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Escherichia coli O157:H7 Infection in Dutch Belted and New Zealand White Rabbits

Aruna Panda,[1,*] Ivan Tatarov,[1] Angela R Melton-Celsa,[4] Krishnan Kolappaswamy,[1] Edwin H Kriel,[1] Daniel Petkov,[1] Turhan Coksaygan,[1] Sofie Livio,[2] Charles G McLeod,[1] James P Nataro,[2] Alison D O'Brien,[4] and Louis J DeTolla[1,3]

Enterohemorrhagic *Escherichia coli* (EHEC) produce one or more types of Shiga toxins and are foodborne causes of bloody diarrhea. The prototype EHEC strain, *Escherichia coli* O157:H7, is responsible for both sporadic cases and serious outbreaks worldwide. Infection with *E. coli* that produce Shiga toxins may lead to diarrhea, hemorrhagic colitis, or (less frequently) hemolytic uremic syndrome, which can cause acute kidney failure. The exact mechanism by which EHEC evokes intestinal and renal disease has not yet been determined. The development of a readily reproducible animal oral-infection model with which to evaluate the full pathogenic potential of *E. coli* O157:H7 and assess the efficacy of therapeutics and vaccines remains a research priority. Dutch belted (DB) rabbits are reported to be susceptible to both natural and experimental EHEC-induced disease, and New Zealand white (NZW) rabbits are a model for the intestinal manifestations of EHEC infection. In the current study, we compared the pathology caused by *E. coli* O157:H7 infection in DB and NZW rabbits. Both breeds of rabbits developed clinical signs of disease and intestinal lesions after experimental infection. In addition, one of the infected DB rabbits developed renal lesions. Our findings provide evidence that both breeds are susceptible to *E. coli* O157:H7 infection and that both may be useful models for investigating EHEC infections of humans.

Abbreviations: EHEC, enterohemorrhagic *E. coli*; HUS, hemolytic uremic syndrome; DB, Dutch belted; STEC, Shiga-toxin–producing *E. coli*; NZW, New Zealand white.

Escherichia coli O157:H7 is the prototype enterohemorrhagic strain of Shiga-toxin–producing *E. coli* (STEC), which cause food- and waterborne outbreaks and sporadic cases of serious intestinal disease that manifest as diarrhea or hemorrhagic colitis (or both).[12,13,31] Enterohemorrhagic *E. coli* (EHEC) are a subset of STEC that, in addition to elaborating Shiga toxins, encode the locus of enterocyte effacement, whose products allow intimate attachment of the bacteria to the epithelium.[16,19] Children infected with STEC are more susceptible than adults and may subsequently develop hemolytic uremic syndrome (HUS) that is characterized by hemolytic anemia, thrombocytopenia, and kidney dysfunction or failure.[29] Shiga toxins are considered to be major determinants involved in the pathogenesis of these *E. coli*-induced infections. Indeed, the capacity of STEC to cause bloody diarrhea and HUS derives from the activity of the Stx.[8,25,30,40] The 2 types of Shiga toxins, Stx1 and Stx2, are quite similar in sequence and structure, although their polyclonal antisera do not crossreact.[7,38,39,42]

A vaccine is currently not available to protect humans from infection or disease caused by STEC. There is a need to define the pathogenic mechanisms by which STEC cause disease and to develop strategies for the prevention and treatment of STEC-mediated HUS. Achieving this goal would benefit from a small animal model that displays gastroenteritis or signs of HUS similar to those occurring in humans. Naturally infected DB rabbits mimic the clinical and pathologic signs (including diarrhea, hemorrhagic colitis, and HUS) produced by STEC in humans.[11] In addition, infant NZW rabbits become infected with EHEC and subsequently exhibit diarrhea and hemorrhagic colitis.[20,28,34,36] The current study compared DB and NZW rabbits for breed-specific differences in response to *E. coli* O157:H7 infection.

Materials and Methods

Bacteria. *E. coli* O157:H7 wild-type strain 933, which produces both Stx1 and Stx2, was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). It was originally isolated during an outbreak of hemorrhagic colitis that occurred in the United States in 1982.[24,33] An overnight culture of strain 933 was prepared as described elsewhere.[9] Luria–Bertani broth and agar were used for culturing of bacteria. Serial 10-fold dilutions of strain 933 were plated and incubated overnight at 37 °C to calculate the number of viable bacteria. Rabbits in the infected groups were inoculated with 10^9 cfu of strain 933.

Animals. The study was conducted at the AAALAC-accredited animal facility in the Program of Comparative Medicine at the University of Maryland School of Medicine. All procedures conformed to the guidelines of the *Guide for the Care and Use of Laboratory Animals*[15] and the policies of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. All procedures complied with the *Biosafety in Microbiological and Biomedical Laboratories*.[3]

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[1]Comparative Medicine Program, Department of Pathology and [2]Center for Vaccine Development, Division of Infectious Diseases and Tropical Pediatrics, Department of Pediatrics, and [3]Department of Medicine (Infectious Diseases) and Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, Maryland; [4]Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland.
[*]Corresponding author. Email: apanda@vetmed.umaryland.edu

SPF male DB and NZW rabbits (*Oryctolagus cuniculus*; age, 5 to 8 wk) were purchased from an approved vendor (Covance Research Products, Denver, PA) and used in this study. The pathogen status of the rabbits was verified through health reports from the vendor prior to study initiation. Rabbits used in this study were free of bacteria including *Escherichia coli*, *Clostridium difficile*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Bordetella bronchoseptica*, *Treponema cuniculi*, *Clostridium piliforme*, cilia-associate respiratory bacillus, *Salmonella* spp., and specific protozoa, ectoparasites, and fungi. Because infection with *C. difficile* can produce clinical signs and pathology similar to those produced by *E. coli* infection,[12,21] fecal samples from all animals used in the study were screened by PCR for *C. difficile* prior to study initiation. In addition, rabbit feces were screened for the presence of bacteria unable to ferment sorbitol because after infection the feces were to be evaluated for the presence of sorbitol-nonfermenting strain 933 on sorbitol–MacConkey agar. Finally, these animals were evaluated for clinical signs such as diarrhea or hemorrhagic colitis. All of the rabbits used in the study were free of *C. difficile*, sorbitol nonfermentors, and clinical signs of illness at the time of inoculation. Animals were acclimated for a period of 48 h before initiation of the study. Animals were cared for and housed individually in cages according to the guidelines of the *Guide for the Care and Use of Laboratory Animals*.[15] Feed and water was available ad libitum to all rabbits throughout the study period.

Experimental design. The 16 rabbits (8 DB and 8 NZW) used in this study were divided by breed into a control group of 2 animals and an experimental group of 6 animals. Rabbits were fasted overnight to reduce the contents of the intestinal tract and promote bacterial colonization. Water was removed from the rabbit cages 2 h before inoculation. Animals were anesthetized with 35 to 50 mg/kg ketamine (KetaVed Ketamine HCl Injection, USP Vedco, St Joseph, MO) combined with 3 to 5 mg/kg xylazine (AnaSed Injection, Ben Venue Laboratories, Bedford, OH) given intraperitoneally through a 25-gauge needle. Before inoculation, blood samples (volume equivalent to 1% of an animal's body weight) were obtained from an ear vein for CBC and blood chemistry analyses. Blood was collected by access of the ear vein. Each animal (including the controls) then was gavaged with 10 mL 10% sodium bicarbonate solution to increase the gastric pH and facilitate colonization by the bacteria in the animals' gastrointestinal tracts.

Anesthetized rabbits were inoculated by intragastric gavage through an infant feeding tube (8 to 14 French). Each animal in the experimental group was gavaged with 10^9 CFU of *E. coli* O157:H7 strain 933 suspended in 1 mL PBS. Rabbits in the control group were gavaged with 1 mL PBS only. After inoculation, rabbits were placed on thermostatically controlled warm-water heating pads for thermal support until they were able to remain sternal after recovery from sedation. The animals then were returned to housing cages, where they were permitted free access to food and water. One DB rabbit (no. 67) in the infected group died shortly after inoculation; necropsy findings indicated acute aspiration pneumonia. Therefore, the study was continued with 5 DB rabbits in the infected group.

Clinical assessment. After inoculation, rabbits were monitored daily for development of clinical signs in the form of diarrhea, hemorrhagic colitis, fever exceeding 104 °F, weight loss, or lethargy. The clinical treatment plan included treating animals with analgesics (Buprenorphine Hydrochloride Injection, PharmaForce, Columbus, OH; 0.02 to 0.05 mg/kg SC every 6 to 12 h) if they displayed signs of disease (that is, diarrhea or hemorrhagic colitis) accompanied with pain and discomfort (lethargy and hunched posture). Subcutaneous Ringers lactate solution (25 mg/kg daily; Lactated Ringers Injection USP, B Braun Medical, Scarborough, Ontario, Canada) was administered if a 5% weight loss occurred that was considered to be due to dehydration secondary to diarrhea in infected animals. Rabbits that did not respond to therapy were euthanized at alternative time points in light of the severity of the clinical signs and on defined alternative endpoints, including weight loss of more than 20% compared with the preinoculation weight, hemorrhagic colitis that was more than intermittent (formed stool present with blood-spotting clots), inability to eat or drink for more than 12 h, and lack of movement around the cage when manually stimulated (toe or ear touch or pinch).

Statistical analysis. Differences in colonization were analyzed by using repeated-measures ANOVA to determine whether a difference among the days was present, followed by one-way ANOVA to evaluate which days showed differences. Clinical and pathologic parameters such as weight loss, diarrhea, enteritis, and fibrin thrombi formation in kidneys in both breeds of rabbits were analyzed by using the Fisher exact test (SAS 9.1, SAS Institute, Cary, NC). Differences were considered significant at a P value of less than 0.05.

Bacterial quantification. Fecal samples were collected from each animal on days 2, 4, and 6 after inoculation to determine shedding and colonization of bacteria in infected rabbits. Serial dilutions of fecal suspensions were spread on Luria–Bertani agar and sorbitol–MacConkey plates and incubated overnight at 37 °C to determine the number of viable bacteria per gram of feces. The level of colonization by strain 933 was calculated from the total number of sorbitol-negative colonies recovered from rabbit feces.

PCR screening of Stx1 and Stx2. Bacterial colonies (3 to 5) were selected from sorbitol–MacConkey plates to confirm the presence of the toxin genes Stx1 and Stx2 by PCR analysis. The colonies were streaked onto Luria–Bertani plates and grown overnight. A single colony was suspended in 500 µL sterile distilled H_2O, and 20 µL of that sample was used in the PCR reaction. The primers for Stx1 were Stx1F (5′ CAG TTA ATG TGG TGG CGA AG 3′)[27] and Stx1R (5′ CTG CAC AGT AAC AAA CCG T 3′).[6] For amplification of Stx2, the primers used (LT9B: 5′ TTT ACG GGA TCC TAT ATC TGC GCC GGG TC 3′; CS2H: 5′AAT TCC AAG CTT ACT GAA TTG TGA CAC AGA TTA C 3′) were chosen in part because they were readily available; each incorporated a restriction site into the amplification product. A standard PCR reaction was performed with Fast*Taq* (Roche Applied Science, Indianapolis, IN) and using 30 cycles of 1 min at 95 °C, 1 min at 52 °C, and 1 min at 72 °C followed by a 10-min extension at 72 °C. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Euthanasia. On day 13 after inoculation (the end point of the study), rabbits were euthanized by intraperitoneal injection of pentobarbital sodium (100 mg/kg). One DB rabbit (no. 66), which reached an alternative endpoint of the study (more than 20% weight loss after inoculation), was euthanized on day 8.

Biochemical and histopathologic assessments. Baseline blood samples were collected from the ear veins before bacterial inoculation. In addition, blood was collected for CBC and biochemical analysis by cardiocentesis of each animal at euthanasia. Blood chemistry measurements included total protein, globulin, al-

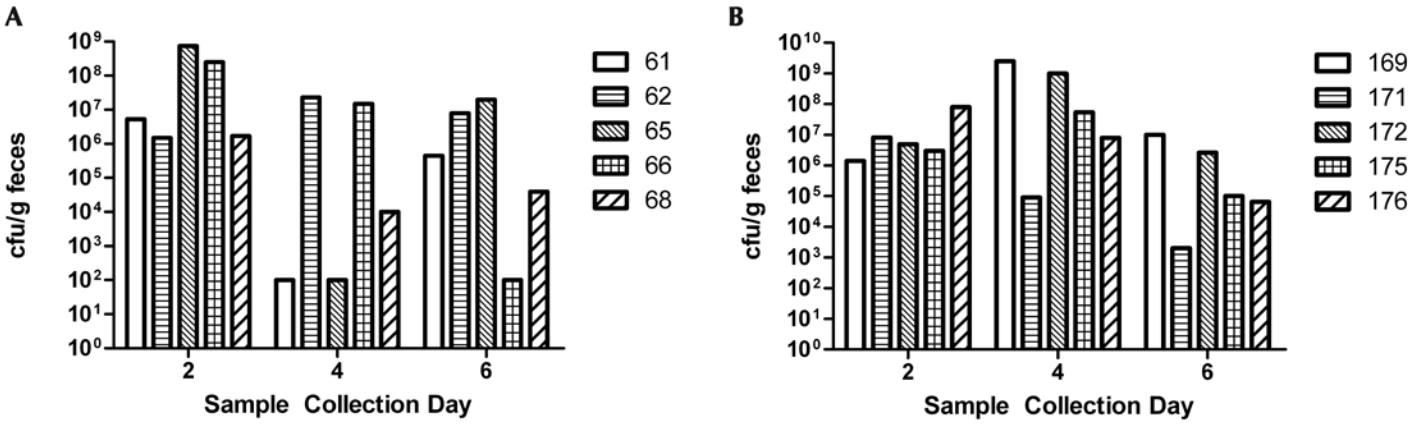

Figure 1. (A) Colonization of *E. coli* O157:H7 strain 933 in fecal samples collected from infected DB rabbits. The identification numbers of the infected animals (nos. 61, 62, 65, 66, and 68) are indicated. The control animals (nos. 63 and 64) showed no infection with strain 933 (limit of detection, 10^2 cfu). (B) Colonization of *E. coli* O157:H7 strain 933 in fecal samples collected from infected NZW rabbits. The identification numbers of the infected animals (nos. 169, 171, 172, 175, and 176) are indicated. We were unable to determine the level of strain 933 in the feces of infected rabbit 170, which shed high numbers of a sorbitol fermentor during the study. The 2 control animals (nos. 173 and 174) showed no infection with strain 933 (limit of detection, 10^2 CFU).

Table 1. Clinical signs and histologic lesions

		No. of rabbits with			
Rabbit strain	*n*; infection status	Weight loss	Diarrhea	Enteritis	Fibrinous renal thrombi
NZW	2; uninfected	0	0	0	0
	6; infected	5	4	6	0
DB	2; uninfected	0	0	0	0
	5[a]; infected	4	3	4	1

[a]One of the 6 animals in this group died shortly after the inoculation procedure, was removed from the study, and is not included in the results. Necropsy findings were indicative of acute aspiration pneumonia.

bumin, albumin:globulin ratio, creatinine, urea, BUN, alkaline phosphatase, AST, ALT, glucose, phosphorus, calcium, chloride, triglycerides, and amylase. Complete blood counts included hemoglobin, hematocrit, WBC, RBC, MCV, MCHC, and platelet counts. Differentials included lymphoctye, eosinophil, monocyte, neutrophil, and basophil counts.

Hematologic and biochemical values derived from blood collected before inoculation and at euthanasia were compared for each animal. Hematologic values were compared with clinical reference data available for rabbits. The cecum and both kidneys from each animal were collected at necropsy for histopathologic analysis. Tissues were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. In addition, fixed sections of kidney were stained with phosphotungstic acid hematoxylin for detection of fibrin. Histopathologic findings were evaluated by a board-certified veterinary pathologist. Infected animals were assessed for the presence of enteric or renal pathology associated with the disease. The presence of enteritis was determined by histopathologic analysis of the cecum. Enteritis was associated with hyperplasia combined with lymphoplasmacytic infiltration, inflammation, and erosion of the cecal mucosa. Renal pathology was associated with renal tubular dilatation and edema combined with the presence of renal thrombi or fibrin in the kidneys.

Results

This study compared 2 breeds of rabbits (DB and NZW) for susceptibility to *E. coli* O157:H7 infection, disease manifestations, selected hematologic and serum chemistry abnormalities, and pathologic damage to intestinal and renal tissues.

Recovery of *E. coli* O157:H7 from feces. Fecal samples were collected on days 2, 4, and 6 after infection from all rabbits, to assess the levels of colonization by strain 933. All of the inoculated rabbits became colonized by *E. coli* O157:H7 strain 933, whereas control rabbits did not become infected (Figure 1 A and B). PCR analysis showed that the strain 933 recovered from the rabbit feces contained both Shiga toxins (Stx1 and Stx2) as expected. Bacterial titers in these animals were as high as 10^7 cfu on day 6 after infection (Figure 1 A). All of the infected NZW rabbits shed recoverable 933 into the feces on days 2, 4, and 6 (Figure 1 B).

Clinical findings. All animals were monitored daily for development of clinical signs of disease (that is, diarrhea, hemorrhagic colitis, fever, lethargy, and weight loss). Control rabbits had no clinical signs, whereas infected rabbits displayed diarrhea and weight loss. None of the rabbits manifested hemorrhagic colitis (Table 1). Clinical signs did not differ significantly between the 2 breeds of rabbits under study—both breeds displayed comparable weight loss and diarrhea after infection. Diarrhea occurred in 67% of the infected NZW rabbits and in 60% of the infected DB rabbits on 1 or more days after infection. Five of the 6 (83.33%)

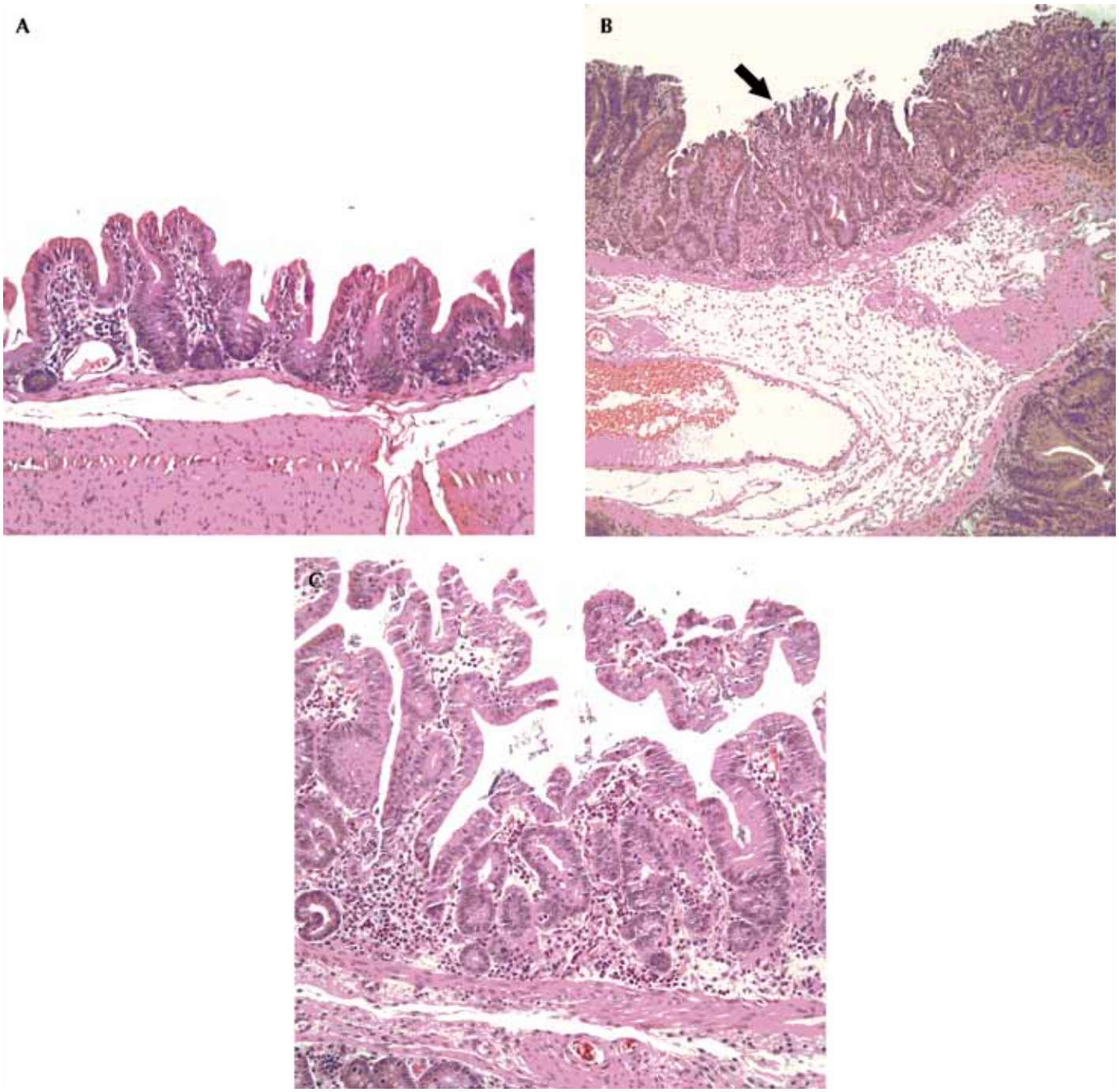

Figure 2. (A) Section of normal cecum from a noninfected DB rabbit. (B) Cecum of a DB rabbit infected with 10^9 cfu of *E. coli* O157:H7 strain 933. Note the inflammation and mucosal erosion (arrow), indicative of chronic enteritis. (C) Diffuse lymphoplasmacytic and suppurative inflammation coupled with hyperplasia of the cecal mucosa in a DB rabbit infected with 10^9 cfu of *E. coli* O157:H7 strain 933. These changes are indicative of chronic enteritis. Hematoxylin and eosin stain; magnification, ×20.

infected NZW rabbits exhibited weight loss, whereas 4 of the 5 (80%) infected DB rabbits lost weight loss after infection (Table 1). One DB rabbit (no. 66) was euthanized on day 8 after infection because it had reached an alternative end point of greater than 20% weight loss.

Histopathology. The kidneys and ceca of all animals appeared grossly normal at necropsy. Control rabbits displayed no enteric or renal pathology. All (100%) of the infected NZW rabbits and 4 of the 5 (80%) infected DB rabbits developed enteritis (Table 1). Rabbits with enteritis displayed inflammation, lymphoplasmacytic infiltration, hyperplasia, and erosion of the cecal mucosa.

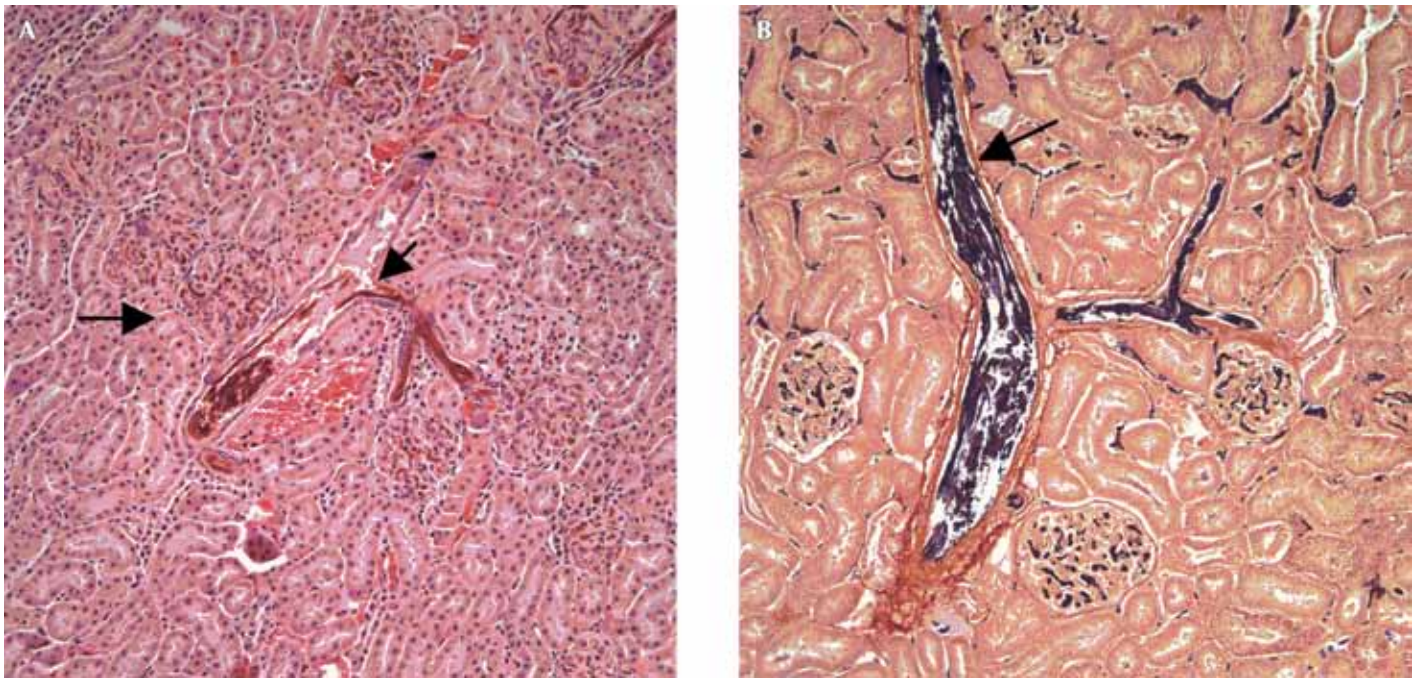

Figure 3. (A) Kidney section from a DB rabbit infected with 10^9 cfu of *E. coli* O157:H7 strain 933. Note the thrombus formation (arrows) in the artery. (B) Kidney section from a DB rabbit infected with 10^9 cfu of *E. coli* O157:H7 strain 933. Note the fibrin deposition (arrow) in the arteries. Phosphotungstic acid hematoxylin stain; magnification, ×20.

(Figure 2 A through C). The affected mucosa had diffuse inflammation and showed infiltration of neutrophils, lymphocytes, and plasma cells mixed with bacteria and cellular debris in the lumen. The kidney of one of the DB rabbits (no. 65) had multiple fibrinous thrombi in the renal arteries combined with tubular dilatation (Figure 3 A and B). Kidney sections of other infected rabbits lacked significant lesions.

Biochemical analysis. The blood counts and serum chemistries of the infected rabbits were not significantly different from those of the control rabbits. Most infected animals displayed eosinophilia and monocytosis by day 13 after infection (data not shown). The DB rabbit that was euthanized on day 8 after infection (no. 66) had high glucose (228 mg/dL; laboratory normal range, 74 to 148 mg/dL), BUN (171 mg/dL; normal range, 5 to 25 mg/dL), and liver enzymes such as AST (210 IU/L; laboratory normal range, 20 to 120 IU/L). This rabbit's CBC revealed high WBC (14×10^3 cells/µL; laboratory normal range, 5.1 to 9.7×10^3 cells/µL) and neutrophils (81%; laboratory normal range, 25% to 46%).

Discussion

We compared 2 rabbit breeds—DB and NZW—for the capacity to develop disease due to *E. coli* O157:H7 infection. Several animal models have been used to evaluate the pathogenesis of *E. coli* O157:H7.[1,2,4,9-11,17,32,35,43] For example, a C57BL/6 mouse model of HUS involves injection of Stx2 in combination with LPS to establish the syndrome.[17] Another study reports a C57BL/6J mouse model that uses Stx2 injection over multiple days and does not require the addition of LPS to cause symptoms of HUS.[35] However, neither of these mouse models[17,35] mimic the pathology found in the intestines of humans with *E. coli* O157:H7. A recent study showed that human Stx2-producing EHEC strains can adhere to the intestinal epithelium of weaned BALB/c mice and produce local damage, leading to systemic disease and death in a percentage of infected mice.[2] The lethality of the EHEC strain was age-dependent and was related to the ability of the bacteria to colonize in the intestines and produce Stx2. The authors concluded that weaned BALB/c mice can be used to study early host responses and the role of bacterial pathogenic factors in the induction of systemic disease.[2] Although murine models of oral infection of O157:H7 are available, they generally require that the mice be germ-free, treated with antibiotics, or given altered diets. In addition, the mouse models do not mimic human disease because they lack a diarrheal aspect and do not exhibit glomerular pathology.[2,5,18,41] Gnotobiotic piglets infected with *E. coli* O157:H7 strains showed diarrhea but did not exhibit renal disease.[4] The need to develop appropriate animal models to study *E. coli* O157:H7 pathogenesis in humans is pressing.

Studies in rabbits have shown their susceptibility to *E. coli* O157:H7 infection. In one report,[1] the effects of *E. coli* Shiga toxins were assessed in NZW rabbits exposed to Shiga toxins alone. About half of the NZW rabbits infused intraperitoneally with Stx2 developed diarrhea.[1] In another study, about 50% of NZW rabbits injected intravenously with Stx1 exhibited mild diarrhea, and none of the infected animals showed any renal histopathology.[32] Recent studies in DB rabbits suggest their promise as a model for EHEC-induced disease.[9,11] Experiments carried out to investigate the pathogenicity of *E. coli* O153 and O157:H7 in DB rabbits[11] provide evidence that the DB rabbit develops naturally occurring EHEC-induced systemic disease that closely resembles human HUS. In addition, DB rabbits injected intravenously with a bolus of Stx2 exhibited severe diarrhea and developed glom-

erular lesions.[10] These findings prompted us to design studies to investigate the pathogenesis of *E. coli* O157:H7 in NZW and DB rabbits.

A model of oral infection is likely to more accurately reflect the disease process as it occurs in humans due to oral ingestion of the *E. coli* O157:H7 and to be useful for evaluation of antibodies or vaccines that could intervene in the disease process. In the present study we used *E. coli* O157:H7 strain 933, which contains both Sx1 and Stx2, to infect DB and NZW rabbits. Our results showed that both DB and NZW rabbits are susceptible to *E. coli* O157:H7 infection and that both breeds exhibit diarrhea, enteritis, and weight loss. Stx1 and Stx2 induce a spectrum of renal pathologic changes.[14,23,26,37] We found that the infected rabbits in our study exhibited diarrhea and weight loss, shed bacteria in their feces, and developed enteritis. One of the infected DB rabbits displayed marked weight loss as well as elevated levels of glucose, BUN, and liver enzymes—findings indicative of renal and liver dysfunction. This rabbit's WBC and neutrophil counts were also above the normal ranges, suggesting acute infection. Histopathology results from another infected DB rabbit showed thrombi formation in renal capillaries, a feature consistent with HUS in humans infected with *E. coli* O157:H7. Pathologic studies of renal biopsy specimens from patients with HUS have revealed that platelets accumulate in this disorder and due to activated endothelial cells and platelet adhesion to the subendothelium, fibrin is deposited, indicating local generation of thrombin.[22] Although only one of the infected DB rabbits in our current study manifested renal pathology, the reproducibility of renal lesions in a larger percentage of infected rabbits would provide us with an opportunity to study EHEC-induced renal pathology. This ability would further help in the development of innovative therapeutics that could reduce renal complications of EHEC infections in humans. Induction of kidney damage in a higher percentage of rabbits may require a higher infectious dose of bacteria.

In summary, we were able to develop challenge models of DB and NZW rabbits for *E. coli* O157:H7 infection. Future studies in DB and NZW rabbits infected with higher doses of *E. coli* O157:H7 may provide new insights into the pathophysiologic events responsible for HUS in humans. Further studies will focus on use of these models to test novel therapeutics against *E. coli* O157:H7 infection.

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