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EXPERIMENTAL ADENOVIRUS HEMORRHAGIC DISEASE IN WHITE-TAILED DEER FAWNS

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ABSTRACT: Infection with a newly described endotheliotropic adenovirus was the cause of a 1993 epizootic reminiscent of hemorrhagic disease in California mule deer (*Odocoileus hemionus columbianus* and *O. hemionus hemionus*). Pulmonary edema and intestinal luminal hemorrhage, or necrotizing stomatitis associated with systemic or localized vasculitis, respectively, were common lesions seen in animals that died during the epizootic. In order to determine if white-tailed deer (*Odocoileus virginianus*) also are susceptible to infection and fatal disease with the deer adenovirus, eight white-tailed deer fawns (4- to 6-mo-old) were inoculated with purified deer adenovirus. Four were inoculated intravenously and four were inoculated through the mucous membranes. Seven days post-inoculation, one of the fawns inoculated intravenously died. Pulmonary edema and hemorrhagic enteropathy were associated with pulmonary and intestinal vasculitis with systemic multiorgan distribution of endotheliotropic adenovirus as demonstrated by transmission electron microscopy and immunohistochemistry. Adenovirus was reisolated from lung homogenates of the fawn that died of adenovirus hemorrhagic disease.

Key words: Adenovirus, experimental infection, hemorrhagic disease, hemorrhagic enteropathy, *Odocolieus virginianus*, pulmonary edema, white-tailed deer.

INTRODUCTION

A novel adenovirus was the cause of an epizootic of a hemorrhagic disease that produced high mortality in mule deer (*Odocoileus hemionus*) throughout northern and central California in 1993 (Woods et al., 1996). Black-tailed deer (*O. hemionus columbianus*) and Rocky Mountain mule deer (*O. hemionus hemionus*) were affected. Most were fawns, although some adults also were affected. Pulmonary edema and intestinal luminal hemorrhage were seen in deer with systemic infection. Pharyngeal necrosis, gingival, lingual and rumenal ulceration, and/or mandibular osteomyelitis were seen in deer with localized infection and occasionally in animals with systemic infection. In previous studies, both the systemic and localized forms of the adenovirus hemorrhagic disease (AHD) were reproduced experimentally in black-tailed deer yearlings and fawns (Woods et al., 1997, 1999).

Lesions in deer that die of the systemic form of AHD are similar to some of the lesions described in deer infected with bluetongue (BT) or epizootic hemorrhagic disease (EHD) viruses (Hoff and Trainer, 1981; Karstad and Trainer, 1967). In fact, a review of tissues from two black-tailed deer that died in a 1987 epizootic of a hemorrhagic disease presumed due to BT virus infection demonstrated adenovirus intimately associated with vascular lesions by transmission electron microscopy and immunohistochemistry (Woods et al., 1996). Since gross lesions of systemic AHD and the orbivirus hemorrhagic diseases (BT and EHD) are similar, all three should be considered in the differential diagnoses in black-tailed deer with pulmonary edema and/or hemorrhagic enteropathy.

Outbreaks of hemorrhagic disease due to BT or EHD viruses have been reported in white-tailed deer (*O. virginianus*) in several regions of the USA (Brannian et al., 1983; Feldner and Smith, 1981; Prest-

wood et al., 1974; Roughton, 1975). Since white-tailed deer are closely related to black-tailed deer, white-tailed deer may also be susceptible to the adenovirus. If so, adenovirus should be ruled out in addition to BT and EHD viruses when a hemorrhagic disease causes mortality in white-tailed deer. This study was therefore designed to determine whether white-tailed deer would develop clinical disease when inoculated with adenovirus isolated from a black-tailed deer that died of AHD.

MATERIALS AND METHODS

Nine orphaned white-tailed deer fawns were collected from east-central Texas near College Station (Texas, USA; 30°37′N, 96°20′W) in early summer. Fawns were housed in a variety of facilities and private homes and fed a variety of diets prior to being assembled at a central location at Texas A&M University (College Station, Texas). Fawns were housed indoors and fed lamb milk replacer (Land O Lakes, Minneapolis, Minnesota, USA) four times daily. Water was provided *ad libitum* and alfalfa hay was offered as the fawns grew. Fawns were shipped to California via airplane and housed indoors at the Veterinary Diagnostic Laboratory (Davis, California, USA; 38°33′N, 121°44′W). Fawns were offered goat milk with kid replacer (Land O Lakes) twice daily, then once daily just prior to the experimental study. Alfalfa hay, water and calf manna (Farmers Warehouse Co., Keys, California) were offered *ad libitum*.

Adenovirus was isolated in black-tailed deer pulmonary artery endothelial (BTDPAE) cells from lung homogenates of a black-tailed deer fawn that died of systemic AHD during the California epizootic in 1993 (Woods et al., 1996). Virus was purified using a CsCl gradient (1.3–1.46 g/cm^3) in phosphate buffered saline (PBS) as previously described (Woods et al., 1997, 1999) and dialyzed overnight in cold PBS (pH 7.4). A well-mixed fraction of the inoculum was negatively-stained with 2% phosphotungstate at pH 7.4 and examined by electron microscopy. Infectious titer was determined as previously described (Woods et al., 1999). Briefly, BTDPAE cells were grown to confluence in 4-chamber microscope slides (Labtek, Nunc, Inc., Naperville, Illinois, USA) and inoculated with log dilutions of the purified viral stock. Four wells were inoculated per dilution. After two weeks, the slides were harvested. As the virus does not produce cytopathic effect in DPAE cells, immunohistochemistry (described below) was used to detect infected cells. The inoculum titer was found to be 1×10 $TCID_{50}$/ml as calculated by the Reed and Muench method (The Subcommittee on Standardized Methods, 1978).

Nine white-tailed deer fawns were randomly assigned to one of three separate isolation rooms (A, B and C) and allowed to acclimate for two months. Room A contained four deer (No. 1–4) which consisted of two males and two females. Room B contained four deer (No. 5–8) which consisted of four males. Deer No. 9, a male, served as the negative control animal and was housed in room C. All animals were administered invermectin (Merck and Co., Inc, Rahway, New Jersey, USA; 10 mg/50 kg SQ) prior to placement in the isolation rooms. On the day of inoculation, all animals received physical examinations which included auscultation, examination of body condition, examination of the oral cavity, and measurement of temperature, pulse and respiration. All animals were 4- to 6-mo-old. Pre-inoculation blood samples were collected for complete blood counts (CBC) and were tested for BT virus by BT virus-specific polymerase chain reaction (PCR; Akita et al., 1993). Pre-inoculation serum samples were collected for serum chemistry panels, and were tested for antibodies to EHD virus and BT virus using the agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Wheat Ridge, Colorado, USA) and the competitive ELISA (Veterinary Diagnostic Technology), respectively. Four deer (Room A, No. 1–4) were each inoculated intravenously, through the cephalic vein, with 2 ml of the sterile deer adenovirus inoculum. Four fawns (Room B, No. 5–8) were each inoculated with 2 ml of sterile deer adenovirus inoculum, equally divided between four different sites by depositing drops on the mucous membranes of the eyes, nose and mouth and via the trachea using a sterile infant feeding tube (8 Fr. × 42″; Mallinckrodt, Inc., Glens Falls, New York, USA) inserted into the trachea (0.5 cc delivered by syringe into the feeding tube followed by air to completely empty the tube into the trachea). The negative control fawn (Room C, No. 9) was inoculated with 2 ml PBS intravenously and with 2 ml of PBS deposited on the mucous membranes. The negative control animal (Room C, No. 9) was inoculated first, followed by the animals in Room A (intravenous inoculation) and then Room B (mucous membrane inoculation). Weekly physical examinations were performed as described previously and post-inoculation blood samples were collected from the jugular vein for CBCs. Inoculation, physical examination, and blood sample collection were per-

formed on the deer immobilized with 1 mg/kg IM xylazine hydrochloride (Miles Laboratory, Shawnee, Kansas, USA) administered via dart (Pneu Dart, Inc., Williamsport, Pennsylvania, USA) delivered through a blow pipe (Pneu-Dart, Inc). Yohimbine hydrochloride (Lloyd Laboratory, Shenandoah, Iowa, USA; 0.25 mg/kg, IV via the cephalic) was used as a reversal. Laboratory animal facilities are rated biosafety level 2, and inoculations, blood collections, feeding and cleaning were performed using biosafety level three precautions (Centers for Disease Control and Prevention, 1999). The room with the negative control fawn (Room C, No. 9) was always entered first, followed by Room A and then Room B. Hazardous agent signs were posted on the doors to Rooms A, B and C, but doors did not have locks. Fawns were observed for signs of clinical disease three times daily. When slight changes in attitude were observed, fawns were euthanatized up to 7 wk post-inoculation (PI) by intravenous phenobarbital injection after immobilization with xylazine hydrochloride. One animal from Room A (intravenous inoculation) died and was necropsied 1 wk PI; one animal from Room B (mucous membrane inoculation) was necropsied 2.5 wk PI; two fawns (one from Room A and one from Room B) were necropsied 5.5 wk PI; and five fawns (two from Room A, two from Room B and the negative control animal) were necropsied 7 wk PI. Complete necropsies were performed on all animals. Fifty-five standard tissue samples representing all major organ systems were collected from each animal during necropsy. Tissues were immersed in 10% neutral buffered formalin and processed routinely for histologic examination. Lung from the affected animal that died (No. 1) and from the negative control animal (No. 9) were immersion-fixed in modified Karnovsky's solution (Nowell et al., 1972), postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, embedded in Spurr's/epon (50:50) resin (Ted Pella, Redding California, USA) and thin sectioned. Ultrathin sections (70–90 nm) of lung were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss 10 C electron microscope (Oberkochen, Germany).

Lungs and tonsils from all fawns were examined using an immunohistochemical method previously described (Woods et al., 1997, 1999). Additionally, intestines from the affected fawn were examined by immunohistochemistry. Formalin-fixed lung from a black-tailed deer with experimental adenovirus infection was used as a positive control (Woods et al., 1999). Lung of a negative control animal from a previous study was used as the negative control. In addition, normal bovine serum (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) diluted 1:1,000 in 10% normal goat serum diluted in PBS-BRIJ 35 (Fisher Scientific, Pittsburgh, Pennsylvania, USA) was substituted for the primary antibody as a negative control.

Virus isolation was performed on lung homogenates from the affected fawn that died (No. 1) and the negative control fawn as described previously (Woods et al., 1999). Briefly, tissue homogenates were inoculated onto BTDPAE cells in Dulbecco's medium (Gibco, Life Technologies, Inc., Grand Island, New York, USA) with 10% FBS (Sigma Chemical Co., St. Louis, Missouri, USA). Cultures were examined daily for cytopathic effect and examined after 14 days using a Zeiss 10 C electron microscope.

Pre- and post-inoculation sera from adenovirus-inoculated fawns and the negative control animal were tested for antibodies to the deer adenovirus. Rabbit antiserum to California deer adenovirus isolate, D94-2569 (Woods et al., 1996), was prepared using virus cultured in newborn white-tailed deer lung (OdL) cells. The viruses were diluted to provide 100 $TCID_{50}$ per well. Serial two-fold dilutions of the deer and positive control sera were made starting at 1:16. Controls included back titration of the reference virus, antiserum prepared in rabbits to the deer adenovirus and a fetal calf serum negative control. Serum antibody titers were expressed as the reciprocal of the highest dilution of serum preventing cytopathic effect (CPE) in 50% of the wells after 7 days incubation. Pre-inoculation serum samples and post-inoculation serum samples (taken at necropsy) were stored at −70 C until tested. The serum-virus neutralization test was done on all pre- and post-inoculation serum samples at the same time.

RESULTS

One of the male fawns (No. 1) died 7 days after intravenous inoculation with deer adenovirus. No clinical signs were noted the previous evening. None of the other fawns developed clinical signs or died. All pre-inoculation blood samples were negative for BT virus by PCR. Antibodies to BT and EHD viruses were not detected in pre- and post-inoculation serum samples. Pre-inoculation clinical chemistry and hematology parameters in all animals were mostly within normal ranges for white-tailed deer except total CO_2 and glucose were generally elevated

in animals likely due to the stress of immobilization and handling, and all fawns were mildly anemic.

Pulmonary edema and hemorrhage within the lumen of the small and large intestines were the significant changes seen on necropsy of the fawn that died (No. 1). No gross lesions were seen in any of the other animals.

Microscopically, pulmonary lobules of fawn No. 1 were separated by interlobular septal edema. Interlobular septal lymphatics were dilated and sometimes had luminal fibrin. Endothelial cells of veins, venules, arteries, arterioles, and peribronchial capillaries were hypertrophic and sometimes had karyorrhectic nuclei. There was margination of leukocytes. Indistinct, eosinophilic-amphophilic endothelial intranuclear inclusions were seen in some vessels.

Vascular changes in the intestines of fawn No. 1 were similar to changes in the lungs affecting all size vessels. Large arteries and veins in the serosa and submucosa were more frequently affected than capillaries in the mucosa. Vascular endothelium was hypertrophic and occasionally had endothelial intranuclear inclusion bodies. There was diffuse hemorrhage in the lamina propria of the mucosa with suffusion into the lumen. No microscopic lesions were seen in the other inoculated animals or in the negative control animal (No. 9).

In the affected fawn, icosahedral adenoviral nucleocapsids (Fenner et al., 1993), 68 to 72 nm in diameter, with electron-dense cores, were loosely arranged in nuclei of endothelial cells exhibiting mitochondrial swelling, dilatation of the endoplasmic reticulum, karyorrhexis and cytoplasmic fragmentation. Some endothelial cells were sloughed into the vascular lumen. Nuclei often had electron dense protein paracrystalline arrays. Virus was not seen in tissues from the negative control animal (No. 9).

Immunohistochemistry on the lung from the fawn that died (No. 1) demonstrated adenovirus antigen in endothelial cells lining large and medium pulmonary vessels while endothelial cells lining the microvasculature in the interalveolar septa were rarely labeled. Capillaries within the adventitia surrounding large airways were frequently labeled, however. Endothelial cell nuclei in vessels in the serosa and submucosa of the intestines stained more frequently than vessels in the mucosa of the intestines. Immunohistochemistry did not detect adenovirus in the lungs or tonsils of the negative control animal (No. 9) or any of the other inoculated deer.

Virus with morphologic characteristics of adenovirus (Fenner et al., 1993) was isolated from the lung tissues of the fawn (No. 1) that died after one passage in BTDPAE cell culture. There was no cytopathic effect (CPE) in the cell culture. Adenovirus was not isolated from tissues of the negative control animal.

Sera from all nine pre-inoculated deer had no antibody at the lowest dilution (1:16) to the deer adenovirus except for No. 7 and No. 8 which had titers of 1:16 and 1:32, respectively. Post-inoculation sera taken at necropsy from all fawns, including the negative control animal, had antibody titers equal to or greater than 1:1,024 except for fawn No. 1 that died 7 days post-inoculation with a titer of 1:16.

No consistent patterns in blood parameters were seen in weekly PI blood samples except a majority of fawns had depressed lymphocyte counts 1 wk PI. Two fawns (one from Room A and one from Room B) had leukocytosis 2 wk PI which then resolved without intervention.

DISCUSSION

Pulmonary edema and hemorrhagic enteropathy were reproduced in one white-tailed deer fawn inoculated intravenously with the adenovirus that causes AHD in black-tailed deer. The rapid fatal course of the disease in fawn No. 1 was similar to that previously described in black-tailed deer with systemic AHD (Woods et al., 1997, 1999). In a previous study, eight of

10 similarly aged black-tailed deer fawns developed clinical disease post-inoculation with this adenovirus (Woods et al., 1999). Black-tailed deer fawns that died were contact animals (uninoculated, exposed) and animals inoculated through the mucous membranes and intravenously. Only one of eight white-tailed fawns died in this study. The infectious dose of the adenovirus was very low and may account for the low mortality in the inoculated white-tailed deer. Comparison of the susceptibility of white-tailed deer with black-tailed deer was not possible in this study because different infectious doses were used in the black-tailed and white-tailed deer studies.

All deer seroconverted developing very high antibody titers to the deer adenovirus except for the fawn that died 7 days post-inoculation. This animal likely died before the immune system could mount a significant antibody response. Unfortunately, the negative control animal also developed a very high antibody titer to the deer adenovirus indicating this animal was exposed to the virus by contamination of the room. As mentioned in the description of the experimental design, biosafety level three precautions were used and the animals were housed in biosafety level two facilities. The rooms were separate, did not share room air, each had anterooms, and the room with the uninfected animal was always entered first and never revisited the same day. It was determined after the termination of the study that unauthorized personnel had entered the rooms. Virus was likely transferred from the inoculation rooms to the negative control room by fomites (shoes, hands). The high antibody titer in the negative control animal demonstrated exposure to the virus, but the animal did not develop disease likely due to extremely low infectious dose. This did, however, demonstrate the contagious nature of the deer adenovirus and that transmission can occur via fomites.

In summary, this study showed that white-tailed deer can develop fatal AHD. Results of physical examination, serum chemistry, CBC, and histopathology indicated that the fawn that died of AHD was healthy prior to inoculation and did not have any concurrent diseases.

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