Alternative Pathway of Complement in Children with Diarrhea-Associated Hemolytic Uremic Syndrome

Joshua M. Thurman,* Russell Marians,† Woodruff Emlen,† Susan Wood,† Christopher Smith,† Hillary Akana,† V. Michael Holers,‡ Martin Lesser,§ Myriam Kline,§ Cathy Hoffman,‖ Erica Christen,‖ and Howard Trachtman‖

**Department of Medicine, Division of Nephrology, University of Colorado, Denver School of Medicine, Aurora, Colorado; †Taligen Therapeutics, Aurora, Colorado; ‡Department of Medicine, Division of Rheumatology, University of Colorado, Denver School of Medicine, Aurora, Colorado; §Biostatistics Unit, Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, New York; and ‖Department of Pediatrics, Division of Nephrology, Schneider Children's Hospital of North Shore-LIJ Health System, New Hyde Park, New York*

Background and objectives: Diarrhea-associated hemolytic uremic syndrome (D+HUS) is a common cause of acute kidney injury in children. Mutations in alternative pathway (AP) complement regulatory proteins have been identified in severe cases of thrombotic microangiopathy, but the role of the AP in D+HUS has not been studied. Therefore, we determined whether plasma levels of markers of activation of the AP are increased in D+HUS and are biomarkers of the severity of renal injury that predict the need for dialysis.

Design, setting, participants, & measurements: Patients were randomly selected from among participants in the HUS-SYNSORB Pk trial. Plasma samples were collected on days 1, 4, 7, and 10 after enrollment and day 28 after discharge from the hospital. Levels of two complement pathway products, Bb and SC5b-9, were determined by ELISA.

Results: Seventeen children (6 boys and 11 girls; age, 5.4 ± 3.5 yr) were studied. Eight (47%) required dialysis support, and two had serious extrarenal events. On the day of enrollment, plasma levels of Bb and SC5b-9 were significantly increased in all patients compared with healthy controls ($P < 0.01$). The elevated concentrations normalized by day 28 after discharge. Circulating levels of complement pathway fragments did not correlate with severity of renal injury or occurrence of complications.

Conclusions: Patients with acute-onset D+HUS manifest activation of the AP of complement that is temporally related to the onset of disease and that resolves within 1 mo. Therapies to inhibit the AP of complement may be useful in attenuating the severity of renal injury and extrarenal complications.

Clin J Am Soc Nephrol 4: 1920–1924, 2009. doi: 10.2215/CJN.02730409

Diarrhea-associated hemolytic uremic syndrome (D+HUS) is one of most common causes of acute kidney injury in previously healthy children (1). It is caused by antecedent infection with Shiga toxin-producing strains of *Escherichia coli* (STEC). These organisms elaborate Shiga toxins (Stx) 1 and/or 2 that bind to the globotriaosylceramide (Gb3) receptor on the surface of endothelial cells, especially in the glomerular microcirculation. After internalization of the toxin, there is retrograde transport to the ribosome, inhibition of protein synthesis, endothelial cell death, and organ hypoperfusion and dysfunction (1,2). In addition, there is activation of numerous inflammatory cytokines and chemokines that have the potential to cause vascular injury and mediate tissue damage (3).

D+HUS is one manifestation of thrombotic microangiopathy (TMA), a histopathologic phenotype characterized by endothelial cell swelling and detachment from the basement membrane and deposition of fibrin-platelet thrombi in the vascular lumen (4). In addition to D+HUS, TMA can occur sporadically in response to various medications, infectious agents, pregnancy, malignancies, rheumatological disorders, and in patients with thrombotic thrombocytopenic purpura. Finally, there is a rare group of patients who develop TMA as a consequence of genetic abnormalities in complement activation and regulatory proteins that lead to uncontrolled activation of the alternative pathway (AP). Recent reviews of TMA have proposed that the disease occurs due to disturbances in one of two distinct pathways—either dysregulation of complement activation or a relative loss of function of ADAMTS13, a protease that modulates the interaction between von Willebrand factor and endothelial cells. Although endothelial damage is the primary step in D+HUS, it has not been definitively attributed to abnormalities in the function of the complement pathway or ADAMTS13.

There are anecdotal reports of low serum C3 levels and C3 deposition in the kidney of children with D+HUS (5). However, there has been no consistent evidence of activation of the

Received April 22, 2009. Accepted September 11, 2009.

Published online ahead of print. Publication date available at www.cjasn.org.

Correspondence: Dr. Howard Trachtman, Schneider Children's Hospital, Division of Nephrology, 269-01 76th Avenue, New Hyde Park, NY 11040. Phone: 718-470-3423; Fax: 718-470-0887; E-mail: trachtma@lij.edu

ISSN: 1555-9041/412–1920

AP of complement in children with D+HUS. We hypothesized that the AP is activated by Shiga toxin-induced endothelial damage in D+HUS. To test this hypothesis, we performed the following study using stored plasma samples from patients who were enrolled in the HUS-SYNSORB Pk multicenter clinical trial to determine whether there was evidence of activation of the AP of complement in this disease and to assess whether it correlated with disease activity or outcome.

Materials and Methods

Patients

The study was approved by the Institutional Review Boards of all of the participating centers in the multicenter trial (see Appendix for a complete list of performance sites). Informed consent for the therapeutic trial and the use of stored samples for future experimental studies was obtained before enrollment.

Children between the ages of 6 mo and 18 yr with D+HUS were eligible for inclusion into the trial. The diagnosis of D+HUS was based on the following four criteria: (*1*) platelet count $<140{,}000/mm^3$; (*2*) fragmentation of erythrocytes on a peripheral smear; (*3*) renal injury as indicated by the presence of hematuria and/or proteinuria and/or azotemia; and (*4*) a diarrhea illness within 7 d before the identification of HUS. Microbiological confirmation of STEC infection was not required before entry to expedite patient enrollment. Exclusion criteria included (*1*) an atypical or nondiarrhea prodrome; (2) family history of hereditary HUS; (*3*) HUS associated with bone marrow transplantation, pneumococcal infection, or HIV infection; (*4*) pre-existing renal disease; and (*5*) pre-existing structural or motility disorder of the gastrointestinal tract.

Study Protocol

After establishment of eligibility and informed consent, patients with D+HUS were randomly assigned to receive either SYNSORB Pk or corn meal placebo. The study was double-blind, and the allocation was performed randomly in a 2:1 ratio. The study medication, 500 mg/kg per day, was administered orally or through a nasogastric tube every 8 h for 7 d. The clinical management of patients, experimental studies, primary and secondary endpoints, and the outcome of this clinical trial has been reported previously (6). The decision to start dialysis was made based on the occurrence of 72 consecutive hours of oligoanuria, defined as a urine flow <0.5 ml/kg per hour after hospitalization at the participating center or the presence of acute symptoms related to azotemia (7).

In the SYNSORB Pk trial, some patients were admitted directly to the participating site where the diagnosis of D+HUS was made, whereas others were transferred to the center after recognition of the disease at an outlying facility. In all cases, the first day of the study and hospitalization, when sample collection began, was defined as the day when randomization to study medication or placebo was accomplished. The course was divided into two phases: hospitalization and recovery after discharge. Clinical and laboratory data were recorded daily, and EDTA–anti-coagulated plasma samples were collected on days 1, 4, 7, and 10 during the hospitalization phase for each child. The day 1 sample was collected after the patient was randomized to a treatment arm but before initiation of dialysis at the participating site. After discharge from the hospital, patients were evaluated on days 7, 14, 28, and 60, and a plasma sample was collected on day 28. Because of the variable duration of hospitalization, the 28-d sample after discharge was obtained between 33 and 90 d after the onset of D+HUS. All biologic samples were stored in aliquots at −70°C.

Bb and SC5b-9 Assays

Complete sets of plasma samples were selected randomly from the Biorepository maintained at the Schneider Children's Hospital, Department of Pediatrics, where the specimens were stored. They were shipped to Taligen Therapeutics (Aurora, CO) for determination of AP complement activation products by ELISA (Quidel, San Diego, CA). To show the stability of the analytes, AP fragments levels were determined in select samples that were thawed once before assay or subjected to two to three freeze thaw cycles. Laboratory personnel performing the complement activation fragment assays were masked as to the identity of subjects and the relative point in the disease process of the sample acquisition.

Statistical Methods

Data are reported as mean ± SD. Differences between groups were compared using the *t* test and the Wilcoxon rank sum test. Association between variables was evaluated with Spearman correlations analysis. Findings were considered significant if $P < 0.05$.

Results

Patients

Seventeen patients were included in this study. The key clinical and laboratory features are summarized in Table 1. The demographic features and the severity of the acute illness in this subgroup were comparable to the full cohort of 145 patients included in the modified intent-to-treat analysis of efficacy (6). In particular, the sample included a preponderance of girls of

Table 1. Clinical and laboratory features of children with D+HUS

Feature	Result [Mean ± SD (Range)]
Age (yr)	5.4 ± 3.5 (1.2–14)
Gender	6 M:11 F
Ethnicity	16 white:1 asian
Nadir hematocrit (vol%)	17.4 ± 4.6 (2.3–22.8)
Nadir platelet count ($/ml^3$)	22,235 ± 14,441 (8000–70,000)
Peak blood urea nitrogen (mg/dl)	95 ± 31 (40–150)
Peak creatinine (mg/dl)	3.8 ± 2.0 (0.8–7.3)
Dialysis	8/17 (47%)
Extrarenal complications	2/17 (12%)

preschool age, and 16 of 17 were white. Forty-seven percent of the children required temporary renal replacement therapy, and all patients had at least partial recovery of kidney function. There were four control patients (two boys and two girls), all white, and the mean age was 6.3 ± 1.5 yr.

Bb and SC5b-9 Levels

In normal plasma, the concentration of Bb and SC5b-9 were 1195 ± 583 and 402 ± 167 ng/ml, respectively. The levels were comparable in control samples that were subjected to two to three freeze-thaw cycles, indicating the results in the samples from the patients with D+HUS were stable despite long-term storage in the HUS-SYNSORB Pk Repository. As shown in Figure 1, on the day of enrollment, there was a marked increase in the plasma concentration of Bb (A) and SC5b-9 (B) compared with healthy controls. The levels of both AP of complement fragments fell to normal levels by day 28 after discharge from the hospital. There were no differences in the patients assigned to SYNSORB Pk compared with those who received placebo. The mean levels of Bb and SC5b-9 have not been tabulated for all tests done during the course of the illness because some patients did not have samples collected at all of the time points. However, all patients had a steady decline over the observation period.

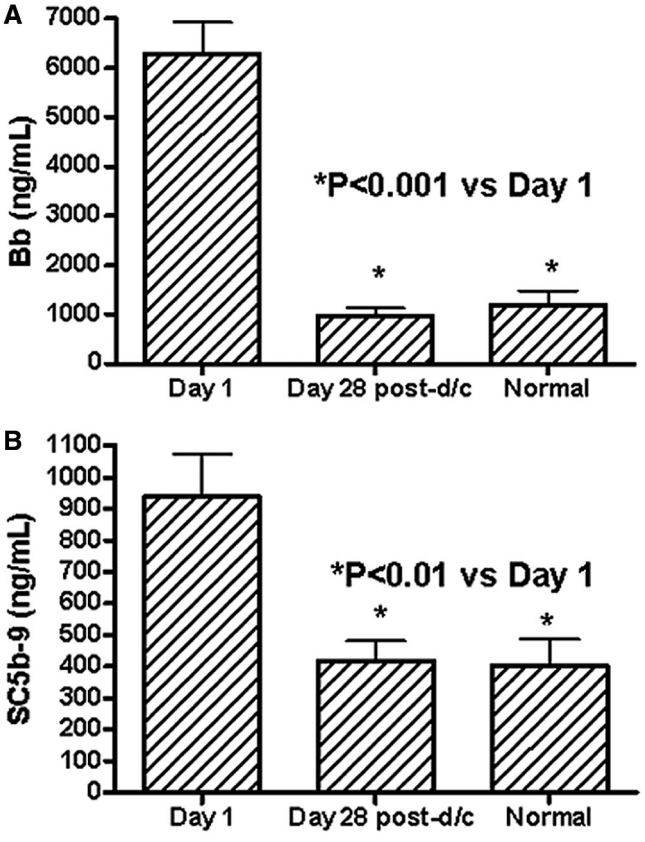

Figure 1. The bar graphs show the mean plasma concentration of Bb (A) and SC5b-9 (B) in patients with D+HUS on day 1 of their illness and at day 28 after hospitalization. The mean value for each AP of complement fragment in normal controls ($n = 4$) is included in each panel.

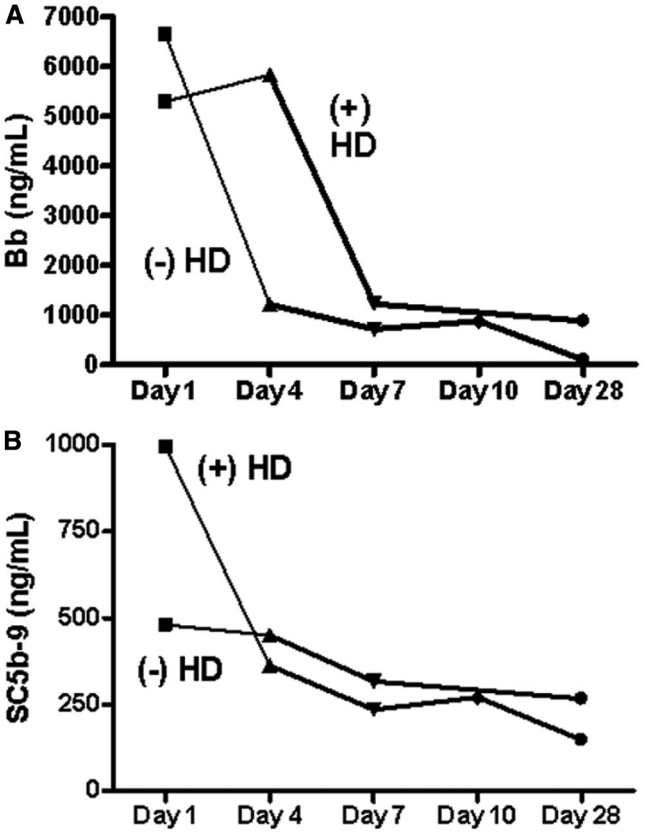

Figure 2. The graphs show the plasma concentration of Bb (A) and SC5b-9 (B) over the course of the D+HUS episode in a patient who required dialysis [(+)HD] and in a patient who was treated with conservative medical management for the acute kidney injury [(−)HD].

Figure 2 shows serial determinations of Bb (A) and SC5b-9 (B) in a representative patient who required temporary dialysis [(+)HD] and another who was managed with conservative medical therapy [(−)HD].

There were no significant correlations between circulating levels of Bb or SC5b-9 and the patient's age, peak blood urea nitrogen and creatinine levels, or nadir hematocrit and platelet count. For Bb, the Spearman correlations ranged from $r = -0.40$ to $r = 0.24$, and for SC5b-9, the Spearman correlations ranged from $r = -0.38$ to $r = 0.45$ (Table 2). In addition, there were no significant relationships between the percent decline in plasma levels of SC5b-9 and Bb from day 1 of the study to day 28 after discharge for any of the four clinical measurements.

Although the mean plasma concentration of SC5b-9 was numerically higher in the patients who required dialysis support (1091 ± 681 ng/ml) compared with those who did not receive this treatment (787 ± 317 ng/ml), the difference was not statistically significant. The peak circulating level of Bb was nearly identical in these two groups of patients (Figure 3). No cut-off value for either marker of complement activation could be defined that predicted which children would require renal replacement therapy or experience serious extrarenal complications during the course of their illness.

Table 2. Bb and SC5b-9: correlations with laboratory tests

Laboratory Test	Bb		SC5b-9	
	Spearman Correlation	*P* Value	Spearman Correlation	*P* Value
Age	−0.23	0.38	−0.21	0.43
Nadir platelet count	−0.20	0.45	0.45	0.08
Nadir hematocrit	−0.40	0.13	−0.38	0.15
Peak BUN concentration	0.24	0.36	−0.07	0.80
Peak creatinine concentration	−0.06	0.83	0.13	0.62

Discussion

The key finding in this report from the HUS-SYNSORB Pk clinical trial is that, in all children with D+HUS presumably caused by antecedent STEC-induced enteritis, there is evidence of significant activation of the complement cascade at the onset of the disease based on high plasma concentrations of SC5b-9 and Bb. The former measurement is a nonspecific marker of complement activation by either the classical, alternative, or lectin pathways, whereas the latter is selective for the AP. These values rapidly return to normal within 4 wk after resolution of the acute episode. The degree of activation of the complement cascade is not related to clinical laboratory indices of the renal injury (peak BUN and creatinine levels) or hematologic disturbances (nadir hematocrit or platelet count) that characterize this illness. Determination of circulating levels of complement fragments, reflective of either general or specific AP activation, is not useful as an index of disease severity. Moreover, in this pilot group of patients, they were not prognostic markers of the need for renal replacement therapy or the occurrence of serious extrarenal events. However, given the fact that the study cohort included only 17 patients and the weak to moderate correlations, we are unable to exclude the possibility of diagnostic or prognostic value to Bb or SC5b-9 levels because of the small sample size and low power. Therefore, we recommend that the correlations between AP of complement fragments and disease activity should be studied in a larger cohort of patients with D+HUS.

It should be noted that the complement activation fragments were assayed in plasma at the time of enrollment in the study and at the time of resolution. Previous studies in this study cohort evaluated the urinary excretion of basic fibroblast growth factor and neutrophil gelatinase associated lipocalcin as markers of renal injury (8,9). The advantage of a plasma marker for D+HUS is the ability to obtain the sample under controlled conditions at a defined time during the course of the disease. It avoids the problems associated with urine sample collection in anuric patients, the confounding effects of administration of diuretic agents, and defining the optimal method of the measurement (*i.e.,* concentration or normalization to urinary creatinine concentration).

In the HUS-SYNSORB Pk clinical trial, measurements of serum C3 and C4 levels were not included in the protocol because of lack of evidence of a role of complement in D+HUS and infrequent detection of low levels. Measurements of classical pathway fragments and C3a and C5a were not done because previous studies in the laboratory indicated that these levels were not stable after prolonged storage like Bb and SC5b-9. Moreover, additional patients could not be studied because the number of complete plasma sample sets had been depleted by prior ancillary projects done using samples stored in the SYNSORB Pk repository. Our findings suggest that measurement of AP of complement fragments may be useful in monitoring the course of disease compared with routine determination of individual complement components.

It is likely that endothelial injury triggered directly by Stx1 and/or Stx2 is responsible for the activation of the complement cascade in general and specifically the AP. It may be exacerbated by secondary damage triggered by various inflammatory mediator and cytokines such as IL-8 and TNF-α that are upregulated in response to Stx in neutrophils and enterocytes (10). Similar to the circumstance in children with atypical HUS related to genetic mutations in complement activation and regulatory proteins, there may be substantial activation of the AP of complement in D+HUS even in the face of a normal C3 level. In this study, terminal complement pathway fragments and markers of the AP were assayed in the plasma rather than simply measuring C3 levels. This may explain the difference between our findings and previous studies that failed to document activation of the AP of complement.

In conclusion, our findings about the AP of complement may potentially be relevant to the treatment of D+HUS. To date, no therapeutic intervention has successfully reduced the severity of D+HUS, decreased the need for renal replacement therapy, or lowered the mortality rate. Treatments that have been tested include corticosteroids, plasma infusions, anti-coagulants, fibrino-

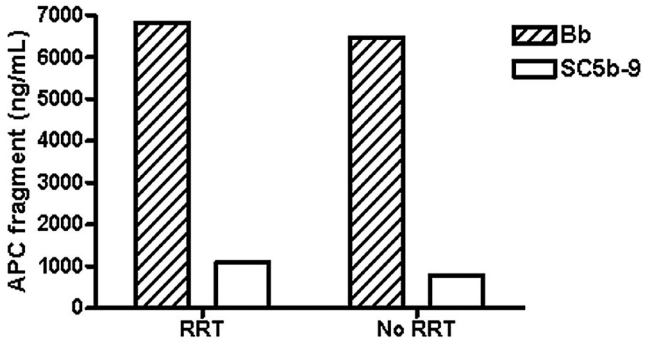

Figure 3. The bar graph shows the mean peak concentration of Bb and SC5b-9 in patients with required renal replacement therapy and those who did not need this supportive treatment.

lytic drugs, and oral Stx binding agents. A number of agents are under development that inhibit or modulate the dysregulated activation of the AP of complement present in human diseases. In addition to protection of the vascular endothelium against complement-mediated injury, blockade of the AP would diminish renal damage caused by proinflammatory fragments released by this cascade (11). Our data suggest that prompt initiation of these agents to children immediately after the onset of D+HUS to prevent aberrant activity of the AP of complement may be able to attenuate the manifestations of this serious illness.

Appendix: List of Participating Centers

Administrative Center: Howard Trachtman, MD, Principal Investigator; Erica Christen, RN, Project Coordinator, Schneider Children's Hospital, New Hyde Park, NY. Data Coordinating Center: Avital Cnaan, PhD (Director), Kathleen Gibbs, MSIS, Children' Hospital of Philadelphia, Philadelphia, PA. Microbiology Core Laboratory: David Acheson, MD (Director), Ramona Chitrakar, Thao Ngo, Fred Smith, Michelle Nieves, Sam Caraballo, Tufts-New England Medical Center, Boston, MA; Jilma Patrick, University of Maryland, Baltimore, MD. Data Safety Monitoring Board: Julie Ingelfinger, MD (Chairperson), Gladys Hirschman, MD, Josephine Briggs, MD, John Kusek, MD, Daniel Cattran, MD, Mitchell B. Cohen, MD, Katherine Freeman, PhD, Thomas Greene, PhD, Solomon Moshe, MD. Participating Centers: Howard Trachtman, MD, Schneider Children's Hospital, New Hyde Park, NY; Seth Schulman, MD, Children's Hospital of Philadelphia, Philadelphia, PA; James Springate, MD, Children's Hospital of Buffalo, Buffalo, NY; Frederick Kaskel, MD, PhD, Montefiore Medical Center, Bronx, NY; Dilys Whyte, MD, State University of New York Hospital at Stony Brook, Stony Brook, NY; Robert Weiss, MD, New York Medical College/Westchester County Medical Center, Valhalla, NY; Charles McKay, MD, duPont Hospital for Children, Wilmington, DE; Lewis Reisman, MD, St. Barnabas Hospital for Children, Livingston, NJ; Eduardo Perelstein, MD, Cornell University Medical Center, New York, NY; Manju Chandra, MD, North Shore University Hospital, Manhasset, NY; Jose Salcedo, MD, St. Joseph's Children's Hospital, Patterson, NJ; Lynne Weiss, MD, Robert Wood Johnson University Hospital, New Brunswick, NJ; William Varade, MD, State University of New York Rochester Medical Center, Rochester, NY; Douglas Ford, MD, Denver Children's Hospital, Denver, CO; James Chan, MD, Medical College of Virginia, Richmond, VA; Irene Restaino, MD, Children's Hospital of the King's Daughters, Norfolk, VA; Shashi Nagaraj, MD, Wake Forest University/North Carolina Baptist Hospital, Winston-Salem, NC; Victoria Norwood, MD, University of Virginia Medical Center, Charlottesville, VA; John Foreman, MD, Duke University Medical Center, Durham, NC; Michael Moritz, MD, Children's Hospital of Pittsburgh, Pittsburgh, PA; John Mahan, MD, Columbus Children's Hospital, Columbus, OH; Marva Moxey-Mims, MD, Children's National Medical Center, Washington, DC; Barry Warshaw, MD, Eggleston Children's Hospital, Atlanta, GA; Verna Yiu, MD, University of Alberta Hospital, Edmonton, AL, Canada; Andrew Brem, MD, Rhode Island Hospital, Providence, RI; Sharon Bartosh, MD, University of Wisconsin Hospital, Madison, WI; Sharon Andreoli, MD, University of Indiana/Riley Children's Hospital, Indianapolis, IN; Lawrence Milner, MD, Tufts New England Medical Center, Boston, MA; Jens Goebel, MD, University of Kentucky Medical Center, Lexington, KY; Dianne Muchant, MD, West Viginia University Medical Center, Morgantown, WV; Coral Hanevold, MD, Medical College of Georgia, Augusta, GA.

Acknowledgments

This work was supported in part by Grants DK52147 (H.T.) and DK76690 (J.M.T.). The findings were presented in part at the Annual Meeting of the Society for Pediatric Research, May 2 through 5, 2009, Baltimore, MD.

Disclosures

J.M.T. is a consultant and V.M.H. is a scientific advisor for Taligen Therapeutics.

References

1. Tarr PI, Gordon CA, Chandler WL: Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 365: 1073-1086, 2005
2. Hughes AK, Ergonul Z, Stricklett PK, Kohan DE: Molecular basis for high renal cell sensitivity to the cytotoxic effects of Shigatoxin-1: Upregulation of globotriaosylceramide expression. *J Am Soc Nephrol* 13: 2239-2245, 2002
3. Proulx F, Toledano B, Phan V, Clermont MJ, Mariscalco MM, Seidman EG: Circulating granulocyte colony-stimulating factor, C-X-C, and C-C chemokines with *Escherichia coli* O157:H7 associated with hemolytic uremic syndrome. *Pediatr Res* 52: 928-934, 2002
4. Besbas N, Karpman D, Landau D, Loirat C, Proesmans W, Remuzzi G, Rizzoni G, Taylor CM, Van de Kar N, Zimmerhackl LB: A classification of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and related disorders. *Kidney Int* 70: 423-431, 2006
5. Robson WL, Leung AK, Fick GHG, McKenna AI: Hypocomplementemia and leukocytosis in diarrhea-associated hemolytic uremic syndrome. *Nephron* 62: 296-298, 1992
6. Trachtman H, Christen E, Cnaan A, et al: Effect of a Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: A randomized controlled trial. *JAMA* 290: 1337-1344, 2003
7. Schulman SL, Kaplan BS: Management of patients with hemolytic uremic syndrome demonstrating severe azotemia but not oliguria. *Pediatr Nephrol* 10: 671-674, 1996
8. Ray P, Acheson D, Chitrakar R, Cnaan A, Gibbs K, Hirschman GH, Christen E, Trachtman H: Basic fibroblast growth factor among children with diarrhea-associated hemolytic uremic syndrome. *J Am Soc Nephrol* 13: 699-707, 2002
9. Trachtman H, Christen E, Cnaan A, Patrick J, Mai V, Mishra J, Jain A, Bullington N, Devarajan P: Urinary neutrophil gelatinase-associated lipocalcin in D+HUS: A novel marker of renal injury. *Pediatr Nephrol* 21: 989-994, 2006
10. Westerholt S, Hartung T, Tollens M, Gustrau A, Oberhoffer M, Karch H, Klare B, Pfeffer K, Emmrich P, Oberhoffer R: Inflammatory and immunological parameters in children with haemolytic uremic syndrome(HUS) and gastroenteritis: Pathophysiological and diagnostic clues. *Cytokine* 12: 822-827, 2000
11. Holers VM: The spectrum of complement alternative pathway-mediated diseases. *Immunol Rev* 223: 300-316, 2008