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IN VITRO REPLICATION OF EPIZOOTIC HEMORRHAGIC DISEASE AND BLUETONGUE VIRUSES IN WHITE-TAILED DEER PERIPHERAL BLOOD MONONUCLEAR CELLS AND VIRUS-CELL ASSOCIATION DURING IN VIVO INFECTIONS

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ABSTRACT: In vitro and in vivo infections were conducted to determine if the epizootic hemorrhagic disease (EHD) and bluetongue (BT) viruses would replicate in peripheral blood mononuclear (PBM) cells of white-tailed deer (*Odocoileus virginianus*). All of the North American EHD and BT viruses (EHD virus serotypes 1 and 2, and BT virus serotypes 2, 10, 11, 13, and 17) replicated in vitro in cultures of white-tailed deer PBM cells. However, this replication appeared to be monocyte-dependent and was not enhanced by lymphocyte blastogenesis induced by the addition of concanavalin A. In white-tailed deer infected with either EHD virus serotype 2 or BT virus serotype 10, virus could be isolated consistently from PBM cells only from post-infection day 4 through 8, although they remained viremic through post-infection day 21. In deer, highest viral titers were associated with the erythrocyte fraction, and in no cases did viral titers detected in the platelet, PBM cell or polymorphonuclear cell fractions approach titers observed in whole blood. In the in vitro infections of white-tailed deer erythrocytes, the EHD and BT viruses were associated with pits in the erythrocyte membrane. This association may be important in the long-term viremia observed in deer.

Key words: Bluetongue virus, cell association, epizootic hemorrhagic disease virus, hemorrhagic disease, lymphocytes, monocytes, *Odocoileus virginianus*, replication, white-tailed deer.

INTRODUCTION

Hemorrhagic disease (HD) in white-tailed deer (*Odocoileus virginianus*), is caused by viruses in either the epizootic hemorrhagic disease (EHD) or bluetongue (BT) virus serogroups, and is characterized by lymphopenia and a cell-associated viremia (Hoff and Trainer, 1974; Howerth et al., 1988). The cause of this lymphopenia is currently unknown, but it may relate to viral replication in lymphocytes. Hoff and Trainer (1974) reported that virus in white-tailed deer infected with an unspecified EHD virus serotype was associated with both erythrocytes and buffy coat cells. However, little information is available regarding potential replication of the EHD or BT viruses in white-tailed deer peripheral blood cells. To date, this has been investigated only with the BT viruses in cattle.

In cattle, a long-term viremia that persists in the presence of neutralizing antibodies is evidence that the BT viruses are cell-associated (Richards et al., 1988). This cell association primarily involves erythrocytes, but virus has been isolated from the buffy coat fraction (Luedke, 1970). Infected monocytes and lymphocytes were detected in BT virus infected cattle by electron microscopy (Morrill and McConnell, 1985). However, the association with the buffy coat fraction appeared to be transient and in cattle experimentally infected with BT virus serotype 10 (BTV-10), virus could be isolated from blood leukocytes only during the first 10 days of infection (Whetter et al., 1989). In sheep infected with BT virus serotype 23, infection of peripheral blood mononuclear (PBM) cells, as detected by polymerase chain reaction, also was transient and was detected from 3 to 14 days post-infection (McColl and Gould, 1994).

In vitro replication of BTV-10 in PBM cells of cattle primarily has been associated with monocytes (Whetter et al., 1989) and

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stimulated lymphocytes (Barratt-Boyes et al., 1992). In other in vitro studies, BT virus serotype 17 (BTV-17) replication was not demonstrated in freshly isolated PBM cells from cattle, even when cultures were stimulated with mitogen (Stott et al., 1990). Replication of BTV-17, however, was observed in established lymphocyte cultures from cattle, and was restricted to cells undergoing blastogenesis. At the cell population level, in vitro BTV-17 replication was demonstrated in T-helper, T-cytotoxic/suppressor, and null cell populations (Stott et al., 1992). Although these results are evidence that the BT viruses can replicate in lymphocyte populations, long-term or persistent lymphocyte infections in cattle currently are lacking (MacLachlan et al., 1990).

In this study, we utilized both in vitro and in vivo techniques to: (1) determine if the North American EHD and BT viruses will replicate in white-tailed deer PBM cells; (2) identify which cell fractions (monocytes and lymphocytes) are necessary for viral replication; (3) evaluate the potential effects of mitogen stimulation on viral replication; (4) determine if these viruses are PBM cell associated during experimental infections of deer; (5) determine the distribution of virus in peripheral blood components, such as platelets, PBM cells, polymorphonuclear (PMN) cells, and erythrocytes in white-tailed deer experimentally infected with EHDV serotype 2 (EHDV-2) and BTV-10; and (6) demonstrate the association between EHD and BT viruses with white-tailed deer erythrocytes by electron microscopy.

MATERIALS AND METHODS

To determine if the BT and EHD viruses would replicate in PBM cells, approximately 20 ml of blood was collected in heparinized tubes from each of 10 hand-reared white-tailed deer fawns (3- to 6-mo-old). All fawns were negative for antibodies to viruses in the EHD and BT virus serogroups as determined by agar gel immunodiffusion (AGID) tests (Pearson and Jochim, 1979). Blood samples were centrifuged at 250 × *G* for 10 min and platelet-rich plasma was removed. The original blood volume was restored with Dulbecco's phosphate buffered saline (DPBS) (Sigma Chemical Co., St. Louis, Missouri, USA) and 15 ml were overlaid on a ficol gradient (15 ml Histopaque 1077, Sigma Chemical Co.). After 45 min of centrifugation at 400 × *G*, the band containing PBM cells was harvested. Harvested cells were washed three times in DPBS, resuspended in 2 ml of maintenance medium consisting of minimal essential medium with non-essential amino acids, supplemented with L-glutamine, 10% fetal bovine serum and antibiotics (100 units penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B/ml) (Sigma Chemical Co.) and cells were counted.

Cells were diluted to a concentration of 5×10^5 cells/ml in maintenance medium. For each deer, 1 ml of the cell suspension was seeded into eight wells of a 24-well tissue culture plate. An additional eight wells were inoculated with maintenance medium only and served as a no-cell negative control. For each deer and no-cell negative control, one well each was inoculated with 50 μl of viral suspension containing approximately 400 median tissue culture infective doses ($TCID_{50}$) of EHD virus serotype 1 (EHDV-1), EHDV-2, BT virus serotype 2 (BTV-2), BTV-10, BT virus serotype 11 (BTV-11), BT virus serotype 13 (BTV-13), or BTV-17. The final well for each animal and the control were inoculated with 50 μl of maintenance medium to serve as a negative (no virus) control. All viruses used in this trial were grown on baby hamster kidney cells (BHK_{21}) (American Type Culture Collection, Rockville, Maryland, USA) from original stocks obtained from the National Veterinary Services Laboratories (NVSL, Science and Technology, Animal and Plant Health Inspection Service, USDA, Ames, Iowa, USA) (BTV-2) and the Arthropod-Borne Animal Disease Research Laboratory (USDA, ARS, Laramie, Wyoming, USA) (all other EHDV and BTV serotypes). All of the test viruses were quantified by endpoint titration in 96-well tissue culture plates using BHK_{21} cells. Eight replicate wells were used for each dilution, and titers were determined as described by Reed and Muench (1938).

At 8 days post inoculation, all wells were scraped with a pipette tip and tissue culture medium and cells were harvested and frozen at −70 C. Upon thawing, tissue culture media and suspended cells were sonicated and viral titers were determined by endpoint titration in BHK_{21} cells as described above. Evidence of viral replication in this and all subsequent trials was based on a 10-fold rise in titer compared with the titer of the original inoculum.

To determine if replication of EHDV-2 and BTV-10 was occurring in lymphocytes and/or

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monocytes, peripheral blood mononuclear cells were harvested as described from four deer. Cells were counted, and evenly divided for inoculation into six 25 cm^2 tissue culture flasks. Four of these flasks, containing 5 ml of maintenance medium, were inoculated directly. Cell counts varied by deer and ranged from 5×10^5 to 2.2×10^6 cells per flask. Cells for the remaining two flasks were first suspended in maintenance medium supplemented with 5 mM l-leucine methyl ester (Sigma Chemical Co.) to deplete monocytes (Wacholtz and Lipsky, 1993). After 45 min at 37 C, these cells were washed once with DPBS and added to the two remaining flasks also containing 5 ml of maintenance medium. Flasks were incubated at 37 C with 5% CO_2 for 24 hr. At this time, the medium containing primarily non-adherent lymphocytes from two of the directly inoculated flasks was transferred into two additional tissue culture flasks. Maintenance medium was then added to the original flasks in which the primarily adherent monocytes remained.

At this point, each deer was represented by eight flasks representing four treatments, two each of total PBM cells (lymphocytes and monocytes), non-adherent cells (primarily lymphocytes), the adherent cells (primarily monocytes), and l-leucine methyl ester monocyte depleted cells (primarily lymphocytes). One flask for each deer and cell type was inoculated with 50 µl of either EHDV-2 or BTV-10 at an initial titer ranging from $1 \times 10^{1.5}$ to $1 \times 10^{2.5}$ $TCID_{50}$/50 µl. For each virus, a control flask containing maintenance medium, but no cells, also was inoculated. At day 8 post-inoculation the contents of each flask was collected and frozen at −70 C. Upon thawing, cells and medium were sonicated and virus titers were determined by endpoint titration as previously described.

The potential effects of mitogen stimulation on BTV-10 and EHDV-2 replication in white-tailed deer PBM cells and lymphocytes was investigated in two additional trials. PBM cells were harvested as previously described from two deer. For each deer, 1 ml of cell suspension containing from 6×10^5 to 1×10^6 cells was inoculated into 16 wells of a 48-well tissue culture plate. An additional 16 wells were inoculated with maintenance medium to serve as a no-cell control. Eight wells representing each animal and the control were inoculated with 25 µl of either EHDV-2 or BTV-10. Four of these eight wells were treated with 5 µl of concanavalin A (Con A) (Sigma Chemical Co.), which represented a final concentration of 5 µg Con A/well as described by Quist et al. (1997). At this Con A concentration, lymphocyte blastogenesis could be easily visualized by light microscopy. Contents of wells were harvested on post-inoculation days 2, 4, 6, and 8, and were frozen at −70 C for future virus titrations.

For the lymphocyte trials, PBM cells were harvested from four deer and monocytes were allowed to adhere for 24 hr in a 25 cm^2 flask. The non-adherent cells (lymphocytes) were used to seed the 48-well tissue culture plate. The experimental protocol was identical to the PBM cell mitogen trial except that cells were harvested for virus titrations on days 2, 4 and 6 post-inoculation.

The distribution of EHDV-2 and BTV-10 in infected white-tailed deer was investigated in nine white-tailed deer which were infected with either EHDV-2 (n = 8) or BTV-10 (n = 1) as part of another study (Quist et al., 1997). Blood was collected in citrated vials from each infected deer for virus isolation on days 4, 8, 14, 17, and 21. The PBM cell fraction was separated on a ficol gradient as previously described. Cells were counted and diluted to a minimum of 2×10^6 cells/ml in DPBS. An additional sample of citrated whole blood was washed three times in DPBS, and cells were resuspended to the original blood sample volume. Blood samples diluted 1:10 in DPBS and PBM samples were sonicated and inoculated onto BHK_{21} cells for virus isolation.

In addition, blood was collected in heparinized vials from eight deer 7 days after they were experimentally infected with either EHDV-2 (n = 7) or BTV-10 (n = 1). To separate individual blood components from these samples, 4 ml of blood from the original sample was centrifuged at 100 × G and the platelet-rich plasma was removed. The platelet fraction was removed by additional centrifugation of the plasma at 1500 × G. Remaining blood cells were resuspended in DPBS to the original 4 ml volume and layered onto a gradient consisting of 4 ml of Histopaque 1077 over 4 ml of Histopaque 1119 (Sigma Chemical Co.). After centrifugation, the PBM cells, PMN and erythrocytes were harvested. The PBM and PMN fractions were resuspended in 1 ml of DPBS and remaining erythrocytes were lysed by the addition of 3 ml of cell lysing solution containing 0.15 M sodium phosphate (Na_2HPO_4), and 0.15 M potassium phosphate (KH_2PO_4). After 50 sec, 3 ml of a restoring solution containing 2.7% (weight per volume) sodium chloride, 0.15 M sodium phosphate and 0.15M potassium phosphate was added. Cells were centrifuged at 1,000 × G, washed in DPBS, and resuspended in 1 ml of DPBS. Harvested erythrocytes were washed twice in DPBS and resuspended in 4 ml of DPBS. Cytocentrifuge preparations were made of each cell fraction and were stained with Diff-Quick (Scientific

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TABLE 1. Replication of epizootic hemorrhagic disease virus and bluetongue virus serotypes in white-tailed deer peripheral blood mononuclear cell cultures.

Serotype	Inoculum titer[a]	Virus titer (Log_{10} $TCID_{50}$/ml on day 8) Deer number 78	79	80	81	83	84	85	86	87	91	Mean[b]	Control[c]
EHDV-1	2.5	4.8	3.6	4.2	4.5	4.6	4.7	4.6	5.5	5.3	3.0	4.5	<2.2[d]
EHDV-2	2.8	5.1	3.6	4.3	3.6	4.6	3.8	4.2	4.3	4.5	3.6	4.2	2.3
BTV-2	3.0	3.3	2.5	3.8	4.6	4.6	3.3	4.8	5.8	4.8	4.2	4.2	<2.2
BTV-10	1.9	4.8	4.2	4.0	4.2	4.7	4.3	4.6	4.8	4.6	3.1	4.3	<2.2
BTV-11	2.9	4.6	4.6	4.0	3.8	4.3	4.8	4.5	4.6	4.8	3.6	4.4	<2.2
BTV-13	2.9	4.5	4.2	4.5	5.0	4.7	4.1	5.1	4.1	5.0	4.2	4.5	<2.2
BTV-17	2.8	3.7	3.2	3.3	3.2	3.8	3.1	4.5	4.6	3.7	2.3	3.5	<2.2
Negative control[e]	0.0	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	—	<2.2

[a] Log_{10} ($TCID_{50}$/ml) of virus inoculated into each well.
[b] Mean titer.
[c] Control wells – virus without cells.
[d] <2.2 = below minimum detectable titer.
[e] Control well – cells without virus.

Products, Stone Mountain, Georgia, USA) to estimate the relative number of each cell type in the preparations.

All cell fractions including platelets, PBM cells, PMNs and erythrocytes were sonicated prior to endpoint titration in 48-well tissue culture plates. Four replicates were used at each dilution, and plates were incubated at 33.5 C for 7 days. At that time, cells were scraped and 100 µl of the resulting suspension was inoculated into a second 48-well plate that was incubated for an additional 7 days. Endpoints were determined based on detected cytopathic effect on the second passage.

To demonstrate an association between the EHD and BT virus and white-tailed deer erythrocytes, blood was washed with DPBS and the cells were resuspended to original volume with DPBS. Approximately 10 µl resuspended cells were incubated at 37 C for 18 hr with 500 µl of either EHDV-2 ($1 \times 10^{6.7}$ $TCID_{50}$/ml) or BTV-11 ($1 \times 10^{6.5}$ $TCID_{50}$/ml). The washed blood and virus mixtures were centrifuged at 1,000 × *G* and the resulting pellet was resuspended in a fixative containing 2% paraformaldehyde (Sigma Chemical Co.), 2% glutaraldehyde (EM Sciences, Gibbstown, New Jersey, USA), and 0.2% picric acid (J.T. Baker, Phillipsburg, New Jersey, USA) in 0.1 M cacodylate buffer (pH 7.2–7.4) (Sigma Chemical Co.), and then refixed in osmium tetroxide, dehydrated in graded alcohols, and embedded in Spurr's Epoxy resin (Spurr, 1969). Thin sections were stained with uranyl acetate and Reynolds' lead citrate (Reynolds, 1963), and examined with a transmission electron microscope.

RESULTS

All EHD and BT viruses tested in vitro replicated in white-tailed deer PBM cells (Table 1). The mean titers for the seven serotypes were very consistent and did not vary by more than 10^1 $TCID_{50}$. The extent of replication for these serotypes, however, varied between deer, and in some cases (BTV-2) more than a 1,000-fold difference in viral titers for a given serotype was apparent between animals. Virus was detected in control (no-cell) wells after 8 days, but in no case did virus titers exceed the titer of the original inoculum.

Viral replication of EHDV-2 and BTV-10 was dependent on the presence of monocytes, and with one exception (deer 82, BTV-10) little or no replication was observed in either the non-adherent or l-leucine methyl ester HCl lymphocyte flasks (Table 2). In contrast, virus replication was detected in all but one (deer 85, EHDV-2) of the combined lymphocyte/monocyte flasks and five of eight flasks containing adherent monocytes.

Viral replication was not enhanced by Con A treatment of PBM cells (Table 3) or lymphocytes (Table 4). As in the previous trial, replication was demonstrated in all but one (deer 82, BTV-10, Con A-sti-

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TABLE 2. Replication of EHDV-2 and BTV-10 in monocyte and lymphocyte fractions of white-tailed deer peripheral blood.

Animal number	Serotype	Inoculum[a]	Virus titer (Log_{10} $TCID_{50}$/ml) post-inoculation day 8			
			Lymphocyte/ monocyte[b]	Lymphocyte[c]	Monocyte[d]	Lymphocyte[e]
82	BTV-10	1.5	4.1	3.6	3.1	<2.2
	EHDV-2	2.5	4.8	2.2	3.6	2.2
85	BTV-10	1.5	3.4	2.2	2.2	l<2.2
	EHDV-2	2.5	<2.2	2.9	2.2	<2.2
11	BTV-10	2.0	4.1	<2.2	4.0	2.9
	EHDV-2	2.2	3.6	2.2	2.4	2.3
13	BTV-10	2.0	4.2	2.6	4.3	<2.2
	EHDV-2	2.2	3.6	<2.2	3.2	2.3

[a] Log_{10} ($TCID_{50}$/ml).
[b] Peripheral blood mononuclear cell cultures.
[c] Non-adherent cells after 24 hr incubation.
[d] Adherent cells after 24 hr incubation.
[e] L-leucine methyl ester HCl monocyte depleted lymphocytes.

mulated) of the PBM cell (lymphocytes and monocytes) cultures; but in only one (deer 17, BTV-10, unstimulated) lymphocyte culture.

Virus was isolated from the experimentally inoculated deer from both washed whole blood cells and the PBM cell fraction (Table 5). Virus isolations from the PBM cells, with two exceptions (deer 77 on day 17 and deer 79 on day 14), were restricted to post-infection days 4 and 8. Viral titers of individual blood cell fractions collected on post-inoculation day 7 on eight experimentally infected deer were highest in erythrocytes (Table 6). In no cases did viral titers detected in platelets, PBM cells, or PMNs approach titers observed in washed whole blood.

Both EHDV-2 and BTV-11 were associated with white-tailed deer erythrocytes as demonstrated in vitro (Fig. 1). With both viruses, virions were embedded in pits on the surface of the erythrocytes.

DISCUSSION

All of the EHDV and BTV serotypes replicated in white-tailed deer PBM cells and with few exceptions this replication appeared to be monocyte-dependent. Viral replication in deer monocytes was consistent with results from cattle (Whetter et al., 1989; Barratt-Boyes et al., 1992), where these cells may be important in the initial replication of BT virus in bovine lymph nodes (Barratt-Boyes and Maclachlan, 1994). Some exceptions to this were encountered, such as the detected replication of BTV-10 in the non-adherent lymphocyte fraction of deer 82 and the minimal or lack of replication observed with EHDV-2 in the monocyte fraction of deer 85 and 11 (Table 2). This variation may be attributable to the crude separation techniques employed in this study, especially since adherence may not occur with all monocytes (Pennline, 1981). For this reason, more detailed work is needed to unequivocally prove that replication is occurring in this specific cell type.

The apparent inability of these viruses to replicate in resting white-tailed deer lymphocytes was consistent with results from cattle (Stott et al., 1990; Barratt-Boyes et al., 1992). The inability of these viruses to replicate in stimulated white-tailed deer lymphocytes, however, was in contrast to results reported from cattle (Barratt-Boyes et al., 1992). Although visible blast cell for-

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TABLE 3. Effects of mitogen stimulation on replication of EHDV-2 and BTV-10 in white-tailed deer peripheral blood mononuclear cell cultures.

Deer	Cells/well	Serotype	Inoculum titer	Treatment	Virus titer (Log_{10} $TCID_{50}$/ml) Days post-inoculation 2	4	6	8
82	6×10^5	EHDV-2	2.5	Unstim.	<2.2	3.3	4.3	>5.1
				Con A	2.2	<2.2	4.0	5.0
82	6×10^5	BTV-10	2.0	Unstim.	2.9	3.9	4.1	4.2
				Con A	<2.2	2.2	<2.2	2.2
85	1×10^6	EHDV-2	2.5	Unstim.	3.8	3.2	3.2	3.9
				Con A	2.2	3.3	>5.1	4.9
85	1×10^6	BTV-10	2.0	Unstim.	3.6	3.1	4.0	4.4
				Con A	<2.2	2.3	4.2	>5.1
Control	None	EHDV-2	2.5	Unstim.	2.4	2.4	<2.2	<2.2
				Con A	3.0	2.4	<2.2	2.2
Control	None	BTV-10	2.0	Unstim.	<2.2	<2.2	<2.2	<2.2
				Con A	2.2	<2.2	<2.2	<2.2

mation was apparent in both Con A-stimulated white-tailed deer PBM cell (Table 3) and lymphocyte cultures (Table 4), no enhancement of viral replication was detected. As observed in our other trials, viral replication occurred only if the adherent cell fraction was present, and in this case, the addition of Con A had little or no effect. This further supports the premise that replication in deer PBM cells is monocyte-dependent. The lack of effect associated with Con A stimulation of deer lymphocytes is puzzling and may relate to experimental protocol rather than a true species difference. In previous studies, lymphocytes were stimulated with both IL-2 and Con A (Stott et al., 1992; Barratt-Boyes et al., 1992; Brewer and MacLachlan, 1994), whereas cells were stimulated with only Con A in this study. In addition, lymphocyte subset differences were apparent in the ability of the virus to replicate in cattle lymphocytes (Stott et al., 1992), and this was not considered in this study.

The detection of EHDV-2 in PBM cells of experimentally infected deer was consistent with results reported from BT virus

TABLE 4. Effects of mitogen stimulation on replication of EHDV-2 and BTV-10 in white-tailed deer lymphocyte[a] cultures.

Deer	Cells/well	Serotype	Inoculum titer	Treatment	Virus titer (Log_{10} $TCID_{50}$/ml) Days post-inoculation 2	4	6
14	2×10^5	EHDV-2	2.5	Unstim.	2.6	2.4	<2.2
				Con A	2.6	<2.2	2.2
25	4×10^5	EHDV-2	2.5	Unstim.	2.4	2.6	<2.2
				Con A	2.3	2.2	2.2
17	3×10^5	BTV-10	2.3	Unstim.	3.2	4.0	3.9
				Con A	<2.2	2.3	3.2
15	1×10^5	BTV-10	2.3	Unstim.	<2.2	2.9	2.3
				Con A	2.6	2.6	2.6
Control	None	EHDV-2	2.5	Unstim.	2.6	2.4	2.3
				Con A	2.6	2.2	2.3
Control	None	BTV-10	2.3	Unstim.	<2.2	<2.2	<2.2
				Con A	<2.2	<2.2	<2.2

[a] Non-adherent cells after 24 h in culture.

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TABLE 5. Detection of EHDV-2 and BTV-10 in peripheral blood mononuclear and washed blood cells from experimentally infected white-tailed deer.

Day post-inoculation	Animal number (serotype of experimental infection)																	
	Deer 76 (EHDV-2)		Deer 77 (EHDV-2)		Deer 79 (EHDV-2)		Deer 80 (EHDV-2)		Deer 81 (EHDV-2)		Deer 83 (EHDV-2)		Deer 84 (EHDV-2)		Deer 91 (EHDV-2)		Deer 87 (BTV-10)	
	WW B[a]	PBM [b]	WW B	PBM	WW B	PBM	WW B	PBM	WW B	PBM	WW B	PBM	WW B	PBM	WW B	PBM	WW B	PBM
4	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	−
8	+	+	+	+	+	+	+	+	Died		+	+	+	+	+	+	Died	
14	+	−	+	−	+	+	+	−			+	−	+	−	Died			
17	+	−	+	+	+	−	+	−			+	−	+	−				
21	+	−	+	−	+	−	+	−			+	−	+	−				

[a] Washed whole blood cells.
[b] Peripheral blood mononuclear cells.

infected cattle (Whetter et al., 1986; MacLachlan et al., 1990) and sheep (McColl and Gould, 1994). Virus could be consistently isolated from the PBM cell fraction only on post-infection days 4 and 8, and in only one deer each was virus detected on post-infection days 14 (deer 79) and 17 (deer 77). This short period of PBM cell infection not only coincided with the period of peak viremia in these animals, but also with initial detection of precipitating and neutralizing antibodies, which were first detected between days 8 and 10 (Quist et al., 1997). In BTV-10-infected cattle, virus was isolated from the PBM cell fraction up to day 10 (Barratt-Boyes and MacLachlan, 1994). It is interesting that in cattle the detection of antibodies was delayed as compared to deer, but still coincided with the inability to isolate virus from the PBM fraction. This implies that either virus or infected cells can be rapidly neutralized or removed by the immune response. There is recent evidence from cattle that cellular immunity may be important in this process (Barratt-Boyes et al., 1995). Regardless of the mechanism involved, results from deer, as with cattle, are evidence that the prolonged viremia is not associated with the PBM cell fraction.

The detection of virus in all blood fractions of deer also was consistent with past studies from cattle (MacLachlan et al., 1990). However, it must be emphasized that collection of blood from deer (Table

TABLE 6. Titers of EHDV-2 and BTV-10 in peripheral blood cell fractions from experimentally infected white-tailed deer.

Deer	Virus	Blood cell fraction (Log_{10} $TCID_{50}$/ml blood equivalent day 7 post-inoculation)				
		Washed whole blood	Platelets	Erythrocytes	Mononuclear cells	Polymorphonuclear cells
79	EHDV-2	6.0	2.4[a]	not done	<1.0 (88%)[b]	2.1 (71%)
80	EHDV-2	6.2	3.1[a]	not done	2.1 (100%)	2.0 (96%)
83	EHDV-2	6.0	3.1[a]	not done	1.6 (100%)	2.6 (82%)
84	EHDV-2	6.0	3.1[a]	not done	2.0 (71%)	<1.0 (98%)
91	EHDV-2	6.0	3.4[a]	not done	3.0 (100%)	3.0 (100%)
87	BTV-10	3.7	2.1[c]	4.0	<1.0[c]	<1.0[c]
411	EHDV-2	5.3	1.4[a]	4.3	2.7 (95%)	1.4 (98%)
70	EHDV-2	5.3	3.2[a]	4.9	2.7 (100%)	2.0 (100%)

[a] Erythrocytes detected in platelet fraction.
[b] (% of desired cell type) Based on 100 cell count.
[c] Cell types not determined.

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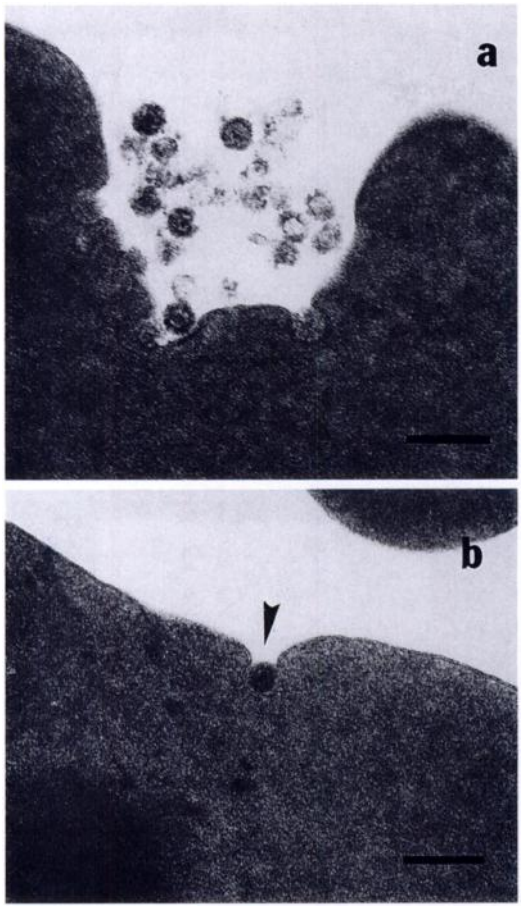


FIGURE 1. White-tailed deer erythrocytes incubated with EHDV-2. a. Erythrocyte forming cup around virions. b. single virion (arrowhead) embedded in pit on the surface of erythrocyte. Bar = 147 nm.

6) coincided with peak viremia, which in the case of EHDV-2 exceeded 1×10^5 $TCID_{50}$/ml blood in all infected animals. In all blood fractions, except the erythrocytes, viral titers were relatively low, and never exceeded $1 \times 10^{3.4}$ $TCID_{50}$/ml blood. In addition, the possibility exists that some or all of this detected virus resulted from infected erythrocyte contamination. Although erythrocytes were lysed, released virus could have persisted in the cell preparations. This was especially evident in the platelet fraction where erythrocytes were detected on preparations from all animals (Table 6).

The association between BTV and bovine erythrocytes is well documented (Brewer and Maclachlan, 1992; 1994) and helps to explain the long-term viremia observed in the presence of neutralizing antibody. This same cell-association was observed with both the EHD and BT viruses and white-tailed deer erythrocytes. In the case of white-tailed deer infected with EHDV-2, viremia in excess of 50 days has been demonstrated (Quist et al., 1997). As with cattle and sheep infected with BT viruses, no evidence of long-term or persistent infection of other blood cell types has been demonstrated in vivo.

The pathogenesis of these viruses in white-tailed deer may be very similar to currently accepted pathogenesis models for cattle where initial viral replication probably occurs in the lymphoid tissue of the lymph nodes and the spleen (Barratt-Boyes et al., 1995). This may represent the combined results of viral replication in both monocytes and some subsets of stimulated lymphocytes. Later in infection, the virus associates with erythrocytes, where it is sequestered and subsequently protected from the antibody and cell-mediated immune response. Results from this study support this pathogenesis model.

Demonstration of replication in PBM cells is consistent with early replication in lymphoid tissue, and in fact, both lymph nodes and spleen are good sites for virus isolation from naturally infected deer (D. E. Stallknecht, unpubl. data). A major difference, however, is the ability of these viruses to infect endothelial cells in vivo. Infected endothelial cells are commonly observed in BT virus infected deer (Howerth et al., 1988), but not in cattle (MacLachlan et al., 1990). In experimental infections of white-tailed deer with BTV-17, virus infection of endothelial cells was demonstrated as early as day 4 (Howerth and Tyler, 1988) and was responsible for extensive microvascular damage and the development of disseminated intravascular coagulation (Howerth et al., 1988). Extensive endothelial infection and damage also has been observed in sheep which, like deer,

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can be highly susceptible to BT virus infection (Mahrt and Osburn, 1986). Such endothelial cell infections may explain very high blood titers and increased severity of the disease seen in these species. In terms of the long-term viremia observed in deer, a similarity again exists with the described pathogenesis in cattle. In both cases, long-term viremia appears to be associated with the erythrocyte fraction.

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