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Coxiella burnetii Infection in C.B-17 *Scid-bg* Mice Xenotransplanted with Fetal Bovine Tissue

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Two from a group of approximately 50 C.B-17 *scid-bg* mice were examined because of lethargy, dehydration, and rough coat. Three months prior to development of clinical signs of disease, mice of this study had been surgically implanted with fetal bovine liver, thymus, and lymph node. At necropsy, marked splenomegaly and mild hepatomegaly were observed in both animals. Large areas of necrosis and inflammation, with associated intracytoplasmic granular basophilic inclusions, were observed in histologic sections of multiple organs. Aerobic and anaerobic culturing of the liver yielded negative results. Six months after the initial case, four more reconstituted *scid-bg* mice from a different fetal donor had identical clinical, gross, and histologic signs of disease. To determine whether the basophilic inclusions represented an infective agent, 4-month-old immune-naïve C.B-17 *scid-bg* mice were inoculated intraperitoneally with a liver and spleen homogenate from an affected mouse. Two weeks after inoculation, mice developed clinical signs of disease and lesions identical to those seen in the signal mice. On ultrastructural examination of the liver, pleomorphic bacteria were found in large cytoplasmic vacuoles of hepatocytes. Bacterial DNA was amplified from the liver, using primers that amplify a segment of the16S rRNA gene from many bacterial species. Sequencing of the polymerase chain reaction (PCR) product revealed gene sequence identical to that of *Coxiella burnetii,* the agent of Q-fever. These results highlight the need to consider infective agents of the donor species when working with xenografted animals.

The SCID-bo mouse was developed as a rodent model to study the bovine immune system. This mouse model is created by surgical implantation of small sections of second-trimester fetal bovine liver, lymph node, and thymus into the peritoneal cavity of a female C.B-17 *scid-bg* mouse (1). The resultant xenochimeric mouse is capable of developing primary and secondary bovine humoral immune responses to T cell-dependent antigens, and long-term self-sustaining multi-lineage bovine hematopoiesis (2). Use of this rodent model allows functional study of the bovine immune system while avoiding many of the problems associated with using large animals in research, such as expense of housing, difficulty of handling, uncertain health status, and non-uniform genetic background.

The advantages of the xenochimeric models come with an inherent risk—the possibility of aberrant infections from the donor species in the recipient animal. We report two cases of *Coxiella burnetii* infection in SCID-bo mice. The infection with *C. burnetii* in SCID-bo mice was most likely due to implantation of tissue from an infected bovine host. Therefore, we identify a major risk associated with fetal bovine xenografts and highlight the need to consider xenoinfection when using this method.

Case History

Case 1. A group of 50, female, six to 10-week-old C.B-17/GbmsIcrHsd-*scid-bg* (SCID) mice (Harlan, Indianapolis, Ind.) were reconstituted with fetal bovine tissue as described (1, 2). Briefly, pieces of fresh second-trimester fetal lymph node, liver, and thymus were aseptically sutured together, then were sutured to the caudoventral abdominal wall. Fetal bovine tissues were collected aseptically from cows at a slaughter house and were placed in cell culture medium containing gentamicin. Tissues were kept on ice and implanted within 18 h of collection. One day prior to surgery, mice received a trimethoprim (0.25 mg/ml of drinking water)-sulfamethoxazole (0.625 mg/ml of drinking water) suspension, which was then continued on alternate days for 14 days after surgery. Approximately 50 mice were implanted with tissue from a single donor bovine fetus.

Three months after reconstitution, two SCID-bo mice were submitted for diagnostic necropsy. The mice had been housed in the same cage from the time of surgery. Three cage-mates had been found dead during the preceding month and had not been necropsied. Other animals in the surgical group of 50 mice were not similarly affected. Other than the surgical reconstitution and a single blood sample collected from the retroorbital sinus, experimental manipulations had not been performed.

The two mice were mildly dehydrated and lethargic and had rough coat. They were euthanized by inhalation of overdoses of CO_2, and necropsy was performed. Both mice had similar lesions, including enlarged liver with irregular surfaces and irregular white depressed foci, with spleen approximately three times normal size but normal in color. Other gross abnormalities were not observed. Bacteria did not grow from sections of liver and spleen homogenized in saline and cultured at 37°C aerobically in Gram-negative broth or on 5% sheep blood and Hektoen chocolate, and MacConkey agars, or anaerobically on 5% sheep blood agar. Additional sections of liver and spleen and xenografts were collected and frozen at –80°C. Sections of brain, heart, lung, liver, gastrointestinal tract, and kidney and the xenograft were fixed in neutral-buffered 10% formalin, processed, and embedded in paraffin. Five-micron sections were stained

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with hematoxylin and eosin and Macchiavello's methods and were examined by use of light microscopy.

Histologically, chronic active hepatitis was seen in both mice. Lesions were characterized by marked Kupffer cell hyperplasia, Ito cell hyperplasia, and sinusoidal neutrophil and macrophage infiltration. (Fig. 1) Acute and chronic stages of necrosis were evident. Acute stages were characterized by coagulative necrosis, whereas chronic stages were characterized by parenchymal collapse, marked vacuolation, macrophage infiltration, and fibroplasia. Numerous basophilic cytoplasmic inclusions were present within Kupffer cells and infiltrating macrophages. (Fig. 2). Cytoplasmic inclusions were colored bright magenta when stained with Macchiavello's stain. Cells with inclusions were most prevalent around portal tracts. In addition to the expected paucity of lymphoid tissue, marked granulopoiesis and vacuolation of various degrees of severity were present in the spleen of both mice. Basophilic cytoplasmic inclusions identical to those in the liver were evident in splenic mononuclear cells. Cells with inclusions were most prevalent in the marginal zone of the depleted white pulp. Other lesions included chronic duodenitis characterized by fibrosis, vacuolation, neutrophil and macrophage infiltration, and mild chronic typhlitis. Inclusions were not evident in the examined sections of the intestines of these two mice.

Sections of formalin-fixed liver were embedded in resin and examined by use of transmission electron microscopy. Pleomorphic organisms, ranging from spherical to rod shaped, with bilaminar walls were evident within spacious vacuoles in the cytoplasm of hepatocytes. (Fig. 3).

Case 2. Approximately six months after the initial case was studied, five mice from a second surgical group were submitted for necropsy. These mice had been reconstituted with fetal bovine tissue from a single donor two months earlier. Four of the submitted mice were dehydrated, lethargic, and poorly groomed; one was clinically normal. These mice were from two separate cages; three of the clinically affected mice were housed together in one cage, and the other affected mouse was housed with the clinically normal animal. All other mice from the surgical group of approximately 50 animals were clinically normal. Gross and histologic lesions similar to those of case 1 were observed in four of five mice. Gross or histologic lesions were not observed in the clinically normal animal.

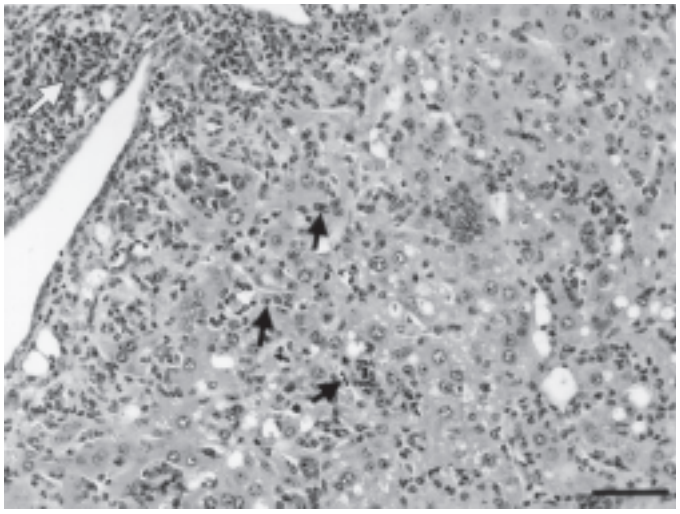

Figure 1. Photomicrograph of a section of liver from a mouse infected with *Coxiella burnetii*. Notice the mixed, predominantly neutrophilic, portal inflammation and accumulation of neutrophils and mononuclear cells in the sinusoids (black arrows). A cytoplasmic inclusion is indicated by the white arrow. Hematoxylin and eosin; bar = 60 µm.

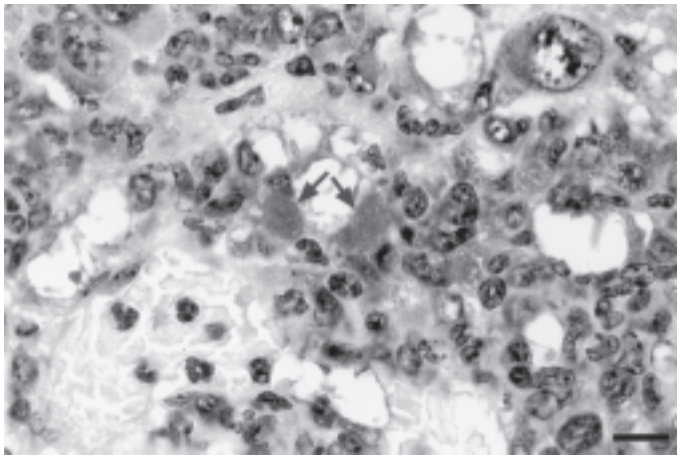

Figure 2. Photomicrograph of a section of liver from a mouse infected with *C. burnetii*. A cytoplasmic inclusion is indicated by the arrow. Macchiavello's stain; bar = 10 µm.

Inoculation Study

An experimentally induced infection study was performed to further characterize the etiologic agent. Seven four-month-old female C.B-17/GbmsIcrHsd-*scid-bg* were inoculated intraperitoneally with 0.5 ml of a tissue homogenate from a clinically affected mouse. The inoculum was prepared by homogenizing approximately 4-mm cubes of frozen liver and spleen in a tissue grinder (Con-Torque; Eberbach, Ann Arbor, Mich.) in 2.5 ml of sterile phosphate-buffered saline (PBS) and passing the resultant homogenate through an 80-µm filter. Three control mice received 0.5 ml of PBS intraperitoneally. To assess transmission, sham-inoculated control mice were housed in the same microisolator cage with experimental mice. One cage housed two control and three experimentally inoculated mice; a second cage housed one control and four inoculated mice. This inoculation experiment was conducted under a protocol approved by the University of Missouri Institutional Animal Care and Use Committee. Mice were housed in Micro-Isolator cages (Lab Products, Inc., Seaford, Del.), five per cage, at an AAALAC-approved facility with a 12/12-h light/dark cycle and room temperature maintained between 19-21°C. Mice were fed an autoclaved commercial rodent chow (Lab diet 5010, PMI Nutrition, Brentwood, Mo.) and were offered autoclaved acidified water (pH 2). The animal room was a negative-pressure room, with HEPA-filtered intake and exhaust and 10 to 15 air changes/h. Cage changes and animal manipulations were carried out in a biosafety level-2 hood.

One of the sham-inoculated controls was found dead five days after inoculation. Peritonitis, presumably a sequella to bacterial contamination from intraperitoneal injection, was evident at necropsy. All seven of the mice inoculated with the tissue homogenate developed lethargy and rough coat of variable severity by 13 days after inoculation. As a result, all experimental and control mice were euthanized by use of CO_2 asphyxiation and were examined at 14 days after inoculation. Gross lesions identical in character and severity to those seen in cases 1 and 2 were present in all experimentally inoculated mice. One sham-

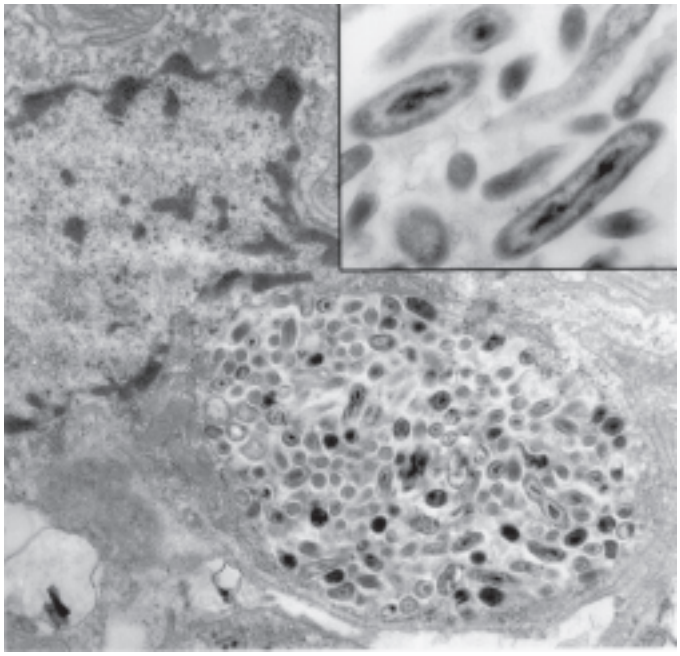

Figure 3. Transmission electron micrograph demonstrating the intracytoplasmic inclusions characterized ultrastructurally by spacious vacuoles that contained pleomorphic bodies. Magnification, 3,500×. The inset shows higher magnification of these bodies. Magnification, 50,000×.

inoculated control mouse was poorly groomed and lethargic at the time of necropsy; thymic lymphoma was diagnosed in this mouse, but other abnormalities were not identified. The other sham-inoculated control was clinically normal at the time of necropsy, and gross or histologic lesions were not evident.

Sections of liver and spleen were collected aseptically in a laminar flow hood; one section of each organ was collected for culture, and another section was frozen at -80°C. Sections of brain, heart, lung, liver, gastrointestinal tract, and kidney were collected for histologic examination. Aerobic and anaerobic culturing and tissue processing were carried out in identical manner to those used for the initial clinical cases. All culture results were negative.

Histologic lesions were similar to those seen in cases 1 and 2, although in general, the lesions were more severe in the experimentally inoculated mice. In addition to acute and chronic stages of necrosis described in the mice of case 1, some livers contained thrombi suggestive of endothelial damage in portal veins. It is conceivable that these thrombi occluded the portal vein, causing infarction and the aforementioned foci of necrosis. However, in the sections examined, thrombi were not adjacent to necrotic foci. Dystrophic mineralization was evident at the periphery of some necrotic foci. Rare hepatocellular mitoses and multiple foci of extramedullary granulopoiesis also were found. The distribution of lesions progressed from portal to diffuse as lesion severity increased. Typhlitis and duodenitis were detected in all experimentally inoculated animals. Basophilic inclusions were seen in mononuclear cells infiltrating the duodenal lamina propria, but inclusions were not seen in the cecum. Cytoplasmic inclusions were seen in low numbers in the renal tubular epithelium, perirenal adipose tissue, and myocardium of several experimentally inoculated mice; in these organs, inflammation was not associated with the cells containing inclusions.

Polymerase Chain Reaction/ Sequencing

A bacterial pathogen was suspected on the basis of pleomorphic bodies visualized in the cytoplasmic vacuoles by use of transmission electron microscopy. To identify the probable bacterial pathogen, polymerase chain reaction (PCR) was performed on DNA extracted from the frozen liver of a mouse that had been experimentally inoculated with a tissue homogenate from a mouse from case 1. The DNA was extracted from an approximately 25-mg tissue section, using a commercially available kit (QIAamp Tissue Kit, Qiagen, Inc. Valencia, Calif.) and stored at –20°C until use. The PCR was performed using a published primer set (13B, p93E) that amplifies a 460-base pair segment of the bacterial 16S ribosomal gene from a broad range of bacteria (3). An amplicon of the appropriate size was obtained. Sequence templates were prepared by use of PCR analysis and were purified on a 3.5% polyacrylamide gel. The gene sequence was determined using the *Taq* dideoxy chain termination method and a commercially available kit (*Taq* Dye Terminator Cycle sequencing kit; Applied BioSystems, Inc., Foster City, Calif.). Sequence analyses were performed by use of a sequence analysis software package (Genetics computing group, Inc. Madison Wis.). The sequence was compared with known bacterial sequences obtained from GenBank, and had 100% identity with GenBank No. M21291 from *Coxiella burnetii*, implicating this organism as the etiologic agent of the syndrome seen in these mice.

A PCR using primers that specifically amplify a segment of the 16S rRNA gene from *C. burnetii* (4) was used to screen peritoneal implant, spleen, and liver of mice from case 1; the liver of mice from case 2; the liver and spleen of the experimentally inoculated mice; and multiple sections of frozen fetal bovine liver, spleen, and lymph node used to reconstitute mice of case 2. All specimens from mice diagnosed with coxiellosis by histologic examination were PCR positive. Sham-inoculated control mice (excluding the mouse found dead on day five after inoculation, which was not examined) and the unaffected cagemate of the infected mice from case 2 were PCR negative. All samples from the bovine donor tissue were PCR negative.

Discussion

Coxiella burnetii is an obligate intracellular bacterium and is the causative agent of Q-fever in humans and domestic animals. Q-Fever is classically considered a disease of ruminants, but can be found in a broad range of domestic and non-domestic mammalian and non-mammalian hosts, including birds and ticks. Geographic distribution of naturally acquired disease is worldwide. Natural transmission is achieved most commonly by inhalation of contaminated aerosols, although disease may also be spread via tick bites or sexual transmission (5). Disease in humans is often asymptomatic. Acute febrile illness or a chronic illness characterized by any combination of endocarditis, granulomatous hepatitis, or osteomyelitis may develop. Infection in domestic animals also is often characterized by lack of clinical signs of infection. Ruminants may develop late-term abortion, infertility, and/or transient anorexia. Importantly, ruminants without clinical signs of disease may carry and shed organisms for long periods (6).

To our knowledge, this is the first report of a xenoinfection in mice implanted with bovine tissue. Identification and elimination of the source of the infection in this case is of academic in-

terest as well as significant financial interest to the producers and users of the xenografted animals. Experimental *Coxiella* infection studies have documented infection of mice by introduction of as few as one organism (7). We, therefore, considered two potential sources of infection: the bovine donor tissue and environmental contamination. The finding that only a few cages of mice were affected suggests that the bacterial burden, irrespective of the source, was low.

The most likely source was contaminated donor bovine tissue. However, *C. burnetii* could not be detected in the donor bovine tissue by use of PCR analysis, and only sporadic infections were seen. Because *C. burnetii* organisms are concentrated in the placental tissue rather than fetal tissue (8, 9), we believe that these discrepancies can be explained by low fetal tissue burden of organisms and/or sporadic contamination of tissue by placental contact during donor tissue harvest. In either situation, not all sections of tissue would be infected, thus explaining the low disease incidence and our inability to detect *C. burnetii* in fetal donor tissue.

Environmental contamination as a source of infection is conceivable because the facility that generated these mice was located on a large animal farm. Because *C. burnetii* is stable in the environment and resistant to many disinfectants, contamination from domestic livestock previously housed in the vicinity could persist. The finding that some cages with affected mice also contained healthy mice is not consistent with environmental contamination. Given the stability of *C. burnetii* in the environment, the immunocompromised status of SCID-bo mice and the low infective dose for this bacterium, one would anticipate that if a cage were contaminated, via a break in microisolator technique, all mice within that cage would be exposed and develop disease. Although we believe that bovine tissue was the source of infection, the actual source of contamination could not be determined.

Presuming infection occurred at surgery, the time to develop clinical disease in the implanted mice was two to three months. This is longer than the time it took to develop disease in our experimentally inoculated mice (< 2 weeks) and other reported experimental studies (10-12). This time difference is most likely a combination of microbial dose and antibiotic activity. The theoretical low concentration of organisms in fetal tissue would result in a small inoculum.

Although attempt was not made to quantify organisms experimentally inoculated into the mice, large quantities of organisms were visible histologically in the organs that were used to prepare the inoculum. A higher number of organisms in the inoculum likely resulted in a more rapid course of disease. An additional factor may have been the trimethoprim-sulfamethoxazole suspension given to the mice for 14 days after surgery. Although not the drug of choice for treatment of *C. burnetii*, this antibiotic has been documented to have some efficacy against the organism (13) and may have delayed replication of the organism and onset of clinical disease.

We emphasize the risks associated with xenografting fetal animal tissue into immunocompromised mice; unexpected disease transmission can occur that poses substantial risks to humans. It is critical that investigators and animal facility staff are aware of these risks and take appropriate precautions. In addition, the diagnostic approach to disease in xenografted animals must include screening for diseases of the donor species in the recipient animal, and tissues from donors of known health status should be used whenever possible.

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