Animal Model

Characterization of the Baboon Responses to Shiga-Like Toxin

Descriptive Study of a New Primate Model of Toxic Responses to Stx-1

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The baboon response to intravenous infusion of Shiga toxin 1 (Stx-1) varied from acute renal failure, proteinuria, hyperkalemia, and melena with minimal perturbation of host inflammatory and hemostatic systems (high-dose group, 2.0 $\mu\text{g/kg}$; $n = 5$) to renal failure with hematuria, proteinuria, thrombocytopenia, schistocytosis, anemia, and melena (low-dose group, 0.05 to 0.2 $\mu\text{g/kg}$; $n = 8$). Both groups exhibited renal shutdown and died in 57 hours or less. Both groups produced urine that was positive for tumor necrosis factor and interleukin-6 although neither of these cytokines was detectable ($\leq 5 \text{ ng/ml}$) in the general circulation. Light and electron microscopy showed organelle disintegration and necrosis of the renal proximal tubular epithelium and of the intestinal mucosal epithelium at the tips of the microvilli, both of which were previously shown to bear Gb₃ receptors. The renal distal tubular epithelium was spared. The renal proximal tubular epithelial changes were accompanied by swelling of visceral epithelial cells (podocytes) and by swelling and detachment of endothelial cells of the glomerular capillaries. In addition, all of the animals receiving low-dose Stx-1 showed microvascular fibrin deposition and throm-

bosis in renal glomerular and peritubular capillaries in association with a fall in hematocrit and platelet count and a rise in schistocyte count. The gastrointestinal villous tip lesions were accompanied by varying degrees of mucosal and submucosal congestion, hemorrhage, or necrosis. Electron microscopic images of cerebral cortex and cerebellum showed diffuse unraveling of myelin sheaths with occasional disintegration of neuronal cell bodies. In contrast to the gastrointestinal mucosal and renal proximal tubular epithelium, the Gb₃ receptor glycolipid of the renal glomerular and neuronal tissues as determined using toxin overlay thin-layer chromatography plates was below the limit of detection ($< 13 \text{ pM/g wet tissue}$). We conclude that, depending on the status of the host and amount of toxin infused, Stx-1 can produce a variety of responses ranging from damage to cells carrying the Gb₃ receptor (renal proximal tubular epithelial cells and gastrointestinal mucosa) to damage to renal glomerular tissues with microvascular thrombosis as a result of the host's inflammatory response localized to the kidney. We conclude that this thrombotic coagulopathy arises from local changes in the kidney because the appearance of host inflammatory mediators was limited to the urine. This suggests that the initial host response is localized in the kidney, and that the systemic thrombocytopenia, anemia, and schistocytosis may arise secondarily. (*Am J Pathol* 1999, 154:1285-1299)

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In the decade following the seminal observation linking hemorrhagic colitis caused by Shiga-like toxin-producing (SLT) *Escherichia coli* with the subsequent development of acute renal failure,¹ our understanding of the structure, genetics, and mode of action of these potent bacterial cytotoxins has advanced rapidly. The *E. coli* SLTs and the Shiga toxin produced by *Shigella dysenteriae* type 1 comprise a family of multi-subunit toxins that are structurally and genetically related. SLTs are classified according to the extent of antigenic similarity to Shiga toxin. SLTs whose cytotoxic activity is neutralized by antisera to Shiga toxin are referred to as type I (Stx-1), whereas SLTs that are not cross-neutralized by anti-Shiga toxin antibodies are classified as type II (Stx-2).² All members of the toxin family 1) consist of pentamers of 7- to 8-kD binding subunits (B-subunits) in noncovalent association with single enzymatic subunits (A-subunits) of approximately 35 kD, 2) bind to target cells via the neutral glycolipid globotriaosylceramide (Gb₃) and globotetraosylceramide (Gb₄), and 3) mediate protein synthesis inhibition in target cells by the specific N-glycosidase activity of A-subunits that act at a site adjacent to adenine-4324 in the 28 S rRNA component of eukaryotic ribosomes.³⁻⁵

Despite impressive advances in the biochemistry and genetics of SLTs, the precise roles of SLTs and host immune response to the toxins in the development of bloody diarrhea and extra-intestinal sequelae remain to be elucidated. Histopathological examination of tissues from patients with SLT-associated acute renal failure (HUS) or thrombotic thrombocytopenic purpura (TTP) usually show striking microvascular lesions. In the kidney, glomerular endothelial cells are swollen and detached from the glomerular basement membrane, and glomerular capillaries frequently contain fibrin thrombi.⁶⁻⁸ Thrombocytopenia and the presence of fragmented erythrocytes (schistocytes) on blood smears also are observed. The constellation of acute-onset oliguria or anuria, azotemia, microangiopathic hemolytic anemia, and reduced platelet counts following prodromal diarrheal illness define the diarrhea-associated hemolytic uremic syndrome (D+ HUS).

Based on these clinical studies, the concept emerged that SLTs specifically target microvascular endothelial cells for damage. However, human endothelial cells derived from umbilical or saphenous veins are not sensitive to low concentrations of purified SLTs unless cultured in the presence of SLTs plus the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) or interleukin-1 (IL-1).^{9,10} Cytokine-mediated sensitization of endothelial cells is associated with increased synthesis and membrane expression of the toxin receptor Gb₃.^{11,12} Monocytes may participate in pathogenesis, as human monocytes also synthesize TNF- α and IL-1 when incubated with purified SLTs *in vitro*.¹³ Although these data suggest that SLTs and/or endotoxin-induced host factors target microvascular endothelial cells for damage, immunofluorescence staining of human kidney tissue for SLT binding suggests that Gb₃ is primarily localized to proximal tubules rather than glomeruli.^{14,15} Although histopathological studies of patients and *in vitro* experiments have been useful in

developing hypotheses regarding the pathogenic role(s) of SLTs in hemorrhagic colitis and D+ HUS, experimental evaluation of these hypotheses is hampered by the lack of experience with a subhuman primate model. Studies carried out using mice, rabbits, and pigs have supported the concept that the sites of elevated toxin receptor expression coincide with sites of tissue damage. Each of these animal models, however, has certain limitations.¹⁶ Experience with a subhuman primate model may provide information under controlled conditions concerning the range and variability of responses to Stx-1 that would be directly applicable to the disease as seen in humans. Using immunofluorescence and thin-layer chromatography techniques, we previously demonstrated that the sites and quantities of Gb₃ in human and baboon (*Papio anubis cynocephalis*) kidney tissues are similar.¹⁵ We report here additional experiments characterizing the baboon as an animal model for the study of Stx-1 (SLT-1) in which the following questions are addressed. 1) What are the responses to high and low concentrations of Stx-1 in the baboon, and do these responses mimic the hemolytic uremic syndrome (ie, acute onset of oliguria, azotemia, microangiopathic hemolytic anemia, and thrombocytopenia with histopathological evidence of glomerular capillary endothelial swelling and fibrin/platelet deposition)? 2) Are the proximal tubular and gastrointestinal mucosal epithelium, which contain the Gb₃ receptors, affected directly by the toxin? 3) Are adjacent structures (eg, glomerular capillary endothelium and podocytes) affected? 4) To what degree are either of these categories of responses (direct toxin *versus* host mediated) limited to the kidney and gastrointestinal tract, and to what extent are other systems, including the hemostatic, inflammatory, and central nervous systems, affected in this model?

Materials and Methods

Toxin

Purified Stx-1 was prepared from cell lysates of *E. coli* transformed with plasmids containing the structural genes under control of the phage T7 promoter.^{10,17} Stx-1 was purified by sequential chromatography over anion-exchange, chromato-focusing, and immunoaffinity columns. To reduce endotoxin contamination, Stx-1 preparations were passed over Cibachrome blue columns. All toxin preparations contained <0.1 ng of endotoxin per ml as assessed by *Limulus* amoebocyte lysate assay. The specific cytotoxicity of the toxin preparations was 2×10^6 CD50/ μ g of protein as assessed by vero cell cytotoxicity and Pierce protein quantitative kits. Toxin preparations were stored at 4°C. Dilutions of Stx-1 were prepared in sterile saline immediately before each experiment.

Pre-Experimentation and Experimentation Procedures

Papio c. cynocephalus or *Papio c. anubis* baboons were purchased from a breeding colony maintained at the University of Oklahoma Health Sciences Center or from

Biomedical Research Foundation, Houston, TX. Animals weighed 3.4 to 15.5 kg, had leukocyte concentrations of 5000/mm³ to 14,000/mm³, and hematocrits exceeding 36%. They were screened for tuberculosis. These animals were held for 30 days at the University of Oklahoma Health Sciences Center animal facility, and the infusion studies were done in this facility. The animals were observed continuously during the first 2 hours after infusion. All animals were followed until they died or were moribund, at which time they were euthanized. The kidney, small and large intestine, heart, lung, liver, spleen, adrenal, pancreas, and brain were examined, and specimens were collected for light microscopy within 1 hour of death. Samples from kidney, small and large intestine, and brain also were collected for electron microscopy. This study protocol received prior approval by the Institutional Animal Care and Use Committee of both the Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center.

Infusion Procedures

The baboons were fasted overnight before the study, allowed water *ad libitum*, and immobilized the morning of the experiment with Ketamine (14 ± 0.5 mg/kg intramuscularly). Sodium pentobarbital (2 mg/kg) then was infused every 20 to 40 minutes. The animals were intubated orally and allowed to breathe spontaneously.

A superficial femoral vein was exposed and cannulated aseptically and the catheter advanced to the inferior vena cava to sample blood and monitor central venous pressure. The femoral artery was cannulated to monitor arterial pressure. Each baboon was placed on its side in contact with a controlled temperature heating pad. Blood pressure and rectal temperature were monitored using a Statham pressure transducer and Gould recorder (Valley View, OH), and a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH), respectively. A percutaneous catheter in the cephalic vein was used to infuse saline or purified Stx-1 and sodium pentobarbital. Either saline, a high dose (2.0 µg/kg), or a low dose (0.05 to 0.2 µg/kg) of Stx-1 was infused as a bolus into 18 animals (see Table 1).

At the conclusion of the initial 2-hour observation period, the central venous line was secured subcutaneously by a heparin lock at its point of insertion into the superficial femoral vein for collection of additional blood samples and monitoring of central venous pressure. The percutaneous catheter in the cephalic vein was removed, and the animal was returned to the cage and allowed to recover.

Sampling and Monitoring Procedures

Blood and urine samples were obtained, and monitoring of vital signs and urine output was recorded at T-0 and at 2, 6, 12, 24, 36, 44 to 48, and 52 to 56 hours. The whole blood samples at each drawing included 1.0 ml anticoagulated with EDTA for complete blood count (CBC); 2.0 ml anticoagulated with 3.8% sodium citrate for fibrino-

gen,¹⁸ prothrombin time, tissue plasminogen activator (t-PA),¹⁹ thrombin-antithrombin complex (TAT),²⁰ factor VIIa,²¹ TNF, and IL-6²²; 1.0 ml in trasylol/thrombin for fibrin degradation products (FDP)²³; and 1.0 ml of clotted blood for blood urea nitrogen,²⁴ creatinine,²⁵ serum glutamic pyruvic transaminase,²⁶ and electrolytes. Not more than 10% of the animal's blood volume was collected during the first 12 hours. Urine was collected over a 1-hour period at each of the time intervals listed above via a Foley catheter, and the volume of urine produced during this hour was measured together with urine protein, hemoglobin, pH, glucose, and acetone. Aliquots of urine (1 to 2 ml) were frozen at -80°C for assay of TNF, IL-6, and FDP. Urine TNF and IL-6 were determined using commercial (Pharmingen, San Diego, CA) ELISA kits.

Precautions against development of hypovolemia were taken by measuring central venous pressure at each of these intervals and replacement of fluids (saline) using the following criteria: 1) weight less than control (initial period) weight: infuse sufficient normal saline to return animal to initial weight (1 g = 1 ml) at 1 ml/kg/minute; 2) blood pressure (BP) low (mean pressure <75): infuse normal saline, 10 ml/kg (1 ml/kg/minute), repeat as needed while following CVP and BP, and stop when mean BP >75, or sooner if CVP rises above 10; 3) CVP <3: infuse normal saline at 10 ml/kg (1 ml/kg/minute), and repeat as needed to raise CVP >3; 4) urine output <2 ml/kg/hour (0.5 ml/kg/15 minute) but weight, BP, and CVP are not low: infuse 10 ml/kg normal saline (1 ml/kg/minute) only once, and do not give if CVP >10.

Light Microscopy (LM) and Transmission Electron Microscopy (TEM)

Fixation

Representative tissue samples of each organ listed above were fixed in 10% neutral buffered formalin for routine histopathology and in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for plastic embedded light and electron microscopy. Sampling protocol for renal tissue included collection of tissue (coronal section) from both kidneys at three different sites (lower, mid, and upper poles). See below for the predetermined scoring system of the light microscopic observations. Sampling protocol for gastrointestinal (GI) tract tissues involved sampling of those areas that were visibly involved.

Processing for LM

For LM, tissues were fixed in neutral buffered formalin for at least 24 hours, processed by standard methods, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and phosphotungstic acid hematoxylin (PTAH).^{27,28} To illustrate the differential responses among animals and among organs within the same animal, tissues from animals exposed to a high concentration of toxin (2.0 µg/kg) were compared with those that received low concentrations of toxin (0.05 to 0.2 µg/kg). Gut microvilli were examined for histopatho-

logical changes in the villous tip, lacteal, villous base, and crypt. Renal cortex was examined for histopathological changes in the glomerular and proximal, distal, and collecting tubules. The outer renal medulla and papilla also were examined. The brain was examined for histopathological changes involving the cerebral cortex and pons.

Processing for TEM

Tissue samples (1 mm thick) fixed in 2.5% glutaraldehyde in PBS, pH 7.4, for 72 hours in the refrigerator were post-fixed in 2% OsO₄ in water for 1 hour, washed in PBS followed by distilled water, and then *en bloc* stained for 60 minutes in 2% uranyl acetate and 0.25% lead citrate in distilled water. The samples were dehydrated in a graded ethanol series, infiltrated overnight with a 50:50 mixture of propylene oxide and Embed 812 (Electron Microscopy Science, Ft. Washington, NY), and then infiltrated with pure resin for 6 to 8 hours.²⁹⁻³² Finally, the specimens were transferred to Beem capsules filled with pure resin and polymerized in a 60°C oven for 16 to 24 hours. Thick (1- μ m) sections from each block, stained with Paragon multi-stain (Paragon C&C Co., Bronx, NY) and mounted in Permount (Fisher Scientific, Pittsburgh, PA) were used to prepare survey photographs and to select areas for EM thin sectioning. Thin sections (silver to gray) stained for 4 minutes in 5% uranyl acetate in 25% methanol and 4 minutes in 0.3% lead citrate in water, both at room temperature, were examined in a Hitachi MET scope or a JOEL JEM 1200 EX-II.

Statistical Analysis and Scoring of Histopathology

Statistical Analysis

The clinical data were analyzed using an analysis of variance with Duncan's multicomparison test to determine significant differences ($P < 0.05$) between groups at given times and for certain variables. An analysis of variance, repeated measure design, with Dunnett's multicomparison test was also used to determine significant differences ($P < 0.05$) between time 0 (T-0) or baseline and subsequent times for a given group.

General Evaluation of Histopathology of All Organs

The pathological lesions of adrenal glands, kidneys, and lungs were analyzed by dividing their description into five categories: congestion, white cell influx, hemorrhage, thrombosis, and necrosis. The tissues were rated according to the severity of the histopathological lesions. The scale ranged from 1 to 4, with 4 being the most severe. All microscopic sections were read by Dr. Kosanke, who was blinded as to which study was being analyzed. The Kruskal-Wallis test, a nonparametric test, was used to determine significant differences ($P < 0.05$) between groups for a given pathological lesion.

Specific Evaluation of Renal Histopathology

One slide from each case was examined at $\times 400$ magnification until 100 consecutive glomeruli were assigned to one of eight categories: no thrombotic microangiopathy (TMA), endothelial swelling (ES), fragmented red blood corpuscles (FR), fibrin thrombi (FT), endothelial swelling with fragmented red blood corpuscles (ES + FR), endothelial swelling with fibrin thrombi (ES + FT), all three lesions (ES, FR, FT). All microscopic sections were read by Dr. Pysher who was blinded as to which study was being analyzed.

Results

Results of the baboon response to high and low dose Shiga-like toxin will be reviewed under the following headings: 1) clinical, routine laboratory, and histopathological findings, 2) detailed renal, GI, and central nervous system pathology by light and electron microscopy on plastic-embedded sections, 3) blood *versus* urinary cytokine response to Shiga-like toxin, and 4) blood inflammatory and hemostatic mediator response to Shiga-like toxin. The first two sections are descriptive and correlate the clinical and chemical aspects of the model with the light and electron microscopic histopathology. The second two sections, although descriptive, also concern mechanistic (inflammatory/hemostatic) aspects of this model.

Clinical, Routine Laboratory, and Histopathological Findings

We studied three different groups. These included a control and two groups receiving high and low concentrations of Shiga-like toxin 1 (Stx-1) (Table 1).

Control Group

The control group showed no significant changes in vital signs or markers of renal function (urine volume, protein and blood, creatinine, and potassium) or hemostatic activity (platelet count and fibrin degradation products). The total white cell count did rise from $8.7 \pm 1.5 \times 10^3$ cells per mm³ at T-0 to a peak 17.6 ± 0.1 at T + 6 hours. The count returned to baseline by T + 24 hours. No schistocytes above 1% were observed, and there were no significant changes in hematocrit. Table 2 shows evidence of only a slight degree of congestion in the tissues examined and, in the case of the lung, a modest influx of white cells. Table 3 shows that an average of $86.6 \pm 3.9\%$ of glomeruli had no evidence of thrombo-microangiopathy (TMA) with only $12.8 \pm 3.7\%$ showing any evidence of endothelial swelling. All animals of this control group were sacrificed at T + 72 hours.

High-Dose Group

Table 4 shows that the average response of animals 1, 2, 13, 14, and 15 to high-dose Stx-1 (2.0 μ g/kg) was

Table 1. Experimental Groups

Experiment	Sex	Weight (kg)	Infused Stx-1 ($\mu\text{g/kg}$)	Survival (hours)
Control group				
1	Male	5.5	Saline	Sacrificed at 72 hours
2	Male	5.2	Saline	Sacrificed at 72 hours
3	Male	14.4	Saline	Sacrificed at 72 hours
4	Male	11.7	Saline	Sacrificed at 72 hours
5	Male	16.2	Saline	Sacrificed at 72 hours
Average		10.6		
SE		2.3		
High-dose group				
1	Female	15.5	2.0	48
2	Male	3.8	2.0	22
13	Male	10.5	2.0	40
14	Male	11.6	2.0	32
15	Female	4.8	2.0	35.1
Average		9.2	2.0	35.4
SE		2.2	0	4.3
Low-dose group				
3	Male	3.4	0.2	45
4	Male	4.5	0.1	52
5	Male	7.6	0.1	48
6	Male	4.5	0.1	52
9	Male	4.8	0.05	57
B9	Male	5.0	0.1	72
B10	Male	5.0	0.1	70
B11	Male	5.1	0.1	60
Average		5.0	0.1	57
SE		0.4	0.0	3.5

characterized by 4+ proteinuria and by a sharp fall in urine volume from 2.3 to 0.7 ml/kg/hour by T + 12 hours followed by anuria. This was accompanied by creatinine and anion gap (GAP) peaking at 5.9 mg/dl and 36 mmol/L, respectively, and by severe hyperkalemia peaking at 15 mmol/L by T + 44 hours. The blood hematocrit, however, was stable at 41% from T-0 to T + 44 hours. There was no increase in schistocytes. In the kidney, the light histopathological changes were less pronounced in this group challenged with high concentrations of toxin than in that receiving low concentrations of toxin. Table 2

shows evidence of significant congestion and white cell influx in the adrenals, lungs, and liver. In addition, tissue from the GI tract (both small and large bowel) exhibited severe mucosal and submucosal hemorrhage, thrombosis, and necrosis. Table 3 shows that an average of $61.8 \pm 4.5\%$ of glomeruli had no evidence of TMA with only 28.7% and 6.3% exhibiting endothelial swelling and fibrin thrombi, respectively. The gross examination of the brains of the high-dose animals at the autopsy showed edema as reflected by flattened sulci. Light microscopy showed little or no evidence of injury to neuronal tissue of

Table 2. General Organ Histopathology of Control and Low- and High-Dose Groups

Organ	Congestion	White cell influx	Hemorrhage	Thrombosis	Necrosis
Control group					
Kidney	0.5 ± 0	—	—	—	—
GI tract	0.5 ± 0	—	—	—	—
Adrenals	0.6 ± 0.2	—	—	—	—
Lungs	1.8 ± 0.3	0.5 ± 0.1	—	—	—
Liver	1.1 ± 0.2	—	—	—	—
High-dose group					
Kidney	1.3 ± 0.3	—	—	—	—
GI tract	2.1 ± 0.3	—	1.8 ± 1.0	1.9 ± 0.9	1.9 ± 0.9
Adrenals	1.1 ± 0.6	0.7 ± 0.4	0.3 ± 0.2	0.5 ± 0.3	0.3 ± 0.1
Lungs	1.4 ± 0.2	1.0 ± 0.3	—	—	—
Liver	2.2 ± 0.3	1.1 ± 0.2	—	—	—
Low-dose group					
Kidney	1.6 ± 0.3	$0.06 \pm .06$	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
GI tract	2.3 ± 0.3	—	1.0 ± 0.3	—	—
Adrenals	1.6 ± 0.6	0.8 ± 0.3	0.7 ± 0.4	0.6 ± 0.4	0.1 ± 0.1
Lungs	1.9 ± 0.3	0.7 ± 0.1	0.2 ± 0.2	0.5 ± 0.4	—
Liver	1.6 ± 0.3	0.7 ± 0.2	—	—	—

Results are scored as follows: 0 to 4+ indicates severity of lesion with 4+ being the most severe; —, no change above background or baseline for that tissue was observed.

Table 3. Specific Renal Histopathology of Control and Low- and High-Dose Groups

Experiment	% of glomeruli with							
	No TMA	Endo swl	Frag RBC	Fib throm	ES + FR	ES + FT	FR + FT	ES, FR, FT
Control group								
1	77	23	—	—	—	—	—	—
2	88	11	—	—	1	—	—	—
3	96	4	—	—	—	—	—	—
4	78	20	2	—	2	3	—	—
5	94	6	—	—	—	—	—	—
Average	86.6	12.8	2	—	1.5	3	—	—
SE	3.95	3.77	—	—	0.50	—	—	—
High-dose group								
1	56	36	1	—	2	3	—	—
2	52	35	—	12	1	—	—	—
13	71*	19*	5*	5*	—	—	—	—
14	68	25	1	2	—	4	—	—
Average	61.75	28.75	2.33	6.33	1.5	3.5	—	—
SE	4.58	4.9	1.33	2.96	0.50	0.50	—	—
Low-dose group								
3	—	8	—	—	70	1	—	21
4	7.1	41	—	—	40	61	—	12
5	15	76	—	—	6	2	1	—
6	12	61	1	—	24	2	—	—
9	1	2	—	—	70	—	—	27
B9	8	54	—	—	11	22	—	5
B10	4	23	2	—	69	—	—	2
B11	4	55	—	—	39	1	—	1
Average	7.3	40.0	1.5	—	41.3	14.8	1	11.3
SE	2.2	9.4	0.5	—	9.3	9.8	—	4.4

See Materials and Methods for description of categories and grading system.

either gray or white matter in the cerebral cortex, pons, or the floor of the fourth ventricle. Survival time of the high-dose group averaged 35.4 ± 4.3 hours.

Low-Dose Group

Table 5 shows that the response of animals 3 to 6, 9, and B9 to B11 to low-dose Stx-1 (0.05 to 0.2 $\mu\text{g/kg}$) also

was characterized by a sharp fall in urine volume from 4.0 and 6.0 ml to 0.7 ml by T + 24 hours. This was accompanied by proteinuria at T + 24 hours and by the creatinine concentration and anion gap peaking at 2.0 mg/dl and 27.7 mmol/L, respectively, at T + 52 hours. The potassium concentration peaked at 5.2 mmol/L at T + 52 hours. This was accompanied by an increase in schistocytes from 2.7% to 8.5% during this interval. In the kid-

Table 4. High Dose (Laboratory Studies)

	T-0	T + 6 hours	T + 12 hours	T + 24 hours	T + 44 hours
Blood					
Platelets (%)	100	99 $\pm$ 8	105 $\pm$ 9	27 $\pm$ 7	12
Schistocytes (% of RBC)	<1	<1	<1	<1*	<1
Hematocrit	41 $\pm$ 1.0	41 $\pm$ 0.3	41 $\pm$ 1.0	41 $\pm$ 2*	41.5
Fibrinogen (%)	100	127 $\pm$ 24	235 $\pm$ 8	130 $\pm$ 12	135
FDP ($\mu\text{g/dl}$)	<10	<10	<10	22 $\pm$ 3	<10
WBC ($\times 10^3/\text{mm}^3$)	13.6 $\pm$ 5.6	14.8 $\pm$ 1.3	7.1 $\pm$ 2.5	5.7 $\pm$ 1.7	4.4 $\pm$ 0
TNF (ng/ml)	<5	<5	<5	<5	<5
IL-6 (ng/ml)	<2.5	<2.5	<2.5	6.7 $\pm$ 1.2	12
Vlla (%)	100	89 $\pm$ 3	90 $\pm$ 2	83 $\pm$ 8*	—
TAT (nM)	<1	<1	<1	<1	<1
t-PA (ng/ml)	4.7 $\pm$ 0.3	7.6 $\pm$ 5.0	16.1 $\pm$ 7.6	110 $\pm$ 8.0*	152
Renal					
Cr (mg/dl)	0.6 $\pm$ 0.6	0.6 $\pm$ 0	0.7 $\pm$ 0	1.7 $\pm$ 0.2	5.9
BUN (mg/dl)	25 $\pm$ 1.9	21 $\pm$ 4.5	23 $\pm$ 5	50 $\pm$ 5	91
GAP (mmol/L)	12.3 $\pm$ 1.0	11.7 $\pm$ 1.7	10.8 $\pm$ 1.4	20.3 $\pm$ 3.3	36.2 $\pm$ 8.8
K ⁺ (mmol/L)	4.2 $\pm$ 0.2	3.5 $\pm$ 0.1	3.8 $\pm$ 0.3	8.6 $\pm$ 1.5*	15
Urine volume (ml/hr/kg)	2.3 $\pm$.32	0.9 $\pm$.37	0.7 $\pm$.25	0	0
Urine protein (0–4+)	0.3 $\pm$ 0.1	2.6 $\pm$ 0.1	4 $\pm$ 0	4 $\pm$ 0	—
Urine blood (0–4+)	0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.6* $\pm$ 0.1	—

At T + 44 hours, only one animal survived for analysis. Results are shown $\pm$ SE.

*Significantly different from that of the same measurement made at the same time shown in Table 5 of the low-dose animals ($P \leq 0.05$).

Table 5. Low Dose (Laboratory Studies)

	T-0	T + 6 hours	T + 12 hours	T + 24 hours	T + 44 hours	T + 52 hours
Blood						
Platelets ($\times 10^3/\text{mm}^3$)	278 $\pm$ 51	257 $\pm$ 27	183 $\pm$ 26	78 $\pm$ 18	45 $\pm$ 9	67 $\pm$ 17
Schistocytes (% of RBC)	2.7 $\pm$ 0.6	—	1.4 $\pm$ 0.8	2.0 $\pm$ 1.2	6.3 $\pm$ 0.1*	8.5 $\pm$ 1.2
Hematocrit (mm)	36 $\pm$ 5.1	37 $\pm$ 0.9	35 $\pm$ 1.1	32 $\pm$ 1.1	30 $\pm$ 0.1*	28 $\pm$ 2.0
Fibrinogen (%)	100	127 $\pm$ 32	138 $\pm$ 29	297 $\pm$ 125	739 $\pm$ 495	1987 $\pm$ 1716
FDP ($\mu\text{g}/\text{dl}$)	<10	12 $\pm$ 4	20 $\pm$ 8.8	22 $\pm$ 8	46 $\pm$ 29	78 $\pm$ 41
WBC ($\times 10^3/\text{mm}^3$)	8 $\pm$ 2.3	9 $\pm$ 1.7	6.6 $\pm$ 1.2	6.0 $\pm$ 1.1	5.5 $\pm$ 1	5.5 $\pm$ 0.6
TNF (ng/ml)	<5	<5	<5	<5	<5	<5
IL-6 (ng/ml)	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Vlla (%)	100	107 $\pm$ 2	84 $\pm$ 2	75 $\pm$ 5	53 $\pm$ 11*	—
TAT (nmol/L)	<1	1.9 $\pm$ 0.9	1.2 $\pm$ 0.2	<1	<1	<1
t-PA (ng/ml)	3.8 $\pm$ 0.7	4.3 $\pm$ 0.4	5.0 $\pm$ 0.4	15.2 $\pm$ 2.6	22.1 $\pm$ 3.5*	42.3 $\pm$ 7.1
Renal						
Cr (mg/dl)	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.2	1.1 $\pm$ 0.2	2.0 $\pm$ 0.5
BUN (mg/dl)	19 $\pm$ 3	17 $\pm$ 4	19 $\pm$ 2	26 $\pm$ 5	35 $\pm$ 5	51 $\pm$ 7
GAP (mmol/L)	11.2 $\pm$ 2.0	9.6 $\pm$ 3.1	11.8 $\pm$ 1.7	13.8 $\pm$ 2.3	20.0 $\pm$ 3.4	27.7 $\pm$ 7.6
K ⁺ (mmol/L)	3.2 $\pm$ 0.5	2.5 $\pm$ 0.6	3.2 $\pm$ 0.2	3.3 $\pm$ 0.2	3.5 $\pm$ 0.3*	5.2 $\pm$ 0.5
Urine volume (ml/hr/kg)	4.0 $\pm$ 0.7	3.6 $\pm$ 1.1	6.0 $\pm$ 1.3	0.7 $\pm$ 0.2	0.3 $\pm$ 0.9	0.2 $\pm$ 0.1
Urine protein (0–4+)	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.7 $\pm$ 0.3	3.3 $\pm$ 0.3	4.0 $\pm$ 0	4.0 $\pm$ 0
Urine blood (0–4+)	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	1.9 $\pm$ 0.5*	2.7 $\pm$ 0.7*	3.7 $\pm$ 0.2

At T + 52 hours, six animals had survived for analysis.

*Significantly different from that of same measurement made at the same time shown in Table 4 of the high dose animals ($P = <0.05$).

ney, the histopathology was more pronounced in animals challenged with low concentrations of toxin than those receiving high concentrations of toxin. Table 2 shows evidence of significant congestion and white cell influx in all organs studied. In addition, hemorrhage was observed in tissues from the GI tract and adrenal glands. The GI tract lesions were much less severe than those observed in the high-dose group. Of particular significance in this group was the evidence of thrombosis and necrosis in the renal tissues. Table 3 emphasizes the importance of these renal findings by showing that on a systematic examination of these tissues for histological markers of renal involvement, only 7.3 $\pm$ 2.2% of glomeruli had no evidence of TMA with 39.9% and 41.3% exhibiting endothelial swelling and combined endothelial swelling and red corpuscle fragmentation, respectively. The brains of low-dose animals had normal gross appearances and weight. They were not submitted to microscopic or ultrastructural examination. Survival time of the low-dose group averaged 57 $\pm$ 3.5 hours.

Members of both groups manifested renal failure and either died or were moribund at times ranging from 22 hours to 72 hours (see Table 1). Fibrinogen concentration of both groups rose and peaked at 44 to 48 hours at concentrations ranging from 135% to 1987% of baseline whereas fibrin split products ranged from a peak of 22 $\mu\text{g}/\text{dl}$ in the high-dose group to 78 $\mu\text{g}/\text{dl}$ in the low-dose group. These changes involving hemostatic components were accompanied by a fall in platelet count to 45 $\times 10^3/\text{mm}^3$ or below in both the high- and low-dose groups.

The adrenals of animal 5 of the low-dose group were the most severely affected of all the animals in this study. This severity of the adrenal lesion was unique to this one animal. The lungs and liver of all animals

showed moderate congestion and mild to moderate leukocyte infiltration.

Light and Electron Microscopic Pathology of Renal, GI, and Central Nervous Systems

Renal Glomeruli

Figure 1 compares the glomeruli of control animals (Figure 1A) with those from the high- and low-dose Stx-1 groups. The glomeruli from the high-dose group (Figure 1B) are congested but exhibit no thrombotic angiopathy, whereas the glomeruli from the low-dose group (Figure 1C) show almost universal obliteration of the capillary lumina, frequent fragmentation of red blood corpuscles, and occasional fibrin thrombi. The tubules of control animals (Figure 1D) show no epithelial necrosis, whereas those of the high-dose group (Figure 1E) show extensive necrosis, and those of the low dose group (Figure 1F) show an intermediate degree of epithelial necrosis. Figures 2A and 3a (1- μm plastic sections) show glomeruli from the high-dose group (animal 13) congested with blood in the capillary loops, some endothelial cell swelling, and generalized edema with reduction of the urinary space within the glomerular capsule.

Figures 2B and 3, b and c (1- μm plastic sections), show glomeruli from the low-dose group (animal 9) with more extensive lesions than the high-dose animals. Fibrin clots were a common finding in the capillary loops; however, there was no swelling of the tuft and there was retention of ample urinary space within Bowman's capsule. By electron microscopy, the glomeruli from this low-dose group showed nonocclusive fibrin

Figure 1. In comparison with glomeruli of control animals (A), those from animals receiving 2 $\mu\text{g/kg}$ Shiga-like toxin (Stx-1) were congested but did not show a thrombotic microangiopathy (B), whereas those from animals receiving 0.05 to 0.2 $\mu\text{g/kg}$ Stx-1 showed almost universal obliteration of capillary lumina, frequent fragmentation of red blood cells, and occasional fibrin thrombi (arrows in C). In comparison with tubules of control animals (D), those from animals receiving 2 $\mu\text{g/kg}$ Shiga-like toxin (Stx-1) showed extensive epithelial necrosis (E), but this lesion was much less prominent in animals receiving 0.05 to 0.2 $\mu\text{g/kg}$ Stx-1 (F). H&E; magnification, $\times 100$.

deposits in the capillary loops and osmophilic inclusions in the visceral epithelial cells. All members of this low-dose group exhibited positive immunostaining for fibrin deposits in renal glomeruli and fragmented red blood corpuscles (Figure 4, A and B).

Renal Tubules

The most severely affected segment of the nephron was the proximal tubule (Figure 5). The figures illustrate the representative changes that occur in the high-dose

animals. The minimal changes seen were increased clear vacuoles in the tubular epithelium (Figure 5a) but with normal-appearing brush borders and peritubular capillaries. Early signs of tubular epithelial degeneration included shortening or loss of the brush border and occasional blebbing of epithelial cytoplasm into the tubule lumen (Figure 5c). One of the more common features of the proximal tubules from animals exposed to high concentrations of toxin was the presence of casts in the lumen of otherwise normal or near-normal proximal tubules (Figure 5e).

Figure 2. Light micrographs of 1- μ m plastic sections of renal glomeruli. **A:** Glomerulus from an animal challenged with high concentration of toxin. Note the congestion of red blood cells in some of the capillary loops (arrow) and the tuft expansion, which has reduced the urinary space within Bowman's capsule. **B:** Glomerulus from an animal challenged with low concentration of toxin. Note the fibrin thrombi in several of the capillary loops (arrows) but the absence of tuft expansion and retention of ample urinary space in the capsule. Also note the osmophilic inclusions (i) in the visceral epithelial cells. Paragon stain; bar, 20 μ m.

Figure 3. Electron micrograph of glomerular capillary loops from animals exposed to high (a) or low (b and c) toxin dose. **a:** Illustrates a minimal change seen in animals exposed to high concentrations of toxin. Note the swelling(s) of capillary endothelium and the numerous red blood cells in the capillary lumen. **b:** Illustrates early changes in animals exposed to low concentrations of toxin (ie, fibrin (f) in the capillary lumen and osmophilic inclusions (i) in the visceral epithelial cells). **c:** A capillary loop with significant fibrin (f) deposits. Uranyl acetate, lead citrate; bar, 5 μ m.

Figure 4. Thrombotic microangiopathy in the 0.05 to 0.2 μ g/kg group was manifested by increased numbers of schistocytes and burr cells in the peripheral blood (A) and by strongly positive staining for fibrinogen in glomerular capillaries (B). Experiment B10. **A:** Wright stain; magnification, $\times 500$. **B:** Fluoresceinated anti-human fibrin reactive antigen; magnification, $\times 50$.

In the low-dose group, the proximal tubules were more severely affected, and the damage was more widespread. The minimal changes seen were numerous osmophilic inclusions in tubular epithelium with intact brush borders (Figure 5b). Frank extrusion of epithelial cytoplasmic blebs was a frequent finding (Figure 5d). These cytoplasmic blebs varied in their size and content (some with cytoplasmic organelles and some without). The principal feature of the tubules in this low-dose group was the widespread damage (Figure 5f) in tubular epithelium that made it difficult to identify the tubule type. This type of damage was observed in all three zones examined (ie, cortex, outer medulla, and papilla). The distal tubules in all three groups were spared.

GI Tract

In contrast to the renal tubules, the degree of damage to intestinal mucosal villi was directly dose dependent (ie, the high-dose animals showed the highest degree of villous damage of both small and large bowel; Figure 6, a and c), and the low-dose toxin group showed significantly less damage (Figure 6, b and d). The most common features of the damaged villi in the high-dose toxin group were the loss of epithelial cells from the tip of the villi (Figure 6a) and blood-filled lacteal (Figure 6c). By comparison, the villi of the low-dose toxin group had

Figure 5. Light micrographs of 1- μ m plastic sections of renal proximal tubules from an animal exposed to high concentrations of toxin (**a**, **c**, and **e**) and a similar animal exposed to low concentrations of toxin (**b**, **d**, and **f**). **a:** Normal clear vacuoles in the epithelium and a normal-appearing brush border and peritubular capillary from an animal exposed to high toxin. **b:** Osmophilic inclusions in the epithelium and an intact brush border. **c:** Early epithelial degeneration manifested by loss of the brush border (**arrows**). **d:** Frank extrusion of epithelial cytoplasmic blebs, some with organelles (**arrow**) and some without. Note that the cells with the more extensive extrusions have lost more of their brush border. **e:** No damage to tubular epithelium. **f:** Widespread tubular epithelial cell damage. Paragon stain; bar, 20 μ m.

normal and intact epithelium even at their tips (Figure 6b) and only minimal changes in the submucosa, ie, mild increase in macrophages near the tip (Figure 6d).

Brain

Electron microscopic studies revealed subtle but significant changes in the brains of high-dose animals, in-

cluding the separation (unraveling) of myelin of the larger nerve fibers in all areas examined (Figure 7, A and B) plus perivascular edema (Figure 7B). The microvessels were normal appearing in all respects. The mitochondria and glial fibrils appeared normal, indicating adequate fixation and preservation during the EM processing. In some areas of the brain large neuronal cells and some glial cells showed degeneration. However, the degener-

Figure 6. Light micrographs of 1- μ m plastic sections of small intestinal villi from animals exposed to high concentrations of toxin (a and c) and an animal exposed to low concentrations of toxin (b and d). **a:** The tip of the villus from a high-toxin-dose animal has deteriorated, the epithelial layer is lost, and a lacteal (L) is filled with blood. Bar, 50 μ m. **b:** A villous tip from a low-toxin-dose animal is essentially normal except for an increase in inflammatory cells near the tip. The lacteal (L) is clear. Bar, 50 μ m. **c:** The base of the villus from a high-toxin-dose animal shows congested capillaries (arrows) and a blood-filled lacteal (L). Bar, 20 μ m. **d:** A villous tip from a low-toxin-dose animal shows macrophages scattered in the lamina propria near the tip. The capillaries (arrows) appear normal with no congestion. The lacteal (L) is near normal with only an occasional red cell in its space. Bar, 20 μ m. Uranyl acetate, lead citrate.

ation was focal and spotty, ie, degenerating neuronal cell body and two adjacent glial cells, one degenerating and one intact. This latter phenomenon was also observed in absence of degeneration of the adjacent neuronal cell body. Here, too, it is important to note that the mitochondria were normal in all neuronal cells but the degenerating ones.

Blood and Urinary Cytokine Responses to Shiga-Like Toxin (Systemic versus Local)

Table 6 shows that concentrations of TNF and IL-6 in the urine increased from 1055 to 4609 pg/ng and 434 to 4463 pg/ng creatinine, respectively, whereas the blood value of IL-6 increased only slightly during the same interval. The T-0 concentrations of TNF and IL-6 in the urine of normal baboons are <25 and 15 pg/ng creatinine, respectively.

Figure 7. Electron micrograph of brain tissue from an animal exposed to high levels of toxin. **A:** Cross section of a normal microvessel (ne, normal endothelial cell) surrounded by normal glial elements. Note the separation of the myelin (arrows), particularly in the large fibers. The preservation of the mitochondria throughout the tissue suggest that the myelin changes are not due to poor fixation. rc, red cell. Bar, 5 μ m. **B:** Longitudinal section of a normal-appearing microvessel with some perivascular edema (e) and myelin separation (arrows). rc, red cell. Bar, 10 μ m. Uranyl acetate, lead citrate.

Blood Inflammatory and Hemostatic Responses to Shiga-Like Toxin

The inflammatory response of the two experimental groups as reflected by the white cell count was remarkably mild (see Tables 4 and 5). Only the high-dose group showed an elevation in white cell count with a shift to the left at T + 6 hours. The lack of appearance of cytokines in the plasma in any of these groups also suggests a limited systemic inflammatory response. The appearance, however, of an acute-phase protein response as reflected by a two- to fivefold rise in fibrinogen in both groups probably reflects a low-grade inflammatory response of increasing intensity over time.

In contrast, the response of the hemostatic components was more revealing. Although no TAT complexes appeared in plasma, there was a reduction in factor VIIa concentrations in the low-dose but not in the high-dose group. The factor VIIa concentration fell as low as 53% of baseline before returning to normal. The fibrinogen concentrations, however, of both groups rose and peaked at 44 to 48 hours at concentrations ranging from 135% to 1987% of baseline while fibrin degradation products ranged from a peak of 22 μ g/dl in the high-dose group to 78 μ g/dl in the low-dose group. The rise in plasma t-PA concentrations, however, was the highest in the high-dose group. This is in contrast to the serum fibrin degradation product concentrations, which were the highest in

Table 6. Fibrin Degradation Product and Cytokine Concentrations in Urine and Blood From Combined Experimental Groups

Assays	T-0	2-6 hours	12-24 hours	36-48 hours
Urine				
TNF (pg/ng Cr)/ study	<25	1055	5060	4609
IL-6 (pg/ng Cr)/ study	<15	434	1111	4463
Blood				
TNF (ng/ml)	<5	<5	<5	<5
IL-6 (ng/ml)	<2.5	<2.5	3.6	3.5

Urine was collected at the beginning and end of each time range and the results were averaged.

the low-dose group. The response of hemostatic factors of neither the high- nor the low-dose group met criteria for disseminated intravascular coagulation (DIC) as the fibrinogen concentration rose and prothrombin times (not shown) remained stable in both groups (the prothrombin times of the high- and low-dose groups ranged from 12.1 to 13.2 and 12.6 to 14.1, respectively, within our normal range of 11.8 to 13.8 seconds). These relatively limited changes in the hemostatic factors, however, were accompanied by a fall in platelet count to $45 \times 10^3/\text{mm}^3$ or below in both groups by 44 to 48 hours.

Discussion

The principal characteristics of the lethal responses of both experimental groups to a single intravenous bolus infusion of Stx-1 were renal failure and varying degrees of injury to the mucosa of the GI tract. More specifically, the target tissues included the proximal tubular epithelium and the mucosa/submucosa of the GI tract, both of which bear the Gb₃ receptors for Stx-1.^{14,15,33} These changes were accompanied by glomerular lesions, including edema of podocytes throughout the glomerulus and focal areas of swelling and desquamation of capillary endothelium. The functional abnormalities associated with these lesions required at least 12 hours to develop and began with decreased urine volume followed within 24 hours by azotemia and proteinuria. Both groups shared these abnormalities, and members of both died in less than 72 hours. The distinguishing features of these two groups included hyperkalemia and hemorrhagic colitis in the high-dose group and hemolytic uremic syndrome in the low-dose group.

The association of proximal tubular epithelial and the GI mucosal/submucosal injury in all groups corresponded with the presence of Gb₃ receptors for Stx-1 and is in agreement with observations of numerous investigators using other animal species.³⁴⁻⁴⁰ In particular, our observation that bolus intravenous infusion of Stx-1 damages villous capillaries with the appearance of red cells in the extravascular space is in accordance with the earlier studies of Fontaine et al.⁴⁰ They showed that macaque monkeys fed intragastrically with a nontoxigenic strain of *Shigella dysenteriae* developed dysentery without

the destruction of capillary loops within the colonic mucosa characteristic of ingestion of wild-type *Shigella dysenteriae*. Our observations of a 12-hour lag period together with an inflammatory response that was limited to the rise in fibrinogen concentration suggests that despite Stx-1 being cleared rapidly³⁷ and having relatively high affinity (4.6×10^{-8} mol/L) for Gb₃,⁴¹ its direct effects on target tissue develop slowly. This may reflect the fact that the toxin through its glycosidase activity may require time to inhibit protein synthesis.⁴²⁻⁴⁵ Recent observations also have shown that verotoxins can induce an increase in prepro-ET-1 mRNA at a concentration of toxin that causes little or no effect on protein synthesis.⁴⁶

The renal glomerular lesions and the gut submucosal hemorrhagic lesions are not as readily explained as are those involving the renal tubular and intestinal epithelial cells that carry the Gb₃ receptors for Stx-1. Studies have shown, however, that human endothelial cells will respond to Stx-1 if they are co-incubated with TNF or IL-1^{9,10} and that these cells under these conditions will synthesize and express Gb₃.¹¹ Other investigators^{47,48} have observed that human renal tubular epithelial cells are capable of producing cytokines in response to endotoxin (LPS), which raises the possibility that cells adjacent to the renal glomerular endothelium could in some as yet described manner supply the cytokines that are thought to be necessary to induce expression of Gb₃ by endothelial cells. Studies by Karpman et al suggest that endotoxin and/or host cytokines work in conjunction with Stx-1 produced by *E. coli* 0157-H7.⁴⁹ They fed LPS-responder mice (C3H/HeN) and LPS-nonresponder (C3H/HeJ) mice *E. coli* 0157-H7 intragastrically and observed that the responder mice developed a severe combination of GI, neurological, and systemic symptoms, whereas the nonresponder strains developed a biphasic course of disease with milder symptoms followed later by neurological symptoms. They concluded that although both strains exhibited symptoms to the toxin, the difference in response between the two strains could be ascribed to the combined effects of toxin and LPS in the responder mice and to toxin alone in the nonresponder strain.⁴⁹ Our studies demonstrate that cytokines (TNF and IL-6) appear in the urine beginning at 12 hours after Stx-1 infusion and that at no time do they appear in quantities above 5 ng/ml in the plasma. This suggests, but does not prove, that these cytokines are produced locally and that there is a local but unequivocal host inflammatory response to Stx-1. This coincides with the above referenced studies suggesting that toxin and host inflammatory mediators could, acting together, expand the specific targeted effect of the toxin to involve adjacent tissues such as the glomerular visceral epithelium and capillary endothelium (in addition to proximal tubular epithelium) in the case of renal lesions, and the GI submucosa (in addition to the epithelium of the mucosal villous tips) in the case of the GI tract lesions. In neither case have these postulated events been established *in vivo*.

The observation that the low-dose group exhibited the hemolytic uremic syndrome suggests that the above described events can be expanded to produce a dysfunctional glomerular capillary endothelium with perturbation

of hematological and hemostatic elements. In addition to the light and electron microscopic pathology described above, this group of animals exhibited irreversible thrombotic lesions of the glomerular capillaries and the most clearly defined systemic signs of perturbation of the hemostatic system. Although much of the glomerular capillary endothelium appeared intact there were foci where the endothelium was edematous, formed blebs, and was either retracted at the intercellular junctions or completely separated from the subendothelium. Fibrin thrombi were observed both on intact endothelium and on exposed basement membrane as reported by others.⁸ There were some instances where platelets were observed outside these thrombi. Changes in the circulating hemostatic factors in the low-dose group did not begin until 24 hours at which time the platelet count and hematocrit fell. This was followed at 36 hours by the appearance of schistocytes and by the fall in concentration of factor VIIa. This suggests that the components of the hemostatic system did not participate in the chain of events leading to renal failure and uremia in the low-dose group. There was, however, evidence of perturbation of endothelium as reflected by the rise in tissue plasminogen activator concentration beginning at 24 hours. This was accompanied by up-regulation of the production of fibrinogen (acute-phase protein) and the appearance of fibrin degradation products, both of which peaked at T + 42 to 52 hours in the low-dose group. These peak fibrinogen concentrations tended toward being higher in the low-dose than in the high-dose group, although they did not meet statistical criteria for significance. In short, the profile of hemostatic activity in this low-dose group reflects a microangiopathic thrombosis with thrombocytopenia, a decline in hematocrit coupled with the appearance of schistocytes, and evidence of fibrinolytic activity (t-PA and FDP) in the absence of any systemic evidence of fibrinogen consumption or the appearance of thrombin-antithrombin complexes. Although there is evidence of systemic hemostatic activity, this appears to be a localized thrombotic coagulopathy as opposed to a systemic consumptive coagulopathy. This low-dose group accurately reflects what is usually seen in childhood post-diarrheal hemolytic uremic syndrome.⁵⁰ The lack, however, of a GI prodrome in this intravenous model of HUS may exclude the potentially important parallel pathophysiological effects of endotoxin originating from the gut. Finally, the observation that factor VIIa concentration fell and reached its nadir at T + 24 to 48 hours is a new observation. It suggests that the tissue factor/VII complex can, together with platelets, contribute to the microangiopathic thrombotic response whether it is expressed by an intact but dysfunctional endothelium or exposed by an endothelium that has retracted or desquamated. This has been observed after infusion of endotoxin into humans⁵¹ and has led to the speculation that tissue factor exposed or expressed as a consequence of reperfusion might act as a sink for factor VIIa during the 24- to 48-hour interval. This decline in factor VIIa concentration has not been observed, however, in patients with hemolytic uremic syndrome.⁵²

A surprising observation was the electron microscopic evidence of lesions in the central nervous system in the absence of focal neurological signs or brain Gb₃ receptors. Although gross examination revealed evidence of cerebral edema in the high-dose toxin group, the light microscopic studies revealed no structural abnormalities. In contrast, electron microscopic studies revealed an unraveling or separation of myelin sheath filaments uniformly throughout the central nervous system and focal areas of neuronal body degeneration. These findings were consistent with those of Fujii et al⁵³ in the mouse and Mizuguchi et al⁵⁴ in the rabbit. Interestingly, when Zoja et al³⁸ performed toxin overlay assays on chromatographically separated rabbit brain neutral glycolipids, they did not detect toxin binding to Gb₃; rather, only faint Stx-1 binding was detected in the ceramide dihexoside fraction. In accordance with this earlier study, our analysis of baboon neuronal tissue for Gb₃ receptor detected as little as 10 ng or 13 pmol/L Gb₃/mg wet weight of neuronal tissue using toxin overlay thin-layer chromatography plates. The affinity of Stx-1 for Gb₃ is estimated as 4.6×10^{-8} mol/L. Thus, assuming Stx-1 pentamer could pass the blood-brain barrier, it is possible that a productive interaction with picomolar quantities of Gb₃ or other neutral glycolipid species could produce the lesions observed. Alternatively, as with the renal pathology, host inflammatory mediators released locally could contribute to neuronal tissue injury. This possibility is supported by the observation that low but detectable concentrations of IL-6 were found in the spinal fluid of rabbits and the fact that astrocytes as well as microglial elements can produce cytokines.⁵⁵⁻⁵⁹ Cytokines themselves can cause inflammatory pathology in the brain.⁵⁹⁻⁶³ They are cytotoxic to oligodendrocytes *in vitro*⁶⁴⁻⁶⁶ and can alter the permeability of the blood-brain barrier capillary endothelium.⁶⁷ The actual steps involved in the production of these lesions are not clear from these studies. The absence of leukocyte infiltration has been described in the early stages of experimental meningitis,⁶⁸⁻⁷⁰ and the absence of elevated blood levels of cytokines, however, does favor injury due to toxin/cytokine interactions localized to the central nervous system.

The following conclusions are emphasized. First, the injury to renal tubular and glomerular structures can occur independently of microangiopathic thrombosis. The injury to these structures appears to arise from direct toxin damage as well as inflammatory mediator activity. Of special interest is the observation that the production of these mediators appears to be localized to the renal tissue. Second, although microangiopathic thrombosis may not be a necessary sole source of renal and GI tract pathology, the response, when it occurs, can amplify the initial injury (irreversibly). This response not only is driven by platelets but may also be driven by the expression of tissue factor by a dysfunctional capillary endothelium as reflected by the consumption of factor VIIa in these studies. Third, involvement of the central nervous system recalls anecdotal observations made in clinical practice⁵⁰ and the observations of Fujii et al⁵³ and Mizuguchi et al.⁵⁴ Again, like the renal phenomenon, the central

nervous system response may also be localized in response to host mediators.

Finally, we believe that this primate model in which a low-dose of Shiga-like toxin is infused as a single bolus mimics the hemolytic uremic syndrome, absent the initial diarrheal prodrome. We believe that it offers a base for future studies of mechanism and of conditions (route, method, duration of delivery, and coincident release of endotoxin) that give rise to variants of this syndrome.

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