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NATURAL DISEASE

Systemic Adenovirus Infection Associated with High Mortality in Mule Deer (*Odocoileus hemionus*) in California

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Abstract. Seventeen counties in northern California experienced epizootics of high mortality in the mule deer (*Odocoileus hemionus*) population during the latter half of 1993. Thirteen deer submitted to the California Veterinary Diagnostic Laboratory System as part of this natural die-off had systemic adenovirus infection. Pulmonary edema was present in all 13 deer. Erosions, ulceration, and abscessation of the upper alimentary tract occurred in 7/13 deer. Four of 13 deer had hemorrhagic enteritis. All 13 deer had widespread systemic vasculitis with endothelial intranuclear inclusions. Fluorescein isothiocyanate-labeled antibody directed against bovine adenovirus type 5 bound to antigen in endothelial cells. Adenovirus was identified by transmission electron microscopy within the nuclei of endothelial cells in 6/6 deer examined. An adenovirus was isolated from lung homogenates of one deer that were cultured on black-tailed deer pulmonary artery endothelial cells. With the exception of the intranuclear inclusions evident on histologic evaluation, gross and histologic changes were similar to those described for bluetongue virus infection and epizootic hemorrhagic disease virus infection in white-tailed deer. Nine additional deer were emaciated and had pharyngeal abscesses with focal vasculitis, which may represent the chronic affects of previous nonfatal adenovirus infection.

Key words: Adenovirus; deer; hemorrhagic disease; vasculitis.

Carcasses or tissue from two subspecies of mule deer (*Odocoileus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), and Rocky Mountain mule deer (*Odocoileus hemionus hemionus*), associated with a large natural die-off of mule deer in northern California, were submitted to the California Veterinary Diagnostic Laboratory by the California Department of Fish and Game during the second half of 1993. At least 1,000 mule deer were estimated to have died in this epizootic, which occurred throughout 17 counties in northern California, extending from the Oregon border to Yosemite National Park. The epizootic lasted from July to December 1993, with sporadic cases occurring in 1994. Histologic and transmission electron microscopic findings in necropsied animals suggest that a previously unrecognized adenovirus infection was responsible for the die-off.

Materials and Methods

Complete necropsies with subsequent histologic evaluation of tissues were performed on 37 deer from July 1993 to May 1994. Extensive diagnostic testing was performed on the first four deer carcasses submitted (Nos. 1–4). Testing was more selective on subsequent submissions (Nos. 5–13), based on prior test results and findings.

Deer Nos. 1 and 2 were screened for pesticides, nitrate/nitrite, and heavy metals. Kidney and liver from two deer were tested for carbamates and organophosphates using high-performance liquid chromatography and gas chromatography, respectively. A rapid colorimetric assay was used to determine nitrate and nitrite levels in eye and rumen contents. Heavy metal analysis for arsenic, molybdenum, zinc, lead, cadmium, copper, iron, and mercury was performed on liver and kidney using inductively coupled plasma emission spectrometry.

Lung, liver, and intestine from four animals (Nos. 1–4)

were cultured aerobically on blood agar. Pharyngeal abscesses were cultured on blood agar under aerobic and anaerobic conditions in two deer (Nos. 14, 15). Pleural fluids from deer Nos. 1–3 were tested for antibodies to epizootic hemorrhagic disease (EHD) virus and bluetongue (BT) virus using the agar gel immunodiffusion (AGID) test. Tissue homogenates from three deer were tested for BT virus by conventional virus isolation in specific-pathogen-free embryonated chicken eggs (ECE) (deer Nos. 2, 3) and by BT virus-specific polymerase chain reaction (PCR) (deer Nos. 2, 3, 12).[1]

Virus isolation

Fresh or frozen (−70 C) homogenates of lung and spleen (20% suspensions in phosphate-buffered saline) from seven affected deer (Nos. 1–7) were centrifuged at 1,500 × *g* for 10 minutes, and supernatant was inoculated onto Vero, baby hamster kidney (BHK-21), bovine turbinate (courtesy of Dr. M. M. Sawyer, University of California), rabbit kidney (RK-13), white-tailed deer (*Odocoileus virginianus*) carotid artery cells (courtesy of Dr. E. Howerth, University of Georgia), and black-tailed deer testicular cells (derived from a healthy young black-tailed deer). Tissue cultures were maintained at 37 C and examined daily for cytopathic effect. After 10 days, tissue culture flasks were frozen at −70 C and then thawed, and the supernatants were used as inoculum for three to six blind passages. Black-tailed deer pulmonary artery endothelial (PAE) cells were cultivated in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS) (MEM) from pulmonary artery explants of a young black-tailed deer euthanatized for a fractured hip. Flasks were frozen and examined for viral contamination by electron microscopy prior to inoculation. Lung homogenates (10% suspension in MEM with antibiotics) from one affected deer (No. 12) were blind-passaged twice in black-tailed deer PAE cells in Dulbecco's medium with 10% FBS. Cultures were examined daily for cytopathic effect. Inoculated PAE cells were frozen 14 days after the second passage and examined using electron microscopy. All tissue culture flasks were frozen at −70 C, thawed at room temperature, and filtered through a series of syringe filters (5.0–0.2 μm), and the ultrafiltrate was centrifuged at 249,000 × *g*. The pellet was resuspended in distilled water, applied to polyvinyl formate-coated copper grids, and stained with 2% phosphotungstate. Preparations were examined with a Zeiss 10 C transmission electron microscope.

Fluorescent antibody (FA) staining

Five-micrometer cryostat sections of lung from one affected deer (No. 12) were fixed in acetone/methanol (3:1) for 20 minutes and stained with porcine anti-porcine adenovirus, porcine anti-equine adenovirus fluorescein-isothiocyanate-labeled antibodies (Natural Veterinary Services Laboratory, Ames, IA), and two serotypes of bovine anti-bovine adenovirus (BAV-3 and BAV-5) fluorescein-isothiocyanate-conjugated antibodies (American BioResearch, Seymour, TN) for 25–30 minutes at 37 C with high humidity. Slides were rinsed for 10 minutes in phosphate-buffered saline (pH 9), coverslipped, and examined using an ultraviolet microscope.

Light and electron microscopy

Sections of brain, heart, lungs, liver, kidney, spleen, adrenal gland, rumen, abomasum, large and small intestine, skeletal muscle, esophagus, trachea, lymph nodes, pharyngeal oral mucosa, pulmonary artery, skin, and thymus were fixed in 10% neutral buffered formalin and processed routinely for histologic examination.

Supernatants of contents from the large and small intestines of deer Nos. 1 and 2 were suspended in distilled water and negatively stained with 2% phosphotungstate. Preparations were examined with a Zeiss 10 C transmission electron microscope.

Tissues from six deer (Nos. 1–3, 5, 12, 13) were examined by transmission electron microscopy (TEM). Specimens of lung, esophagus, or abscessed pharyngeal mucosa (2 × 2 mm) were immersion-fixed in modified Karnovsky's solution (1% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate), postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, embedded in Medcast resin (Ted Pella, Redding, CA), and sectioned. In selected cases where lesions or tissues were limited (deer Nos. 1, 5), 2 × 2 mm tissue fragments were excised from paraffin blocks of lung, placed in a paraffin bath at 60 C to remove excess wax, and deparaffinized in several changes of xylene at 24-hour intervals for 3 days. The tissue was then rehydrated through a reverse ethanol series (100–30%) and immersed in 0.2 M sodium cacodylate at pH 7.4. After rehydration in cacodylate buffer, the tissue was fixed in modified Karnovsky's solution for 1 hour, washed twice in 0.2 sodium cacodylate, and postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate before ethanol dehydration, propylene oxide transition, infiltration, and embeddment in Medcast resin. Additionally, paraffin-embedded esophagus from a black-tailed deer with similar gross and microscopic changes, which was examined in 1987 at the California Veterinary Diagnostic Laboratory in Petaluma, was excised from the paraffin blocks and processed in a similar manner. Ultrathin sections (70–90 nm) of lung or esophageal tissue were mounted on copper grids and stained with uranyl acetate and lead citrate.

Results

Thirteen mule deer examined had systemic vasculitis with endothelial intranuclear inclusions (deer Nos. 1–13). All deer with systemic vasculitis, except for one juvenile and one adult (Nos. 5, 6), were fawns. Two of the affected deer were Rocky Mountain mule deer, and the remaining deer were black-tailed deer. Disease, when observed, followed a rapid course. Signs in affected deer included ptyalism, diarrhea, regurgitation of rumen contents, seizures, and recumbency prior to death. Nine additional deer were emaciated and had bilateral or unilateral pharyngeal or gingival abscesses (deer Nos. 14–22). The diagnoses of the remaining 15 of the 37 deer examined from July 1993 to May 1994 included starvation, verminous pneumonia, trauma, bacterial septicemia, and systemic neosporosis.

Results of diagnostic tests are summarized in Table 1. All toxicologic tests (deer Nos. 1, 2), bacteriologic cultures (deer Nos. 1–4), and serologic tests for antibody to BT virus and EHD virus (deer Nos. 1–3) were negative. BT virus was not isolated by conventional virus isolation in ECE (deer Nos. 2, 3) or in BHK-21

Table 1. Results* of diagnostic tests performed on black-tailed deer with inclusion body systemic vasculitis (deer Nos. 1–13).

Test	Deer No.									
	1	2	3	4	5	6	7	8–11	12	13
Aerobic culture	NSF	NSF	NSF	NSF	nt	nt	nt	nt	nt	nt
Toxicology	NSF	NSF	nt	nt	nt	nt	nt	nt	nt	nt
BT/EHD virus AGID	Neg	Neg	Neg	nt	nt	nt	nt	nt	nt	nt
BT virus isolation	Neg†	Neg†‡	Neg†‡	Neg†	Neg†	Neg†	Neg†	nt	nt	nt
BT virus PCR	nt	Neg	Pos	nt	nt	nt	nt	nt	Neg	nt
Virus isolation (black-tailed deer PAE)	nt	nt	nt	nt	nt	nt	nt	nt	AV	nt
Adenovirus FA	nt	nt	nt	nt	nt	nt	nt	nt	AV	nt
TEM	AV	AV	AV	nt	AV	nt	nt	nt	AV	AV

* NSF = no significant findings; nt = not tested; AV = adenovirus.
† BHK-21, white-tailed deer carotid cells.
‡ ECE.

or carotid artery cells (deer Nos. 1–7). BT virus was identified in tissue from one deer (No. 3) by BT virus-specific PCR but was not identified in tissue from the other two deer (Nos. 2, 12). Viruses resembling enteric pathogens were not seen on direct electron microscopic examination of negatively stained supernatants of intestinal contents from deer Nos. 1 and 2. Virus isolation attempts on tissues from the fawn examined in 1987, which were performed at the time of necropsy, were negative for EHD virus and BT virus after two blind passages on C6/36 cells (mosquito-derived cells) and big horn fetal tongue explant cells.

Significant gross pathologic findings in 13 deer (Nos. 1–13) were most consistently present in the lungs and alimentary tract. In all 13 deer, the lungs were dark pink and edematous, with broad, edematous interlobular septa (Fig. 1). Petechial and paint brush hemorrhages were seen in the endocardium. There was focal hemorrhage at the base of the pulmonary artery in two deer (Nos. 3, 9). Mucosal erosion, ulceration, and abscessation were observed in the oral cavity and esophagus in seven deer (Nos. 3–6, 8, 10, 13) (Fig. 1). Segmental or diffuse mucosal or transmural congestion and hemorrhage with bloody contents were seen in the jejunum of two deer (Nos. 2, 3).

Light microscopy

Microscopic changes are summarized in Table 2. An acute to subacute systemic vasculitis with eosinophilic and amphophilic intranuclear inclusions in endothelial cells (Fig. 2) was present in 13 deer (Nos. 1–13). The vasculitis occurred in vessels ranging from capillaries to large arteries and veins. Vessels exhibited a range of histopathologic changes from endothelial cell hypertrophy to endothelial cell necrosis and fibrinoid necrosis of the tunica intima with intramural infiltrates of neutrophils, lymphocytes, and macrophages. Smooth muscle hyperplasia was present in the tunica media of pulmonary arterioles and small arteries of the single adult deer examined (No. 5). Large eosinophilic or amphophilic intranuclear inclusion bodies, surrounded by a thin rim of marginated chromatin, were found in hypertrophied and necrotic vascular endothelial cells in decreasing order of frequency in the lungs, brain,

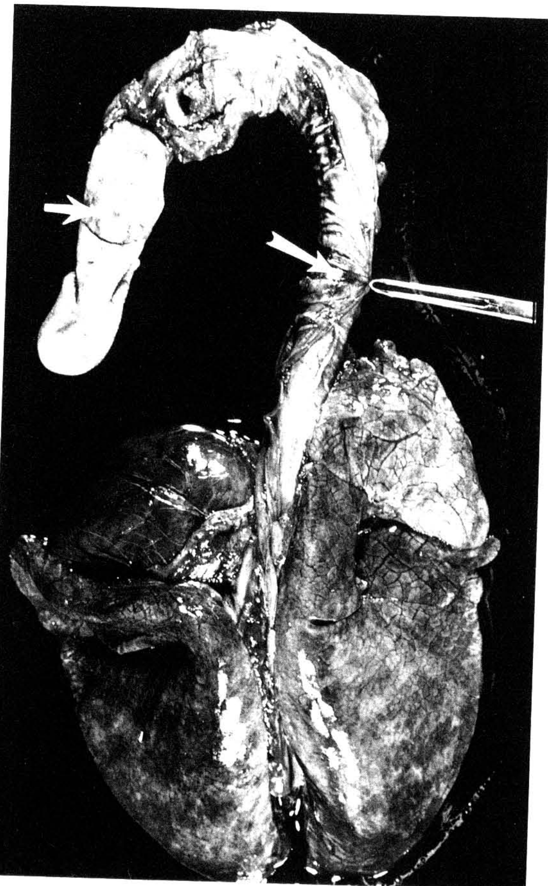

Fig. 1. Lungs, alimentary tract; black-tailed fawn No. 3. Systemic adenovirus infection. Lungs are edematous and there is multifocal ulceration of the tongue and esophagus (arrows).

Table 2. Histopathologic changes in deer with adenovirus infection.

Lesion	Deer No.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14–22
Systemic vasculitis/endothelial intranuclear inclusions	X	X	X	X	X	X	X	X	X	X	X	X	X	
Nonsuppurative interstitial pneumonia		X			X		X	X	X	X	X	X		
Glossal/esophageal ulceration			X					X		X				
Hemorrhagic enteritis/enteropathy		X	X											
Nonsuppurative encephalitis			X									X		
Lymphoid depletion/necrosis (spleen, lymph nodes)					X		X	X	X	X	X	X		
Hepatic necrosis (centrilobular, paracentral)					X		X		X					
Pharyngeal/gingival abscess with focal vasculitis													X	X

alimentary tract, heart, liver, kidney, spleen, pulmonary artery, uterus, urinary bladder, pharynx, and thymus (Fig. 2-inset). In one deer (No. 11), vasculitis and endothelial intranuclear inclusions were found only in the brain.

Additional significant histologic changes were consistent with vascular damage. All deer had a variable degree of pulmonary alveolar, pleural, and interlobular edema with fibrin exudation. Alveolar septal infiltrates of lymphocytes, plasma cells, and macrophages were also present in the lungs of eight deer (Nos. 2, 5, 7–12). Glossal and esophageal erosions and ulcerations

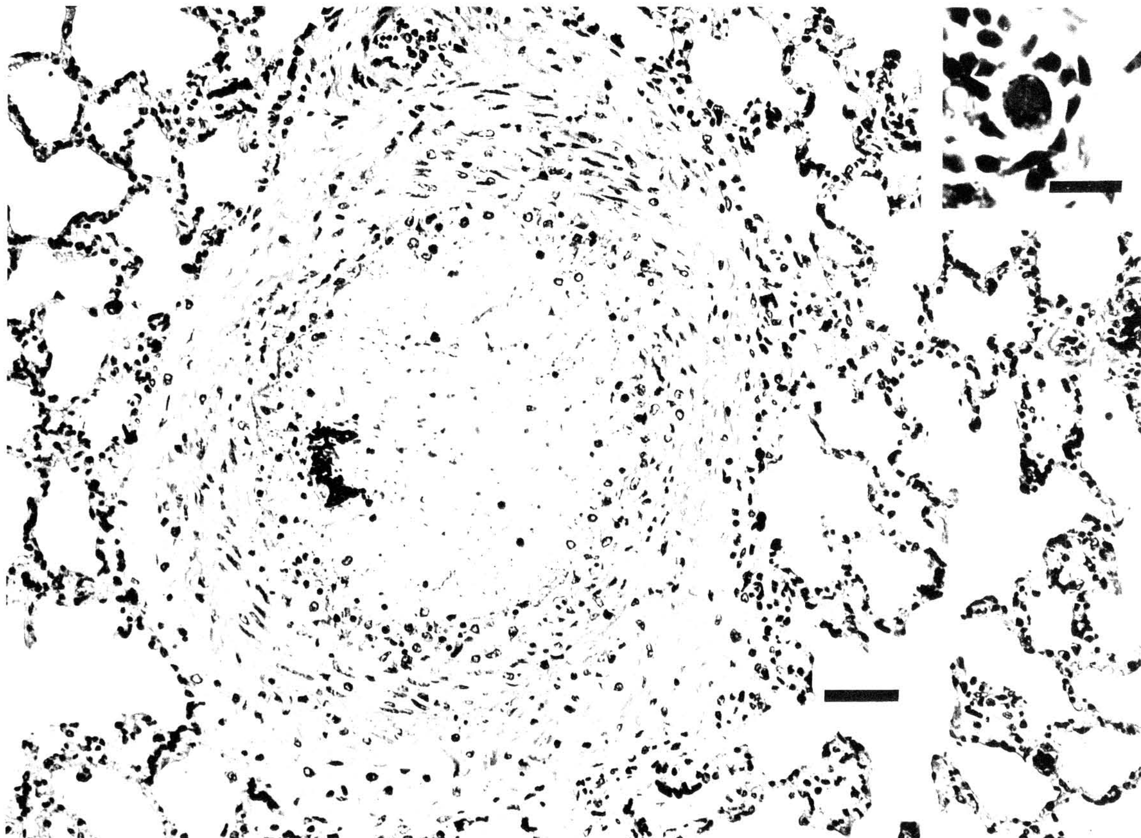

Fig. 2. Lungs; black-tailed fawn No. 4. Systemic adenovirus infection. There is endothelial degeneration and loss and disruption of the tunica intima by lymphocytes and macrophages, which ocasionally extend into the tunica media. HE. Bar = 70 μm. *Inset:* The endothelial intranuclear inclusion extends out to the peripheral rim of marginated chromatin. HE. Bar = 17 μm.

(deer Nos. 3, 8, 10) were accompanied by focal dense infiltrates of macrophages and neutrophils. Subjacent to the erosions and ulcers, the lamina propria and submucosa, respectively, were edematous and hemorrhagic with multifocal or diffuse infiltrates of macrophages, plasma cells, and lymphocytes and focal collections of neutrophils. There was endothelial hypertrophy and necrosis with intranuclear inclusions in capillaries and small arterioles in the intestines in two deer (Nos. 2, 3). Affected vessels were associated with segmental to diffuse intestinal mucosal or transmural edema, congestion, hemorrhage, and necrosis. Lesions in the brain were seen in four deer. In two deer (Nos. 2, 11), changes were limited to the vessel walls, whereas in two other deer (Nos. 3, 12), perivascular lymphocytic cuffs accompanied vascular changes.

The spleen, Peyer's patches, and lymph nodes exhibited lymphoid depletion (deer Nos. 5, 7–10). In many lymph nodes, multifocal hemorrhage was associated with endothelial hypertrophy, necrosis, and intranuclear inclusions in capillaries and venules. Three deer (Nos. 5, 7, 9) had hepatic centrilobular or paracentral necrosis. Large arteries were most commonly affected in the liver, kidney, and spleen.

Deer No. 13 and nine additional black-tailed deer fawns and adults (Nos. 14–22) had abscesses in the pharyngeal and gingival mucosa with evidence of chronic wasting. Bacterial isolates from the abscesses included *Actinomyces pyogenes, Fusobacterium necrophorum, Peptostreptococcus indolicus, P. anaerobius, Pasteurella multocida,* and *Streptococcus* sp. In deer Nos. 14–22, subacute to chronic vasculitis was apparent in tissue subjacent to the abscesses, but systemic vasculitis was not present. Intranuclear inclusions within hypertrophic vascular endothelial cells associated with vasculitis in the tissue subjacent to pharyngeal abscesses were present only in deer No. 13.

Transmission electron microscopy

Transmission electron microscopy demonstrated pulmonary and esophageal endothelial intranuclear inclusions from six deer (Nos. 1–3, 5, 12, 13) associated with the 1993 epizootic and from the deer necropsied in 1987. The inclusions were composed of paracrystalline arrays or loosely dispersed hexagonal viral nucleocapsids (68–73.5 nm in diameter) with central electron-dense cores (Fig. 3). In addition, there were long, needlelike crystal lattices of granular material associated with the viral nucleocapids. The size and morphology of the nucleocapsids are consistent with those of members of the Adenoviridae.[6,16]

Virus isolation

Adenovirus was isolated from lung homogenates of deer No. 12 in black-tailed deer PAE cells after the second passage, with no associated cytopathologic effect. Negatively stained virions were icosahedral and 77.6–79.6 nm in diameter, with triangular facets and four capsomeric structures between the vertices (Fig. 3, inset left). Adenovirus was not identified in negatively stained preparations of any other cell cultures inoculated.

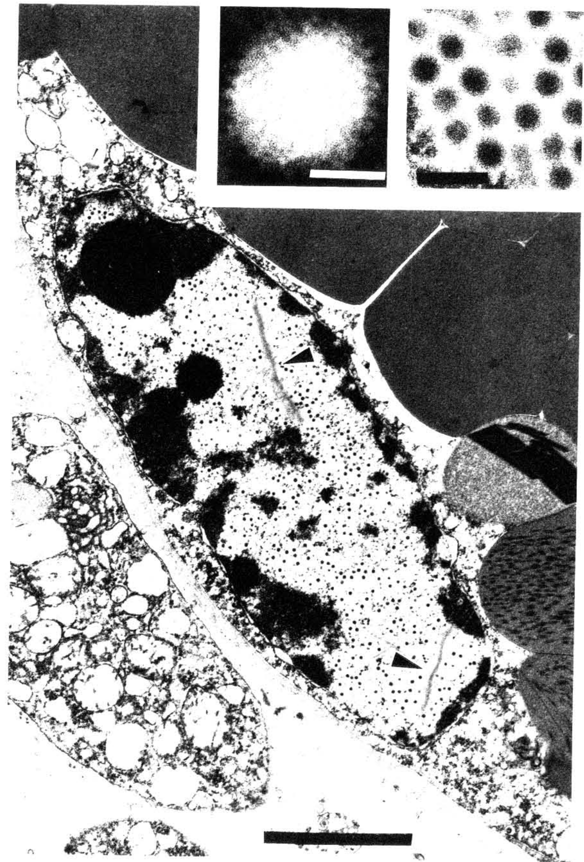

Fig. 3. Transmission electron photomicrograph. Pulmonary vessel; black-tailed fawn No. 3. Systemic adenovirus infection. Endothelial cell nucleus contains numerous adenovirus particles. Note the long, granular needlelike crystal lattices in the nucleus (arrows). Lead citrate and uranyl acetate. Bar = 4 μm. *Inset, left:* Negatively stained black-tailed deer pulmonary artery endothelial cell culture supernatants (deer No. 12) showed icosahedral virions, 77.6–79.6 nm in diameter, with triangular facets. Phosphotungstic acid. Bar = 40 nm. *Inset, right:* Lung; black-tailed fawn No. 3. Icosahedral virions, 68–73.5 nm in diameter, with electron-dense central cores within endothelial cell nuclei. Lead citrate and uranyl acetate. Bar = 160 nm.

Fluorescent antibody test

Fluorescein-isothiocyanate-labeled antibody directed against BAV-5 was found to bind to antigen in endothelial cells (deer No. 12) (Fig. 4). Fluorescein-isothiocyanate-conjugated antibodies directed against porcine adenovirus, equine adenovirus, and BAV-3 did not bind to any endothelial cells.

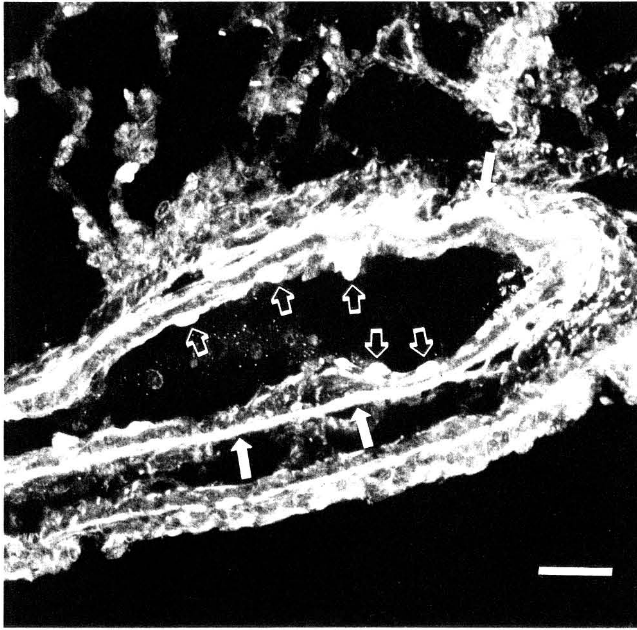

Fig. 4. Lung; black-tailed fawn No. 12. Systemic adenovirus infection. Endothelial nuclei reacting positively with bovine adenovirus type 5 fluorescein-labeled antibody appear white (open arrows). The color of the positive reaction was actually apple green. Solid arrows show distinct white elastic fibers that have nonspecifically reacted with Evan's blue counterstain and were actually orange in color. Ultraviolet light. Bar = 70 μm.

Discussion

We believe adenovirus was the cause of the fatal epizootic that occurred in mule deer in northern California in 1993. This conclusion is based on the predominance of vascular lesions in the tissues of affected deer, the direct association between these vascular lesions and the consistent vascular endothelial location of adenovirus, the positive reaction of these viral inclusions with anti-BAV-5 serum, and the isolation of adenovirus from an affected deer. The gross and microscopic features of this disease are strikingly similar to those of hemorrhagic disease attributed to EHD and BT viruses in white-tailed deer. The endothelial intranuclear inclusions, however, are a distinguishing feature. Association of BT virus and EHD virus infections with hemorrhagic disease in white-tailed deer is well documented.[3,7–10,12,14,17,18,20] Lesions in white-tailed deer associated with infection with either virus include pulmonary edema, hemorrhage throughout the digestive tract and in the urinary bladder, heart, aorta, and pulmonary artery, erosion and ulceration in the oral cavity, and coronitis.[9,14,18] The cause of hemorrhagic disease in mule deer is not as well documented. Seroprevalence studies of BT virus infection in different mule deer populations have reported infection of some herds to be 0–40%.[4,13] From 0% to 73% of different mule deer populations in California and Montana have antibody to EHD virus.[5,13] The association of BT virus infection with hemorrhagic disease in mule deer is based primarily on seroprevalence studies, the similarity to lesions reported in white-tailed deer infected with BT virus, and a single case report in which BT virus was isolated from the splenic homogenates of a fawn and an adult mule deer with bluetongue-like postmortem findings.[15] Investigators, however, have been unable to produce clinical disease or gross and microscopic lesions in fawns or adults following experimental inoculation with BT virus or EHD virus.[19,21] Outbreaks of hemorrhagic disease in mule deer in California have been attributed to BT virus despite inconsistent evidence of infection of affected deer with BT virus. Detection of adenovirus-like particles in archived tissues from a deer necropsied in 1987 with bluetongue-like lesions suggests that some of these outbreaks may have, in fact, been due to systemic adenovirus infection.

In other species, clinical disease associated with adenovirus is often limited to neonates or immunocompromised animals.[6] In some instances, coinfection with a helper virus is required. Deer with systemic adenovirus infection were predominantly fawns, although some juveniles and adults were also affected. We found no evidence of any consistent viral coinfections in these cases. In particular, attempts were made to rule out the possibility of BT virus coinfection. BT virus was not isolated from any deer in embryonated chicken embryos or in cell culture but was detected by PCR in one of three deer examined. The absence of BT virus from the remaining deer would suggest that BT virus coinfection is not a requirement for the manifestation of this adenovirus-induced disease.

Adenovirus or adenovirus-like infection in deer has previously been reported.[2,11] Adenovirus-like particles were identified in bronchiolar epithelium associated with a bronchiolitis in a red deer (*Cervus elaphus*) in New Zealand.[11] Bovine adenovirus type 6 was isolated from the lung of a fallow deer (*Dama dama*) from the Budapest Zoological and Botanic Gardens with histologic changes similar those reported in the red deer.[2] In both cases, the primary target cell was the bronchiolar epithelial cell; therefore, these adenoviruses may be different from the adenovirus identified in the California outbreak described here. There are no previous reports of a highly pathogenic adenovirus that is tropic for endothelial cells and causes systemic vasculitis and high mortality in deer.

Nine additional deer (Nos. 14–22) had pharyngeal abscesses and evidence of chronic wasting. All nine had an associated subacute to chronic vasculitis in the tissue subjacent to the abscesses, but there was no direct evidence that adenovirus was present in tissues

from these deer. Adenovirus was detected by transmission electron microscopy in endothelial cells associated with vascular lesions deep to a pharyngeal abscess in only one deer (No. 13). Ulceration with vasculitis in subjacent tissue was present in the oral cavity of several deer with systemic adenovirus vasculitis. Based on the presence of deep vasculitis in all deer with pharyngeal abscesses, light microscopic evidence of endothelial intranuclear inclusions and transmission electron microscopic evidence of endothelial adenovirus particles in vessels in tissues deep to an abscess in one deer (No. 13), and information that these deer came from herds that had experienced heavy losses due to systemic adenovirus infection, we speculate that abscessation of the oral cavity with subsequent chronic wasting may be a sequela of previous nonfatal adenovirus infection in these deer.

The extent of deer mortality in northern California is unknown but it is estimated that more than 1,000 deer died as a result of this epizootic. Statewide herd composite counts will help determine the effects of the virus on the deer population. Initial results indicate that the ratio of fawns to does is less than half of normal in some herds (Maddox, personal communication).

Attempts to reproduce the disease are currently in progress at the California Veterinary Diagnostic Laboratory System. Reproduction of the disease is crucial to determine if adenovirus is solely responsible for systemic vasculitis in black-tailed deer or if the disease requires coinfection of the adenovirus with another agent, as is often the case with adenovirus infections. Molecular and serologic studies are in progress to determine the nature of the virus and its genomic relationship to currently recognized adenoviruses. In addition, epidemiologic studies are planned at the California Department of Fish and Game to determine the prevalence of adenovirus infection in mule deer in California. Epidemiologic and laboratory studies may help to elucidate why systemic adenovirus infection was associated with heavy losses in the mule deer population in northern California during 1993 and if adenovirus is the major cause of hemorrhagic disease in black-tailed deer in California.

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