The Role of Lipopolysaccharide and Shiga-like Toxin in a Mouse Model of Escherichia coli O157:H7 Infection

Diana Karpman, Hugh Connell, Majlis Svensson, Flemming Scheutz, Per Alm, and Catharina Svanborg

The role of lipopolysaccharide (LPS) and Shiga-like toxin (SLT) in the pathogenesis of hemolytic uremic syndrome (HUS) was studied in a mouse model. Mice inoculated intragastrically with Escherichia coli O157:H7 developed gastrointestinal, neurologic, and systemic symptoms, necrotic foci in the colon, glomerular and tubular histopathology, and fragmented erythrocytes. LPS-responder (C3H/HeJ) mice developed a combination of neurologic and systemic symptoms, whereas LPS-nonresponder (C3H/HeN) mice had a biphasic course of disease, first developing systemic symptoms and later severe neurologic symptoms. Mice inoculated with SLT-II-positive strains developed severe neurotoxic symptoms and a higher frequency of systemic symptoms and glomerular pathology compared with SLT-II-negative strains. Anti-SLT-II antibodies protected against these symptoms and pathology. These results demonstrate that this model could be used to study aspects of human HUS and that both LPS and SLT are important for disease development.

Hemolytic uremic syndrome (HUS) is characterized by a prodrome of hemorrhagic colitis followed by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure [1]. Neurologic manifestations are found in up to 30% of patients [2, 3]. Renal pathology involves predominantly the glomerular endothelial cell, but tubular epithelial cells may also be damaged [1].

Escherichia coli O157:H7 has been associated with outbreaks of diarrhea, hemorrhagic colitis, and HUS [4–7]. This strain belongs to the family of enterohemorrhagic E. coli (EHEC), which produce intimin [8], Shiga-like toxins (SLTs; also known as verotoxins) [9], lipopolysaccharide (LPS), and enterohemolysins [10, 11]. SLT and LPS have been suggested to be involved in the pathogenesis of HUS [12, 13]; the role of other bacterial properties has not been extensively studied.

A variety of animal models has been used to study the symptoms and histopathologic changes associated with human HUS. SLT-producing strains caused gastrointestinal, neurologic, or systemic symptoms (or a combination) and death in gnotobiotic piglets [14–16], rabbits [17], and mice [18, 19]. Histopathologic lesions, such as inflammatory colitis [15, 16], endothelial cell necrosis in the brain [14], and acute tubular necrosis of the kidneys [18, 19], were found in inoculated animals, but glomerular pathology was not observed. The role of SLT for disease was studied by injection of purified SLT; gastrointestinal, neurologic [20], renal, and systemic effects were reproduced [21–23]. Strains differing in SLT production have been studied in the piglet and rabbit colitis model [15, 17], but other HUS-associated symptoms and pathology were not compared.

LPS has been shown to cause renal changes and to act in synergy with SLT. Renal glomerular endothelial cell lesions mimicking those seen in human HUS have been demonstrated in rabbits intravenously infected with LPS [24, 25]. The lethal effects of LPS in C3H/HeN mice were enhanced by pretreatment of mice with Shiga toxin [22] or SLT-II [26]. LPS hypersensitivity was associated with a longer time to death in mice injected with SLT-II [27], but the susceptibility to E. coli O157:H7 infection has not been compared between LPS-responder and -nonresponder mice.

The aim of this study was to assess the relative importance of SLT-II and LPS in E. coli O157:H7 infection, using LPS-responder (C3H/HeN) and -nonresponder (C3H/HeJ) mice and SLT-II-positive and -negative strains.

Materials and Methods

Mice. C3H/HeN and C3H/HeJ mice were bred in the animal facilities at the Department of Medical Microbiology, University of Lund. C3H/HeN mice (Lps^a, Lps^b) differ from C3H/HeJ (Lps^a, Lps^b) mice in the ability to respond to LPS [28, 29]. Female and male mice were used at 8–16 weeks of age.

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This study was approved by the Animal Ethics Committee, University of Lund, Sweden.
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Bacteria. Strains of E. coli O157:H7 used for infection are listed in table 1. E. coli 86-24 and 87-23 were isolated during an outbreak of hemorrhagic colitis and HUS in Walla Walla, Washington, in 1986 and were provided by A. D. O'Brien (Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD) [30, 31]. E. coli 134 was isolated from a child with HUS and was provided by R. Möllby (Microbiology Tumor Center, Karolinska Institute, Stockholm). E. coli 86BL and 87BL were isolated from the blood of mice infected intragastrically with strains 86-24 and 87-23, respectively. Stock cultures were maintained in 15% glycerol at −70°C. E. coli FN414 was isolated from the stool of a healthy child [32]. This strain was selected as a fecal control strain since it lacked genotypic and phenotypic traits associated with the SLT-producing E. coli (table 1).

Before mouse inoculation, the strains were grown in Luria broth [33] overnight, harvested by centrifugation, and resuspended in 0.06 M PBS, pH 7.2, at a concentration of 10^9 cfu/mL. Strains used for inoculation and strains recovered from infected mice were subjected to genotypic and phenotypic analyses.

Genotyping by DNA-DNA hybridization. DNA probes specific for the SLT-I and SLT-II DNA sequences were derived from the recombinant plasmids pNTP705 and pDEP28, respectively [34, 35] (supplied by H. Smith, Division of Enteric Pathogens, Central Public Health Laboratory, London). The 0.75-kb EcoRI/HindIII fragment of pNTP705 contained the DNA sequences specific for SLT-I, and the 0.85-kb SmaI/PstI fragment of pDEP28 contained the DNA sequences specific for SLT-II. Isolates that hybridized with these probes were designated as SLT-positive. The DNA probe specific for eae (E. coli attaching and effacing) was the ~1-kb SaI1-KpnI fragment of the recombinant plasmid pCVD434 [8]. The 3.4-kb HindIII fragment of the recombinant plasmid pCVD419 was used as a probe for the EHEC 60-MDa plasmid [36]. The ~1-kb Hincll-KpnI fragment of pCVD432 was used as a probe for enteroaggregative E. coli [37]. The ~1-kb BamHI-SaI fragment of pJPN16 was used for detection of enteropathogenic E. coli adherence factor [38], and the 390-bp PstI fragment of pSLM862 was used for detection of diffuse adherence E. coli [39]. These probes were provided by J. B. Kaper (Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore).

Probe DNA was prepared by digestion with the appropriate restriction endonucleases. The fragments were separated using low melting agarose gel electrophoresis and isolated from the gel. The DNA fragments were labeled with [α-32P]dCTP using the Rediprime random primer labeling kit (Amersham, Solna, Sweden) or with digoxigenin as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Individual strains were taken from overnight cultures on tryptic soy agar plates, spotted on a Hybond N filter (Amersham) placed on a tryptic soy agar plate, and cultured for 4 h at 37°C. The filters were then washed twice in 2× SSC (0.3 M NaCl, 0.03 M TRIS, pH 8.0) and neutralized in 1.5 M NaCl/0.5 M TRIS (pH 7.2). The filter was dried and cross-linked by UV light treatment for 2 min. The prehybridizations were performed in 2× SSC containing plasmids R1 (62 MDa) and RP4 (34 MDa) [41] (provided by S. Molin and K. Nordström, Department of Microbiology, University of Odense, Odense, Denmark). The filters were then washed twice in 2× SSC and 0.1% SDS at room temperature for 5 min. This was followed by two washes in 0.1× SSC and 0.1% SDS for 15 min at 50°C. Filters probed with [α-32P]DNA were then exposed to Kodak X-Omat AR film for at least 24 h at −70°C. Filters probed with digoxigenin-DNA were detected per manufacturer's instructions.

Plasmid content. Plasmids were extracted from E. coli 86-24, 86BL, 87-23, 87BL, 134, and FN414 according to Kado and Liu [40]. Briefly, cells from 1 mL of an overnight Luria broth culture grown at 37°C were harvested and resuspended in 20 μL of TE (50 mM TRIS-Cl, pH 8.0; 1 mM EDTA). The cells were lysed by the addition of 200 μL of lysis buffer (3% SDS; 50 mM TRIS, pH 12.46; 100 mg/mL herring sperm DNA for 20 min. An equal volume (200 μL) of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the lysate and mixed by inversion. The aqueous phase was recovered by centrifugation at 15,000 g for 10 min. An aliquot of the supernatant was electrophoresed on 0.5% agarose. The following standard plasmid reference strains were used for size determination: E. coli J5-3 containing plasmids R1 (62 MDa) and RP4 (34 MDa) [41] (provided by S. Falkow, Department of Microbiology and Immunology, Stanford University, Stanford, CA) and E. coli 1100 with plasmid pSF2124 (7.3 MDa) [43] (provided by S. Molin and K. Nordström, Department of Microbiology, University of Odense, Odense, Denmark).

Phenotyping. Hemolysin production was examined on blood agar plates by using 5% washed defibrinated sheep blood [10].
Cytotoxicity was tested by a modified Vero cell assay [44–46]. The 50% cytotoxic dose was given as the toxin concentration that caused ≥50% of the Vero cells to detach from the plastic as assessed by dye uptake at A500 (Multiscan Plus, Labsystems, Helsinki).

**Infection protocol.** Mice were fasted (but given water) for 20–24 h before inoculation. The mice were then placed under ether anesthesia during which bacterial suspensions (0.1 mL of $10^8$–$10^9$ cfu/mL) were deposited intragastrically through a soft polyethylene catheter (outer diameter, 0.61 mm; Clay Adams, Parsippany, NJ). Immediately after inoculation, the catheter was removed and no further manipulations were performed. The controls received 0.1 mL of PBS.

Experiments with *E. coli* 86-24, 87-23, 134, and FN414 were carried out once. Experiments with *E. coli* 86BL and 87BL were done four times.

Mice were examined for gastrointestinal (changes in fecal color, frequency, and consistency), neurologic (paresis, paralyses, ataxia, tremor, rigidity, spasticity, convulsions, coma), and/or systemic (lethargy, anorexia, dehydration, anuria, ruffled fur, shivers, tachypnea, restlessness, body swelling) symptoms every 12 h after inoculation and sacrificed by cervical dislocation when signs of terminal disease were evident or 10 days after inoculation.

**Quantitation of infection.** Blood cultures were obtained by sterile heart puncture of infected and control mice just before sacrifice. Blood was drawn aseptically and immediately transferred to a BBL SEPTI-CHECK tryptic soy broth (Becton Dickinson, Cockeysville, MD) blood culture bottle (20 mL/bottle). The bottle was then incubated at 37°C and observed for turbidity and color change for 10 days. Positive cultures were subcultured on 4% sodium polypeptide agar (blood agar base 2; Oxoid, London). Blood agar plates were cultured under aerobic and anaerobic conditions (40%–60% N₂, 30% CO₂, 10% H₂). *E. coli* O157 was initially identified by a latex agglutination test (Oxoid Unipath, Basingstoke, UK), after which the somatic (O), capsular (K), and flagellar (H) antigens were determined [47].

**Histologic studies.** Kidneys and colons were surgically removed immediately after sacrifice or death of the mice and examined for gross pathologic changes. Tissues were then fixed in 4% formaldehyde in PBS (pH 7.4), embedded in paraffin, and sections were assessed for histopathologic abnormalities by light microscopy.

**Hematologic analyses.** Blood was obtained from the ophthalmic arteries or by cardiac puncture of infected and control mice just before sacrifice and kept in EDTA tubes. Blood films were prepared within 1 h, fixed in pure methanol, and stained with May-Grünwald-Giemsa solution.

**Passive protection by monoclonal antibody (MAb) to the SLT-II A subunit.** The hybridoma cell line 11E10 (ATCC CRL 1907) secretes IgG1κ specific for the A subunit of SLT-II but does not react with the B subunit [48]. For purification of this antibody, cells were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10% fetal calf serum (GIBCO Life Technologies, Täby, Sweden) at 37°C with 5% CO₂. Larger quantities of antibody were produced in a miniPERM Bioreactor (Heraeus Instruments, Hanau, Germany) by placing a 30-ml cell suspension (2 × 10⁶ cells/mL) in prewarmed culture medium in miniPERM culture vessels. These were incubated at 37°C with 5% CO₂ for 7–10 days with one change of medium. Cells were harvested by centrifugation at 600 g for 10 min at 4°C, and the antibody was purified from the supernatant using protein G-Sepharose 4 Fast Flow (20 mL; Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 20 mM sodium phosphate, pH 7.2, and washed with 20 mM sodium phosphate, pH 7.2. Antibody was eluted with 0.1 M glycine at pH 3.0 into 200 µL of 2 M TRIS. Fractions were pooled and dialyzed against PBS (0.06 M, pH 7.2). Protein was quantitated by BCA protein assay reagent (Pierce, Rockford, IL) with endoglobulin as the standard. The concentration of MAb that neutralized cytotoxin in the Vero cell assay was ~700 µg/mL.

The MAb to human CD40 S2C6, isotype IgG1κ, was used as a control (Mabtech, Nacka, Sweden). This antibody did not neutralize cytotoxin in the Vero cell assay.

Mice were injected intraperitoneally with 1 mL of MAb to SLT-II or to human CD40 at dilutions of 70 or 400 µg/mL, at 24 h and 1 h before intragastric inoculation.

**Statistics.** Differences between C3H/HeN and C3H/HeJ mice, between SLT-II–positive and SLT-II–negative strains, and between mice pretreated and not pretreated with MAb were evaluated with Fisher’s exact test. *P < .05* was considered significant.

**Results**

**Symptoms of disease in inoculated mice.** Mice (*n = 129*) were inoculated intragastrically with $10^7$–$10^8$ cfu of *E. coli O157:H7*. The mice were monitored for gastrointestinal, neurologic, and systemic symptoms. Gastrointestinal symptoms developed within 5–95 h after inoculation and consisted of loose, watery, bulky, or discolored stools. Mice did not shed any feces during the terminal stage of disease. Neurologic symptoms developed within 17–120 h after inoculation and consisted of hindleg weakness or paralysis, rigidity, jerky rhythmic motions, intention tremor, ataxia, spastic paralysis, convulsions, coma, and death. Systemic symptoms developed within 4–96 h and included lethargy, anorexia, dehydration, anuria, body swelling with edematous pouches, ruffled fur, loss of fur, shivering, tachypnea, and restlessness. Symptoms were followed by terminal disease and death in 51 mice (40%); the remainder recovered.

Mice (*n = 8, C3H/HeN*) inoculated intragastrically with the control strain *E. coli* FN414 did not develop gastrointestinal and neurologic symptoms. Four mice developed systemic symptoms with ruffled fur and weakness within 6 h. These mice had recovered partially after 9 h, and by 21 h after inocula-
Figure 1. Photomicrograph of hematoxylin-eosin–stained colon from C3H/HeJ mouse inoculated with E. coli O157:H7 87BL. This mouse developed systemic symptoms 45 h after inoculation and died 7 h later. Colon was removed immediately after death. Macroscopically, colon was swollen, with lack of feces. Preserved mucosal crypts lined with intact epithelium (with numerous black nuclei) are seen at left. Total coagulative necrosis of mucosa with no viable stromal or epithelial cells and absence of nuclei is demonstrated at right (arrow). Preserved muscularis propria is shown at bottom. Magnification, x200.

Figure 2A, B. In renal sections from symptomatic mice, thrombi involving the glomeruli were noted (figure 2A, B). Vascular congestion (an abundance of red blood cells [RBC] in the blood vessels), and diffuse interstitial inflammation were also noted (figure 2C, table 2). Necrosis of tubular cells was noted in the immediate vicinity of inflammatory infiltrates. In 1 mouse, interstitial abscesses and pus casts were observed in the tubular luminae of the renal cortex.

Two or more of these changes occurred in kidney sections from all sick mice (table 2). In contrast, microscopic changes were not observed in the kidneys of asymptomatic mice (5 C3H/HeN and 7 C3H/HeJ), in mice inoculated with E. coli FN414 (n = 4, C3H/HeN), or in PBS-treated mice (n = 6, 3 C3H/HeN and 3 C3H/HeJ).

Macroscopic inspection of kidneys (n = 47) removed from sick mice (n = 27) showed pronounced luminal dilatation, prominent congested blood vessels, and a lack of feces. Microscopic examination revealed inflammatory infiltrates, thinning of the intestinal wall, or necrotic foci involving the entire intestinal wall (figure 1). Histopathologic changes were not observed in colon from symptomatic mice (n = 8; 4 C3H/HeN and 4 C3H/HeJ), from mice inoculated with E. coli FN414 (n = 4), or from PBS-treated mice (n = 4; 2 C3H/HeN and 2 C3H/HeJ).

Macroscopic inspection of kidneys (n = 47) removed from sick mice (n = 35) and asymptomatic mice (n = 12) was normal in all but 1 mouse. The kidney of this C3H/HeN mouse was pale and swollen. Histopathologic changes in the kidney sections from symptomatic mice included focal proliferation of glomerular mesangial cells and increased deposition of mesangial matrix (figure 2A, B), vascular congestion (an abundance of red blood cells [RBC] in the blood vessels), and diffuse interstitial inflammation (figure 2C, table 2). Necrosis of tubular cells was noted in the immediate vicinity of inflammatory infiltrates. In 1 mouse, interstitial abscesses and pus casts were observed in the tubular luminae of the renal cortex.

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Increased deposition of fibrinogen was demonstrated in the glomeruli of 13 kidneys taken from symptomatic mice. Increased fibrinogen deposition was not demonstrated in the glomeruli of asymptomatic mice (3 C3H/HeN) or PBS-treated mice (1 C3H/HeN and 1 C3H/HeJ). Glomerular thrombi were not noticed in any of the mice.

LPS genotype and susceptibility to infection. The role of the Lps genotype for susceptibility to infection was examined by comparing C3H/HeN (Lpsn/Lpsn) and C3H/HeJ (Lpsd/Lpsd) mice (tables 3, 4). The mice differed with regard to the course of disease. C3H/HeN mice inoculated with SLT-II–positive strains developed a combination of severe neurologic and systemic symptoms within the first 4 days after inoculation. Mice that recovered from these symptoms did not develop any other symptoms. C3H/HeJ mice inoculated with SLT-II–positive strains had a biphasic course of disease; they first developed milder systemic symptoms than C3H/HeN mice (5 mice developed only severe restlessness). Mild neurologic symptoms such as hindleg weakness and rigidity were also noted initially. Mice recovered from these mild symptoms, but 5 days after inoculation they developed severe neurologic symptoms consisting of jerky motions, ataxia, intention tremor, spastic paralysis, convulsions, coma, and death. The biphasic course was not observed in C3H/HeN mice inoculated with the same strains. C3H/HeN mice inoculated with SLT-II–negative strains developed hindleg paralysis, not observed in C3H/HeJ mice inoculated...
with SLT-II-negative strains. There were no other differences in symptomatology between C3H/HeN and C3H/HeJ mice inoculated with SLT-II-negative strains.

There were no differences between C3H/HeN and C3H/HeJ mice with regard to the frequency or severity of gastrointestinal symptoms or with regard to histopathologic findings in the colons or kidneys.

The effect of SLT production on susceptibility to infection. SLT-II-positive strains caused more severe symptoms and more pronounced histopathologic changes than SLT-II-negative strains. The differences between SLT-II-positive and SLT-II-negative strains are summarized in table 4. A difference in the frequency of systemic symptoms was noted in C3H/HeN mice (P < .05) and in C3H/HeJ mice (P < .002). Severe neurologic symptoms developed 5 days after inoculation with SLT-II-positive strains in 9 C3H/HeJ mice and 1 C3H/HeN mouse. These symptoms were not observed in mice inoculated with SLT-II-negative strains, but mild neurologic symptoms such as hindleg weakness and paralysis were noted even in mice inoculated with SLT-II-negative strains. There were no differences between SLT-II-positive and SLT-II-negative strains with regard to gastrointestinal symptoms. Glomerular mesangial changes were noticed only in mice inoculated with SLT-II-positive strains (table 2). There were no other differences in histopathology between SLT-II-positive and -negative strains.

Similar differences in symptoms and pathology related to SLT-II expression were observed in subgroups including smaller numbers of animals.

Protection by anti-SLT-II antibodies. Mice were injected intraperitoneally with MAb 11E10, specific for the A subunit of SLT-II, or with the control antibody S2C6 and infected with the SLT-II-positive strain 86BL. C3H/HeN mice were protected from neurologic and systemic symptoms by pretreatment with 400 μg/mL MAb (3/15 mice developed systemic symptoms vs. 23/36 mice not pretreated with antibody, P < .01). Two kidneys from symptomatic mice showed vascular congestion and few interstitial inflammatory infiltrates, but glomerular changes were not observed. Asymptomatic mice exhibited normal histology (4 kidneys examined). A lower concentration of MAb (70 μg/mL) did not influence the frequency of neurologic and systemic symptoms (10/16 mice developed systemic symptoms); however, symptoms developed ~30 h later in antibody-treated mice compared with mice not pretreated with MAb. The kidneys from the antibody-treated mice (n = 5) showed the same histopathologic changes as those not pretreated with antibody.

The lower concentration of MAb (70 μg/mL) was sufficient to protect C3H/HeJ mice from symptoms (2/10 vs. 18/25 mice not pretreated with antibody, P < .01). The 2 symptomatic mice did not develop neurologic symptoms. Kidneys from these mice (n = 2, both asymptomatic when sacrificed) showed very few interstitial inflammatory infiltrates.

Figure 2. Photomicrographs of silver methenamine-stained sections. A. Renal cortex of C3H/HeN control mouse given 0.1 mL of PBS intragastrically and sacrificed 10 days later. Renal cortex was histologically normal. Arrowhead, normal glomerulus. Magnification, ×400. B. Renal cortex of C3H/HeN mouse inoculated with E. coli O157:H7 86BL. This mouse developed systemic symptoms and was sacrificed 24 h after inoculation. Arrowhead, glomerulus exhibiting proliferation of mesangial cells and increased deposition of mesangial matrix. Tubular cells are normal. Magnification, ×400. C. Renal cortex of C3H/HeJ mouse inoculated with E. coli O157:H7 86-24. This mouse developed systemic symptoms and died 5 days after inoculation. Kidneys were removed immediately after death. Arrowhead, focal influx of inflammatory cells in interstitium. Surrounding tubular cells show signs of necrosis with disintegration of cytoplasm and absence of nuclei. One histologically normal glomerulus is seen. Magnification, ×400.
Table 2. Histopathologic changes observed in mice with systemic symptoms.

<table>
<thead>
<tr>
<th>Mouse genotype, bacterial strain</th>
<th>Observed changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glomerular mesangial</td>
</tr>
<tr>
<td></td>
<td>Symptomatic</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td></td>
</tr>
<tr>
<td>SLT-II–positive*</td>
<td>+ (16/18)</td>
</tr>
<tr>
<td>SLT-II–negative†</td>
<td>–</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td></td>
</tr>
<tr>
<td>SLT-II–positive†</td>
<td>+ (7/7)</td>
</tr>
<tr>
<td>SLT-II–negative†</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE. +, histopathologic changes observed; −, histopathologic changes not observed.
* 34 of 48 mice developed systemic symptoms; 21 kidneys were examined, 18 from sick mice and 3 from asymptomatic mice.
† 9 of 22 mice developed symptoms; 7 kidneys were examined, 5 from sick mice and 2 from asymptomatic mice.
‡ 21 of 34 mice developed symptoms; 12 kidneys were examined, 7 from sick mice and 5 from asymptomatic mice.
§ 5 of 25 mice developed systemic symptoms; 7 kidneys were examined, 5 from sick mice and 2 from asymptomatic mice.

Table 3. Clinical and laboratory observations in mice.

<table>
<thead>
<tr>
<th>Mouse genotype, E. coli strain</th>
<th>Total no. of mice</th>
<th>GI</th>
<th>Neurologic</th>
<th>Systemic</th>
<th>Positive blood culture</th>
<th>Fragmentation of RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sick</td>
<td>Recovered*</td>
<td></td>
</tr>
<tr>
<td>C3H/HeN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLT-II–positive</td>
<td>48</td>
<td>23</td>
<td>21</td>
<td>34 (71%)</td>
<td>14</td>
<td>9/17 (55%)</td>
</tr>
<tr>
<td>SLT-II–negative</td>
<td>22</td>
<td>4</td>
<td>4</td>
<td>9 (41%)</td>
<td>2</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLT-II–positive</td>
<td>34</td>
<td>9</td>
<td>11</td>
<td>21 (62%)</td>
<td>11</td>
<td>5/8 (65%)</td>
</tr>
<tr>
<td>SLT-II–negative</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>5 (20%)</td>
<td>0</td>
<td>1/2 (65%)</td>
</tr>
</tbody>
</table>

NOTE. GI, gastrointestinal.
* No. of mice that had systemic symptoms from which they recovered; remaining mice died or were sacrificed due to terminal disease.
† Blood cultures and films taken from mice that did not recover, not available from all mice since some died during observation period; no. of positive assays/ no. of assays available from sick mice. Blood cultures (n = 11) and films (n = 9) taken from E. coli O157:H7–inoculated mice that remained asymptomatic were negative.
Table 4. Effect of LPS genotype and SLT production on susceptibility to infection.

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>SLT-II—positive</th>
<th>SLT-II—negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeN (Lpsa/Lpsa)</td>
<td>34/48 (71%)*</td>
<td>9/22 (41%)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>C3H/HeJ (Lpsa/Lpsa)</td>
<td>21/34 (62%)</td>
<td>5/25 (20%)*</td>
<td>.002</td>
</tr>
</tbody>
</table>

NOTE. NS, not significant. Data show no. of sick mice (systemic symptoms)/total no. of mice inoculated with this strain.

* C3H/HeN mice inoculated with SLT-II—positive strains vs. C3H/HeJ mice inoculated with SLT-II—negative strains: \( P < .001 \). Differences were evaluated by Fisher’s exact test.

infected with strains of \( E. coli \) O157:H7. Blood cultures were positive only in symptomatic mice and were not related to bacterial SLT production or mouse genotype (table 3). Three of the positive cultures were obtained from C3H/HeJ mice that developed neurologic symptoms \( \sim 120 \) h after inoculation. Blood cultures from 4 C3H/HeN mice inoculated with the control strain \( E. coli \) FN414 (taken 10 days after inoculation when mice were asymptomatic) were negative.

Discussion

\( E. coli \) O157:H7 causes hemorrhagic colitis and HUS [49]. These strains express virulence factors such as SLT and LPS that have been proposed to contribute to the induction of HUS [12, 13]. This study examined the relative roles of SLT-II and LPS in a mouse model of \( E. coli \) O157:H7 infection. Neurotoxic symptoms and glomerular changes were induced only by SLT-II—positive strains. C3H/HeN (LPS-responder) mice developed a severe combination of gastrointestinal, neurologic, and systemic symptoms, whereas C3H/HeJ (LPS-nonresponder) mice developed a biphasic course of disease with milder systemic symptoms followed by severe neurologic symptoms. The results indicate that both SLT and LPS are important for the pathogenesis of disease in this mouse model and that these factors act in a synergistic manner.

HUS is characterized by a prodrome of gastroenteritis consisting of watery or bloody diarrhea, followed by the development of acute renal failure manifest by oligo/anuria, hypertension, and edema [1]. Patients may develop extrarenal manifestations, predominantly neurologic symptoms such as ataxia, seizures, and coma [2, 3]. Pathologic findings in the colons of patients with bloody diarrhea and HUS include thrombosis and ischemia leading to necrosis of the mucosa and gangrene in severe cases [50]. Histopathologic examination of kidneys from these patients demonstrates glomerular and tubular damage [51] induced primarily by injury to endothelial cells. Mesangial swelling has been observed.

We were able to reproduce the gastrointestinal, renal, and neurologic symptoms and to find fragmented RBC and glomerular pathology. Other aspects of human disease were not investigated. Urine was not examined for hematuria and proteinuria because sick mice were anuric. Blood samples were not ana-

Figure 3. Photomicrograph of May-Grünwald-Giemsa—stained blood film from C3H/HeN mouse inoculated with \( E. coli \) O157:H7 86-24. Fragmented red blood cells are demonstrated. Arrows, helmet cells. Magnification, \( \times 1000 \).
lyzed for creatinine, erythrocyte volume fraction, and platelet and reticulocyte counts because the volume of blood left after cultures and blood films were taken did not suffice, and the formation of small blood clots in the tube lead to low levels of measurable RBC and platelets (a value that may be false positive).

The gastrointestinal symptoms consisted of loose stools or watery diarrhea. Focal necrosis was observed in the colons. Similar changes have been noted in previous animal models for EHEC infection in rabbits [17] and gnotobiotic piglets [16, 52] but not in mice pretreated with peroral antibiotics [19]. Colitis may be essential for the further development of renal, neurologic, and systemic symptoms. Symptoms indicating renal involvement consisted of body swelling with edematous pouches and anuria. These symptoms have not been reported in previous animal models. Anuria could indicate renal failure but may also be due to dehydration. The pathologic changes in the kidney included glomerular mesangial proliferation, deposition of fibrinogen in glomeruli, and tubular necrosis in areas surrounding inflammatory infiltrates. Earlier studies of EHEC infection or verotoxemia have demonstrated tubular but not glomerular changes [18, 19, 21, 22, 53, 54]. We observed severe neurologic symptoms, including rigidity, intention tremor, ataxia, spastic paralysis, convulsions, and coma. While ataxia, convulsions, and hindlimb paralysis have been previously observed [14, 15, 54, 55], this combination of severe symptoms has not previously been demonstrated.

RBC fragmentation is common in patients with HUS [5] but has not been previously demonstrated in animal models of E. coli O157:H7 disease. In this study, fragmentation of RBC was found in sick mice. It has been previously assumed that fragmentation is due to mechanical breakdown of RBC secondary to endothelial cell injury [56]. Recent studies have suggested that fragmentation can result from oxidative damage to RBC [57]. The presence of fragmented RBC in the mouse model without signs of endothelial cell damage may indicate that fragmentation does not require changes in the vascular endothelium.

We chose to use C3H/HeJ mice to examine the influence of LPS responsiveness on the symptoms and histopathology induced by E. coli O157:H7. C3H/HeN mice inoculated with SLT-II-positive strains developed a simultaneous combination of severe neurologic and systemic symptoms. In contrast, C3H/HeJ mice inoculated with SLT-II-positive strains had a biphasic course of disease and developed isolated severe neurologic symptoms at a later stage. These results indicate that C3H/HeJ mice may be protected from the initial severe disease by lack of response to LPS but susceptible to a prolonged course of disease due to defective clearance of bacteria.

C3H/HeJ mice have previously been shown to have an increased susceptibility to infection with gram-negative bacteria. These mice are highly susceptible to Salmonella typhimurium infection [58] and show defective bacterial clearance from the kidneys of both E. coli and S. typhimurium [59]. Thus, a hyporesponsiveness to LPS may influence the clearance of bacteria from the local site of infection. In the present study, such a defect might encourage bacterial proliferation and explain the prolonged biphasic course of disease seen in C3H/HeJ mice. In contrast, activation of the host response in C3H/HeN mice during the initial phase of disease would lead to a quicker clearance of bacteria from the local site and a faster recovery. It should be emphasized, however, that C3H/HeJ mice do not have a selective defect in LPS responses. Recent studies have suggested that the mice have a dysfunction in the ceramide signaling pathway and that they respond poorly to all agonists that activate this pathway [60].

SLT is cytotoxic for HeLa and Vero cells and inhibits protein synthesis in eukaryotic cells [44], including endothelial cells of the kidney [61] and brain [20]. This study compared strains differing in SLT production. The EHEC strains 86-24 (SLT-II-positive) and 87-23 (SLT-II-negative) were isolated during the same outbreak of diarrhea [30], suggesting a common origin. Although the strains were not shown to be isogenic, they did not differ in other known traits, including the eae<sup>+</sup>, eaf<sup>+</sup>, ehec<sup>+</sup>, agg<sup>−</sup>, da<sup>−</sup> genotype, and both carried 22-, 22-, and 22-MDa plasmids. Isolates of these strains were obtained from blood cultures after intragastric inoculation and were used for subsequent experiments with the assumption that in vivo passage might activate factors that are essential for the induction of disease and pathology. The symptoms and pathology found only in mice inoculated with SLT-II-positive strains were therefore attributed to SLT production. The most severe neurologic symptoms and glomerular mesangial changes developed only in mice inoculated with SLT-II-positive strains. Furthermore, the frequency of systemic symptoms was higher in mice infected with SLT-II-positive strains than SLT-II-negative strains. In agreement with previous studies [15, 62], gastrointestinal symptoms were not shown to be related to the presence of SLT. Furthermore, fragmentation of RBC and bacteremia were also not dependent on SLT production of the inoculated strain.

These results were confirmed by passive protection with anti-SLT-II antibodies. A higher concentration of antibody protected C3H/HeN mice from neurologic and systemic symptoms and glomerular pathology, indicating that these changes were caused by SLT-II. A lower concentration of antibody was sufficient to protect C3H/HeJ mice, suggesting that LPS may enhance the effect of SLT in C3H/HeN mice. The control antibody did not have a protective effect. The results are in agreement with previous studies that used MAbS to SLT-II to protect mice from the effects of E. coli O157:H7 and SLT-II [18, 21].

The results of this study suggest the following pathogenetic sequence: The bacteria establish in the colon, where they cause colitis with diarrhea. Focal necrosis of the gut wall may allow bacteria or bacterial components to cross the damaged intestinal...
wall and reach the bloodstream. It has been discussed whether *E. coli* O157 strains can invade through an intact intestinal mucosal barrier [63, 64]; this was not examined in the present study. The bacteriaemia observed here may not be relevant to human disease, since positive blood cultures are rarely found in patients with HUS. Furthermore, bacteriaemia did not appear to be essential for the development of gastrointestinal, neurologic, or systemic symptoms, since many symptomatic mice did not have positive blood cultures.

Symptoms and pathology from extraintestinal organs may be caused by the spread of bacterial constituents. The mechanisms by which LPS and SLT enhance disease at these sites are not well understood, but previous studies suggested that LPS and SLT act in synergy [22, 26]. In agreement with these observations, we found the highest frequency of symptoms in C3H/HeN mice infected with SLT-II-positive strains. This group of mice also developed the most severe combination of renal and extrarenal symptoms, suggesting that SLT and LPS had an additive or synergistic effect. This model is appropriate for the study of how these virulence determinants, singularly and in concert, cause the clinical and pathologic aspects of EHEC infection.

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References


