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HOST DEFENSE RESPONSES ASSOCIATED WITH EXPERIMENTAL HEMORRHAGIC DISEASE IN WHITE-TAILED DEER

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ABSTRACT: Our objectives were to examine the immunity conferred by epizootic hemorrhagic disease virus serotype 2 (EHDV-2) infection in white-tailed deer (*Odocoileus virginianus*) and determine if this immunity was protective during challenge with homologous (EHDV-2) or heterologous (bluetongue virus serotype 10; BTV-10) virus. Trials were conducted in the fall of 1992 and 1993. In the first experiment, naive white-tailed deer were infected intradermally and subcutaneously with EHDV-2 and monitored via physical examinations, complete blood counts, α and β interferon (IFN) assays, viral isolation, and serology. Infected deer had a wide range of clinical signs in response to infection. Eleven of the 16 deer had body temperature elevations ≥0.5 C between post-infection day (PID) 4 and 8. Infected deer had decreased lymphocyte counts between PID 6 and 10 that returned to normal levels by PID 17. Severely lymphopenic animals had the most severe clinical signs; five of 10 deer with lymphocyte counts less than 1000 cells/μl succumbed to the infection. Viremia was detected in all 16 EHDV-2 infected animals by PID 4, and peak viremias occurred between PID 4 and PID 10. Three deer remained viremic until PID 56, the study endpoint. Interferon was first detected between PID 2 and 6. Peak α and β IFN levels coincided with peak viremia in 11 deer. Precipitating and neutralizing antibodies were detected in infected deer by PID 10. In the second experiment, convalescent deer were challenged subcutaneously and intradermally with either EHDV-2 or BTV-10 and similarly monitored. Virus was detected in the blood of all four deer challenged with BTV-10, but viremia was not detected in three EHDV-2-challenged deer. Temperature fluctuations, blood cell parameter changes, and IFN and antibody responses seen in BTV-10-challenged deer were similar to those seen in the initial experiment. Deer challenged with EHDV-2 had mildly increased temperatures, but minimal IFN response and lymphocyte alterations.

Key words: Experimental infection, epizootic hemorrhagic disease virus, EHDV, bluetongue virus, BTV, white-tailed deer, *Odocoileus virginianus*, humoral immunity, interferon

INTRODUCTION

Hemorrhagic disease of white-tailed deer (*Odocoileus virginianus*) and other wild ruminants is caused by orbiviruses in the epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) serogroups. Epizootic hemorrhagic disease virus serotypes 1 and 2 (EHDV-1, EHDV-2) and BTV serotypes 2, 10, 11, 13, and 17 (BTV-2, BTV-10, BTV-11, BTV-13, and BTV-17) may act either singly or concurrently causing morbidity and mortality, not only in white-tailed deer but also in other wild and domestic ruminant species. Based on prevalence estimates using precipitating antibodies, exposure to EHDV and BTV appears to be is widespread in white-tailed deer populations in the southeastern United States (Stallknecht et al., 1991); EHDV-2 is enzootic throughout much of the region with occasional serologic evidence of EHDV-1 and BTV serotypes (Stallknecht et al., 1995). Epizootics may be related to low herd immunity combined with the periodic introduction of disparate serotypes of EHDV or BTV in susceptible populations of deer (Stallknecht et al., 1995).

While the resultant clinical signs and lesions are well-described (Hoff and Trainer, 1978), little is known about host defense mechanisms of white-tailed deer infected with either EHDV or BTV, or how the immune response is modulated by subsequent infections with other serotypes of EHDV or BTV as can occur under natural

conditions. The available information from experimental challenge infections is conflicting and limited in scope. Vosdingh et al. (1968) indicated that two white-tailed deer that survived BTV infection were immune to EHDV. Yet when Hoff and Trainer (1974) inoculated an EHDV-vaccinated deer with BTV and a BTV-vaccinated deer with EHDV, both animals became ill with signs compatible with hemorrhagic disease. Interferon (IFN) production in deer has not been evaluated yet α and β IFNs are recognized as potent antiviral agents that are important in the initial antiviral response in BTV-infected sheep (Foster et al., 1991) and cattle (MacLachlan and Thompson, 1985).

Our objectives were to evaluate the temporal relationship between clinical response, viremia, α and β IFN production, and antibody response in naive white-tailed deer infected with EHDV serotype 2 (EHDV-2). Subsequently, these same host defense parameters were measured after convalescent deer were challenged with homologous (EHDV-2) or heterologous (BTV-10) virus to determine if the immunity conferred by the initial EHDV-2 infection was protective.

MATERIALS AND METHODS

Twenty-two hand-raised, 3-to-4-mo-old white-tailed deer fawns obtained from the North Carolina (USA) Wildlife Resources Commission were used in the study. Deer ranged in weight from 9.1 to 13.6 kg (median = 11.6 kg) with 11 males and 11 females. Prior to the beginning of the study, all fawns were determined to be serologically negative to EHDV and BTV via agar-gel immunodiffusion (AGID) test kits as described by the manufacturer (Veterinary Diagnostic Technology, Inc., Wheatridge, Colorado, USA). Animals were housed indoors to prevent contact with *Culicoides* spp. vectors.

The study was divided into two trials that were conducted from September to November of 1992 and 1993. Ten deer were used in 1992 and 12 deer were used in 1993. In the first experiment in each trial, eight deer were infected with EHDV-2, and two deer served as uninfected sham-inoculated controls. In the second experiment, which was conducted after 28 days, surviving convalescent deer were divided into two groups. In 1992, three of the five surviving convalescent deer were challenged with EHDV-2 while two deer served as convalescent controls. In 1993, four surviving convalescent deer were challenged with BTV-10 and two deer served as convalescent controls. The two uninfected control animals from each year were monitored throughout the trial. In 1993, two additional fawns were held until the challenge infection to test the BTV-10 inoculum.

Deer were sampled for a total of 56 days (28 days in each trial). During both experimental infections, animals were manually restrained every other day for the first 14 days and biweekly thereafter for physical examination; body temperature evaluation; and blood collection for complete blood counts, viral isolation, IFN assays, serology, and blood coagulation analyses. Complete blood counts were performed on a Baker System 9000 automated cell counter (Baker Instrument Corporation, Allentown, Pennsylvania, USA). Parameters evaluated included white blood cell counts, red blood cell counts, and hematocrits. The white blood cell differential counts were performed manually. From post-infection day (PID) or post-challenge day 4 to 10, body temperatures were taken daily via digital thermometer rectally. All blood collections were done via jugular venipuncture. Samples were collected at approximately the same time daily to reduce any diurnal variation in blood component values. Additional blood was taken weekly for studies of cell-mediated immunity. Hemostasis studies and cell-mediated immune function will be reported elsewhere. All surviving animals were euthanized with sodium pentobarbital at 1 ml/5 kg (Butler Company, Columbus, Ohio, USA) and necropsies were performed after 8 wk.

The EHDV-2 inoculum for the initial infections was prepared from an EHDV-2 isolate obtained from a white-tailed deer lymph node cultured on baby hamster kidney cells (BHK_{21} cells) (American Type Cell Culture, Rockville, Maryland, USA). The original isolate was obtained from a white-tailed deer submitted to Southeastern Cooperative Wildlife Disease Study as a diagnostic case from Clarke County, Georgia USA, in 1990. To prepare the inoculum, a serologically negative yearling white-tailed deer was subcutaneously inoculated with 2.2 ml of a sonicated BHK_{21} cell suspension containing $10^{8.7}$ 50% tissue culture infective doses ($TCID_{50}$). On PID 6 and 7, blood from this infected animal was collected in sodium citrate and pooled. Blood cells were washed and resuspended three times with Dulbecco's phosphate-buffered saline (DPBS), sonicated, and frozen in multiple aliquots at −70 C. The viral

titer of the resulting EHDV-2 inoculum, as determined by endpoint titration, was $10^{4.5}$ $TCID_{50}$ per ml. The inoculum used in the homologous challenge experiment was similarly prepared by giving 10 ml of blood from the first deer subcutaneously to a second serologically negative yearling white-tailed deer. On PID 5, 6, and 7, blood from this second deer was collected and processed as described. The titer of that second inoculum was $10^{2.0}$ $TCID_{50}$ per ml. The inoculum used in the heterologous viral challenge was prepared by giving BTV-10-infected blood from an experimentally infected deer to a third serologically negative white-tailed deer. Blood was collected and aliquoted as described for the EHDV-2 inocula. The titer of the BTV-10 inoculum was $10^{2.1}$ $TCID_{50}$ per ml.

In both experiments, deer were sedated with 0.5 mg/kg xylazine (Rompun^R, Miles, Inc., Shawnee Mission, Kansas, USA), and the inoculation sites were clipped. Approximately half of the 10 ml inoculum was administered subcutaneously and half intradermally at multiple sites along the both sides of the neck. Control animals were similarly inoculated with uninfected deer blood that had been washed and sonicated.

For virus isolation, blood samples collected in acid citrate were washed three times with DPBS, then sonicated. Samples were diluted 1:10 in minimal essential media (MEM) and adsorbed onto BHK_{21} cell cultures for 1 hr at 33.5 C. Monolayers were washed with DPBS and overlaid with maintenance media consisting of MEM supplemented with 3% heat-inactivated fetal bovine serum (FBS), 200 mM/l L-glutamine, and antibiotics (100 U penicillin, 0.1 mg dihydrostreptomycin, and 0.25 μg amphotericin B/ml) (Sigma Chemical Company, St. Louis, Missouri, USA). Cultures were incubated in 5% CO_2 at 33.5 C. After 7 days, cells were scraped and 100 μl of cell suspension were inoculated onto an additional BHK_{21} culture. Cultures with no cytopathic effect after 7 days in the second passage were considered negative.

Viral titers of positive blood samples were determined by endpoint titration on BHK_{21} cells. All titrations from an individual animal were done on the same day, and included samples collected 1 day before the initial virus isolation to 1 day after the final virus isolation. Blood was diluted 1:10 in maintenance media (as described) and sonicated. Ten-fold dilutions were made up to 10^{-6} and 100 μl of each dilution were placed in four wells of a 48-well plate. Each well was seeded with 1 ml of maintenance media and approximately 5×10^4 BHK_{21} cells. Plates were incubated for 7 days at 33.5 C in 5% CO_2. On day 7, individual wells were scraped and 100 μl of cell suspension were passed into another 48-well plate for an additional 7 days. Titers were calculated as described by Reed and Muench (1938).

All isolated viruses were identified by virus neutralization against all North American EHDV and BTV serotypes. Infected tissue culture media from culture attempts with a cytophatic effect were diluted 1:10 in maintenance media, and 25 μl were inoculated into eight wells of a 96-well tissue culture plate. Each of seven wells were inoculated with 25 μl of a 1:10 dilution of heat-inactivated bovine antisera to BTV-2, BTV-10, BTV-11, BTV-13, BTV-17, EHDV-1 or EHDV-2 (National Veterinary Services Laboratory, Ames, Iowa, USA). The final well served as a positive control and received 25 μl of maintenance medium. Plates were incubated for 2 hr at 37 C in 5% CO_2, and 150 μl (approximately 1×10^4 cells) of cattle pulmonary artery endothelial (CPAE) cells (American Type Cell Culture) were added to each well. Plates were read from 48 to 72 hr to detect cytopathic effect.

Primary cell cultures were prepared from fetal white-tailed deer kidneys via trypsin digestion for use in the IFN assays. A white-tailed deer fetus was obtained within 1 hr of the death of the female. Kidney sections weighing approximately 2 g were minced into 1 mm^2 pieces and washed three times in 37 C DPBS. The tissue was then placed in 30 ml of a 0.25% trypsin solution (Sigma Chemical Company) maintained at 37 C and stirred for 10 min. The resultant supernatant was harvested and retained at 4 C. The volume was replaced with 37 C trypsin solution and the samples were again stirred. This process was repeated four times. The resultant cell solution was centrifuged at $1000 \times G$ for 10 min. The cell pellet was resuspended in 25 ml of MEM supplemented with 10% FBS and antibiotics (as described). Cell aliquots were frozen at −70 C in cell freezing media with dimethyl sulfoxide (Sigma Chemical Company) and stored in liquid nitrogen vapor pending use. Upon thawing, the growth media for cells was MEM-supplemented with 10% FBS, 200 mM/l L-glutamine, 1×10^6 U/l penicillin, and 10 mg/l dihydrostreptomycin. Cell cultures were incubated at 37 C in 5% CO_2. Cell cultures used in the IFN assay were fourth to sixth passage.

The α and β IFN was quantitated using a microtiter assay of cytopathic effect as described by Lewis (1987), with vesicular stomatitis virus (VSV) (New Jersey serotype; National Veterinary Services Laboratory) as the indicator virus. Prior to assay, serum samples were dialyzed overnight at 4 C in pH 2 acetic acid in

distilled water to destroy inciting virus and to eliminate gamma IFN activity (Joklik, 1991). Samples were redialyzed overnight at 4 C in DPBS to pH 7.2. Selected undialized samples were measured for gamma IFN activity to insure inactivation. Duplicate two-fold dilutions of sera in MEM from 1:2 to 1:1024 were made in 96-well plates. All samples from an individual deer were run at the same time. Human recombinant α IFN (Hoffman-LaRoche, Inc., Nutley, New Jersey, USA), which was active on deer cells, was used at an initial concentration of 100 U/ml as a positive control in the assay. Fetal deer kidney cells were added at approximately 3×10^4 cells/well and plates were incubated for 24 to 30 hr at 37 C in 5% CO_2 until cells were confluent. Well supernatants were removed and cell monolayers were washed with sterile DPBS. Vesicular stomatitis virus-NJ diluted in 100 μl of cell growth media was added at 10^4 $TCID_{50}$/well, and plates were incubated until virus control wells had 100% cytopathic effect (24 to 48 hr). Well supernatants were decanted, cell monolayers were rinsed with tap water then stained as per Meager (1987) for 30 min with 0.1 ml/well of a 0.05% amido black in 9% acetic acid and 0.1 M sodium acetate solution. The cells were then fixed for 15 min with 0.1 ml/well of a 10% formaldehyde in 9% acetic acid with 0.1 M sodium acetate solution. Plates were rinsed with tap water and air dried. To allow for measurement of optical densities via a spectrophotometer, the dye was extracted for 5 min with 150 μl/well of a 50 mM sodium hydroxide solution. Plates were read via an ELISA microplate reader set at 600 nm. Cell staining and artifactual disturbances in cell monolayers were evaluated manually. Interferon titers were expressed as the reciprocal of the dilutions that caused a 50% protection from cytopathic effect in cell monolayers as described by Meager (1987).

Precipitating antibodies to BTV and EHDV serogroups were detected using agar-gel immunodiffusion (AGID) test kits as described by the manufacturer (Veterinary Diagnostic Technology, Inc.). Serotype specific antibodies were quantitated via serum neutralization as described by Stallknecht et al. (1995).

Bacterial cultures performed on deer with secondary bacterial infections were done at the Athens Diagnostic Laboratory, College of Veterinary Medicine, University of Georgia, Athens, Georgia. Samples were initially inoculated onto blood agar plates (Difco Company, Detroit, Michigan, USA) and thioglycolate broth (Difco). After 24 hr or when bacterial growth was observed, bacteria were tentatively identified based on colony morphology and Gram stains. Individual bacterial colonies were subcultured on appropriate media for final identification (Carter and Cole, 1990).

For statistical analysis, the data collected during the first experiment of EHDV-2 infection of naive deer were analyzed separately from the data collected during the second experiment on the viral challenge of convalescent deer.

Body temperatures, blood cell parameters, virus titers, and serum neutralizing antibody titers were analyzed using a two-factor analysis of variance using animal group and PID as factors (Zar, 1974). In the first experiment, EHDV-2-infected deer were divided into control animals and three infected groups (mild, moderate, or severe) based on clinical signs (Table 1) to allow for more in-depth comparisons of response to infection. During the second experiment, the statistical groups used consisted of the three EHDV-2-challenged, four BTV-10-challenged, four convalescent-controls and four non-infected controls. Significance was located using a Dunnett's multiple comparison test at $\alpha \leq 0.05$. This was a specialized multiple comparison test where the only comparisons desired were between the appropriate control group and the various experimental groups. Since the sample size was widely varying, a standard error was calculated for each comparison.

To determine the relationship between the variable alive/dead and other continuous variables, a point-biserial calculation was made (Bruning and Kintz, 1987). To determine the relationship between pairs of continuous variables, a Pearson product-moment correlation (r) was calculated. Data with significant correlations were further analyzed using an analysis of variance for linear regression.

RESULTS

Experiment 1: EHDV-2 infection of naive white-tailed deer

Based on the scoring system used to rate the clinical signs observed in affected deer, five deer had mild clinical signs (increased body temperature, subtle redness of thinly haired regions: ear pinna, perineum, nares, periorbital zones), five deer had moderate clinical signs (increased body temperature, noticeable redness of thinly haired regions: ear pinna, perineum, nares, periorbital zones; mild depression and lethargy), and six deer had severe clinical signs (increased body temperature, marked redness of thinly haired regions:

TABLE 1. Experiment 1: Comparison of clinical morbidity rating (MR), virus titer on peripheral blood (in log 10), and antibody titers (dilution reciprocal) during initial EHDV-2 infections. None of the control deer had VI or antibody titers. Serum samples were initially screened for precipitating antibodies by AGID. All positive samples were titered by serum neutralization.

Deer number	Year	MR[a]	Test	Post-infection day 0	2	4	6	8	10	12	14	17	21	24
30	92	3	Vir Tr[b]	—[c]	—	4.7	5.0	3.7	(Died)					
			Ab Tr[d]	—	—	—	—	80						
44	92	3	Vir Tr	—	—	5.3	5.7	(Euthanized)						
			Ab Tr	—	—	—	—							
48	92	3	Vir Tr	—	—	5.7	5.7	4.7	4.0	4.0	3.3	(Died)		
			Ab Tr	—	—	—	—	10	10	NA[e]	40			
49	92	3	Vir Tr	—	—	4.7	3.5	3.4	2.0	2.7	1.7	1.7	2.0	1.7
			Ab Tr	—	—	—	—	640	2,560	2,560	5,120	5,120	5,120	2,560
76	93	3	Vir Tr	—	—	5.7	5.5	6.0	6.5	5.0	3.3	3.3	3.3	3.0
			Ab Tr	—	—	—	—	—	10	20	40	40	20	40
77	93	3	Vir Tr	—	—	5.7	6.3	6.7	5.5	4.7	4.5	4.5	3.7	2.3
			Ab Tr	—	—	—	—	—	40	40	40	320	320	160
46	92	2	Vir Tr	—	—	2.5	2.5	2.0	—	—	—	—	—	—
			Ab Tr	—	—	—	—	1,280	1,280	2,560	1,280	2,560	320	320
80	93	2	Vir Tr	—	—	5.5	6.3	5.7	5.7	+	4.5	4.5	4.3	4.0
			Ab Tr	—	—	—	—	—	40	80	160	80	320	160
81	93	2	Vir Tr	—	1.7	5.3	6.5	(Euthanized)						
			Ab Tr	—	—	—	—							
84	93	2	Vir Tr	—	—	3.5	6.0	5.5	4.5	+	4.0	4.3	3.7	—
			Ab Tr	—	—	—	—	—	40	80	80	40	40	40
91	93	2	Vir Tr	—	2.5	6.3	6.7	6.5	4.7	(Died)				
			Ab Tr	—	—	—	—	80	20					
31	92	1	Vir Tr	—	1.7	4.3	5.3	3.5	2.3	1.7	2.5	2.3	2.0	—
			Ab Tr	—	—	—	—	—	20	40	40	20	40	80
33	92	1	Vir Tr	—	—	3.5	4.3	3.0	—	—	1.7	<1.7	—	—
			Ab Tr	—	—	—	—	—	40	160	320	160	320	640
36	92	1	Vir Tr	—	—	1.7	2.7	<1.7	<1.7	1.7	NA	<1.7	—	—
			Ab Tr	—	—	—	—	—	10	40	80	80	80	80
79	93	1	Vir Tr	—	—	3.7	5.5	5.3	4.5	3.5	2.7	3.3	3.3	2.0
			Ab Tr	—	—	—	—	—	80	160	40	80	80	80
83	93	1	Vir Tr	—	—	4.3	6.3	5.7	5.5	5.3	4.5	4.0	4.0	3.7
			Ab Tr	—	—	—	—	—	20	20	80	160	160	160

[a] MR = mortality rating; 0 = no clinical signs, 1 = mild clinical signs, 2 = moderate clinical signs, 3 = severe clinical signs. See text for specific details of rating methods.

[b] Vir Tr, virus titer.

[c] Dash (—) indicates lack of detectable titer.

[d] Ab Tr, antibody titer.

[e] Information not available.

ear pinna, perineum, nares, periorbital zones; severe depression and lethargy, dehydration, subcutaneous swelling about head, bleeding tendencies) (Table 1). Clinical signs were initially noticed on PID 4 or 5 and generally were most severe by PID 7 to 8. Deer that survived gradually returned to normal over the next 1 to 2 wk; however, the three severely affected deer that survived were thin and appeared weak 4 wk after infection.

Three deer died and two deer were euthanized because of the severity of clinical signs during the acute phase of infection. Two euthanized deer and one deer that died succumbed in the first 10 days of infection. Lesions typical of hemorrhagic disease were minimal to extensive pete-

chial and ecchymotic hemorrhages on various internal organs, but uniformly at the base of pulmonary artery and over the pylorus. Other common lesions were hyperemia in the forestomachs and buccal papillae, subcutaneous and pulmonary edema, and pericardial and pleural effusions. The two animals that died at PID 12 and 15 had secondary bacterial infections. Both animals had bacterial bronchopneumonia; one also had necrotic laryngitis. A mixture of bacterial pathogens were isolated from the lungs of these deer including *Pasteurella multocida*, *Streptococcus* sp., *Actinomyces pyogenes*, *Escherichia coli*, and *Enterococcus* sp.

Body temperatures in infected deer varied with degree of excitement or activity prior to temperature monitoring making interpretation of temperature changes difficult. However, when assessed in conjunction with other clinically apparent signs of fever, such as cutaneous hyperemia, 11 of the 16 EHDV-2-infected deer developed body temperature increases of ≥0.5 C (range 39.5 C to 40.1 C) by PID 4 to 8, as compared to baseline temperatures and temperatures of control deer; increased body temperatures lasted 1 to 6 days. One deer had little temperature change, but had to be euthanized at PID 7 because of severe bloody diarrhea and dehydration. Seven moderately or severely affected deer became hypothermic with body temperatures dropping to <38.5 C between PID 8 and 12. Baseline levels and temperatures of control deer never dropped below 38.5 C during the same time period. Three of the hypothermic animals from the severely affected group died.

While no statistically significant differences were found in the mean total leukocyte counts of EHDV-2-infected deer, distinct trends were apparent. Mean total leukocyte counts of infected deer decreased until PID 6, and returned to baseline levels by PID 8 or 10. At its nadir on PID 6, the mean (± SD) total leukocyte counts seen in mildly, moderately, and severely affected groups were similar: 2,760 ± 984 cells/ml, 2,680 ± 687 cells/ml, and 2,920 ± 1,246 cells/ml, respectively. In comparison, the mean (± SD) leukocyte count of control animals on the same day was 4,500 ± 2,043 cells/ml.

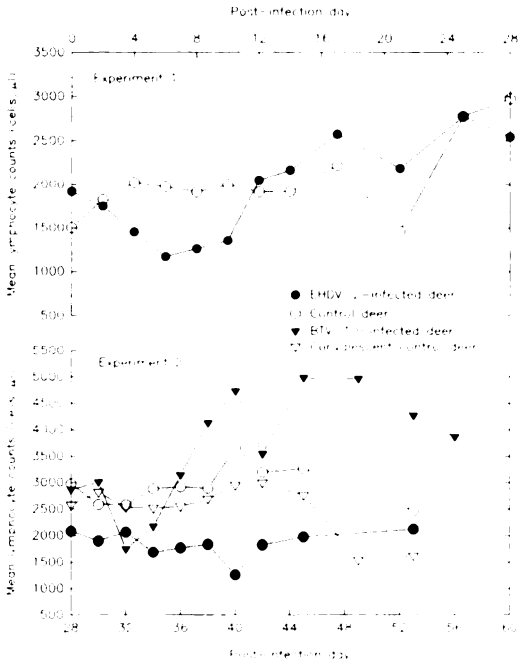

FIGURE 1. Average lymphocyte counts of infected and control white-tailed deer during Experiment 1 (initial EHDV-2 exposure) and Experiment 2 (challenge with BTV-10 or EHDV-2). During initial EHDV-2 infections, the number of animals decreased from 16 at PID 0 to 11 by PID 28. Four animals were challenged with BTV-10; one animal died. Three animals were challenged with EHDV-2; no animals were lost. Control animals = 4. Convalescent control animals = 4.

The most notable leukocyte changes in infected deer were decreased average lymphocyte counts, which generally developed 6 to 10 days after infection (Fig. 1). Overall, 10 of 16 infected animals had absolute lymphocyte counts of less than 1000 cells/μl at some point between PID 4 and PID 10; five of these 10 deer died. The degree of decrease in the average lymphocyte count varied between groups of infected animals with severely affected animals having significantly lower lymphocyte counts on PID 6 and 8 ($P = 0.01$) than control animals or baseline levels for those ani-

mals. No association between minimum number of lymphocytes and death was found; however, animals with more marked clinical signs or high virus titers had low lymphocyte counts. The degree of clinical signs and lymphocyte counts were inversely correlated (Pearson product-moment correlational coefficient = −0.0581, $P = 0.02$) as were the peak virus titers and lymphocyte counts (correlational coefficient = −0.49, $P = 0.05$). Similarly, the six mildly lymphopenic deer (lymphocyte counts below control average but >1,000 cells/ml) during this same period had milder clinical signs than profoundly lymphopenic deer.

No significant changes were detected in neutrophil counts of EHDV-infected or control animals. However, the deer that developed secondary bacterial infections had increased neutrophil counts for 1 to 2 days prior to death ($\bar{x} \pm$ SD = 3,698 ± 1,763 cells/ml) when compared to the mean baseline values for those animals (2,188.67 ± 275 cells/ml).

Mean hematocrits of infected deer decreased between PID 8 and 20, then gradually rebounded but never returned to baseline levels (Fig. 2). While there were no significant differences between infected versus control groups of deer during the first 2 wk of infection, the three severely affected deer that survived the first 14 days of infection had significant decreases ($P \leq 0.01$) in hematocrits during the second 2 wk of the study. Mean (± SD) hematocrits for these three animals dropped from a baseline of 38.4 ± 4% to 30.33 ± 10% by PID 28. Other erythrocyte indices remained within range of normal as compared to control animals. On an individual basis, hematocrits of nine of the EHDV-2-infected deer decreased after initial EHDV-2 infections from a baseline of 38.73 ± 5.8% to 27.68 ± 4.9% by PID 28, yet the hematocrits of four infected animals and the four control animals stayed relatively constant throughout the study. Mean (± SD) hematocrits of three animals that died during the acute phase of infection increased gradually from a baseline average of 40.1 ± 1.5% to an average of 49.7% ± 3% by PID 6, reflecting hemoconcentration secondary to dehydration which was noted clinically.

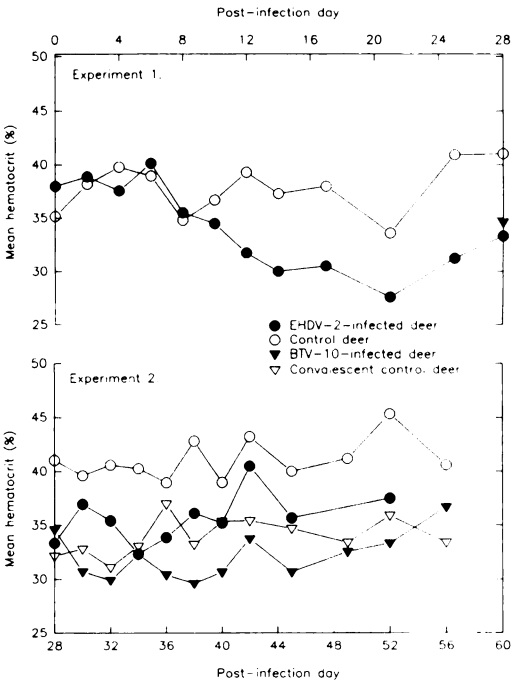

FIGURE 2. Average hematocrits of infected and control white-tailed deer during Experiment 1 (initial EHDV-2 exposure) and Experiment 2 (challenge with BTV-10 or EHDV-2). During initial EHDV-2 infections, the number of animals decreased from 16 at PID 0 to 11 by PID 28. Four animals were challenged with BTV-10; one animal died. Three animals were challenged with EHDV-2; no animals were lost. Control animals = 4. Convalescent control animals = 4.

All naive EHDV-2-infected animals were viremic by PID 4 (Table 1). Three of the 16 infected deer were viremic by PID 2; two of these three animals later died. There was a significant ($P = 0.001$) difference in peak virus titers the second year of the study ($\log_{10}6.31 \pm 0.40$ $TCID_{50}$/ml) as compared to the first year of the study ($\log_{10}4.50 \pm 1.26$ $TCID_{50}$/ml).

Viremia was last detected in surviving deer from PID 9 to PID 56. Two deer in the second year of the study that were unchallenged by BTV were still viremic at PID 56. The longest viremia found in the

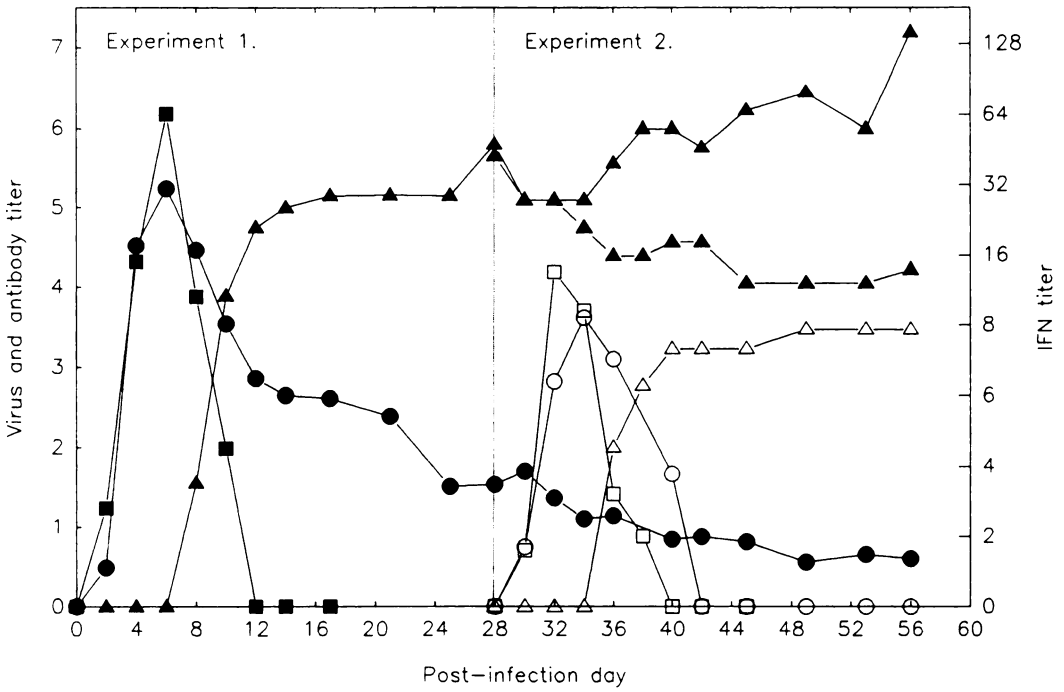

FIGURE 3. Temporal correlation of the virus titers ($log_{10}TCID_{50}$), antibody titers (log_{nat} reciprocal dilution), and α and β interferon titers (reciprocal dilution) of white-tailed deer during Experiment 1 (initial EHDV-2 exposure) and Experiment 2 (challenge with BTV-10 or EHDV-2). Legend: ▲ = EHDV-2 antibody titer (in Experiment 2: lower line is convalescent control deer titer, upper line is EHDV-2 challenged deer titer), △ = BTV-10 antibody titer, ■ = IFN titer to EHDV-2, □ = IFN titer to BTV-10, ● = EHDV-2 virus titer, and ○ = virus titer during BTV-10 challenge.

first year of the study was in one animal that was still viremic at PID 30. This animal was challenged with EHDV-2 at PID 28, but no increase in viral titer was detected post-challenge. The duration of viremia was longer in deer with higher peak viral titers than in animals with lower titers ($P = 0.007$). Although the peak virus titer was lower in animals with mild clinical signs ($\bar{x} \pm SD = log_{10}4.92 \pm 1.46$ $TCID_{50}$/ml) than deer showing severe clinical signs ($log_{10}5.71 \pm 0.79$ $TCID_{50}$/ml), a statistical difference was not detected. Based on point-biserial correlation, there was no association between the degree of clinical signs and duration of viremia in surviving deer or between the degree of viremia and death.

Interferon levels rose abruptly in all infected deer after initial EHDV-2 infection, peaked between PID 4 and 6 with levels varying between deer (range 1:8 to 1:512), and fell rapidly with no IFN activity detected after PID 10. In 11 deer, peak IFN levels and peak viremia occurred simultaneously followed by initial decline of the viremias (Fig. 3). Control animals produced no detectable IFN. Deer with moderate or severe clinical signs generally had peak IFN levels that developed on about Day 4 ($\bar{x} \pm SD$ = PID 4.8 ± 1.8 and PID 4.67 ± 1.0, respectively). In contrast, peak IFN titers in deer with mild clinical signs occurred slightly later (Day 6; PID 6 ± 0), but the differences in levels of IFN between these groups were not significant. There was no correlation between peak virus titers and peak IFN levels. Temporally, the mean peak viremia coincided with the mean peak IFN titers on PID 6, and preceded the presence of detectable antibodies (Fig. 3).

By PID 10, all EHDV-2-infected animals developed precipitating and neutralizing antibodies to EHDV-2 that persisted for the duration of the study (Tables 1, 2). The appearance of antibodies in peripheral blood was associated with a continued reduction of viremia, yet viremia persisted despite these antibodies. Statistically, there was no correlation between the degree of peak viremia and the height of the antibody titer produced later. Similarly, there were no significant differences detected between peak antibody levels between mild, moderate, or severely affected deer.

Experiment 2: Viral challenge of convalescent white-tailed deer

Clinical signs were not detected in the three deer that were challenged with homologous virus. A range of clinical signs similar to those noted in the initial EHDV infection were seen in all four animals challenged with BTV-10 (Table 2). A previously uninfected deer given the BTV-10 inoculum developed clinical signs at the same time as, and similar to, the BTV-10-challenged animals. This deer and one severely affected challenged animal were euthanized on post-challenge day 7. Lesions seen at necropsy in both deer were compatible with hemorrhagic disease as described during initial infections. The severely affected BTV-10-challenged animal also had cranioventral bronchopneumonia; a mixture of bacterial pathogens including *Enterococcus* sp., *Escherichia coli, Enterobacter* sp., and *Actinobacter* sp., were isolated from its lungs.

Three of 10 white-tailed deer necropsied at PID 56 (challenge day 28) had mild pericardial effusions; one animal had been challenged with EHDV-2, one animal had been challenged with BTV-10, and one animal received the initial EHDV-2 but no challenge inoculum.

Body temperature elevations seen in EHDV-2-challenged animals were not as marked as initial temperature increases. All three deer challenged with homologous virus had mild temperature elevations ranging from 39.2 C to 39.6 C between challenge day 2 and 8. Body temperature elevations seen in BTV-10-challenged deer were similar to elevations seen in those same animals during initial EHDV-2 infections. All four deer challenged with heterologous virus had temperature elevations ≥39.5 C between challenge day 4 and 8.

Overall during the second experiment, total leukocyte and lymphocyte counts of both EHDV-2 and BTV-10 infected deer were slightly higher than those seen during the initial EHDV-2 experiment. Hematocrits were lower during the challenge infections than during initial infections, but the average initial count at PID 0 was lower (34% compared to 38% initially). Neutrophils counts were similar during the two experiments.

Mean (±SD) leukocyte counts of both EHDV-2- and BTV-10-challenged deer decreased by challenge days 4 to 3133±503 cells/μl and 3500±945 cells/μl, respectively, while leukocyte counts of convalescent control deer remained stable. However, these changes were not significantly different from either baseline levels or control deer. Mean (±SD) lymphocyte counts in EHDV-2-challenged deer decreased progressively from an initial count of 2,080±413 cells/μl on challenge day 0 to 1,259±627 cells/μl on challenge day 12, which was significantly lower ($P = 0.01$) than baseline or control counterparts (3,656±1,024 cells/μl) (Fig. 1). Deer challenged with BTV-10 had decreased lymphocyte counts by challenge day 4 (1,752 ± 799 cells/μl), which rebounded rapidly; however, the changes were not significant when compared to baseline or control animals (Fig. 2).

Mean neutrophil counts of both EHDV-2-challenged and BTV-10-challenged deer decreased slightly during the first 8 days post-infection. The mean neutrophil counts of the EHDV-2 and BTV-10 challenged groups were significantly lower on challenge day 4 than non-infected controls ($P = 0.01$), but the counts were not sig-

TABLE 2. Experiment 2: Comparison of clinical morbidity rating (MR), virus titer in peripheral blood (in log 10), and antibody titer (dilution reciprocal) of deer challenged by infection with either EHDV-2 or BTV-10. None of the control deer had virus or antibody titers. Serum samples were initially screened for precipitating antibodies by AGID. Samples with detectible antibodies were titered by serum neutralization. Post-challenge day is listed; post-infection day of initial study is in parenthesis.

Deer number	Year	MR[a]	Test	Post-challenge day 0 (28)	2 (30)	4 (32)	6 (34)	8 (36)	10 (38)	12 (40)	14 (42)	17 (45)	21 (49)	24 (52)	28 (56)
EHDV-2-challenged deer															
31	92	1	Vir Tr[b]	—[c]	—	—	—	—	—	—	—	—	—	—	—
			Ab Tr[d]	40	40	40	40	40	40	80	40	80	40	40	NA[e]
46	92	1	Vir Tr	—	—	—	—	—	—	—	—	—	—	—	—
			Ab Tr	640	640	640	320	640	320	160	320	320	1280	640	1280
49	92	1	Vir Tr	<1.7[f]	<1.7[f]	—	—	—	—	—	—	—	—	—	—
			Ab Tr	1280	160	160	320	640	5120	5120	2560	5120	5120	2560	1280
BTV-10-challenged deer															
76	93	3	Vir Tr	3.0[f]	2.5[f]	3.7[g]	4.0[g]	(Died)							
			EHD Ab	80	320	320	160								
			BTV Ab	—	—	—	—								
79	93	2	Vir Tr	2.0[f]	2.5[f]	3.7[g]	3.5[g]	NA[g]	NA[g]	2.3[f]	—	—	—	—	—
			EHD Ab	80	40	80	80	40	40	20	40	40	40	20	40
			BTV Ab	—	—	—	—	20	20	40	40	40	40	20	40
83	93	1	Vir Tr	3.3[f]	3.5[f]	3.7[f]	3.3[g]	3.5[g]	NA[g]	2.7[h]	2.5[f]	2.5[f]	2.5[h]	2.0[f]	1.7[h]
			EHD Ab	160	320	320	80	40	160	160	160	80	320	320	160
			BTV Ab	—	—	—	—	—	20	20	20	20	40	40	40
84	93	1	Vir Tr	—	3.0[g]	3.0[g]	3.3[g]	2.7[g]	—	—	—	—	—	—	—
			EHD Ab	80	40	40	80	80	80	80	80	160	40	80	80
			BTV Ab	—	—	—	—	20	10	20	20	20	20	20	20
Convalescent controls:															
33	92	0	Vir Tr	—	—	—	—	—	—	—	—	—	—	—	—
			EHD Ab	1280	640	640	640	160	160	80	160	160	80	80	80
36	92	0	Vir Tr	—	—	—	—	—	—	—	—	—	—	—	—
			EHD Ab	80	80	80	40	40	80	160	160	80	80	40	80
77	93	0	Vir Tr	4.0[f]	3.7[f]	3.5[f]	4.0[f]	4.0[f]	NA[f]	2.3[f]	3.3[f]	3.3[f]	3.0[f]	2.0[f]	2.0[f]
			EHD Ab	320	160	160	80	40	20	80	40	40	40	40	40
80	93	0	Vir Tr	3.7[f]	4.0[f]	3.7[f]	3.7[f]	4.0[f]	NA[f]	3.0[f]	3.0[f]	2.3[f]	2.0[f]	2.5[h]	1.7[f]
			EHD Ab	160	80	80	80	160	160	80	80	20	40	80	80

[a] MR = mortality rating; 0 = no clinical signs, 1 = mild clinical signs, 2 = moderate clinical signs, 3 = severe clinical signs. See text for specific details of rating methods.
[b] Vir Tr, virus titer.
[c] Dash (—) indicates lack of detectable titers.
[d] Ab Tr, antibody titer.
[e] NA = Information not available.
[f] EHDV-2 isolated.
[g] BTV-10 isolated.
[h] No positive identification of isolated virus made.

nificantly different from convalescent controls.

Mean hematocrits of the EHDV-2-challenged, BTV-10-challenged, and convalescent control groups remained lower than the control group through challenge day 28. Mean hematocrits of BTV-10-challenged deer decreased slightly by challenge day 2 (Fig. 2). No significant differences between groups could be located with Dunnett's test.

No increase in viremia was detected in the EHDV-2 challenge inoculation (Table 2). Viremia increased in all four animals challenged with BTV-10. Since three of the four animals challenged with BTV-10

still had EHDV-2 titers at the time of challenge (Table 2), viral titers could not be attributed to BTV-10 alone and may have represented the combined effect of EHDV-2 and BTV-10. However, BTV-10 was isolated from the blood of all four deer and, in two deer, both EHDV-2 and BTV-10 were intermittently isolated from blood during the period of BTV viremia. Detectable BTV-10 viremias were of shorter duration than those seen with initial EHDV-2 infections as isolations identified as BTV-10 were last detected on challenge day 8 in one animal and on challenge day 10 in two of the animals. However, it is possible that later unidentified isolates in one animal (Deer 83) may have been BTV-10.

Only one of the three animals challenged with EHDV-2 developed detectable IFN, which was a low level (dilution of 1:8) that was only seen on PID 2 and 4 with a peak on PID 4. All animals challenged with BTV-10 produced detectable IFN with peak levels occurring either on challenge days 4 or 6. The three animals with EHDV-2 titers at the time of BTV-10 challenge had lower peak titers (range 1:2 to 1:32) than did the single animal (Deer 84; IFN peak at a dilution of 1:128) that had no detectable EHDV-2 titer at the time of BTV-10 challenge.

Two of three EHDV-2-challenged deer had an increase in neutralizing antibody titers, at challenge day 10 and 21, respectively (Table 2). The third animal (Deer 31) that had the IFN response had no meaningful increase in antibody titer. The average antibody titers of convalescent controls gradually decreased. As was seen during initial EHDV-2 infections, BTV-10-challenged animals developed neutralizing antibodies to BTV-10 by challenge day 10; antibody titers peaked on or before challenge day 21 (Table 2). Both BTV and EHDV antibodies persisted in all deer until the end of the study. Control animals did not seroconvert to either EHDV or BTV.

DISCUSSION

Optimally, the initial and challenge inocula should have had the same virus titer. Unfortunately, the inoculum used in the EHDV-2 challenge experiment had a lower titer ($10^{2.0}$ $TCID_{50}$ per ml) than did the initial EHDV-2 inoculum ($10^{4.5}$ $TCID_{50}$ per ml). The reason for this difference was that two deer were used to prepare the inocula to avoid possible Type II hypersensitivity reactions due to repeated injections of blood from a single donor deer. In spite of this difference, the challenge inoculum was capable of causing infection; it successfully caused viremia in naive white-tailed deer used in a separate study (Smith et al., 1995). Thus, it was felt that the difference in the inoculum titer was not the cause of the lack of detectable viremias seen in EHDV-2 challenged deer.

Although EHDV can have a significant impact on white-tailed deer populations through widespread morbidity and mortality (Nettles and Stallknecht, 1992), early studies of hemorrhagic disease in white-tailed deer resulted in extremely high estimates of mortality rates, often greater than 90% (Hoff and Trainer, 1978). However, based on the presence of chronic foot lesions and serologic studies, it is apparent that many deer survive infections (Couvillion et al., 1981; Nettles and Stallknecht, 1992). Our study is supportive of field observations that there is tremendous individual variability in clinical response in deer infected with either EHDV or BTV. While our initial EHDV-2 infection produced 100% morbidity in naive deer, death occurred in only 31% of the deer. Subtle clinical signs, that probably would have gone undetected under field conditions, were seen in another 31% of the animals. This individual variation in clinical response may be reflective of an outbred population with wide genetic variability in resistance to infection. Nettles and Stallknecht (1992) hypothesized that genetic resistance to infection has evolved in wild ruminants in areas where hemorrhagic disease is endemic.

The degree of lymphopenia seen in EHDV-2-infected animals was directly correlated with morbidity and mortality. In previous studies, decreased lymphocyte counts occurred in EHDV-infected white-tailed deer (Wilhelm and Trainer, 1969) and BTV-17-infected white-tailed deer (Howerth et al., 1988), but no correlations were made between the degree of lymphopenia and morbidity or mortality. Lymphopenia also was seen in sheep infected with BTV (Ellis et al., 1990). In contrast, cattle infected with BTV became neutropenic versus lymphopenic (MacLachlan and Thompson, 1985). The pattern of lymphopenia seen during the BTV-10 challenge resembled initial EHDV-2 infections. While no clinical illness was seen in EHDV-2-challenged animals, mild lymphocyte decreases were present. These decreased lymphocyte counts, combined with slight temperature elevations, are evidence that the virus entered the lymphoid system and stimulated an immune response prior to containment.

Several mechanisms may be involved in development of the lymphopenia. Since virus can be isolated from peripheral blood mononuclear cells of EHDV-2-infected deer for approximately 2 wk after infection (Stallknecht et al., 1997), lymphopenia may relate to direct virus destruction of lymphocytes or to viral-induced lymphocytic apoptosis. Alternatively, the lymphopenia may be a manifestation of cytokine-induced alterations in lymphoid trafficking in lymph nodes and other sites of viral replication similar to that shown in BTV-infected sheep (Ellis et al., 1991) and cattle (Barratt-Boyes et al., 1995). Regardless of the mechanisms, the development of secondary infections during the subacute phase of disease is further evidence for possible immune dysfunction in EHDV- and BTV-infected deer. Concurrent studies of cell-mediated immune function in these EHDV-2 and BTV-10 infected white-tailed deer showed a significant reduction of lymphocyte proliferative responses to concanavalin A during the acute phase of infection in naive animals (Quist et al., in press). The combined data are evidence that white-tailed deer have transient compromise of cell-mediated immune function during the first few weeks of EHDV-2 or BTV-10 infection.

The anemias observed by the second week of the study in severely affected EHDV-2-infected deer are compatible with the vascular damage and resultant hemorrhage that is a hallmark of severe hemorrhagic disease caused by either EHDV (Debbie and Abelseth, 1971) or BTV (Howerth et al., 1988). In contrast, mild and moderately affected animals developed no significant decrease in hematocrits, possibly reflecting minimal vascular damage. Iatrogenic blood loss during the study was an initial concern as a contributory cause of anemia, but the stability of hematocrits of four infected and the four control deer is evidence that blood sampling was not a major factor.

Serum neutralizing antibodies were detectable by the 10th day after both the initial EHDV-2 and the BTV-10-challenge infections; however, viremia persisted despite these antibodies. This is in contrast to the previous observation that white-tailed deer experimentally infected with EHDV and BTV had no detectable viremia within 2 to 8 days after neutralizing antibodies appeared in circulation (Hoff and Trainer, 1974). The persistence of viremia despite the presence of circulating neutralizing antibodies has been reported in BTV-infected cattle (MacLachlan et al., 1990) and sheep (Luedke, 1969). In BTV-infected cattle, protection from immune elimination may be due to the association of virus particles with red cell membranes (MacLachlan et al., 1990). In in vitro studies with BTV, virus was found sequestered in pits in bovine erythrocyte membranes (Brewer and MacLachlan, 1992). Stallknecht et al. (1997) found EHD virions similarly sequestered within infoldings of white-tailed deer red blood cell membranes infected in vitro. This close association with the red cell membrane may

protect the viruses from circulating antibodies and immune clearance (Barratt-Boyes and MacLachlan, 1995).

The higher viremias found in fawns during the second trial of the 2-yr study remain unexplained, but could involve differences in the fawns, in experimental factors between years, or in the deers' environment. While the fawn groups were similar, the fawns used the second year were possibly slightly younger as they weighed less as a group, though not significantly so. In regard to experimental factors, because virus isolations were performed on different years, there were differences in other uncontrollable factors such as cell culture media. Biting insects were a problem in the animal facility the second year of the study, and it is possible, though not probable, that they contributed to the higher virus titers seen that year. The facility was closed during the entire experiment to minimize ingression of insects, and *Culicoides* spp., the known vectors of EHDV and BTV, typically do not frequent covered enclosures (Blanton and Wirth, 1979). Furthermore, no *Culicoides* spp. were found using light traps near the deer pens during the study. However, while the *Culicoides* spp. midge is the only known biological vector of EHDV and BTV, mechanical transmission of EHDV and BTV may be possible. Transmission of BTV by sheep keds (*Melophagus ovinus*) (Luedke et al., 1965) and *Ornithodoros* spp. ticks (Stott et al., 1985) has occurred. Fleas (resembling *Ctenocephalides felis*) and biting stable flies (probably *Stomoxys* sp.) were observed on the deer early in the study, necessitating treatment of the deer and premises with insecticides. It is conceivable that fleas and stable flies may have been the source of the EHDV-2 that infected a deer that was being held separately in the same facility and may have transmitted additional virus to infected deer.

For several statistical analyses, deer were combined into subgroups based on severity of clinical signs. The resultant small group sizes made it difficult to statistically verify differences that were observed. Nonetheless, statistical correlations were detected between the severity of clinical disease and some parameters such as decreased lymphocyte counts and decreased hematocrits. A correlation also existed between high virus titers and low lymphocyte counts. However, only trends were observed in these same categories with mild disease. For several parameters, such as hematocrits and body temperatures, temporal differences in the occurrence of increased or decreased values among animals hampered the ability to identify statistically significant points. Failure to identify certain associations also was deemed important; there was no correlation between peak virus titer and disease outcome or subsequent peak antibody levels. Thus, while some meaningful correlations or lack of correlation were made, no single factor or combination or factors could be identified as invariably associated with a particular level of disease.

The prolonged viremias seen in some of the deer showed that white-tailed deer can maintain EHDV for much longer than previously realized, albeit at low titers. In earlier studies with EHDV in experimentally infected white-tailed deer, Hoff and Trainer (1974) documented viremias only to PID 20; yet three of our deer remained viremic until the end of the study (PID 56). This interval is similar to that seen in BTV-infected cattle where viremias of 59 days have been reported (MacLachlan et al., 1984). Erythrocytes from BTV-infected cattle may harbor viral nucleic acid for their lifespan (Barratt-Boyes and MacLachlan, 1995), as a polymerase chain reaction detected BTV nucleic acid in naturally infected cattle up to 160 days postinfection (Katz et al., 1994). Certainly, this may be the case in deer as well, although the lifespan of deer erythrocytes is unknown. The polymerase chain reaction-based assays that have been performed on deer (Aradaib et al., 1995) have not determined the length of time viral nucleic acid

can be detected post-infection. However, the biological importance of positive polymerase chain reaction findings may be insignificant because detected viral nucleic acid does not correlate with infectivity (MacLachlan et al., 1994). While endpoint viremias were quite low (range from $\log_{10}$ 1.7 to 2.0 $TCID_{50}$/ml) in our deer, *Culicoides variipennis* can become infected while feeding on EHDV-2-infected deer with virus titers of $\log_{10}$ 3.0 $TCID_{50}$/ml (Smith et al., 1996).

Deer in our study had a widely variable initial clinical response to infection with EHDV-2, and there was with no indication that immunity to EHDV-2 tempered subsequent infection with BTV-10. In contrast, Hoff and Trainer (1978) reported that a prolonged incubation occurred when white-tailed deer vaccinated with EHDV or BTV were challenged 75 days later with heterologous virus. In our study, equivalent 4-day incubation periods were seen in the initial EHDV infection and the BTV challenge, which gave no evidence that virus activity was diminished due to previous exposure to heterologous virus. Our findings are similar to those of Jeggo et al. (1983) where heterotypic viral challenge of sheep 66 days after initial BTV infection resulted in a course of pyrexia and viremia indistinguishable from that seen in naive animals.

The outcome of hemorrhagic disease in white-tailed deer appears to be multifaceted, involving an interaction of many host defense systems. The pattern of concurrent fever, viremia and IFN production observed between PID 4 and 6 in deer given EHDV-2 and BTV-10 was similar to that reported in BTV-infected sheep (Foster et al., 1991), but differed from BTV-infected cattle in which IFN production occurred prior to PID 4 and was unassociated with peak viremia (MacLachlan and Thompson, 1985). How these differences and similarities relate to species susceptibility are unknown and should be explored.

Cattle rarely have clinical illness from BTV, yet the prolonged viremias seen in cattle have led to their consideration as the natural reservoir host of BTV (MacLachlan et al., 1990). While sufficient vector studies have not been done with white-tailed deer to determine the actual role non-symptomatic yet viremic deer play in the epizootiology of hemorrhagic disease, the prolonged viremias seen here are evidence that white-tailed deer may act as reservoirs of EHDV up to 56 days after infection.

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