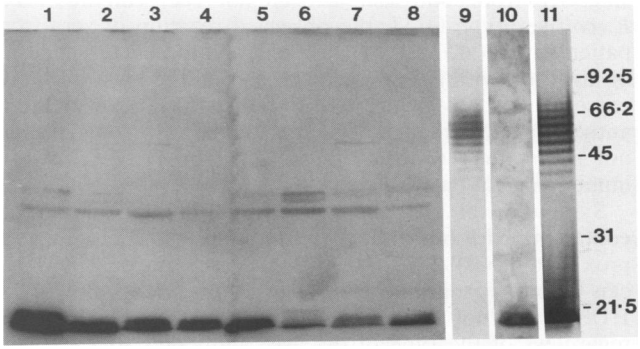

FIG. 2. SDS-PAGE OM profiles of *E. coli* strains from non-O157 serotypes (lanes 1 to 8) reacted weakly with sera from HUS patients. Sera from HUS patients reacted with the LPS from *B. abortus* (lane 9) but did not react with the O antigen of *Y. enterocolitica* (lane 10). The LPS of *E. coli* serotype O157 is included (lane 11) for comparison. A 30-μg protein sample was applied in each lane. The LPSs from 100 μg of proteinase K-digested bacteria were applied per lane. Molecular size standards are given in kilodaltons.

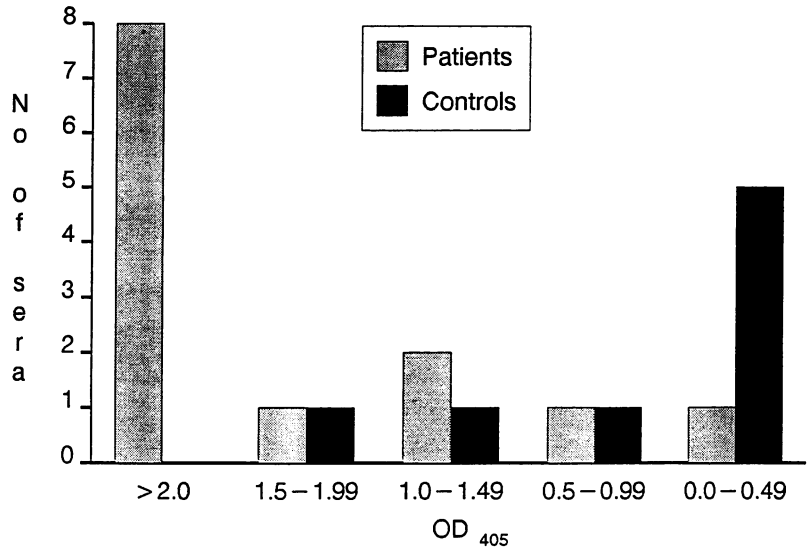

FIG. 3. Histogram showing reaction of sera with O157 LPS by ELISA. Sera from HUS patients gave a generally stronger reaction with LPS than control sera did. OD_{405}, Optical density at 405 nm.

profile (lane 11) shows the migration of long-chain LPS molecules.

DISCUSSION

VT-producing *E. coli* belonging to serotypes other than O157 have been shown to cause HUS (12, 24), and assaying antisera from patients for anti-VT antibodies might provide evidence of infection by strains of VT-producing *E. coli* in the absence of a causative organism. However, since a suitable assay for detecting antibodies to VT has not been encountered and because *E. coli* serotype O157:H7 has been frequently associated with cases of HUS, we examined sera from patients known to be infected with this organism and sera from healthy control subjects to investigate their immune response to this organism. The main immunological response to the bacteria was high levels of IgM antibodies to the LPS of *E. coli* serotype O157 and some reaction with OMPs. Only one antiserum from a patient gave a strong reaction with the IROMPs. Since sera from HUS patients did not react with the LPS of non-O157 strains of *E. coli*, and since sera from control subjects did not react with the LPS of *E. coli* serotype O157, the observed reaction of sera from patients with O157 LPS was considered to be a direct result of infection with *E. coli* serotype O157. The lack of an IgG reaction with any of the sera to either O157 LPS or the H antigen suggests that HUS patients and healthy controls had not been infected with *E. coli* serotype O157:H7 within immunological memory.

Symptoms of HC occur 4 days after consumption of contaminated foods and, if self-limiting, subside within approximately 8 days (22, 23). In some cases, the onset of HUS can follow a prodrome of HC; in others, the symptoms of HUS appear not to be preceded by those of HC. However, regardless of the type of disease, antibodies to LPS were detected in sera obtained up to 75 days postinfection. Although no clear correlation could be made between antibody titer and the number of days postinfection sera were obtained, patients sampled shortly after onset of disease had generally higher titers to LPS. Notenboom et al. (20) reported that HUS patients developed agglutinating antibodies to *E. coli* serotype O157. Since the pentameric structure of IgM facilitates agglutination reactions, as compared with monomeric immunoglobulins, the results described by Notenboom et al. (20) would also indicate a strong IgM response of patients to *E. coli* serotype O157. In addition, the observed lack of antibodies to H7 flagella and the fact that LPS tends to prevent antibodies binding to surface-exposed OMPs (33) further suggest that the antibody reactions reported by Notenboom et al. (20) were indeed directed against LPS.

The strong reaction of sera from HUS patients specifically to O157 LPS makes this antigen potentially useful for a diagnostic immunological test for assessing the role of *E. coli* serotype O157 in cases of HUS (and HC) in which VT-producing *E. coli* has not been isolated. Diluting serum (100 times) before its addition to ELISA plates failed to completely separate HUS patients from healthy controls, although sera from patients generally contained higher antibody titers. A high serum antibody titer to O157 LPS would therefore clearly implicate *E. coli* serotype O157 in the disease. However, a weak anti-O157 LPS reaction with antiserum from a patient cannot exclude the involvement of *E. coli* serotype O157:H7.

When examining the antibody responses of patients to the LPS of *E. coli* serotype O157, due consideration must be given to the possible involvement of nontoxigenic strains of *E. coli* belonging to serogroup O157 but not of H-type 7 (which have been isolated from human infections [27]) and also to antigenic cross-reactions with the LPS of other bacteria. The LPSs of certain serogroups of *B. abortus* and *Y. enterocolitica* are known to serologically cross-react (2, 5, 18), and *E. coli* serotype O157 has been shown to cross-react with strains of *B. abortus* (20, 30). However, lack of cross-reaction between *Y. enterocolitica* serotype O9 and *E. coli* serotype O157 has also been reported (31). Our data confirmed a cross-reaction between *E. coli* serotype O157 and *B. abortus* by immunoblotting, but a reaction was not detected with the LPS of *Y. enterocolitica*. The LPSs of these bacteria contain identical carbohydrate repeating subunits (N-acylated 4-amino-4,6-dideoxy-α-mannopyranosyl residues) (21), and cross-reactions would be expected; this

makes the lack of cross-reaction between *E. coli* serotype O157 and *Y. enterocolitica* difficult to explain.

Since the symptoms of disease mediated by *B. abortus* are quite distinct from those of HC or HUS, misdiagnosis based on serum antibody reactions would seem unlikely. Similarly, serum antibodies to O157 LPS would probably not be attributed to an infection by *Y. enterocolitica* since cross-reacting antibodies appear not to occur.

Antibody cross-reactions would have to be considered when interpreting serological test data. However, using isolated bacterial antigens such as O157 LPS in an ELISA would eliminate antibody cross-reactions with protein epitopes common to many enteric bacteria that might occur with, for example, whole cell bacterial agglutination tests. We conclude that using a serological test alone to implicate a particular organism as a cause of HUS or HC is inconclusive in the absence of detection of a causative organism and that every effort should be made to isolate the infectious agent and use available serological results to support any bacteriological findings. In cases in which the causative organism cannot be detected, serological data should be considered as being subjective and any antibody reactions should be considered carefully and interpreted in relation to the symptoms of the patient. A potential diagnostic test system based on an ELISA for *E. coli* serotype O157 LPS is currently being investigated for use with sera from patients with HC or HUS. The value of using an ELISA for investigating patients suspected of having HUS and the problems associated with bacterial cross-reactions will be assessed in due course.

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