### Gnotobiotic Piglets Develop Thrombotic Microangiopathy After Oral Infection With Enterohemorrhagic Escherichia coli

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**Key Words:** Escherichia coli; Shiga toxin; Hemolytic uremic syndrome; Endothelium; Thrombotic microangiopathy; Disease models, animal

#### Abstract

Oral infection with enterohemorrhagic Escherichia coli (EHEC) may cause severe enteritis, followed in up to 10% of cases by an extraintestinal complication, the hemolytic uremic syndrome (HUS). HUS is characterized by a triad of symptoms: anemia, thrombocytopenia, and acute renal failure due to thrombotic microangiopathy. EHEC produces several virulence factors, among which a family of phage-encoded cytotoxins, called Shiga toxin 1 and Shiga toxin 2, seems to be most important. However, since an appropriate animal model is not available, pathogenicity of these emerging enteric pathogens is still poorly understood. Germ-free gnotobiotic piglets infected orally with an O157:H7 or an O26:H11 EHEC wild-type isolate, both producing Shiga toxin 2, developed intestinal and extraintestinal manifestations of EHEC disease, including thrombotic microangiopathy in the kidneys, the morphologic hallmark of HUS in humans. Thus, gnotobiotic piglets are suitable to further study the pathophysiology of EHEC-induced HUS. It can be expected that data obtained from this animal model will improve our current standard of knowledge about this emerging infectious disease.

The term hemolytic uremic syndrome (HUS) was first used in 1955 by Gasser et al1 of Switzerland, who described a syndrome, encountered in 5 children, that consisted of hemolytic anemia, thrombocytopenia, and acute renal failure. The patients had cortical necrosis of the kidneys, and cerebral symptoms also were observed. Since the original description, it has become apparent that HUS also may occur in adults.2 Today the disease is classified as typical and atypical HUS.3 The typical form is, in most cases, caused by Shiga toxin–producing Escherichia coli and has a prodromal episode of diarrhea (diarrhea-positive HUS) followed by the onset of hemolytic anemia, thrombocytopenia, and renal failure. The atypical form, which also is known as diarrhea-negative or sporadic HUS, manifests without prodromal diarrhea in both children and adults. Vascular lesions, described as thrombotic microangiopathy (TMA), are the morphologic hallmark of all forms of HUS.2

In 1983, Riley et al4 described 2 outbreaks of bloody diarrhea that were linked to meat contaminated with E coli O157:H7, a serotype that rarely was isolated before. The same year, Karmali and coworkers5 showed that diarrhea-associated HUS was caused by E coli strains of the same serotype, producing potent cytotoxins, now called Shiga toxins.6 The respective E coli strains were described as enterohemorrhagic E coli, or EHEC. EHEC is a subset of Shiga toxin–producing E coli strains, frequently causing severe disease in humans. Although E coli O157:H7, the prototype EHEC, still accounts for up to 70% of the strains from patients with hemorrhagic colitis or HUS, non–O157 EHEC is emerging,7 with E coli O26, O55, or O111 frequently being isolated. Shiga toxins comprise a family of multisubunit protein toxins that are classified according to...
their antigenic similarity to Shiga toxin produced from Shigella dysenteriae type 1 strains. Toxin molecules that are neutralized by antisera to Shiga toxin are referred to as Shiga toxin 1 (Stx1), whereas Shiga toxins that are not cross-neutralized by anti– Shiga toxin antibodies are called Shiga toxin 2 or Shiga toxin 2 variants (Stx2, Stx2v).8 Stx2e is an Stx2v that is produced by E coli isolates causing edema disease in pigs.9 All members of this family of E coli toxins are AB5 protein molecules. Five receptor-binding B subunits are linked to an enzymatically active A subunit. Toxin uptake into sensitive target cells is mediated via the neutral glycosphingolipids (GSLs) globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4) as specific receptors. After retrograde transport through the Golgi apparatus,10 the toxins exhibit protein synthesis inhibition by a specific N-glycosidase activity on the 28S ribosomal RNA component of eukaryotic ribosomes.11-13 Although compelling evidence indicates that EHEC is linked causally to typical HUS, no experimental animal model has been described in which the animal developed the classic syndrome after infection by the natural oral route.

We report the results of experiments characterizing gnotobiotic piglets as an animal model for the study of oral infection with EHEC. The response of these animals very closely resembled intestinal and extraintestinal features of human EHEC disease. This model is well-suited to further study the pathogenicity of EHEC-induced illness in humans, and it also might be used as a tool for other studies with respect to EHEC research, such as immunization and challenge trials.

Materials and Methods

Bacterial Strains

Two EHEC wild-type strains were used in this study. E coli 86-24 is an O157:H7 meat isolate that originated from an outbreak in Walla Walla, WA.14 E coli 126814 is positive for O26:H11 antigens and was isolated at the Hannover Medical School, Hannover, Germany, from a 4.5-year-old girl with severe HUS. Both strains produce Stx2.14,15 An apathogenic E coli isolate (Nissle 1917) was used as the control strain.16

Delivery and Rearing of Gnotobiotic Piglets

Piglets were derived from conventional sows in a German breeding program by cesarean section into a sterile surgical isolator. The sows were treated with gestagens (chlormadinone acetate, Gestafortin, Bayer, Leverkusen, Germany) at the 105th, 108th, and 112th days of gestation to prevent spontaneous farrowing. Azaperone (2 mg/kg) given intramuscularly and ketamine (10 mg/kg) given intravenously were used for general anesthesia. In addition, a spinal anesthetic of 0.7 mL of 2% lidocaine per 10 cm crown rump length was given epidurally. At 4 to 6 hours after surgery, when the piglets seemed healthy and started drinking, they were transferred pairwise from a transport isolator into rearing isolators. Every 2 hours they were given a milk diet supplemented with vitamins,17 distributed by an automatic feeding system. All isolators were gamma-irradiated with 60Co (Beta-Gamma-Service, Wiehl, Germany) 1 month before the cesarean sections were performed.

Oral Infection and Experimental Design

A total of 17 animals derived from 2 litters were divided into 3 experimental groups. Six piglets were given the E coli O157:H7 wild-type strain, 7 piglets were infected with the EHEC O26:H11 isolate, and 4 animals, which served as control animals, received the apathogenic E coli strain Nissle 1917. They all were inoculated perorally 10 to 12 hours after delivery with doses ranging from 5 × 10⁸ to 1.8 × 10¹⁰ colony-forming units of viable bacteria. Strains were grown at the day of surgery from overnight cultures diluted 1:100 in Luria Bertani broth. Liquid EHEC cultures were washed once to remove any toxin released into the supernatant and then diluted. The bacterial inoculum was applied in 5 mL of Luria Bertani broth. The animal experiments reported herein were approved on April 16, 1998, by the district authority of Hannover (509i-42502-98/56) and were conducted according to the German animal protection law.

Clinical Monitoring and Collection of Samples

A visual examination of all piglets was performed 6 times daily. Each animal received a score of 0 (normal), 1 (slight increase), or 2 (severe increase), based on the incidence and degree of diarrhea, locomotor disorders, and lethargy. Piglets assessed to have a clinical score of 2 were killed. During necropsy, tissue samples were obtained for light microscopy (LM), electron microscopy (EM), and immunohistochemical analysis. Feces were obtained directly from the large intestine for bacterial counting and the detection of Shiga toxins by an enzyme immunoassay (Ridascreen Verotoxin, R-Biopharm, Darmstadt, Germany). If possible, blood and urine samples were obtained immediately before infection and at different time points thereafter. Urine was obtained by cystocentesis.

Light and Electron Microscopy

Specimens and Fixation

All tissue specimens were immediately fixed in 4% neutral buffered formalin for LM and in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for EM. Kidney samples...
were obtained from 2 different locations of both organs for LM. A small cortical specimen from each kidney was obtained for EM. Gut sections (small and large intestine) were obtained from areas visibly inflamed or randomly, if no alteration was detectable macroscopically. From the central nervous system (CNS), samples were obtained from the cerebrum, the cerebellum, and the pons. Representative tissue samples also were obtained from the lung, liver, heart, pancreas, and adrenal gland.

**Tissue Processing and Staining for LM**

Fixed specimens were processed in a Hypercenter 2 instrument (Shandon, Frankfurt am Main, Germany) and embedded in paraffin by standard methods. Kidney sections were stained with periodic acid–Schiff and elastic van Gieson, tissues from the gastrointestinal tract with periodic acid–Schiff and Giemsa, samples from the CNS with H&E and Masson Goldner, and all other organs with H&E and elastic van Gieson.

**Tissue Processing for EM**

Small pieces of fixed tissue from the renal cortex, the colonic mucosa, and the cerebellum were washed in PBS and postfixed in 2% osmium tetroxide in double-distilled H₂O for 90 minutes. The samples were rinsed in 25% ethanol and dehydrated in a graded ethanol series. They then were infiltrated twice with toluol for 10 minutes, followed by a 30-minute incubation with a 1:1 mixture of toluol and Epon (Epon 812, Fluka, Buchs, Switzerland) at 40°C and 2 final incubation steps of 45 minutes each in pure Epon at the same temperature. For polymerization, the specimens were transferred to Beem capsules filled with pure Epon and incubated in a 40°C oven for 20 hours followed by a 60°C incubation step for another 40 hours. Thick sections (1 µm) from each block were stained with toluidine blue and used to select areas for EM thin sectioning. Thin sections (silver to gray) were stained for 5 to 10 minutes in 5% uranyl acetate and 5 minutes in 0.5% lead citrate and then examined using a Zeiss EM 10 electron microscope (Carl Zeiss, Oberkochen, Germany).

**Tissue Processing for Immunohistochemical Analysis**

Frozen sections were fixed in acetone (–20°C for 10 minutes) and air dried. After blocking endogenous biotin by incubation with avidin and biotin (Vector Laboratories, Burlingame, CA), the slides were incubated with a monoclonal rat anti-CD77 IgM antibody (clone 38-13; dilution, 1:10; Beckman Coulter, Unterschleissheim, Germany), which specifically recognizes the terminal galabiose residue from Gb3Cer, or with affinity-purified Stx2 (1 µg/µL) for 60 minutes at room temperature. Purity of the Stx2 preparation had been confirmed by polyacrylamide gel electrophoresis and silver staining. A biotinylated rabbit anti-rat IgM antibody (dilution, 1:250; Dianova, Hamburg, Germany) was detected by an alkaline phosphatase–conjugated streptavidin complex (DAKO, Hamburg, Germany). The slides were subsequently incubated with biotinylated tyramine (TSA Renaissance, R-Biopharm). The slides were subsequently incubated with biotinylated tyramine (TSA Renaissance, R-Biopharm). The slides were subsequently incubated with biotinylated tyramine (TSA Renaissance, R-Biopharm).

### Table 1

<table>
<thead>
<tr>
<th>Group/Animal No.</th>
<th>Infective Dose (CFUs)</th>
<th>CFUs per Gram of Stool</th>
<th>Weight (kg)</th>
<th>Day Killed or Died</th>
<th>Average Clinical Score</th>
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<tbody>
<tr>
<td>EHEC 86-24 (serotype O157:H7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5 x 10⁸</td>
<td>8.3 x 10¹⁰</td>
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<td>8.4 x 10⁹</td>
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<td>4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
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<td>1.9 x 10¹⁰</td>
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<tr>
<td>9</td>
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<td>11</td>
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</tr>
<tr>
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<td>14</td>
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<td>7.2 x 10⁸</td>
<td>6.1 x 10⁹</td>
<td>2.80</td>
<td>33</td>
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</table>

CFU, colony-forming unit; EHEC, enterohemorrhagic Escherichia coli.

* The CFUs per gram of stool and their weight were measured at the time of necropsy.
NenLifeScience, Boston, MA) in 0.003% hydrogen peroxide for 10 minutes at room temperature. The tyramine-bound biotin was again detected with an alkaline phosphatase–conjugated streptavidin complex (DAKO). Fast red (Sigma-Aldrich, Taufkirchen, Germany) served as substrate and hemalauna as counterstain. Appropriate negative controls were processed at the same time.

Evaluation

From each kidney of each animal, areas containing at least 20 artery cross-sections and parts of the cortex with at least 200 glomeruli, as well as parts of the medulla and the pelvis, were screened by LM. By EM, at least 3 glomeruli per renal specimen were evaluated. From the gastrointestinal area, varying locations of the small and large intestine were studied by LM, and additionally a small piece of tissue from the large intestine was studied by EM. Representative specimens from the cerebrum containing white and grayish substance, from the cerebellum, and from the pons were evaluated by LM. The cerebellum also was examined by EM. The other organs (lung, liver, heart, pancreas, adrenal gland) all were studied by LM.

GSL Extraction

Pork-kidney pieces of about 700 to 800 mg wet weight were homogenized with an Ultra-Turrax (IKA-Werke, Staufen, Germany) in 5 mL of water and then vacuum dried. By using an ultrasonic bath, the lyophilized material was extracted 2 times with acetone (6 and 3 mL, 30 minutes at room temperature). Both supernatants were discarded. The residual pellet then was extracted 3 times with 5 mL of methanol. The neutral GSL fraction was dried as described, resuspended in 10 mL of distilled water, and vacuum dried. Acidic GSLs were separated with the running solvent chloroform/methanol/0.2% calcium chloride (aqueous; 60/35/8). The dried plate was cut into halves. One part was stained with a solution of 2% orcinol in 2N sulfuric acid at 110°C for 30 minutes to detect GSLs and to quantify them. The other half was subjected to an immuno-overlay procedure with Stx2. This plate was bathed for 2 minutes in 0.5% Plexigum P28 (Röhm & Haas, Darmstadt, Germany) in chloroform/n-hexane (1/9) and then dried and blocked with buffer (PBS with 1% bovine serum albumin) for 1 hour at room temperature. It then was incubated overnight at 4°C in 2 mL of an Stx2 solution containing affinity-purified Stx2 at a concentration of 2 µg/mL. Bound toxin was detected with a polyclonal rabbit anti-Stx2-antibody (dilution, 1:1,000) and stained with an alkaline phosphatase–conjugated antirabbit IgG antibody (dilution, 1:5,000; Dianova), using fast red (Sigma-Aldrich) as substrate.

Quantification of Gb3Cer

The Gb3Cer band (Stx2 receptor) was quantified densitometrically using a dual wavelength TLC scanner (CS 910, Shimadzu, Duisburg, Germany) at a wavelength of 440 nm for orcinol-H₂SO₄–stained plates. Lactosylceramide (LacCer) of a known concentration was used as standard, taking the amount of sugar residues of LacCer and Gb3Cer into account. TLC-immuno-overlay and Gb3 quantification were repeated twice.

Results

Clinical Outcome of EHEC Infection in Gnotobiotic Piglets

All animals in the EHEC group developed watery diarrhea 2 to 3 days after infection. The first signs of extraintestinal disease were locomotor disorders, especially in the hind limbs, swaying back and assuming a dog-sitting posture. Finally, piglets showed apathy and lateral recumbency. In general, infection with the E coli O157:H7 strain 86-24 was much more severe than the disease caused by the E coli O26:H11 isolate 126814. Five of 6 animals in the EHEC O157 group died or had to be killed within 6 days of infection. Four of them had a clinical score of 2. From the EHEC O26 group, however, only 1 of 7 animals had to be killed 15 days after infection because of a severely deteriorated medical condition; this animal also had a clinical score of 2. The remaining 6 became accustomed to the illness. Clinical signs of extraintestinal disease were only light and

TLC and TLC Immuno-overlay With Stx2

By using a Linomat IV dispenser (CAMAG, Berlin, Germany), samples were loaded onto TLC plates on which the neutral GSLs were separated with the running solvent chloroform/methanol/0.2% calcium chloride (aqueous; 60/35/8). The dried plate was cut into halves. One part was stained with a solution of 2% orcinol in 2N sulfuric acid at 110°C for 30 minutes to detect GSLs and to quantify them. The other half was subjected to an immuno-overlay procedure with Stx2. This plate was bathed for 2 minutes in 0.5% Plexigum P28 (Röhm & Haas, Darmstadt, Germany) in chloroform/n-hexane (1/9) and then dried and blocked with buffer (PBS with 1% bovine serum albumin) for 1 hour at room temperature. It then was incubated overnight at 4°C in 2 mL of an Stx2 solution containing affinity-purified Stx2 at a concentration of 2 µg/mL. Bound toxin was detected with a polyclonal rabbit anti-Stx2-antibody (dilution, 1:1,000) and stained with an alkaline phosphatase–conjugated antirabbit IgG antibody (dilution, 1:5,000; Dianova), using fast red (Sigma-Aldrich) as substrate.
disappeared completely in 4 animals. A clinical score around 0.7 at the day animals were killed reflected the moderate state of disease in these animals. However, the average daily weight gain of all EHEC animals of about 36 g was only 38% of the average daily increase in body weight of 95 g for the control animals.

Clinical Chemistry and Hematologic Findings

Means and SDs of laboratory parameters analyzed in the blood and serum samples obtained from the animals at the day of birth and at different time points thereafter are given in Table 2 and Table 3. None of them reached statistically significant differences between the 2 experimental groups and the control animals (data not shown). However, in individual animals, the following parameters were greatly different from the mean. One EHEC 86-24 animal (animal 1), which died on the 5th day after infection, had a markedly elevated creatinine level of 232 µmol/L, a sign of kidney failure. The EHEC 126814 animal that had to be killed 15 days after infection (animal 11) had a dramatic increase in serum creatinine level, from 74 to 504 µmol/L between day 13 and day 14. Another EHEC O26 animal (animal 8) exhibited a peak in the serum creatinine level of 175 µmol/L on the second day of the experiment, which had decreased again to 85 µmol/L 4 days later, when the animal was killed. At the beginning of the experiment, values for hemoglobin, hematocrit, and platelets were low for all animals. Thrombocytopenia, a characteristic sign of HUS in humans, could not be observed. Fragmentocytes in peripheral blood smears, another characteristic sign of HUS, could not be assessed either, because all animals had severe poikilocytosis. Of 13 EHEC infected piglets, 7 had elevated serum creatinine levels at the beginning of the experiment.

Microbiologic Findings

Bacterial counts in the contents of the large intestine, obtained during necropsy, ranged from $1.6 \times 10^9$ to $8.3 \times 10^{10}$ colony-forming units of pure cultures, indicating that all E. coli strains were colonizing well and that sterility of the isolator system was maintained throughout the duration of the experiments. Results of enzyme-linked immunosorbent assay from the stool samples were positive for Shiga toxin with all EHEC animals and negative with the control animals. Spectrophotometric absorbance values of the Shiga toxin ELISA in the EHEC O157 group were generally higher (mean, 2.41) than those in the EHEC O26 arm of the trial (mean, 0.97), indicating higher amounts of toxin produced by the E. coli wild-type strain 86-24.

Pathologic Findings

Kidneys

Four animals from the EHEC O157 group and 1 animal from the EHEC O26 group showed petechiae on the surface of their kidneys. The kidneys of the control animals appeared normal. Microscopically, all animals of both EHEC groups

| Table 2 |
| Mean ± SD Blood Values Obtained at the Day of Birth (T0) and the Fourth (T4) and 11th (T11) Days of the Experiment for All Animals and the 33rd day (T33) for Animals From the EHEC 126814 Group |

<table>
<thead>
<tr>
<th>Variable</th>
<th>EHEC 86-24</th>
<th>EHEC 126814</th>
<th>E. coli Nissle 1917</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0 (n = 6)</td>
<td>T4 (n = 4)</td>
<td>T11 (n = 1)</td>
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<tr>
<td>Hematocrit (L/L)</td>
<td>0.19 ± 0.04</td>
<td>0.20 ± 0.04</td>
<td>0.28</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>59 ± 14</td>
<td>63 ± 13</td>
<td>93.0</td>
</tr>
<tr>
<td>Erythrocytes (× 10¹²/L)</td>
<td>3.88 ± 0.93</td>
<td>4.10 ± 0.75</td>
<td>5.70</td>
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<tr>
<td>Leukocytes (× 10⁹/L)</td>
<td>6.14 ± 1.54</td>
<td>5.29 ± 2.82</td>
<td>13.56</td>
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<tr>
<td>Lymphocytes (× 10⁹/L)</td>
<td>1.46 ± 0.43</td>
<td>2.22 ± 1.26</td>
<td>4.30</td>
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<tr>
<td>Segmented neutrophils (× 10¹⁰/L)</td>
<td>4.46 ± 1.11</td>
<td>1.70 ± 1.67</td>
<td>7.20</td>
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<tr>
<td>Unsegmented neutrophils (× 10¹⁰/L)</td>
<td>0.12 ± 0.07</td>
<td>0.48 ± 0.23</td>
<td>0.70</td>
</tr>
<tr>
<td>Monocytes (× 10⁹/L)</td>
<td>0.07 ± 0.10</td>
<td>0.38 ± 0.24</td>
<td>0.88</td>
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<tr>
<td>Platelets (× 10⁹/L)</td>
<td>394 ± 71</td>
<td>738 ± 151</td>
<td>825.0</td>
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EHEC, enterohemorrhagic Escherichia coli.
(n = 13), infected with either the *E coli* O157:H7 strain (n = 6) or the *E coli* O26:H11 isolate (n = 7), had diffuse glomerular endothelial swelling and glomerular congestion with narrowing of the capillary lumina. TMA was present in 5 of 6 EHEC O157 and 4 of 7 EHEC O26 piglets. Blood vessels showed typical morphologic criteria of TMA-like narrowing of the lumen with concentric intimal thickening, endothelial proliferation, and hyaline fibrin thrombi occluding the lumen Image 1A and Image 1B. Afferent arterioles were involved preferentially, but small arteries also showed the alterations, while larger arteries were unaffected.

Fragments of RBCs frequently were found in blood vessels and also in glomerular capillary loops, which were occluded focally by hyaline thrombi Image 2. Ultrastructurally, all animals infected with any of the EHEC strains showed diffuse and nearly global narrowing of the glomerular capillaries due to prominent endothelial swelling. Endothelial cells were detached segmentally from the basement membrane. The widened subendothelial space was filled with fibrin-like fragments, aggregates of platelets, and cell debris. These deposits were separated from endothelial cells by a thin layer of basement membrane–like material giving

<table>
<thead>
<tr>
<th>Variable</th>
<th>EHEC 86-24</th>
<th>EHEC 126814</th>
<th>E coli Nissle 1917</th>
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<tr>
<td></td>
<td>T0 (n = 6)</td>
<td>T4 (n = 4)</td>
<td>T11 (n = 1)</td>
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<tr>
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<td>T0 (n = 7)</td>
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<td>T33 (n = 3)</td>
<td>T0 (n = 3)</td>
<td>T4 (n = 4)</td>
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<td></td>
<td>T11 (n = 2)</td>
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<tr>
<td>Plasma protein (g/L)</td>
<td>23.1 ± 1.8</td>
<td>36.0 ± 3.9</td>
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<td>GLDH (U/L)</td>
<td>77 ± 6.7</td>
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<td>Creatinine (µmol/L)</td>
<td>152 ± 81</td>
<td>82 ± 8</td>
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<td>Blood urea (mmol/L)</td>
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<td>5.8 ± 2.1</td>
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<td>LDH (U/L)</td>
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<td>489 ± 87</td>
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<td>Sodium (mmol/L)</td>
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<td>147 ± 7</td>
<td>151.0</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5.7 ± 1.4</td>
<td>6.4 ± 0.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

EHEC, enterohemorrhagic *Escherichia coli*; GLDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; T0, day of birth; T4, T11, and T33, days 4, 11, and 33 of the experiment, respectively.

**Image 1** Thrombotic microangiopathy in small renal preglomerular arteries. **A**, The lumen is occluded by a hyaline fibrin thrombus (arrow) (periodic acid–Schiff, ×630). **B**, The lumen is narrowed substantially by activated endothelial cells, detached from the basement membrane and accompanied by an abundant intimal proliferation, giving the vessel a glomerulus-like appearance (arrow). Next to it a larger artery with an open lumen and a regular wall structure is visible (arrowhead) (elastic van Giesen, ×400).
rise to a double-contour appearance \textbf{Image 3}. Inside the capillaries, dysmorphic RBCs were observed (Image 3). Along with these changes, endothelial fenestration was missing. In 5 of 6 \textit{E coli} O157 and 4 of 7 \textit{E coli} O26 animals, mesangiolysis with disruption of the mesangium and loss of both anatomic boundaries and stainable matrix could be observed focally, mostly affecting subcapsular glomeruli. All EHEC-infected animals showed various degrees of mild to moderate tubular damage. Renal morphologic features of the control animals were shown as normal by LM and EM.

**Gastrointestinal Tract**

Five animals, 2 from each EHEC group and 1 control animal, showed mesenteric petechiae at the time of necropsy. Erosive colitis with loss of the surface epithelial cells was found in all EHEC-infected animals \textbf{Image 4A}. Crypt abscesses containing large amounts of neutrophils destroying the crypt also were observed. No mucosal damage was seen in the small intestine. Both EHEC strains colonized the colon heavily, while in the small intestine, colonization was lacking or only focal and mild. By LM, the surface epithelium appeared flat and irregular, and enormous numbers of bacteria invaded apoptotic or necrotic epithelial cell groups \textbf{Image 4B}. Ultrastructurally, both EHEC serotypes showed characteristic attaching and effacing lesions in the colon with bacteria intimately adhering to the enterocyte membrane, sometimes sitting on a pedestal-like structure \textbf{Image 5}, as described by Moon et al\textsuperscript{20} and Tzipori et al.\textsuperscript{21} In control animals, a regular mucosal architecture without inflammation was revealed.

**Central Nervous System**

The gross examination of the brains showed edema as reflected by flattened sulci in 4 EHEC O157 and 3 EHEC O26 animals. In 5 of 6 animals infected with \textit{E coli} O157, LM revealed focal microhemorrhages in the cerebellum \textbf{Image 6}. In contrast, in only 1 of 7 \textit{E coli} O26 animals were cerebellar microhemorrhages found. No case of TMA in the CNS could be detected. One EHEC O157 animal, 6 EHEC O26 animals, and all control animals had regular CNS morphologic features. Ultrastructurally, all EHEC-infected animals showed focal myelin degeneration with unraveling of myelin sheaths around the nerve fibers \textbf{Image 7A}. In 2 animals infected with the EHEC O157 strain, apoptosis of single cells was found in the cerebellum \textbf{Image 7B}. All control animals had normal CNS histologic features.

**Immunohistochemical Findings**

**Distribution of the Gb3 Receptor**

In the kidneys, specific staining for Gb3/CD77 was found in small preglomerular arterioles. No expression was detectable in large arteries, glomerular endothelial cells, or tubular epithelial cells. In the CNS, the arachnoid and endothelial cells of small arteries were receptor-positive \textbf{Image 8}. No specific binding of the antibody was observed in the colon \textbf{Image 9A}. 

When using Stx2 instead of the Gb3/CD77–specific antibody, the binding pattern was identical in these organs, as described. However, in the colon, the endothelium of submucosal small arteries exhibited strong staining Image 9B.

**Other Organs**

The gross examination of 4 EHEC O157 animals and 3 animals from the O26 group revealed subcutaneous edema.

**Liver**

One *E coli* O157 and 1 *E coli* O26 animal had shock reactions of their livers with marked centrilobular necrosis. No typical signs of TMA could be seen in the liver specimens studied. Seven animals, 2 from the EHEC
O157 group, 4 from the EHEC O26 group, and 1 control animal had regular morphologic features of the liver.

**Lung**

No TMA could be detected in the lung specimens studied. However, in 4 EHEC O157 and 5 EHEC O26 animals, shock lungs were found with marked interstitial and intra-alveolar edema. Two animals of each EHEC wild-type group and all control animals had regular morphologic features of the lungs.

**Pancreas, Heart, Adrenal Gland**

The other organs of all animals included in the study appeared normal. No TMA or necrosis could be detected.

**Quantification of Gb3Cer and TLC Immuno-overlay With Stx2**

TLC results with neutral GSLs extracted from kidney tissues of gnotobiotic piglets are depicted in **Image 10**. Densitometric estimation of Gb3Cer concentrations revealed quantities of 18.4 (± 2.3) µg/g of tissue wet weight. In the Stx2 immuno-overlay, the toxin bound strongly only to Gb3Cer and Gb4Cer. Weak binding to a yet uncharacterized glycolipid, migrating between Gb3Cer and the LacCer standard, can be observed in lanes 2 and 3 of Image 10. This band most likely is galabiosyleramide, which bears the same disaccharide epitope as Gb3Cer and Gb4Cer and which is known to separate very similar to LacCer in TLC.

**Discussion**

TMA of the kidneys is the morphologic hallmark of HUS in humans and is an absolute requirement for any animal model that can be used for in vivo infection studies with EHEC. Gnotobiotic and conventional piglets have been used as a model system for oral infections with diarrheagenic *E. coli* strains. But, for EHEC, pathologic observations...
were focused only on the gut and the CNS. Our observations also are at variance with those of Francis et al, who stated that glomerular damage was not observed in piglets infected with *E coli* O157, and they further stated that glomerular damage was not seen in swine afflicted with edema disease. Moreover, Kurtz et al, investigating the pathologic changes of pigs with edema disease several years earlier, also made no mention of any glomerular damage. Taylor et al have shown that baboons developed renal TMA as a response to the intravenous administration of Stx1. Our gnotobiotic piglets showed identical kidney histologic features; oral infection of the animals, however, resembled
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Strain of E. coli O157 is the high quantity of Stx2 expressed by our O157 strain of E. coli, 86-24, which leads to a high rate of Stx2 absorption from the gut, a hypothesis supported by the recent findings of Siegler et al. When studying the responses to single and divided doses of Stx1 in a primate model of HUS, these authors found that disease expression was modulated by the rate of intravenous Stx1 administration.

Immunohistochemical analysis revealed an interesting observation. Stx2 bound specifically to submucosal small arteries of the colon, although they stained negative for the Gb3 receptor. Despite impressive damage to the mucosa of the large intestine caused by both EHEC strains, no TMA was observed in colonic arterioles of these animals. Therefore, this seemingly Gb3-independent specific binding of Stx2 to submucosal colonic arteries may be part of a Shiga toxin uptake mechanism different from the receptor-mediated transport known so far. The presence of a vascular receptor for Stx2 gives an explanation of why gnotobiotic piglets are susceptible to infections with Shiga toxin–producing E. coli. Cattle, in contrast, that reportedly lack such a receptor are tolerant reservoir hosts for these E. coli strains.

The estimated Gb3Cer concentration in piglet kidneys of 18.4 µg/g of tissue wet weight was about 15- to 50-fold lower than values reported by Boyd and Lingwood for humans, ranging from 330 to 990 µg/g wet weight. Thus, it may be explained that all EHEC animals developed TMA histologically and ultrastructurally but had substantially fewer clinical symptoms than humans who have HUS, especially regarding kidney function.

A drawback of our animal model is the inconclusive results obtained with the laboratory parameters. In addition to morphologic changes, HUS in humans is characterized by anemia, thrombocytopenia, and renal failure. These symptoms could not be demonstrated convincingly with our model, except for single parameters in individual animals. One reason is certainly the very young age and immaturity of the piglets, which may lead to adverse reactions. Newborn piglets have immature kidneys, and nephrogenesis continues during the first months of life. This may be a reason for the inadequate response of the animals, regarding kidney function, to the EHEC-mediated renal endothelial damage. The inability of creatinine to equilibrate across the placental barrier was the reason for elevated serum creatinine levels found in 7 EHEC-infected piglets at the beginning of the experiment. This phenomenon has been discovered in neonatal foals and also can be observed with pigs. Elevated serum creatinine levels normalized with all animals that survived the initial days of the experiment. Thrombocytopenia, another classic parameter of humans with HUS, could not be detected in any animals. Rather, we observed thrombocytosis. This may be because the piglets were slightly dehydrated because of the heavy watery diarrhea, which increased the number of platelets relative to the blood volume. The low RBC counts and values of hemoglobin and hematocrit measured at the beginning of the experiment are most likely due to umbilical hemorrhage that occurred during the cesarean section.

In addition to observations made by other investigators, we showed that gnotobiotic piglets have vascular receptors for Stx2 and that they develop TMA on oral infection with Stx2-producing E. coli. Based on the data obtained by LM, immunohistochemical analysis, and ultrastructural examination, it will be possible to identify differences, even subtle ones, when isogenic EHEC deletion mutants are used for infection. Compared with their wild-type isolates, such strains are tools to identify the role of individual EHEC virulence factors in disease, thus providing new insights into the mechanisms of EHEC pathogenicity. This is a prerequisite for developing new strategies for therapy and prevention of this emerging infectious disease.

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Financial support was provided by a Hochschulinterne Forschungsförderung (HiLF) grant from the Hannover Medical School (to Dr Gunzer).

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Acknowledgments: We thank Dieter Bitter-Suermann for continuous support and encouragement of our work and Jochen H.H. Ehrich for helpful discussions. We acknowledge the excellent technical assistance of Elke Siever in performing the animal studies, Antje Müller and Jaqueline Wolfse, who analyzed blood and serum samples, and Benita von Tümpling-Radosta for GSL extraction.
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