Response to Single and Divided Doses of Shiga Toxin-1 in a Primate Model of Hemolytic Uremic Syndrome

RICHARD L. SIEGLER,* THEODORE J. PYSHER,† VERNON L. TESH,‡ and FLETCHER B. TAYLOR, JR.§

*Department of Pediatrics, Division of Nephrology and Hypertension, University of Utah, and †Department of Pathology, Primary Children’s Medical Center, Salt Lake City, Utah; ‡Department of Medical Microbiology and Immunology, Texas A&M University, Health Science Center, College Station, Texas; and §Cardiovascular Biology Research, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma.

Abstract. Postdiarrheal hemolytic uremic syndrome is caused by Shiga toxin (Stx)-producing Escherichia coli. It was shown previously that the baboon, like the human, has glycolipid receptors for Stx in the gut and the kidney and that a single 50- to 200-ng/kg intravenous dose of purified Stx-1 results in thrombocytopenia, hemolytic anemia, and renal thrombotic microangiopathy. For further characterization of factors that modulate disease expression, the baboon’s response to the intravenous administration of 100 ng/kg Stx-1 given either rapidly as a single bolus or slowly as four 25-ng/kg doses at 12-h intervals was compared. Animals that received the Stx-1 as a single dose developed thrombocytopenia, schistocytosis, and acute renal failure. Urinary but not plasma tumor necrosis factor-α concentrations rose significantly by 6 h and then declined rapidly. Urinary and plasma interleukin-6 concentrations rose later. Glomeruli showed reduced patency of capillary loops, fragmented red blood cells, fibrin and platelet microthrombi, necrosis and detachment of endothelial cells, and accumulation of flocculent material in subendothelial spaces. Damage to tubular epithelium and peritubular capillary endothelium also was seen. Animals that received four divided doses of Stx-1 developed no clinical or histologic features of hemolytic uremic syndrome. It is concluded that in the primate model, disease expression is modulated by the rate of Stx administration, and it is speculated that in the human, the rate of Stx absorption from the gut is one determinant of disease severity.

Most, if not all, cases of postdiarrheal hemolytic uremic syndrome (HUS) are caused by Shiga toxin (Stx)-producing enterohemorrhagic Escherichia coli (EHEC), such as E. coli O157:H7. These bacteria cause a colitis that usually becomes hemorrhagic (1,2). The resulting severe bowel inflammation is thought to facilitate absorption of Stx, a family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins that are composed of a single A subunit and five B subunits that are similar to the prototypic family of protein holotoxins. Urinary but not plasma tumor necrosis factor-α concentrations rose significantly by 6 h and then declined rapidly. Urinary and plasma interleukin-6 concentrations rose later. Glomeruli showed reduced patency of capillary loops, fragmented red blood cells, fibrin and platelet microthrombi, necrosis and detachment of endothelial cells, and accumulation of flocculent material in subendothelial spaces. Damage to tubular epithelium and peritubular capillary endothelium also was seen. Animals that received four divided doses of Stx-1 developed no clinical or histologic features of hemolytic uremic syndrome. It is concluded that in the primate model, disease expression is modulated by the rate of Stx administration, and it is speculated that in the human, the rate of Stx absorption from the gut is one determinant of disease severity.

Abstract. Postdiarrheal hemolytic uremic syndrome is caused by Shiga toxin (Stx)-producing Escherichia coli. It was shown previously that the baboon, like the human, has glycolipid receptors for Stx in the gut and the kidney and that a single 50- to 200-ng/kg intravenous dose of purified Stx-1 results in thrombocytopenia, hemolytic anemia, and renal thrombotic microangiopathy. For further characterization of factors that modulate disease expression, the baboon’s response to the intravenous administration of 100 ng/kg Stx-1 given either rapidly as a single bolus or slowly as four 25-ng/kg doses at 12-h intervals was compared. Animals that received the Stx-1 as a single dose developed thrombocytopenia, schistocytosis, and acute renal failure. Urinary but not plasma tumor necrosis factor-α concentrations rose significantly by 6 h and then declined rapidly. Urinary and plasma interleukin-6 concentrations rose later. Glomeruli showed reduced patency of capillary loops, fragmented red blood cells, fibrin and platelet microthrombi, necrosis and detachment of endothelial cells, and accumulation of flocculent material in subendothelial spaces. Damage to tubular epithelium and peritubular capillary endothelium also was seen. Animals that received four divided doses of Stx-1 developed no clinical or histologic features of hemolytic uremic syndrome. It is concluded that in the primate model, disease expression is modulated by the rate of Stx administration, and it is speculated that in the human, the rate of Stx absorption from the gut is one determinant of disease severity.

Received January 25, 2000. Accepted November 28, 2000.
Correspondence to Dr. Richard L. Siegler, Professor and Division Chief, Pediatric Nephrology and Hypertension, 50 North Medical Drive, #2B441, Salt Lake City, UT 84132. Phone: 801-581-7609; Fax: 801-581-8043; E-mail:dick.siegler@hsc.utah.edu
No reprints available.
1046-6673/1207-1458
Journal of the American Society of Nephrology
Copyright © 2001 by the American Society of Nephrology
Materials and Methods

Toxin Preparation

Stx-1 was purified as described previously (12). Briefly, purification consisted of sequential ion exchange, chromatofocusing, and immunoaffinity chromatography. Purified toxin was assessed for homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with the use of silver staining and Western blots. LPS contaminants were removed by passage through ActiClean Etox columns (Sterogene Bioseparations, Inc., Carlsbad, CA). The final Stx-1 preparation contained less than 0.1 ng of LPS/ml as determined by Limulus amoebocyte lysate assay. The protein content of the toxin was determined with the use of a Pierce protein quantitative kit. The specific cytotoxicity, as verified by Vero cell cytotoxicity assay, was 2 x 10^6 CD50/µg protein.

Experimental Protocol

Baboons (Papio c. anubis or Papio c. cynocephalus) were obtained from the breeding colony at the University of Oklahoma Health Sciences Center or from Osage Research (Osage Beach, MO). Juvenile males were used for the Stx experiments (Table 1) because of the difficulty in catheterizing the urinary bladders of females. The animals weighed between 4 and 7 kg and developmentally were comparable to 3- to 6-year-old humans. They were in good health without evidence of tuberculosis and were observed for 30 d before experiments.

Solid food was withheld for 12 h before the experiments in preparation for general anesthesia, but free access to water was allowed to prevent dehydration and subsequent oliguria and prerenal azotemia. Animals were anesthetized with intramuscular ketamine (14 mg/kg) and orally intubated, and a percutaneous venous catheter was placed in a cephalic vein. They were kept in a light plane of general anesthesia, but free access to water was allowed to prevent dehydration and subsequent oliguria and prerenal azotemia. Animals were anesthetized with intramuscular ketamine (14 mg/kg) and orally intubated, and a percutaneous venous catheter was placed in a cephalic vein. They were kept in a light plane of general anesthesia with intravenous pentobarbital (2 to 3 mg/kg every 30 min, as needed) administered through the venous catheter. A warming pad was used, and body temperature was monitored via a rectal probe and monitor. A femoral artery and vein were surgically exposed with the use of aseptic technique and cannulated for BP and central venous pressure (CVP) measurements and blood sampling. The cannulas were attached to heparin locks (10 U of heparin/ml) and buried under the skin to prevent access by the animals. Arterial BP and heart rate were monitored with the use of a multichannel Hewlett Packard monitor (Palo Alto, CA); CVP was measured via a glass manometer. Ceftriaxone (50 mg/kg) was administered by intramuscular injection once every 24 h. Blood samples were obtained from the femoral venous catheter, and urine was obtained via a Foley catheter temporarily inserted through the urethral meatus.

Before the intravenous administration of Stx-1 (or 0.9% saline in the control animals), vital signs were recorded and baseline (T-0) blood and timed urine samples were collected. After the intravenous administration of Stx-1 or 0.9% saline, the animals were maintained in a light plane of anesthesia and observed on the surgical table for 2 h. The animals then were returned to their cages. Additional blood and urine samples were collected at 6 h to capture early cytokine release and then again at 12 h and every 12 h thereafter. At the beginning of each collection period, animals were weighed and vital signs were obtained. For prevention of hypovolemic (prerenal) oliguria and azotemia, 0.9% saline was administered intravenously if the weight was less than baseline (in a volume sufficient to bring weight back to baseline). One or more infusions of 10 ml/kg were given if the CVP was <3 cm of water, if the mean BP was <75 mmHg, or if the urine output was <2 ml/kg per h. Fluids were withheld, however, if the animal’s CVP was >10 cm/water. Timed urine collections were started after any required intravenous fluid had been administered.

Automated blood cell counts were performed on a Technicon H-1 System (Miles, Inc., Diagnostics Division, Naperville, IL), and Wright-stained peripheral blood smears were examined microscopically. Routine blood chemistry studies were performed on a Vitros 700 Chemistry Analyzer (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY). Urine was analyzed, with the use of reagent strips (Multistix 10 SG; Bayer, Elkhart, IN), for blood, protein, specific gravity, pH, nitrites, and leukocyte esterase. After centrifugation, the urinary sediment was examined microscopically.

Experiments usually were terminated at 72 h (sooner if the animal became moribund) by the intravenous injection of pentobarbital (100 mg/kg). Two animals that received the toxin slowly as divided doses were allowed to survive for 192 and 312 h, respectively, to exclude the possibility of delayed onset HUS. A complete post-mortem examination was performed after death.

Tissue Preparation

Light Microscopy. Sections of cortex and medulla from the mid-lateral aspect of both kidneys were immersed in 10% neutral buffered formalin for at least 6 h and then dehydrated and paraffinized with the use of an automated tissue processor (TissueTek; Sakura; Finetek, Torrance, CA). Sections 3 to 4 µm thick were stained with hematoxylin and eosin and with periodic acid-Schiff and other stains when necessary.

Electron Microscopy. Small portions of cortex from the mid-region of the left kidney, obtained within 5 min of death, were placed in 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate with 2.4% sucrose, and 8 mM calcium chloride for at least 1 h and then processed through graded alcohols and embedded in Spur’s low-viscosity embedding medium. One µm of semithin sections was stained with toluidine blue for light microscopy, and thin

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gender</th>
<th>Weight (kg)</th>
<th>Survival (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Stx (100 ng/kg × 1)</td>
<td>Male</td>
<td>4.95</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.0</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.1</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4.9</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.4</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4.8</td>
<td>56</td>
</tr>
<tr>
<td>Divided Stx (25 ng/kg × 4)</td>
<td>Male</td>
<td>6.3</td>
<td>Killed at 192</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>7.0</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.4</td>
<td>Killed at 312</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>6.3</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>6.5</td>
<td>Died at 120</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>Male</td>
<td>5.45</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.2</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.6</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4.0</td>
<td>Killed at 72</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5.1</td>
<td>Killed at 72</td>
</tr>
</tbody>
</table>

a Stx, shiga toxin; HUS, hemolytic uremic syndrome.

b Animals allowed to survive for >72 h to ensure that late onset HUS did not occur.

c Found dead at approximately 120 h. Necropsy showed no signs of thrombotic microangiopathy.
sections were stained with lead citrate and uranyl acetate and examined with a Hitachi H-700 transmission electron microscope (Tokyo, Japan).

**Immunofluorescence Microscopy.** Sections of renal cortex were flash-frozen in OCT-embedding compound, and frozen sections were stained with a fluorescein-conjugated IgG fraction of goat anti-human fibrinogen (ICN Pharmaceuticals, Aurora, OH) and examined with a fluorescence microscope.

**Scoring of Glomerular Histopathology**
The histologic findings in 100 consecutive glomeruli from animals in each group (i.e., single-dosed [n = 3], split-dosed [n = 3], saline controls [n = 2]) were scored in a blinded manner as described previously (12).

**Cytokine Assays**
Solid-phase enzyme-linked immunosorbent assay commercial kits (R&D Systems, Inc., Minneapolis, MN) were used to measure cytokines in urine and plasma.

**Statistical Analyses**
Because the sample sizes were small and therefore not normally distributed, data were expressed as medians and ranges, and the nonparametric Kruskal-Wallis test was used to determine significance. A P < 0.05 was considered to be significant.

**Results**

**Clinical Features**
Animals (n = 6) that received the 100 ng/kg dose of Stx-1 rapidly as a single bolus (Table 2) seemed to be well for the first 36 h, although all exhibited transient abdominal distention. They then developed progressive lethargy and variable degrees of anorexia. Urinary output remained normal in all of the single-dosed animals for the initial 24 h, but oliguria (<0.5 cc/kg per h) developed in four of the six by 48 h. Three became severely oligoanuric (<0.1 cc/kg per h). As thrombocytopenia progressed (see below), many experienced oozing and/or petechia by approximately 60 h. Three of the six single-dosed animals experienced grand mal seizures by 48 h. Death was preceded by coma, and all died or were moribund by 72 h. Median survival was 58 h (range, 52 to 72 h).

No clinical signs of HUS were observed in the saline control animals (n = 5) or in the animals that received the Stx as four divided doses (n = 5). One split-dosed animal that manifested no signs of clinical illness through 96 h of observation, however, was found dead at 120 h.

**Laboratory Data**

**Cytokine Values.** Urinary (pg/mg creatinine) tumor necrosis factor-α (TNF-α) median values rose in the single-dosed animals from 0 at baseline (hour 0) to 4.39 at 6 h, compared with values of 0.94 in control animals (P = 0.024) and 1.46 in split-dosed animals (P = 0.0495). Levels then declined rapidly (Figure 1A).

Interleukin-6 (IL-6) urine values (Figure 1B) in the single-dosed animals rose from 0.73 at baseline to 4.9 at 24 h, which were significantly higher than those of control (0; P = 0.043) or split-dosed animals (0.64; P = 0.045). Values continued to rise and by 48 h were 101.28, compared with 0 in controls (P = 0.013) and 1.4 in split-dosed animals (P = 0.027). Sixty-h values remained elevated (259) relative to control values (0; P = 0.028) and split-dosed animal values (0.79; P = 0.0495). Insufficient numbers of single-dosed animals survived to 72 h to allow meaningful analysis.

Plasma TNF-α concentrations generally were undetectable. Plasma IL-6 concentrations (pg/ml), however, rose in the single-dosed group from 9.3 at baseline (Figure 1C) to 72.94 by 36 h, which was significantly higher (P = 0.011) than that of control animals (18.1). By 48 h, the median value in the single-dosed group was 102.4, compared with 18.3 (P = 0.028) and 19.6 (P = 0.018) in control and divided-dosed animals, respectively. By 60 h, it had risen further to 132.6, a value that did not achieve statistical significance when compared with controls (10.6; P = 0.05) or split-dosed animals (17.4; P = 0.077).

**Urinalysis.** Granular casts (Figure 2A) were abundant in all but one of the single-dosed animals by 24 h and occasionally were noted as early as 12 h after Stx-1 infusion. Casts were infrequent in the split-dosed group and were absent in the control group. Proteinuria appeared within 24 h in the animals that received the single dose of toxin and became pronounced (i.e., 4+) by 36 h (Figure 2B); hematuria began by 36 h and was prominent by 48 h (Figure 2C). Hematuria and proteinuria in the split-dosed and control groups were only intermittent and modest in amount, with the exception of the split-dosed animal that died at 120 h; this animal had 4+ proteinuria at 48 h that decreased to 1+ by 60 h.

**Renal Function.** Acute renal failure began approximately 24 h after the onset of proteinuria, i.e., by 48 h, in the animals that received the single intravenous bolus dose of Stx-1. The blood urea nitrogen and serum creatinine concentrations are depicted in Figure 3. The blood urea nitrogen (median) in the single-dosed group at 24 h was 10.5 mg/dl, which was significantly greater (P = 0.017) than that of control animals (7 mg/dl). By 36 h, it was 15.5 mg/dl, compared with 8 mg/dl in

**Table 2. Clinical features of animals (n = 6) that received 100 ng/kg Stx-1 rapidly as a single bolus dose**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Time from Stx-1 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal</td>
<td></td>
</tr>
<tr>
<td>urinary casts</td>
<td>24</td>
</tr>
<tr>
<td>proteinuria</td>
<td>24</td>
</tr>
<tr>
<td>hematuria</td>
<td>36</td>
</tr>
<tr>
<td>oliguria</td>
<td>48</td>
</tr>
<tr>
<td>azotemia</td>
<td>48</td>
</tr>
<tr>
<td>Hematologic</td>
<td></td>
</tr>
<tr>
<td>thrombocytopenia</td>
<td>36</td>
</tr>
<tr>
<td>schistocytosis</td>
<td>36</td>
</tr>
<tr>
<td>CNS</td>
<td></td>
</tr>
<tr>
<td>seizures</td>
<td>48</td>
</tr>
<tr>
<td>Survival [median (range)]</td>
<td>58 (52–72)</td>
</tr>
</tbody>
</table>

* In three of six animals.
the divided-dosed animals ($P = 0.006$). The serum creatinine concentration was significantly greater ($P = 0.011$) than that of control animals at 36 h (0.65 mg/dl versus 0.5 mg/dl). By 48 h, it was significantly higher than that of divided-dosed animals (0.75 mg/dl versus 0.6 mg/dl; $P = 0.021$).

**Platelets.** Animals that received the 100 ng/kg Stx-1 as a single intravenous bolus dose developed progressive thrombo-

---

**Figure 1.** Urinary tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (A) and interleukin-6 (IL-6) (B) median values and ranges (pg/mg creatinine) at various timed intervals before (T-0) and after administration of Shiga toxin-1 (Stx-1; or saline). By 6 h after Stx infusion, TNF-$\alpha$ values were significantly greater in the animals that received the Stx-1 rapidly as a single dose than in control animals ($P = 0.024$) or those that received the Stx-1 slowly as divided doses ($P = 0.049$). Urinary IL-6 values at 24 h were significantly higher ($P < 0.04$) than those of control or split-dosed animals; these significant differences persisted through hour 60. (C) Plasma IL-6 median concentrations and ranges (pg/ml). Single-dosed animals' median values were significantly higher than those of control animals ($P = 0.011$) by 36 h and higher than those of control ($P = 0.028$) and split-dosed animals ($P = 0.018$) by 48 h. Differences between single-dosed, split-dosed, and control animals at 60 h did not achieve statistical significance ($P = 0.05, 0.08$, respectively). Plasma TNF-$\alpha$ levels (not shown) remained low and similar to controls. *, significance ($P < 0.05$) between single- and split-dosed groups; **, significance between single-dosed and control groups.

**Figure 2.** Urinalysis results (median values and ranges) for the three groups of animals at various timed intervals (T-0 = baseline, before administration of Stx-1). Granular casts (A) were more frequent in those that received Stx as a single bolus dose by 36 h. Proteinuria (B) preceded hematuria (C) and was significantly greater than in split-dosed and control animals by 24 h. Hematuria was greater than in split-dosed and control animals by 48 h. *, single dose of Stx; **, significance ($P < 0.05$) between single- and split-dosed groups; ***, significance between single-dosed and control groups.
cytopenia. By 36 h, the platelet count was $126.5 \times 10^9/L$ compared with $217 \times 10^9/L (P = 0.01)$ in the control group and $171 \times 10^9/L (P = 0.028)$ in the split-dosed animals (Figure 4A). Thrombocytopenia was severe ($23.5 \times 10^9/L; P = 0.032$) by 60 h. Animals in the divided-dosed group showed only a modest and transient decrease in platelet count (median nadir, $156 \times 10^9/L$) that was not significantly different from that of controls.

**Red Blood Cells.** Schistocytes (Figure 4B) in the single-dosed group were significantly more numerous than in the control ($P = 0.044$) and split-dosed ($P = 0.045$) groups by 36 h. They were abundant (median, 30.0%) by 60 h. Fragmented cells were only occasionally seen in the divided-dosed or control groups. Although hematocrit values (not shown) fell in all animals as a result of multiple blood sampling, the median hematocrit value at 60 h in the single-dosed group was 27.3% compared with 34.4% in the control animals and 31.7% in the animals that received divided doses of Stx. These differences did not achieve statistical significance.

**Necropsy Findings**

Five of the six animals that received the single intravenous dose of Stx-1 underwent necropsy immediately after death; in one, necropsy was performed approximately 8 h after death. The saline control animals and four of the five that received the divided doses of Stx-1 underwent necropsy immediately after the administration of the euthanizing agent. Necropsy was delayed approximately 72 h in the split-dosed animal that died unexpectedly at 120 h.

No renal TMA or other significant gross, histologic, or ultrastructural abnormalities were seen in control animals or in those that received divided doses of Stx-1, except for the...
split-dosed animal that died at 120 h. This animal had marked renal medullary congestion and patchy hemorrhage plus extensive tubular injury but no TMA. The lungs also showed diffuse congestion, patchy edema, and focal hemorrhage, but no significant gastrointestinal, cardiac, or brain abnormalities were found.

The kidneys of the animals that received the Stx-1 as a single intravenous bolus showed congestion and/or hemorrhage at the corticomedullary junction by gross examination. The median indexed weight of the kidneys (kidney weight/body weight × 100) was 0.88 compared with 0.54 (P = 0.011) and 0.52 (P = 0.025) in the control and split-dosed groups, respectively. The adrenal glands were congested or hemorrhagic in five of the six animals that received Stx as a single intravenous bolus. The small and, more notably, the large intestines in four of these six showed mucosal congestion. Two had pulmonary hemorrhages, and all exhibited myocardial petechiae. Fluid that had the appearance of transudate was found in the peritoneal, pericardial, and, occasionally, the pleural spaces. Mild to moderate cerebral edema was noted in three, but no evidence of hemorrhage or infarction was found on gross examination of the intact brain or the cut sections.

Histologically, glomeruli from animals that received Stx as a single intravenous bolus showed reduced patency of capillary lumens and fragmentation of red blood cells (Figure 5C), and abundant fibrin could be demonstrated by immunofluorescence microscopy (Figure 5D). Electron microscopy revealed platelet and fibrin thrombi within lumens, necrosis and detachment of endothelial cells, and accumulation of finely granular electronlucent material in subendothelial spaces (Figure 6B). Renal tissue from animals that received four divided doses of Stx (Figures 5, A and B, and 6A) showed no abnormalities. Scoring of 100 consecutive glomeruli in one kidney from the animals in the single-dosed, split-dosed, and control groups (Table 3) revealed that approximately 50% of the glomeruli in the single-dosed group showed evidence of TMA in the form of reduced patency of capillary loops, fragmented red blood cells, and/or fibrin thrombi, compared with 1% or less in the split-dosed and control groups, respectively.

Renal tubules in the single-dosed group showed vacuolation, apical blebbing, and detachment by light (Figure 5C) and electron microscopy (Figure 7A). Peritubular capillaries showed damage to the endothelial cells and adherent platelets (Figure 7B).

None of the brains showed hemorrhage, infarction, herniation, or large vessel thrombi; light and immunofluorescence

1 The split-dosed animal that died unexpectedly and had renal hemorrhage/congestion and tubular injury but no TMA was excluded from analysis.

Figure 5. Glomeruli and tubules in the split-dosed group were indistinguishable from those of control animals by light (A) and immunofluorescence (B) microscopy. Glomeruli in the single-dosed group showed obliteration of capillary lumens, fibrin thrombi, and fragmented red blood cells (black circle), and tubules showed frequent apical blebs and patchy nuclear dropout (white circle) or exfoliation of epithelial cells (C). Immunofluorescence of glomeruli in the single-dosed group showed extensive accumulation of fibrin (D). Magnification, ×120 (hematoxylin and eosin in A and C; FITC-conjugated anti-human fibrinogen in B and D).
revealed no intravascular fibrin/thrombi. Toluidine blue-stained 0.5-μm sections, however, showed variable expansion of cortical perivascular spaces (edema) that was more prominent in the animals that had had seizures, and transmission electron microscopy showed endothelial swelling and vacuolation in some of the vessels. No consistent differences were noted between groups, including those that experienced seizures, however.

**Discussion**

There has been need for an animal model of D+ (Stx-mediated) HUS that closely approximates the syndrome in the human. Of the numerous subprimate models that have been described, the racing greyhound dog probably provides the best simulation (13). It develops an HUS-like illness (Alabama Rot) after the ingestion of raw hamburger contaminated by *E. coli* O157:H7. Necrotic skin ulcers as a result of skin TMA are the hallmark. Thrombocytopenia and microangiopathic hemolytic anemia also occur, and approximately one half of the animals develop acute renal failure. This naturally occurring disease can be replicated by the parenteral administration of Stx (14).

The baboon’s response to a single 100-ng/kg intravenous bolus infusion of purified LPS-free Stx-1 is similar to that seen in children whose HUS follows Stx-producing *E. coli* enteric infection. That is, both the baboon and the human develop severe thrombocytopenia, microangiopathic hemolytic anemia, and an acute nephropathy characterized histologically by glomerular endothelial cell injury and fibrin-platelet microthrombi.

The split-dosed and control animals did not develop HUS and experienced in their platelet counts only a mild, nonprogressive decrease that probably was due to platelet activation secondary to the surgery and placement of indwelling femoral vessel catheters. They had no schistocytosis or renal TMA. The death of one of the five split-dosed animals at 120 h was unexpected because there had been no progressive thrombocytopenia, schistocytosis, or azotemia throughout the 60 h of blood sampling and no clinical signs of illness throughout 96 h of observation. The animal did exhibit 4+ proteinuria without casts or hematuria at 48 h, but it had decreased to 1+ by 60 h. Although the precise cause of death could not be determined by necropsy, there was marked pulmonary congestion and severe renal medullary congestion and tubular damage but no TMA.

The animal’s clinical and histologic response to the rapid administration of the 100-ng/kg dose was associated with an early transient rise in urinary but not plasma, TNF-α concentrations. This observation suggests that the source of the TNF was the kidney. Stx specifically induces TNF production in the kidney of transgenic mice that have a chloramphenicol acetyltransferase reporter gene that is coupled to a TNF promoter.

**Table 3. Histologic lesions in glomeruli**

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Only ES</th>
<th>ES and FR and/or FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 2)</td>
<td>83.0 (77 + 88)</td>
<td>17 (23 + 11)</td>
<td>0.5 (0 + 1)</td>
</tr>
<tr>
<td>Split-dose (n = 3)</td>
<td>79.7 (67 + 84 + 88)</td>
<td>19.3 (32 + 15 + 11)</td>
<td>1.0 (1 + 1 + 1)</td>
</tr>
<tr>
<td>Single-dose (n = 3)</td>
<td>5.3 (8 + 4 + 4)</td>
<td>44.0 (54 + 23 + 55)</td>
<td>50.7 (38 + 73 + 41)</td>
</tr>
</tbody>
</table>

*Percentage of 100 glomeruli in one section showing endothelial swelling (ES), fragmented red blood cells (FR), and/or fibrin thrombi (FT).*
but not in other tissues (15). Moreover, it induces release of TNF and mRNA when incubated with cultured human proximal renal tubular cells (16). TNF-α has many biologic properties that could be important in the pathogenesis of HUS and is elevated in the urine (17,18) of children during the acute phase of HUS. TNF acts on endothelial cells to induce procoagulant activity (19) and to promote white blood cell adhesiveness, probably through increased production of soluble intracellular adhesion molecule (20). It also induces the release of von Willebrand factor (21), which facilitates formation of platelet thrombi and increases the sensitivity of cultured human glomerular endothelial cells to the cytotoxic effects of Stx-1 (22). It also may play a role in triggering programmed cell death, in that it induces apoptosis in cultured bovine glomerular endothelial cells (23). Even so, it is not yet known whether TNF-α is required for disease expression, only augments the pathogenic cascade, or is merely an epiphenomenon.

We also found a progressive increase in both urine and plasma IL-6 concentrations in the animals that received the toxin as a single dose. Levels became pronounced approximately 48 h after Stx-1 administration and continued to increase until death. IL-6 is a cytokine that, like TNF-α, has prothrombotic properties (24). It is produced by macrophages and mesangial, endothelial, and other cells and is elevated in the urine and plasma of children with HUS (18). Its role in the pathogenic cascade is unknown, however, and it could be merely a marker of disease activity.

Three of the six that received the 100-ng/kg Stx-1 as a single dose developed seizures, not an uncommon occurrence in children with HUS (25). The seizures were not associated with hypertension or marked abnormalities in the serum concentration of glucose, calcium, or electrolytes; azotemia was no more severe in those that seized than in those that did not. Although children with HUS-related seizures generally have more severe electrolyte abnormalities and azotemia than those without convulsions (26), that often is not the case and illustrates our limited understanding of HUS encephalopathy. Two of the three baboons with seizures were found by gross examination to have mild to moderate cerebral edema at necropsy and electron microscopy showed perivascular edema. It seemed to have been part of a more generalized capillary leak disorder, because the majority of animals had fluid in the pleural, pericardial, and/or peritoneal spaces that had the appearance of a transudate. Even though we were unable to determine whether the cerebral edema played a role in the pathogenesis of the convulsions, it is notable that severe generalized cerebral edema and associated convulsions are frequent causes of death in children with HUS (26).

Intestinal involvement was limited to mucosal congestion. We showed previously, however, that the baboon (and human) have GB3 receptors in the large intestine and that when larger amounts (2000 ng/kg) of Stx-1 are given intravenously to the baboon, severe gut lesions occur (12). We speculate that Stx given intravenously in this model is preferentially bound to GB3 receptors in the kidney. When toxin is given in amounts sufficient to saturate renal GB3 receptors, however, Stx-mediated damage also is seen in the gut. Intestinal damage may be severe in the human model because the bacterial colitis facilitates absorption of Stx directly into the gut microcirculation.

The variation in the incidence and severity of HUS after EHEC colitis is not well understood. Although the subcutaneous injection of small amounts of either Stx-1 or Stx-2 are approximately of equal potency in causing HUS in the greyhound model (B. W. Fenwick, Department of Pathology and Microbiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, personal communication, December 1999), this may not be true in the human. There are epidemiologic data suggesting that enteric infection with Stx-2–producing E. coli is more likely to lead to HUS (27) than is

**Figure 7.** The tubulointerstitial compartment in the split-dosed group (A) was indistinguishable from that of control animals by electron microscopy. The brush border was well preserved in proximal tubules (P), and peritubular capillaries showed widely patent lumens lined by intact endothelial cells (*). Whereas some proximal tubules in the single-dosed group (B) were intact (P), other tubules showed epithelial injury (I) in the form of epithelial flattening and intercellular edema, and peritubular capillaries showed endothelial injury and adherent platelets (*). Magnification, ×2671 (lead citrate and uranyl acetate).
infection with bacteria that produce only Stx-1. Moreover, Stx-2 is markedly more cytoxic to cultured human glomerular endothelial cells than is Stx-1 (28). Therefore, although Stx-1 and Stx-2 both interrupt protein synthesis in a similar manner, Stx-2 seems to be more efficient in doing so. We elected to use Stx-1 because it is easier to purify and was available in our laboratory. We recognize that smaller amounts of Stx-2 probably would elicit the same biologic response. Studies to compare the baboon’s response to both Shiga toxins are planned.

It is common for EHEC colitis to affect multiple family members, yet only a minority develop HUS. Moreover, there is often wide variation in disease severity in those family members who progress to HUS. For example, we treated 9 families in whom postdiarrheal HUS developed concurrently in two family members, presumably after exposure to the same strain of Stx-producing E. coli (9). The severity of the HUS differed markedly between members in approximately one half of families; severe dialysis-dependent renal failure (and occasionally death) occurred in one member, but only mild disease occurred in the other.

Although numerous factors probably modulate the clinical response to Stx, our observations suggest that both the amount and the rate of toxin absorption are important. We showed previously that single doses as low as 50 ng/kg of Stx-1, having a specific cytotoxicity of 2 × 10^6 CD50/μg protein, predictably causes severe HUS (12). In the present study, we found that a dose of 25 ng/kg does not result in HUS. The threshold for HUS after a single rapidly administered dose of Stx-1, therefore, seems to be between 25 and 50 ng/kg. Moreover, a dose (e.g., 100 ng/kg) that produces severe HUS when administered rapidly does not when given slowly. In humans, there may be a threshold, relative to both quantity and rate of Stx absorption from the gut, that must be reached for HUS to develop.

Our primate model observations may have therapeutic implications relative to Stx-mediated HUS in humans. Strategies designed to block or slow the rate of Stx absorption from the gut, such as the oral administration Synsorb Pk (Sysorb Biotech, Inc., Calgary, Alberta, Canada) (29), should be helpful.

On the basis of cell culture studies (30) and experience with a mouse model (31), there is evidence that both Stx and LPS are important in the pathogenesis of postdiarrheal HUS. Although that may be true, LPS is not required in our primate model. We speculate that a sufficient dose of Stx alone is able to cause HUS in humans as well. That is not to say, however, that LPS might not amplify the response to Stx, and primate experiments are under way to study the role of LPS in Stx-mediated disease.

Acknowledgments

This research was supported by the National Institutes of Health (Grant no. 5R01 DK52083, awarded to R.S., T.J.P., V.L.T., and F.B.T. and Grant no. A1 34530 awarded to V.L.T.).

The authors gratefully acknowledge Andrew T. Pavia, M.D., Randall Lou, M.D., Nathaniél D. Denkers, Brett D. Welch, Mary Ann Harmon, the personnel of the University of Utah’s Animal Resource Center, and Jana Johnson, who assisted with the experiments, data collection, statistical analysis, and/or preparation of this manuscript.

References


---

Access to UpToDate on-line is available for additional clinical information at [http://www.jasn.org/](http://www.jasn.org/)