

THE MECHANISM OF APOPTOSIS AND THE ROLE OF INFLAMMATORY MEDIATORS
IN HEMORRHAGIC DISEASE IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

by

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ABSTRACT

Epizootic hemorrhagic disease (EHD), an important disease of white-tailed deer (WTD) (*Odocoileus virginianus*), is caused by EHD viruses serotypes 1 and 2 in the United States. These viruses replicate in monocytes and, more importantly in endothelial cells (ECs), and endothelial damage coupled with subsequent disseminated intravascular coagulation is thought to be the primary pathogenesis of the disease. However, factors other than direct viral damage, like inflammatory mediators, are thought to be involved in the pathogenesis of EHD. The goal of this research was to better characterize the role of tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6) in the pathogenesis of EHD in WTD and to evaluate the mechanism of EHDV-induced apoptosis.

Cytotoxicity assays for TNF did not show a significant upregulation of circulatory levels of TNF- α in deer experimentally infected with EHDV. TNF was not detected in tissues during natural or experimental EHDV infection, as determined by immunohistochemistry. RT-PCR showed that EHDV failed to upregulate TNF mRNA in either EHDV-infected WTD or their blood mononuclear cells (MNCs). It is unlikely that TNF- α plays a significant role in the pathogenesis of EHDV infection in WTD.

Using in situ nick end-labeling, caspase-3 (cleaved) immunohistochemistry, flow cytometry, annexin staining, and agarose electrophoresis of DNA we documented apoptosis as a mechanism of EC death in EHDV infected bovine carotid artery ECs. The apoptosis appeared to be directly related to viral replication. Apoptosis was also demonstrated in the microvasculature of WTD infected with EHDV. Assessment of p53 gene regulation and protein expression in infected ECs suggested that apoptosis was p53 independent.

Circulatory levels of IL-1 were mildly increased but not significantly upregulated in EHDV infected WTD as determined by ELISA. IHC demonstrated IL-1 expression in lymph nodes, but not other tissues, of EHDV infected deer. RT-PCR analysis showed a mild upregulation of IL-1 and a significant upregulation of IL-6 in the lymph nodes of infected deer and in blood MNCs in vitro. These observations suggest that IL-1 and IL-6 may be involved in the pathogenesis of EHD in WTD and have potential as markers of disease severity or clinical outcome.

INDEX WORDS: Epizootic hemorrhagic disease virus, interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha, mononuclear cells, bovine endothelial cells, p53, *Odocoileus virginianus*, white-tailed deer.

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DEDICATION

To Balu for his love and support.

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CHAPTER 1:INTRODUCTION

Hemorrhagic disease caused by viruses in the epizootic hemorrhagic disease (EHD) and bluetongue (BT) virus serogroups (*Reoviridae: Orbivirus*) is the most important viral disease complex of white-tailed deer (*Odocoileus virginianus*;WTD). The causative viruses are transmitted by biting midges of the genus *Culicoides* (Nettles and Stallknecht, 1992) and infect a wide variety of domestic and wild ruminants (Sellers, 1991), although susceptibility to these viruses varies among species. Intraspecies susceptibility to these viruses also varies, and, in WTD, infection with either virus can result in non-apparent disease to depression, fever, respiratory distress, and death (Nettles *et al.*, 1992). Reasons for differences in intra- and interspecies susceptibility are poorly understood.

These viruses replicate in cells of the monocyte/macrophage system and endothelial cells (MacLachlan *et al.*,1990; Barrat-Boyes and MacLachlan, 1994; Tsai and Karstad, 1973; Howerth and Tyler, 1988). Although direct viral damage to both cell types could explain the manifestations of disease, several findings suggest that factors other than direct viral damage are also involved in the pathogenesis of these infections and could help explain variability in susceptibility. These findings include: 1) the severe degree of microvascular damage and disseminated intravascular coagulation in WTD that is out of proportion to the relatively small amount of viral antigen that can be detected in endothelial cells by immunohistochemistry or in situ hybridization, 2) differences in clinical disease expression in WTD varies in animals with similar viremias and antibody responses, and 3) the ability for these viruses to infect and replicate efficiently in endothelium and monocytes of species that do not develop disease

(Howerth, unpublished data; DeMaula *et al.*, 2001; Work *et al.*, 1992; McLaughlin *et al.*, 2003; Whetter *et al.*, 1989).

Inflammatory mediators produced in response to infection could potentiate or modulate the expression of disease. Of particular interest in infections with BT/EHD virus are the cytokines IL-1, IL-6 and TNF- α which are produced by macrophages and endothelial cells and could be stimulated by either direct viral infection of these cells or indirectly. IL-1 induces an acute-phase response and has endothelial effects that could potentiate disease (Yao *et al.*, 1995; Dewberry *et al.*, 2000). It is also a primary endogenous pyrogen (Dinarello, 1991) and severe fever is a typical response in WTD with clinical disease due to BT or EHD virus infection. IL-6, which can be induced by IL-1, may induce either a pro- or and inhibitory inflammatory response (Dinarello, 1991; Schindler *et al.*, 1990). TNF has long been recognized as an important agonist in a number of inflammatory and immunologically mediated responses (Larrick and Kunkel, 1988) and its uncontrolled production can potentiate infectious diseases by causing fever, hypotension, intravascular coagulation (Strieter *et al.*, 1993).

A number of viruses carry genes encoding apoptosis-inducing proteins (Teodoro and Branton, 1997) and, depending on the virus, these may either enhance viral infection or serve to limit viral replication or spread (Devireddy and Jones, 1999). Limited information suggests that EHD and BT viruses induce apoptosis in bovine, deer and sheep endothelial cells (Howerth and Murphy, 1999; DeMaula *et al.*, 2001; Howerth, unpublished data), but the viral proteins and mechanisms involved remain to be elucidated. Likewise, the biological significance of EHD/BT virus-induced apoptosis is unknown, but variations in the development of apoptosis among animals might help explain why hemorrhagic disease viruses can infect a wide variety of ungulates but not cause clinical disease in all.

The goal of this research was two-fold: 1) to better characterize the role of inflammatory mediators in EHD in WTD and 2) to evaluate the mechanism of EHD virus induced apoptosis and evaluate the potential role of apoptosis in the pathogenesis of hemorrhagic disease in WTD.

Specific objectives were:

- 1) To determine if tissue and circulating levels of the inflammatory mediators IL-1 and IL-6 are upregulated in WTD infected with EHD virus and if they vary between potentially susceptible and resistant subspecies of WTD.
- 2) To determine if tissue and circulating levels of tumor necrosis factor-alpha are upregulated in WTD infected with EHD virus.
- 3) To determine if IL-1, IL-6, and TNF are upregulated in WTD monocytes infected with EHD viruses in vitro.
- 4) To determine if EHD viral-induced apoptosis in bovine endothelial cells is directly related to viral replication and if it is p53 dependent.
- 5) To determine if EHDV-induced apoptosis occurs in EHDV infected WTD.

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CHAPTER 2: LITERATURE REVIEW

Bluetongue viruses (BTV) and epizootic hemorrhagic disease viruses (EHDV) are closely related orbiviruses that are transmitted by *Culicoides* midges and cause disease in both domestic and wild ruminants (Sellers 1991). BTV and EHDV infect a wide variety of ruminants, but the outcome of infection by either of these viruses differs among species. For example, BTV infection often results in overt clinical disease with high morbidity and mortality in sheep (Mahrt and Osburn, 1986) and white-tailed deer (*Odocoileus virginianus*; WTD) (Howerth and Tyler, 1988), but disease is usually subclinical in cattle (Barratt-Boyes and MacLachlan, 1995; MacLachlan, 1994). While WTD are highly susceptible to EHDV infection (Nettles and Stallknecht, 1992), disease is typically subclinical in cattle (Inaba, 1975), and disease due to EHDV infection has never been reported in sheep (Thompson *et al.*, 1988; Uren *et al.*, 1986). In WTD, disease caused by either BTV or EHDV was collectively termed hemorrhagic disease because of the inability to identify the causative agent at the time when the based on pathologic findings were first described (Prestwood *et al.*, 1974).

White-tailed deer also vary in susceptibility to BT and EHD viruses (Davidson and Doster, 1997), and the infection with either virus results in a spectrum of clinical disease from non-apparent disease to depression, fever, respiratory distress, and death (Nettles *et al.*, 1992). In experimental infections, two different subspecies of WTD, one from Pennsylvania (*O. v. borealis*) and the other from Texas (*O. v. texanus.*), varied markedly in susceptibility to EHDV infection. Although these two subspecies of deer had similar viremias, severe clinical disease and mortality was seen in fawns from Pennsylvania but only mild or undetectable disease in fawns from Texas (Gaydos *et al.*, 2002). Because the humoral immunue response was similar

for both subspecies, innate resistance was thought to play a role in the difference in clinical disease severity; however, exact reasons for differences in intraspecific susceptibility was not determined.

Severe microvascular damage and disseminated intravascular coagulation are associated with hemorrhagic disease in WTD, but the relatively small amount of viral antigen detected in endothelial cells by immunohistochemistry or in situ hybridization (Howerth, unpublished data), and the variation of clinical disease in animals, ranging from subclinical to fulminating disease, in animals with similar viremias (Gaydos *et al.*, 2002), make it appear that factors other than direct viral damage are involved in the pathogenesis of these virus infections. As monocytes and endothelium are the primary cells of replication for both BTV and EHDV (MacLachlan *et al.*, 1990; Barrat-Boyes and MacLachlan, 1994; Tsai and Karstad, 1973; Howerth and Tyler, 1988), it is reasonable to assume that the production of inflammatory mediators released from infected monocytes and endothelium and the response of endothelium to these mediators may contribute to expression of disease. Of particular interest in BT/EHD virus infection are interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α).

Viral agents

EHDV and BTV belong to the genus *Orbivirus*, of the family *Reoviridae*, which contains 12 serologically distinct groups of double stranded RNA viruses (Gould and Hyatt, 1994). EHDV and BTV have widespread distribution along the temperate regions of the world. Ten serotypes of EHDV are known to exist worldwide (EHDV-1 through 9 and Ibaraki) (Monath and Guirakhoo, 1996), with only two serotypes occurring in the United States (EHDV-1 and EHDV-2) (Nettles and Stallknecht, 1992). Five serotypes of BTV (2, 10, 11, 13, 17) are present in the

United States (Stallknecht *et al.*, 1995). Two serotypes of EHDV (EHDV 1 and 2) and four serotypes of BTV (BTV-10, 11, 13, and 17) have been associated with clinical disease and mortality in wild ungulates in the United States (Shope *et al.*, 1990; Thomas *et al.*, 1974; Barber and Jochim, 1975; Howerth *et al.*, 1988; Pearson *et al.*, 1992; Southeastern Cooperative Wildlife Disease Study unpublished). BTV-2 has only been isolated from cattle in Florida and has caused minimal disease (Gibbs *et al.*, 1983). Of the two serogroups, the EHDV are most often associated with hemorrhagic disease in WTD populations in the southeastern United States (Nettles *et al.*, 1992).

The EHDV and BTV are spherical virions that exhibit icosahedral symmetry with a diameter of 60-80 nm. They possess a ten segmented, double-stranded RNA genome and are not enveloped. Bluetongue virus is the prototype virus for structural analysis. The icosahedral structure is composed of 10 proteins that are coded for by 10 genomic segments; the first seven code for structural proteins, named VP1 through VP7 according to decreasing molecular weights. The remaining three genomic segments code for nonstructural proteins and are labeled NS1, NS2, and NS3. The largest structural protein, VP1, is a minor internal core protein that is bound to VP3, a major core protein. Together, VP1, VP3, and VP7 make a protein complex with VP3 serving as the framework for the capsomeres of VP7, the second major core protein (Louden *et al.*, 1992). VP2 and VP5 are the primary components of the outer capsid layer of the virus. Immunologically, VP7 is the major antigen associated with group-specificity, and VP2 is important in eliciting neutralizing antibodies. Recent studies have demonstrated that VP5 in BTV-2, BTV-10, BTV-11, BTV-13, and BTV-17 has an N- and possibly an O-glycosidically-linked carbohydrate chain and, thus, should more appropriately be termed GP5 (Louden *et al.*,

1992). The functional significance of this glycosylation is unknown. The remaining structural proteins, VP4 and VP6 also are minor proteins (Louden *et al.*, 1992).

Clinical disease in WTD

The clinical response of WTD to infection with EHDV and BTV can vary from inapparent to death. Signs of disease include fever, mucosal congestion, inappetence, drooling, dyspnea, recumbency, and terminal convulsions (Howerth *et al.*, 1988; Roughton, 1975; Fischer *et al.*, 1995). In WTD experimentally infected with EHDV and BTV, peak viremia during the first week of infection coincides with leukopenia, lymphopenia, and severe clinical signs (Quist *et al.*, 1997; Howerth *et al.*, 1988).

Gross pathologic changes in peracute cases of hemorrhagic disease are characterized by pulmonary congestion, hemorrhage and edema. In animals that survive longer the disease progresses and gross changes are typified by congestion, hydropericardium, oral erosions, necrotizing glossitis, and hemorrhage in many tissues, especially the gastrointestinal tract and heart. Chronic cases may have healed or healing erosions in the oral cavity, forestomach and abomasum; atrophy and fibrosis of rumen papillae; and partial or complete hoof sloughing (Couvillon *et al.*, 1981). Severe emaciation and death is seen in the winter and spring following initial infection due to these complications (Roughton, 1975).

Multiplication takes place in the cytoplasm of vascular endothelial cells (ECs) (Tsai and Karstad, 1973; Howerth and Tyler, 1988) and damage to ECs results in subsequent activation of coagulation and fibrinolysis culminating in disseminated intravascular coagulation (DIC). This is characterized by thrombocytopenia, prolonged activated partial thromboplastin time, progressive decrease in factor VIII and factor XII activities, decreased plasminogen activity,

increased fibrinogen degradation products and decreased plasma protein (Howerth *et al.*, 1988) and hematocrit (Quist *et al.*, 1997). Vascular damage, thrombosis, and DIC are thought to be directly responsible for the hemorrhage and local tissue necrosis that is typical of HD in deer (Howerth *et al.*, 2001, Howerth and Tyler, 1988; Howerth *et al.*, 1988).

Pathogenesis

The pathogenesis of BTV has been well studied via experimental infections of sheep (Pini , 1976), cattle (MacLachlan *et al.*, 1990; Barrat- Boyes and MacLachlan, 1994) and WTD (Howerth *et al.*, 1988). While EHDV has not been studied in such detail, the pathogenesis of EHDV in susceptible species is probably similar to that of BTV. Experimental infections have shown that once introduced into the skin of the host, localized inflammation ensues, and the virus is carried to the regional lymph node draining the inoculation site where viral replication probably occurs in macrophages (Pini ,1976; Barratt-Boyes and Mac Lachlan, 1994; Barratt-Boyes *et al.*, 1995; MacLachlan *et al.*, 1990). The virus then enters the circulation via infected efferent lymph mononuclear cells causing a low titer viremia and these cells carry the virus to sites of secondary replication, generally the spleen (MacLachlan *et al.*, 1990; Barratt-Boyes and MacLachlan, 1994). Virus subsequently released into circulation from secondary sites of replication results in a high titer secondary viremia and dispersion to other tissues where it replicates in endothelial cells (Barrat-Boyes and MacLachlan, 1994; MacLachlan *et al.*, 1990). At this point, both EHDV and BTV are strongly erythrocyte-associated, although the virus is also carried by other blood components, like mononuclear cells, except granulocytes, in both white-tailed deer (Hoff and Trainer, 1974) and cattle (MacLachlan *et al.*, 1990; Barratt-Boyes and

MacLachlan, 1994). Except for erythrocytes, most BTV-infected cells are removed from circulation soon after circulating antibodies develop (Barratt-Boyes *et al.*, 1995).

In both natural and experimental infections of WTD with BTV and EHDV, clinical disease and mortality is primarily associated with microvascular damage associated with viral replication in endothelium (Howerth *et al.*, 1988). It is believed that disseminated intravascular coagulation secondary to degenerative changes and necrosis of vessel walls is a primary pathogenic event in EHDV and BTV infections (Hoff and Trainer, 1981; Howerth and Tyler, 1988; Howerth *et al.*, 1988) and that the variation in susceptibility to BTV and EHDV among the various ruminant species probably reflects the extent of infection and subsequent virus-mediated destruction of the vascular endothelium (MacLachlan *et al.*, 1990). However, recent *in vitro* studies in juvenile cattle, sheep, WTD and black-tailed deer suggest that other factors like inflammatory and vasoactive mediators released by ECs and other cells could also influence the outcome of BTV or EHDV infection *in vivo* (McLaughlin *et al.*, 2003).

IL-1beta, IL-6, TNF-alpha and disease

Recovery from a viral infection is associated with an early and well-regulated inflammatory response, which may be crucial in controlling viral replication and inducing specific immunity, whereas defective inflammatory responses are associated with fatal outcome (Baize *et al.*, 2002). It is thought that it is the triggering of a cascade of events involving the release of cytokines, and not direct viral infection, that causes lesions observed in viral diseases (Hensley *et al.*, 2002). Of particular interest in EHD and BT are IL-1, IL-6 and TNF- α which are known to induce many of the pathophysiologic and clinical changes seen in several other viral diseases (Linderholm *et al.*, 1996; Baize *et al.*, 2002; Hensley *et al.*, 2002).

During inflammation, the inflammatory cytokines TNF- α , IL-1 and IL-6 are secreted in that order (VanDeventer *et al.*, 1990). IL-6 then inhibits the secretion of TNF alpha and IL-1 (Schindler *et al.*, 1990), activates the production of acute-phase reactants from the liver (Heinrich *et al.*, 1990), and stimulates the hypothalamic-pituitary-adrenal axis (Vankelecom *et al.*, 1989) to help control inflammation. Interleukin-1 beta is produced principally by mononuclear phagocytes and also by various other cells types including endothelium, keratinocytes, epithelium and cells of the CNS (Dinarello, 1991). IL-1 beta produces a wide variety of effects, including either the suppression or induction of proteins that include polypeptide interleukins, other cytokines like TNF- α and IL-6, and growth factors. IL-1 beta is a critical early mediator of the inflammatory and overall immune response and is believed to play an important role in the development of pathological conditions that result in chronic inflammation, septic shock, and hematopoietic abnormalities (Dinarello, 1991). Interleukin-6 is produced by many cells including monocytes/macrophages and endothelial cells and is one of the principal mediators of the clinical manifestations of tissue injury, fever, cachexia, leukocytosis, thrombocytosis, increased plasma levels of acute phase proteins, and decreased plasma levels of albumin (Wilder, 1998). TNF- α is produced by several types of cells, but especially by macrophages, and has an important role in the host immune response to bacterial, and certain fungal, viral, and parasitic infections, as well as in the necrosis of specific tumors (Rink and Kirchner, 1996). It also acts as a key mediator in the local inflammatory immune response (Strieter *et al.*, 1993). TNF- α is an acute phase protein, which initiates a cascade of cytokines and increases vascular permeability, and recruits macrophages and neutrophils to a site of infection (Beutler and Cerami, 1989). High levels of TNF- α have also been shown to correlate with increased risk of mortality (Rink and Kirchner, 1996).

As IL-1 beta, TNF- α and IL-6 are often secreted together; their serum levels exhibit a significant correlation with each other; and are known to induce many of the pathophysiologic and clinical changes (Hensley *et al.*, 2002; Linderholm *et al.*, 1996). Their combined roles have been investigated for several viral diseases, especially in regards to prognosis. While the presence of IL-1 beta and elevated concentrations of IL-6 in plasma in acute hemorrhagic fever caused by Ebola virus subtype Zaire indicates non-fatal infection, release of IL-1 receptor antagonist (IL-1RA) in plasma is indicative of a fatal outcome (Baize *et al.*, 2002). Severity of infection correlated with increased plasma levels of TNF-1 and IL-6 in Nephropathia epidemica, a European form of hantavirus-induced hemorrhagic fever with renal syndrome (Linderholm *et al.*, 1996), and with increased plasma levels of IL-6 in Ebola virus-infected monkeys (Hensley *et al.*, 2002). In addition, guinea pigs suffering from arenavirus disease had increased TNF levels in serum, liver and spleen which is believed to play a part in the pathogenesis of this disease (Aronson *et al.*, 1995). Likewise, dengue-infected patients had high serum values of TNF and IL-6 (Hober *et al.*, 1993) and patients with Argentine hemorrhagic fever (Marta *et al.*, 1999) had increased levels of IL-6 during severe infection.

In vitro studies in endothelial cells cultures of sheep and cattle suggest that inflammatory mediators like IL-1 and IL-6 have a potential role in the pathogenesis of BT. Inflammatory mediators either stimulated or influenced the type of cell death seen in sheep and cattle pulmonary artery and microvasculature endothelial cultures in vitro (DeMaula *et al.*, 2001). When inflammatory mediators derived for BTV infected microvasculature endothelial cells or IL-1 were added to BTV infected sheep or bovine microvascular endothelial cultures, the predominant type of cell death switched from necrosis to apoptosis. A similar switch was seen when IL-1 was added to BTV infected sheep and bovine pulmonary artery cultures.

Transcription of genes encoding IL-1 and IL-6 was up-regulated in microvascular endothelial cell cultures of both sheep and cattle when infected with purified BTV; however, there were species differences (DeMaula *et al.*, 2002a, 2002b). In sheep endothelial cultures, transcription of IL-1 was greatly increased while IL-6 was only minimally induced and the addition of inflammatory mediators suppressed the production of both while in cattle endothelial cell cultures, transcription of both cytokines was greatly increased and expression was potentiated by the addition of inflammatory mediators. Thus, species differences in cytokine production in response to viral infection and/or inflammatory mediators could help explain species-specific differences in the susceptibility to BTV. A variation in the production of inflammatory mediators by infected or noninfected endothelium and the response of various types of endothelium to such mediators could also be involved in the difference in susceptibility to EHDV infection seen in potentially different subspecies of WTD.

Apoptosis in viral disease

Apoptosis was initially recognized in 1972 by its distinctive morphology and named after the greek designation for “falling off”. It is a form of cell death designed to eliminate unwanted host cells through activation of a coordinated, internally programmed series of events effected by a dedicated set of gene products (Cotran *et. al.*, 1998). For the vast majority of normal cells undergoing apoptosis, little is known about the signals that commit cells to death. Cells may emanate signals that cause neighboring cells to die or systemic factors or diseased states may cause cells to die prematurely. Apoptosis can also be initiated in response to a variety of viral infections (Devireddy and Jones, 1999), including, but not limited to, human immunodeficiency syndrome virus (Weiss, 1993); classical swine fever virus (Choi *et al.*, 2004); bovine herpes virus 1 (Devireddy and Jones, 1999) and chicken anemia virus (Noteborn *et al.*, 1994).

Viral proteins may interact with apoptotic pathways in the host at various points to inhibit or induce apoptosis. Induction of early host cell death severely limits virus production and reduces or eliminates spread of progeny virus in the host, so most animal viruses have evolved strategies to evade or delay early apoptosis to allow production of high yields of progeny virus (Teodoro and Branton, 1997) while also evading host immune inflammatory responses and protecting progeny virus from host enzymes and antibodies. Viruses may prevent apoptosis in host cells by expressing caspase inhibitors such as cytokine response modifier A or p35 (e.g., cowpox virus and baculovirus) or by expressing FLIPs (Fas-associated death domain-like IL-1 β -converting enzyme-inhibitory proteins) that interact with the adapter protein FADD (Fas-associated death domain) (e.g., human molluscipoxvirus and human herpes virus-8) and block caspase-8 activation and cell death (Thome *et al.*, 1997; Hu *et al.*, 1997). Viral proteins may also inhibit receptor-mediated cytotoxicity (Krajcsi *et al.*, 1996; Shisler *et al.*, 1997); prevent E1A-induced apoptosis, permitting viral replication (Boulakia *et al.* 1996); suppress the death-promoting activity of Bik (Bcl-2 interacting killer) (Boyd *et al.*, 1995); or inactivate the protein products of tumor suppressors such as p53 (Teodoro and Branton, 1997) to inhibit apoptosis of host cells.

Some viruses also encode for proteins that induce apoptosis. Viral-induced apoptosis occurs by both kinds of mammalian apoptotic pathways described in the literature: (a) Pathways that mainly use the initiator caspase-9 to trigger effector caspases (Saikumar *et al.*, 1999), as seen with avian encephalomyelitis virus (Liu *et al.*, 2004) and West Nile virus (Yang *et al.*, 2002), and (b) Pathways that mainly use the initiator caspase-8 to trigger effector caspases (Saikumar *et al.*, 1999), as seen with Sendai virus (Bitzer *et al.*, 1999) and HIV-1 (Bartz and Emerman, 1999). Viruses can induce apoptosis in a wide variety of host cells: bovine herpes virus-1 in lymphocytes and kidney cells (Griebel *et al.*, 1990; Devireddy and Jones, 1999); African swine

fever virus in fibroblasts, smooth muscle cells and endothelial cells (Gomez-Villamandos *et al.*, 1995); herpes simplex virus in immature dendritic cells (Müller *et al.*, 2004); and BTV in bovine and ovine endothelial cell cultures (DeMaula *et al.*, 2001; DeMaula *et al.*, 2002). Apoptosis has also been identified in EHDV infected cattle endothelium (Howerth and Murphy, 1999) and WTD endothelium (Howerth, unpublished data), however, the mechanism behind it and the proteins involved in the process are unknown.

p53 and apoptosis

p53 is an important trigger of apoptosis (Shaw *et al.*, 1992) and may be activated following infection by a variety of stimuli including viruses (Teodoro and Branton, 1997). It is a transcription factor, which contains both sequence-specific DNA binding activity and an independent acidic activation domain (Teodoro and Branton, 1997). p53 monitors cell cycle checkpoints (Kastan *et al.*, 1992), senses DNA damage (Lee *et al.*, 1995), assembles DNA repair machinery (Wang *et al.*, 1995) and modulates gene amplification (Yin *et al.*, 1992). A link between p53 induction and activation of interleukin-1-converting enzyme-like proteases, or caspases, following DNA damage has also been established (Chandler *et al.*, 1997). When DNA is damaged p53 binds to DNA and may induce apoptosis by activating certain apoptosis-inducing genes like Bax which is one of the cellular targets for p53 activation and encodes a downstream regulator of apoptosis (Miyashita and Reed, 1995).

Viruses may induce apoptosis via p53 dependent or p53 independent mechanisms. In p53 dependent apoptosis, p53 levels generally increase (Bates and Vousden, 1996). A number of viruses of veterinary importance have been shown to induce apoptosis via a p53 dependent mechanism. These include, bovine herpes virus 1 and Sendai virus which induce programmed

cell death by activating the p53 apoptotic pathway in bovine kidney cells and primary mouse embryonic fibroblasts, respectively (Devireddy and Jones, 1999, Huang *et al.*, 2004). Other examples include adenovirus (E1A region product) (Debbas and White, 1993; Lowe and Ruley, 1993) and human papilloma virus (E7 protein) (Pan and Griep, 1994)..

Though p53 may play an important role in triggering apoptosis, mounting evidence suggests that infection by many viruses can induce apoptosis independent of p53. It is becoming increasingly apparent that a number of viral proteins have evolved which function downstream of p53 in the common apoptotic pathway (Teodoro and Branton, 1997). Apoptin, a gene product derived from the chicken anaemia virus, has a p53-independent pro- apoptotic activity (Oro and Jans, 2004) and can employ cellular factors for induction of apoptosis. Activation of caspase-3 and possibly other downstream caspases are observed and apparently the cause of apoptin-induced apoptosis (Danen-van Oorschot *et al.*, 2000). Other examples of viruses inducing p53 independent apoptosis are dengue virus (Thongtan *et al.*, 2004) and adenovirus (E1B protein) (Subramanian *et al.*, 1995).

EHDV has been shown to induce apoptosis in bovine and WTD endothelium, but its biological significance, exact mechanism, and whether this process is dependent or independent of p53 upregulation are unknown.

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CHAPTER 3:
TUMOR NECROSIS FACTOR-ALPHA EXPRESSION IN WHITE-TAILED DEER
INFECTED WITH EPIZOOTIC HEMORRHAGIC DISEASE VIRUS ¹

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ABSTRACT: Hemorrhagic disease caused by either epizootic hemorrhagic disease (EHD) or bluetongue viruses is the most important viral disease complex of white-tailed deer (WTD). However, little is known about the role of inflammatory mediators in its pathogenesis. The objectives of this study were to characterize the expression of tumor necrosis factor-alpha (TNF- α) in: 1) WTD with experimental or natural EHD and 2) WTD blood mononuclear cells infected with EHD virus (EHDV) serotype 2 in vitro. Circulating levels of TNF- α were evaluated in deer experimentally infected with EHDV using a cytotoxicity assay. Tissue expression of TNF- α , in deer naturally and experimentally infected with EHDV, was evaluated via immunohistochemistry. Semi-quantitative RT-PCR was used to assess upregulation of TNF messenger RNA in tissues from deer experimentally infected with EHDV and in deer mononuclear cells infected with EHDV in vitro. Circulating levels of TNF were not significantly increased in infected animals and TNF was not detected in tissues during EHDV infection. EHDV failed to upregulate TNF mRNA in either EHDV infected WTD or their blood mononuclear cells. It is unlikely that TNF- α plays a significant role in the pathogenesis of EHDV infection in WTD.

Key words: Hemorrhagic disease, epizootic hemorrhagic disease, epizootic hemorrhagic disease virus, bluetongue, white-tailed deer, *Odocoileus virginianus*, tumor necrosis factor-alpha.

INTRODUCTION

Epizootic hemorrhagic disease (EHD) viruses and the closely related bluetongue (BT) viruses cause the most important viral disease complex of white-tailed deer (WTD), known as hemorrhage disease (Prestwood et al., 1974). Although these viruses, particularly EHD viruses, are an important cause of morbidity and mortality in WTD, clinical disease expression is variable and ranges from nonapparent to depression, fever, respiratory distress, and death (Nettles et al., 1992). Despite its importance, little is known about the pathogenesis of or reasons for variations in susceptibility to EHD virus infection in WTD.

In WTD with EHD or BT, clinical disease and mortality are primarily associated with virus replication in endothelial cells and subsequent microvascular damage and the development of disseminated intravascular coagulation (Howerth and Tyler, 1988; Howerth et al., 1988; Tsai and Karstad, 1973). However, the severity of vascular damage is often out of proportion to the relatively small amount of viral antigen typically detected in endothelial cells by immunohistochemistry, in situ hybridization or electron microscopy (Howerth, unpublished data). In addition, EHDV can replicate in endothelial cells from species that vary in susceptibility to infection (McLaughlin et al. 2003). Thus, endothelial damage does not appear to directly correlate with viral replication in the endothelium and other factors, such as inflammatory and vasoactive mediators, probably potentiate endothelial damage and/or contribute to manifestations of disease and susceptibility.

Gaydos et al. (2002) experimentally defined two groups of deer representing two subspecies of WTD, *Odocoileus virginianus borealis* and *Odocoileus virginianus texanus*, which varied in susceptibility to EHD virus infection despite having similar viremias and humoral immune responses. This suggested that variable expression of disease in WTD was due to innate

resistance; however this “innate resistance” has not been defined. In other viral diseases, including BT virus infection in sheep and cattle, differences in susceptibility have been attributed to variable expression of inflammatory mediators (DeMaula et al., 2001). This is also a plausible explanation for innate resistance to EHD viruses

Tumor necrosis factor is a cytokine derived from mononuclear phagocytes and T-lymphocytes that is stimulated by a variety of exogenous and endogenous factors from bacteria, viruses, and parasites (Rink and Kirchner, 1996). It is an important agonist in a number of inflammatory and immunologically mediated responses (Larrick and Kunkel, 1988) orchestrating the inflammatory response and initiating a cascade of mediators (Beutler and Cerami, 1989), which are directly responsible for the various events associated with inflammation, e.g. increased vascular permeability (Beutler and Cerami, 1989), chemoattraction of circulating leukocytes (Deventer, 1997) and proteolysis (Azuma et al., 1997). An exaggerated production of TNF under these conditions may result in pathophysiologic events and the generation of symptoms associated with disease (Strieter et al., 1993). In addition, the uncontrolled and overexuberant production of TNF has been associated with high morbidity and mortality rates in certain diseases (Strieter et al., 1993) including, but not limited to, hepatitis in humans (Nagaki et al., 2000), hemorrhagic viral enteritis in turkeys (Rautenschlein and Sharma, 2000), and experimental dengue virus type 2 infection in mice (Atrasheuskaya et al., 2003).

Because EHDV replicates in monocytes, it could stimulate the production of TNF resulting in TNF accentuated endothelial damage. Thus, we hypothesized that TNF- α may play a role in the pathogenesis of HD in WTD. The goal of this study was to characterize the expression of TNF- α in WTD infected with EHDV. The three objectives were: 1) to characterize circulating levels of TNF- α in WTD experimentally infected with EHDV via a cytotoxicity assay; 2) to

characterize tissue levels of TNF in WTD experimentally and naturally infected with EHDV via immunohistochemistry; and 3) to characterize TNF messenger RNA expression in tissues of deer and their peripheral blood monocytes when experimentally infected with EHDV via semiquantitative RT-PCR.

MATERIALS AND METHODS

Circulating TNF levels

Development of a cytotoxicity assay for white-tailed deer TNF alpha

To develop a cytotoxicity assay, WTD were intravenously inoculated with bacterial lipopolysaccharide to stimulate the production of TNF- α and three cell lines were compared (L929 murine fibrosarcoma line, WEHI-161 clone 13 murine cell line, PK-15 porcine kidney cell line). Two white-tailed deer (deer # 17 and 82) were given LPS from *E. coli* (Sigma, St. Louis, MO, USA) as a single bolus (0.1 mg/kg body wt., deer# 17; 0.2 mg/kg body wt., deer# 82) and serum samples were collected from each deer at 20-30 minute intervals over a period of 2-3 hours. Target cell lines L929 (ATCC), WEHI 161 (ATCC) and PK-15 (ATCC), 2.2×10^4 cells per well, were seeded in 96-well plates and allowed to attach for 24 hours. Serum samples were subsequently assayed in two-fold serial dilutions down the columns of the plate. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-s-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (Promega, Madison, WI, USA) after incubation of samples for an additional 24 hours.

Cell survival was quantified by scanning each well with an automated plate reader equipped with a 570nm filter. For each duplicate dilution, the percentage of cytotoxicity was determined

according to the formula: $\frac{(\text{OD negative control} - \text{OD sample}) \times 100}{(\text{OD negative control} - \text{OD positive control})}$

where, OD = optical density. The TNF titer (in units/ml) was estimated as the reciprocal of the dilution that caused a 50 % reduction in cell survival.

Cytotoxicity assay for TNF- α in EHDV-2 infected WTD

Serum samples collected in a previous experimental study (Quist et. al, 1997) were used to evaluate circulating TNF- α levels in WTD infected with EHD serotype 2 (EHDV-2). The study was conducted in two trials in the fall of 1992 and 1993. In each year, eight deer were infected with EHDV-2 and two sham-inoculated deer were used as controls. Serum samples collected every other day during the course of infection (post inoculation days 0 through 18) were used to evaluate TNF levels by cytotoxicity assay. Circulating TNF levels were compared between infected and uninfected deer.

Wells of 96-well flat bottomed 96-well plate were seeded with 90 μ l of L929 cells, diluted to a concentration of 2.22×10^5 cells/ml in TNF dilution media (RPMI-1640 medium containing 5% fetal bovine serum) and incubated for 24 hours at 37°C. Beginning with undiluted samples, serial dilutions of standard and test samples were obtained in duplicate by pipetting 90 μ l across the wells of these plates. For a negative control a column of 8 wells containing cells incubated in medium alone (100% survival) was used. For positive controls, 8 wells on each plate were treated with 0.005 μ g mouse TNF (Roche, Indianapolis, IN, USA) which caused 100% cytotoxicity. Sample dilutions were adjusted to 10 μ g actinomycin D/ml (Sigma, St. Louis, MO, USA). Plates were incubated for additional 24 hrs at 37°C and then 15 μ l MTT dye (Promega) was added to all the wells, and the plate incubated for 4 hrs at 37°C. 100 μ l of stop

solution (Promega stabilization solution) was added to each well and the plate was incubated overnight at 37°C and cell survival was quantified by using an automated microplate reader at a wavelength of 570 nm. The TNF titer was estimated by reference to the curve for cytotoxicity versus sample concentration as the reciprocal of the dilution that caused a 50% reduction in cell survival. Percentage cell death was converted to probit values by use of computer software and dose-response curves were generated by plotting probit values against log₁₀ dilution of the sample. Titers were converted to TNF units/ml by comparing them with the activity of the TNF- α standard in the assay system.

Tissue TNF levels

Collection of tissue samples for RT-PCR and immunohistochemistry

Tissues were from WTD either experimentally or naturally infected with EHDV-2. Fresh and formalin-fixed tissues for RT-PCR and immunohistochemistry, respectively, were collected from six experimentally infected WTD inoculated with $10^{7.03}$ mean tissue culture infected doses (TCID₅₀)/ml EHDV-2 and two mock-infected controls. The deer were inoculated intradermally and subcutaneously with the virus or mock-inoculum on the right side of the neck in multiple sites. 4.0 mm punch-biopsies of buccal mucosa were taken on day 0, 2, 4, 6 and 8 post inoculation. One infected deer died due to EHD on day 7 post inoculation. The remaining six deer were euthanized with pentobarbital on day 8 post inoculation; all had clinical signs and gross lesions compatible with EHD. Brain, skin, buccal mucosa, right prescapular lymph node, which drained the inoculation site, and left prescapular and prefemoral lymph nodes, gastrointestinal tract, lung, heart, liver and spleen were taken at necropsy. Samples for RT-PCR were collected over ice, snap frozen in liquid nitrogen and then stored at -80°C. The tissue

samples collected for immunohistochemistry were fixed in 10% formalin and embedded in paraffin within 24-48 hours. Formalin-fixed, paraffin-embedded tissue samples of gastrointestinal tract, lymph node, buccal mucosa, liver, heart and lung from three naturally infected WTD that died of EHDV-2 were also used for immunohistochemistry.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Semiquantitative RT-PCR was done using Titan One Tube RT-PCR System (Roche) to evaluate levels of TNF- α mRNA in right prescapular lymph nodes and buccal mucosa of the experimental deer using 0.1 μ g RNA with primers at a concentration of 10 μ M and cycling parameters as follows: 50°C for 60 minute, 94°C for 1 minute, 34 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute and 20 seconds, and then 68°C for 7 minutes. To ensure the integrity of RNA, the expression of housekeeping gene beta-actin was assessed in each sample. The nucleotide sequence of the primers were TNF-forward primer: 5'-CCCATCTACCAGGGAGGAGT and TNF-reverse primer: 5'-GGCGATGATCCCAAAGTAGA; Actin-forward primer: 5'-CGCACTACTGGTATTGT and Actin-reverse primer: 5'-AGAGCTTCTCCTTGATGTC. The actin primers were designed based on deer nucleotide sequences. The TNF primers were designed based on bovine, ovine and red deer nucleotide sequences present in genBank. PCR products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide. Location of a predicted PCR product was confirmed using a DNA ladder (Promega) as a standard-size marker. Specific PCR products for TNF- α and beta-actin were seen at 119 bp and 212 bp, respectively, on a 2% agarose gel. The PCR product (TNF- α) was extracted from 2% agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sequenced to confirm identity. Images of

the RT-PCR ethidium bromide-stained agarose gels were acquired under ultraviolet illumination and scanned at 600 dots per inch (DPI). The mean luminosity of the bands was measured in Adobe Photoshop 5.5 and the band intensity was expressed as relative intensity units. The ratio between the mean of sample RNA (TNF- α) and beta-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency.

Immunohistochemistry

Paraffin-embedded tissue sections (3 μ m) were heated at 63°C for 30 minutes and then deparaffinized in xylene, and rehydrated through a series of graded ethanol baths to distilled water. The slides were treated with 1X Trypsin/EDTA (Sigma) at 37°C for 30 minutes and then blocked with Protein Block (Dako) for 8 minutes at room temperature. Primary antibody, mouse anti-TNF antibody (Clone 52B83, HyCult Biotechnology) diluted 1:400 in antibody diluent (Dako) was applied for one hour at room temperature. Biotinylated anti-mouse antibody (Vector), at 5 μ g/ml, was applied for 25 minutes at room temperature followed by avidin-biotin peroxidase complex (Vectastain kit, Vector) for 30 minutes at room temperature.

Diaminobenzidine-tetrahydrochloride (DAB; Vector) was used as the chromogen for the peroxidase reaction. The slides were counterstained with Mayers hematoxylin, dehydrated and cover-slipped. Tissue samples of lung from a clinical case of bovine pasturellosis were used as a positive control. Tissue samples from the bovine lung treated with mouse negative control serum in place of primary antibody were used as negative control. A WTD lymph node that drained an area of tissue necrosis, inflammation, and bacterial infection associated with urethral blockage and rupture was used to confirm that the primary antibody could detect deer TNF- α .

In vitro TNF production

Preparation of viral inoculum

The EHDV-2 was originally isolated in baby hamster kidney (BHK) cells from spleen of a WTD from Kansas that died in a natural outbreak of epizootic hemorrhagic disease in 2000. 100µl of passage 1 tissue culture media (MEM with 3% FBS, 50 units penicillin/ml, 5 µg streptomycin/ml, 12 µg amphotericin B/ml) was inoculated into two T75 flasks containing confluent monolayers of BHK cells. Cells were incubated at 37°C until cytopathic effect was greater than 50% (96 hour post inoculation). Cells were scraped and the contents were centrifuged at 1500xG for 15min. The media was removed and the cell pellet was resuspended in 5 ml of media and sonicated. The sonicated cells were centrifuged at 1500xG for 15 min and the supernatant was added to the previously removed media. The inoculum was frozen in aliquots at -70°C. The titer of the frozen stock was $10^{6.78}$ TCID₅₀/ml. Uninfected BHK cells were prepared similarly for the mock-inoculum.

Isolation and infection of mononuclear cells

Blood was collected from WTD using sodium citrate as anticoagulant. Peripheral blood mononuclear cells (PBMNCs) were isolated using Fico/Lite LymphoH 1.077 (Atlanta Biologicals) and approximately 2×10^6 cells were seeded per well in a 48-well plate with 1 ml RPMI-1640 media (RPMI (Sigma) with 1% 100X antibiotic/antimycotic (Sigma) and 10% fetal bovine serum). Plates were incubated for 48 hours at 37°C to facilitate the adherence of macrophages. After 48 hours the cells were inoculated with EHDV-2 (multiplicity of 0.02 TCID₅₀/cell). Peripheral blood mononuclear cells inoculated with bacterial lipopolysaccharide (L4391; Sigma) at a total dose of 20 ng/ml (L4391; Sigma) were used as positive controls.

Mock-infected blood mononuclear cells from the same deer were used as negative controls. At 0 hr, 24 hr and 48 hr post inoculation, the adherent and non- adherent cells were harvested for RNA extraction. The supernatant from each well was removed and centrifuged to isolate non-adherent cells. The adherent cells were lysed directly by adding 200 μ l RNA-Bee (TEL-TEST, Inc.). The cell pellet of non-adherent cells was resuspended with lysed adherent cells in RNA-bee and RNA was extracted for RT-PCR as per the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Semiquantitative RT-PCR for TNF- α was performed as above using 0.1 μ g RNA.

Statistical analysis

Data from the cytotoxicity assays were analyzed using a mixed effect model. Observations from the same deer were recognized by the model. Infected group, year of the study and interaction of infected group by year were included as categorical fixed effects. Days post inoculation were included as a continuous variable. Different slopes for infected groups and year were tested by modeling their interaction effects with days post inoculation. The data was analyzed in log scale to meet the assumption of normality of residuals. Analysis was performed using SAS PROC MIXED procedure. A p-value of <0.05 was considered statistically significant. Analysis of RT-PCR results for buccal mucosa, lymph nodes and peripheral blood mononuclear cells was done by calculating mean values and standard deviations for each assay and a student's t-test was applied using Graphpad Prism software (Graph Pad software, San Diego, CA) to determine differences between mean values of the data, with $p < 0.05$ considered significant.

RESULTS

Cytotoxicity assay

White-tailed deer produced low levels of TNF- α in response to the experimental administration of endotoxin (LPS) (Fig. 3.1). TNF was detected by both L929 and PK-15 cell lines at relatively the same concentration, but the WEHI-161 cell line did not appear as sensitive to deer TNF- α . We chose to use L929 cells for detection of TNF in the samples from the experimentally infected deer.

A significant increase in circulating TNF was not detected in EHDV-2 infected WTD in either trial (Fig. 3.2). The slope of TNF over the days post inoculation was not statistically significant either between control and infected groups or between year 1 and year 2 ($P>0.05$). None of the effects (infected group and interaction of infected group by year) were statistically significant, except for year. Overall, the average TNF value for year 2 was significantly higher than for year 1 ($P=0.0400$).

Immunohistochemistry

Tissue samples from brain, skin, buccal mucosa, prescapular and prefemoral lymph nodes, gastrointestinal tract, lung, heart, liver and spleen taken at necropsy from WTD, experimentally infected with EHDV-2 and gastrointestinal tract, lymph node, buccal mucosa, liver, heart and lung taken at necropsy from natural cases of EHD, showed no expression of TNF- α protein by immunohistochemistry. Macrophages in the lymph node from the WTD with urethral blockage and associated tissue damage, inflammation, and infection had intense cytoplasmic staining for TNF- α protein.

Semiquantitative RT-PCR

TNF- α mRNA was detected in lymph nodes draining the site of inoculation of both mock and all six EHDV-2 infected deer on post inoculation day 7 or 8; there was no significant difference in the level of expression between control and infected deer (Fig. 3.3) ($P=0.5216$). One deer that died on post inoculation day 7 appeared to have less mRNA expression than the other infected animals that had clinical disease but survived and were euthanized on day 8. There was also TNF mRNA expression in buccal mucosa in both control and infected deer on all days sampled; there was no observable increase in levels of expression between control and infected deer (Fig.3.4).

TNF- α mRNA was detected in WTD PBMNCs at 0 hours post inoculation and in untreated, LPS treated, and EHDV-2 inoculated groups at 24 and 48 hrs post inoculation, but significant up-regulation was not observed with EHDV-2 infection ($P=0.9284$) or LPS stimulation (Fig. 3.5).

DISCUSSION

TNF- α can be involved in disorders of both inflammatory and non-inflammatory origin (Strieter et al., 1993) and may be involved in the pathogenesis of certain viral diseases. For example, plasma levels of TNF- α in patients hospitalized with nephropathia epidemica, a European form of hantavirus-induced hemorrhagic fever with renal syndrome, were increased in all patients at admission (Linderholm et al., 1996) and high serum values of TNF- α were observed in sera of dengue-infected patients obtained during the 1989-1990 outbreak of dengue-3 in Tahiti (Hober et al., 1993). However, the results of our in vivo and in vitro studies suggest

that TNF- α does not play a major role in EHD for we could not detect TNF in either circulation or tissues of EHDV infected WTD.

In a comparison study of three cells lines typically used to detect TNF, namely L929, PK-15 and WEHI cell lines, we determined that L929 cells would most likely detect low levels of TNF in WTD serum. Using the L929 cell assay, increased levels of circulating, biologically active TNF were not detected in WTD infected with EHDV-2 in two different trials. There was 100% morbidity and 31% mortality in the EHDV-2 infected deer in these trials so lack of circulating TNF cannot be attributed to lack of disease. Because samples were collected every other day, it is potentially possible that a surge in circulating TNF was missed; however, such a short spike in circulating levels is unlikely based on information from other viral infections. Increased plasma levels of TNF- α in children infected with dengue virus lasted for 1 to 3 days after the onset of fever (Laur et al., 1998) and concentrations of TNF- α were increased up to one week after the onset of disease in patients hospitalized with nephropathia epidemica (Linderholm et al., 1996). Differences in technicians performing the assays for the two trials probably explains the higher overall TNF values for the second study year.

None of the tissue samples from WTD naturally and experimentally infected with EHD viruses expressed TNF- α protein as determined by immunohistochemistry. Lack of anti-TNF antibody cross-reactivity with WTD TNF is unlikely as the primary antibody used is suppose to cross-react with a wide variety of species, including mouse, guinea pig, rhesus and chimpanzees (Hycult Biotechnology), and we had intense cytoplasmic staining for TNF- α in macrophages in a lymph node from a WTD deer with urethral blockage, rupture and sepsis. Likewise, we have shown that this antibody will detect TNF in macrophages in lung from cattle with pasteurellosis indicating that this antibody cross-reacts with TNF of a closely related species (Sharma,

unpublished data). Lack of TNF protein expression is not likely related to the type or source of tissue examined for a variety of different tissues were examined and these were from deer that died of EHD and deer with varying degrees of clinical disease. Time of tissue sampling is probably not a concern as buccal mucosa, which commonly has lesions in EHD, was sequentially biopsied during the acute phase of the disease (post inoculation day 0- 8) and none of these biopsies had detectable TNF.

There was no detectable up-regulation of genes transcribing TNF in tissues of EHDV infected WTD which could explain the lack of TNF protein expression in EHD virus infection. One deer that died on post inoculation day 6 had decreased tissue expression of TNF mRNA suggesting that EHDV infection may actually depress transcription of genes encoding TNF or the viral infection may degrade the mRNA. Because TNF mRNA appears to be constitutively expressed in WTD tissues, one would expect rapid production of TNF protein upon stimulation, so either EHDV does not stimulate translation of the TNF mRNA or translation is blocked. BT viruses lead to the rapid inhibition of cellular protein synthesis *in vitro* that is suspected to be due to degradation of polyribosomes (Huisman, 1971); such degradation could abolish the translation of constitutively expressed mRNA.

Stallknecht et al. (1997) has shown that EHDV replicates in WTD monocytes. Because other monocytopathic viruses, such as African swine fever virus (Gomez et al., 1999), are known to upregulate TNF production in PBMCs *in vitro* we were surprised that this was not the case with EHD virus. The viral dose used to infect our PBMC cultures in this study was rather low and it is possible that a higher viral dose would have stimulated TNF production in the deer mononuclear cells *in vitro*. However, the viral dose used in this *in vitro* study was previously shown to increase the expression of IL-2 receptor in bovine monocytes infected with BTV

(Barratt-Boyes, et al., 1992) and can upregulate other cytokines, specifically IL-1 and IL-6 beta, in WTD PBMCs (Sharma, unpublished data).

In general, it appears difficult to up-regulate TNF in WTD even in response to strong inducers such as endotoxin (Morris et al., 1990). In our studies, LPS failed to upregulate TNF in WTD PBMCs; however, the same dose has been shown to upregulate IL-1 beta, TNF- α and IL-6 in bovine alveolar macrophages and PBMCs (Ito & Kodama, 1996) and IL-1 beta and IL-6 in deer PBMC's (Sharma, unpublished data). Likewise, when LPS was injected into deer to produce TNF for the development of a cytotoxicity assay, only small amounts of circulating TNF were detectable. The doses of LPS used were based on work in other species in which similar or lesser amounts caused a significant increase in circulating TNF (MacKay et al., 1991; Allen et al., 1993; Perkowski et al., 1996). This includes cattle, which are closely related to WYD, in which these doses would be expected to cause a significant production of TNF (Gerros et al., 1993). Therefore, maybe it is not unexpected that TNF- α does not appear to play a major role in the pathology or disease caused by EHDV.

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Figure legends.

Fig. 3.1. Comparison of three cell lines (WEHI-161, PK-15, and L929) used for developing TNF cytotoxicity assay in two white-tailed deer; **(A)** shows performance of the three cell lines in deer no. 17, inoculated with 0.1 mg/kg b.wt. LPS. **(B)** shows performance of the three cell lines in deer no. 82 inoculated with 0.2 mg/kg b. wt. LPS.

Fig. 3.2. **(A)** Means and standard deviations of circulating TNF for year 1 for control and infected groups. Control n=2; infected n=8. **(B)** Means and standard deviations of TNF for year 2 for control and infected groups. Control n=2; infected n=8.

Fig. 3.3. Semiquantitative determination of TNF- α mRNA expression in right prescapular lymph nodes, from mock-infected control deer and deer experimentally infected with EHDV-2, taken at day 7 or 8 p.i. (control deer n=2; infected deer n= 6). **(A)** Example of agarose gel electrophoresis of PCR products showing TNF and actin PCR products. Lane 1-ladder; lane 2-water control; lane 3, 4-control deer 1 and 2 respectively; lanes 5, 6, 7, 8, 9 and 10-lymph nodes from deer experimentally infected with EHDV-2. Specific PCR product size for beta-actin and TNF- α is 212 bp and 119 bp, respectively. **(B)** Comparison of relative intensities for TNF production by right prescapular lymph nodes in control (890, 962) and infected deer (891, 888, 961, NJ1, NJ2 and NJ4).

Fig. 3.4. Semiquantitative determination of TNF- α expression in buccal mucosa in control and infected white-tailed deer (control deer n=2; infected deer n=2). **(A)** Example of agarose gel electrophoresis of TNF and actin PCR products. Lane 1-ladder; lane 2-water control; lane 3-positive control; lanes 4, 5, 6, 7 and 8-buccal mucosa from mock-infected control deer taken on day 0, 2, 4, 6 and 8 p.i., respectively; lanes 9, 10, 11, 12 and 13-buccal mucosa from a white-tailed deer experimentally infected with EHDV-2, taken on day 0, 2, 4, 6 and 8 p.i. respectively. Specific PCR product size for beta-actin and TNF- α is 212 bp and 119 bp, respectively. **(B)** Comparison of relative intensities for TNF production in buccal mucosa in the control and infected deer.

Fig. 3.5. Semiquantitative determination of mRNA expression levels by RT-PCR of TNF- α and β -actin in peripheral blood mononuclear cells isolated from white-tailed deer and inoculated with mock-inoculum/ bacterial lipopolysaccharide/or EHDV-2 (n=1). **(A)** Example of agarose gel electrophoresis of TNF and β actin PCR products. Lane 1-ladder; lane 2-water control; lane 3-0 hours p.i.; lanes 4-Mock-infected cells (24 hr -ve), 24 hours p.i.; lane 5-mock-infected cells (48 hr-ve), 48 hours p.i.; lane 6-cells inoculated with LPS (positive control), 24 hours p.i.; lane 7-cells inoculated with LPS (positive control), 48 hours p.i.; lane 8-cells inoculated with EHDV-2, 24 hours p.i.; lane 9-cells inoculated with EHDV-2, 48 hours p.i.. Specific PCR product size for β -actin and TNF- α is 212 bp and 119 bp, respectively. **(B)** Comparison of relative intensities of various samples from the in vitro study done on peripheral blood mononuclear cells.

Figure 3.1.

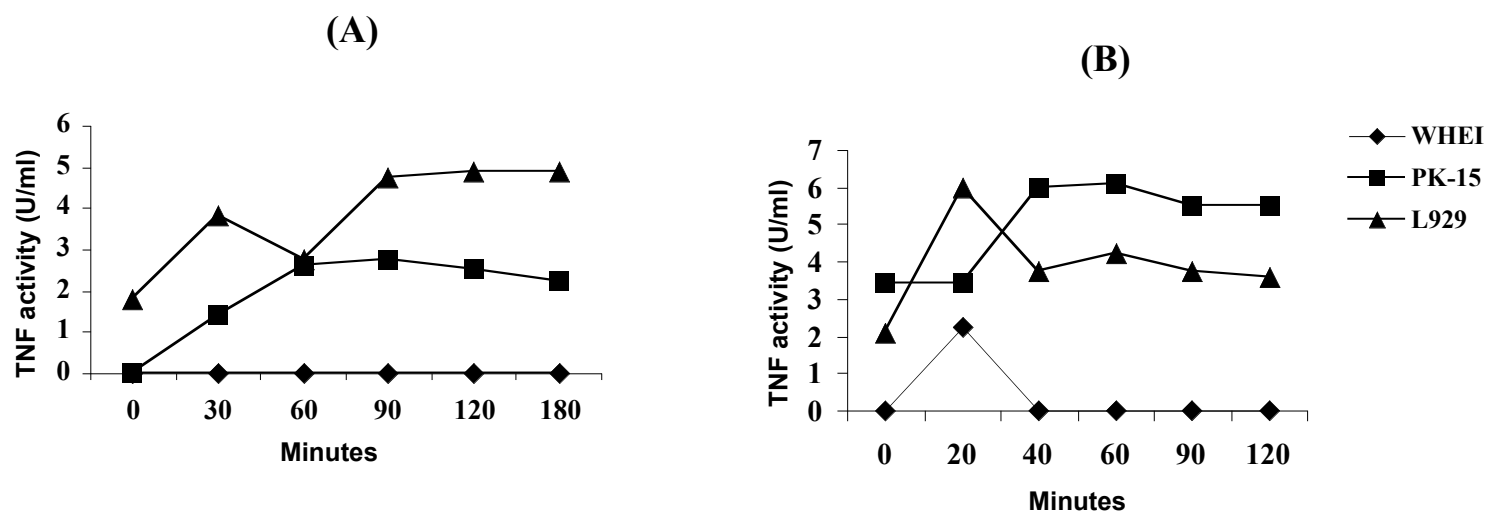


Figure 3.2.

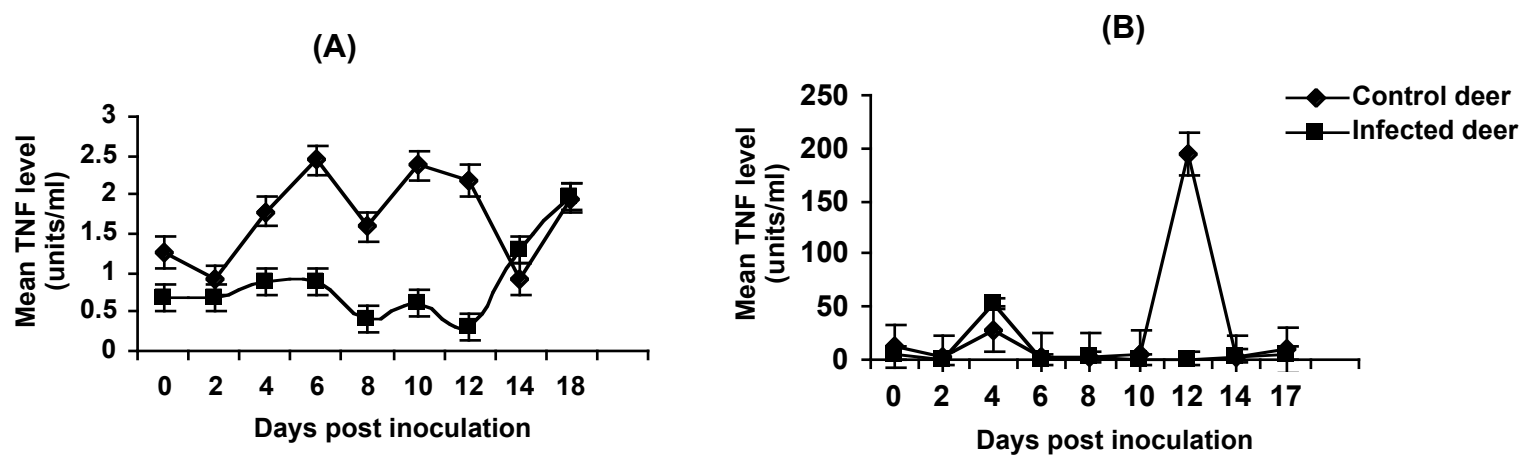


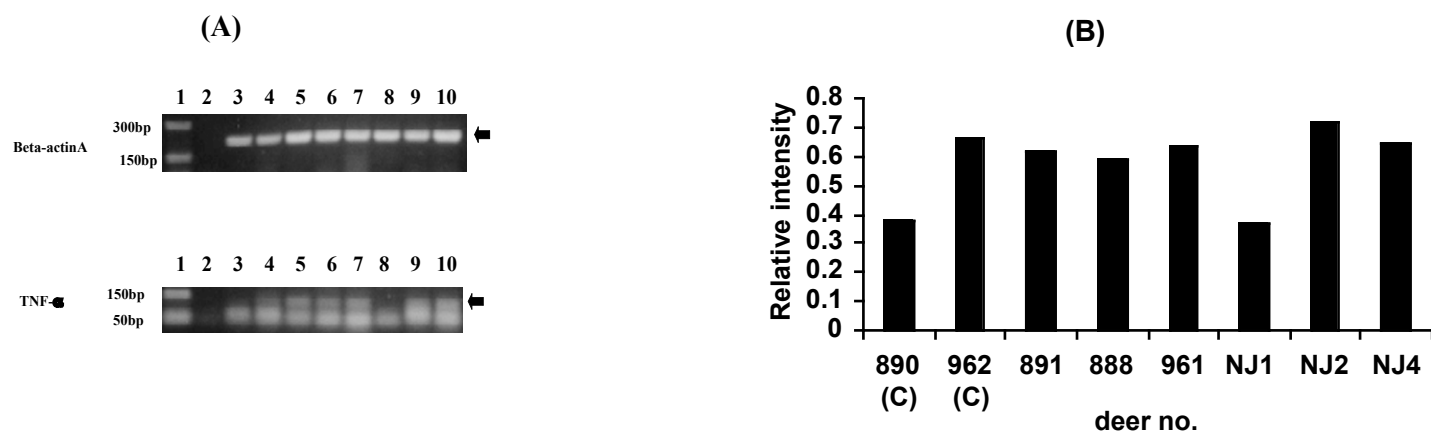
Figure 3.3.

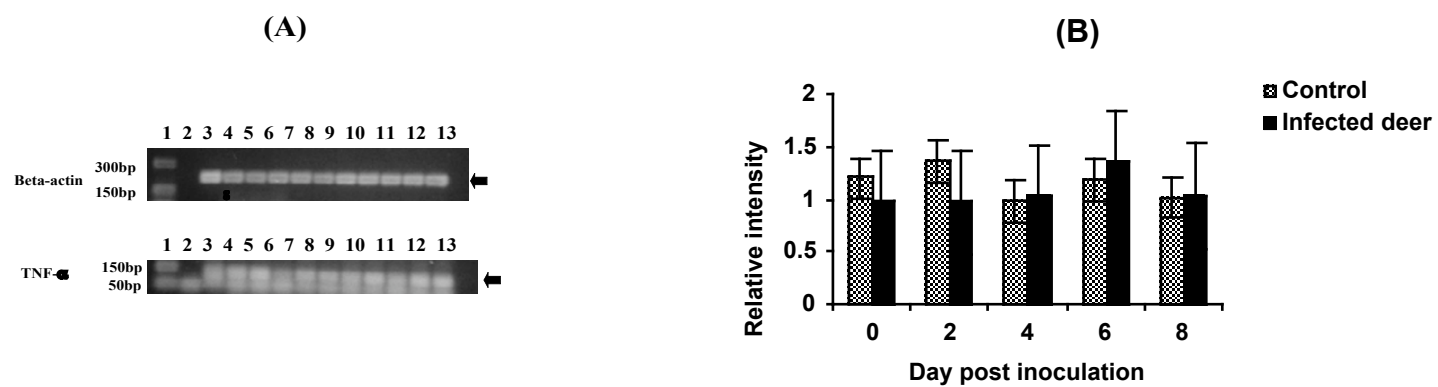
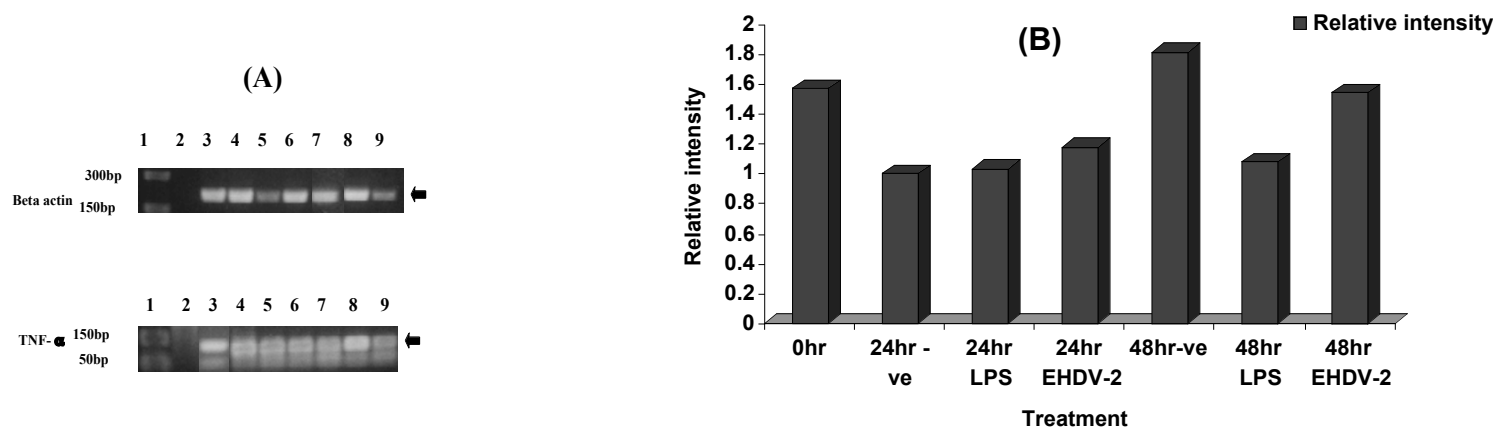
Figure 3.4.

Figure 3.5.

CHAPTER 4:
EPIZOOTIC HEMORRHAGIC DISEASE VIRUS INDUCED APOPTOSIS IN BOVINE AND
DEER ENDOTHELIAL CELLS¹

¹ Sharma, P., D.E. Stallknecht, and E.W. Howerth. To be submitted to the *Journal of General Virology*.

Abstract: Epizootic hemorrhagic disease (EHD) viruses cause disease in both domestic and wild ruminants, particularly white-tailed deer, which is associated with viral replication in endothelial cells and subsequent endothelial damage. Using in situ nick end-labeling (TUNEL), caspase-3 (cleaved) immunohistochemistry, flow cytometry and annexin staining, and agarose electrophoresis of DNA we document apoptosis as a mechanism of endothelial cell death in EHD virus infected bovine carotid artery endothelial cells. Furthermore, apoptosis appears to be directly related to viral replication. Assessment of p53 gene regulation and protein expression in infected endothelial cells suggest that apoptosis is p53 independent. Apoptosis was also demonstrated in the microvasculature of white-tailed deer infected with EHD virus.

INTRODUCTION

An increasing number of viruses have been shown to cause cell death by apoptosis (Shen and Shenk, 1995). In viral diseases, apoptosis may either serve to limit (e.g. simian immunodeficiency virus) or enhance (e.g. bovine herpes virus 1) the spread of the virus (Devireddy & Jones, 1999). Viral encoded proteins may also interact with apoptotic pathways to either inhibit or initiate apoptosis (Danen-van Oorschot *et al.*, 2000; Martin & Berk, 1998; Teodoro & Branton, 1997). Examples of this include, inhibitor of apoptosis (IAP) family of proteins of cowpox virus (Deveraux *et al.* 1999) and adenoviral protein E1B-55K which inhibit apoptosis (Martin and Berk 1998) and the chicken anemia virus protein apoptotin (Danen-van Oorschot *et al.*, 2000) and envelop glycoprotein of feline immunodeficiency virus (Garg *et al.* 2004) which induce apoptosis.

p53 is an important protein transcription factor involved in regulating apoptosis (Teodoro *et al.*, 1997). Viruses may induce apoptosis by a p53 dependent mechanism, e.g. bovine herpes virus 1 (Devireddy & Jones, 1999) and herpes simplex virus (Müller *et al.*, 2004) or by a p53 independent system, e.g. chicken anemia virus (Danen-van Oorschot *et al.*, 2000) and dengue virus (Thongtan *et al.*, 2004).

Epizootic hemorrhagic disease viruses (Genus *Orbivirus*) are *Culicoides* transmitted viruses that infect a wide variety of wild and domestic ruminants (Sellers 1991) although susceptibility to these viruses varies among species. White-tailed deer (*Odocoileus virginianus*) are particularly susceptible to these viruses, and these viruses commonly cause morbidity and mortality in this species (Hourrigan & Klingsporn 1975; Nettles & Stallknecht, 1992). When these viruses cause disease it is characterized by endothelial damage, primarily of the

microvasculature, that results in edema, hemorrhage, tissue necrosis, and disseminated intravascular coagulation (Howerth *et al.* 1988; Howerth & Tyler 1988; Fischer *et al.*, 1995; Roughton, 1975; Fletch & Karstad, 1971). While these viruses primarily replicate in endothelial cells (Tsai & Karstad 1973; Howerth & Tyler, 1988), the mechanism of endothelial damage is not completely understood. Ultrastructural studies demonstrate death of EHDV infected endothelial cells (Tsai & Karstad 1973), but whether cell death is due to apoptosis and/or necrosis has not been fully established.

Preliminary data indicates that EHDV can induce apoptosis of bovine endothelial cells *in vitro* (Howerth & Murphy, 1999). Likewise, the closely related bluetongue viruses have been shown to induce apoptosis, as well as necrosis, of sheep and bovine endothelial cells (DeMaula *et al.* 2001). The objectives of the present study are to : 1) document that EHDV can cause death of endothelial cells via apoptosis, 2) determine whether EHDV induced apoptosis is receptor mediated or directly related to virus replication, and 3) to determine if EHDV induced apoptosis is p53 dependent.

METHODS

Experimental design

Documentation of EHDV-induced apoptosis:

Monolayers of bovine carotid artery endothelial cells were grown in 25 cm² flasks. Fourth passage EHD virus serotype 2 (originally from tongue of an experimentally infected deer) was grown on baby hamster kidney (BHK) cells and prepared as previously described (Gaydos *et al.*, 2002). The inoculum was frozen in aliquots at -70 C; the titer was 10^{5.7} TCID₅₀/25µl.

The monolayers were inoculated with either $10^{5.7}$ TCID₅₀ EHDV serotype 2 (EHDV-2), tumor necrosis factor (TNF) (100ng/ml), or left untreated and incubated for 24 to 48 hours. The monolayers inoculated with TNF were used as positive control for the experiment. Cells were dissociated with trypsin/EDTA and used for either annexin V binding or electrophoretic DNA fragmentation assays. Monolayers of endothelial cells in 4 well-chamber slides were inoculated with $10^{5.3}$ TCID₅₀ EHDV or untreated, incubated for 24 to 48 hours, then fixed in 10 % buffered formalin for 10 minutes, and then DNA fragmentation typical of apoptosis was detected by fragment end labeling (FragEL).

Correlation between EHDV-induced apoptosis and viral replication and p53 regulation:

Bovine carotid artery endothelial cell monolayers were grown in 25cm² flasks with a seeding density of 7×10^5 cells. EHDV-2 was originally isolated in BHK cells from spleen of a white-tailed deer from Kansas that died in natural outbreak of epizootic hemorrhagic disease in 2000. The unpurified inoculum was prepared from passage 1 tissue culture media as previously described (Gaydos *et al.*, 2002). The inoculum was frozen in aliquots at -70°C ; the titer of the frozen stock was $10^{6.78}$ TCID₅₀/ml. EHDV-2 inoculum was partially purified as described by De Maula *et al.* (2001). Aliquots of the virus were frozen at -70°C ; the titer of the frozen stock was $10^{7.3}$ TCID₅₀/ml. The unpurified and partially purified EHDV-2 inoculums were inactivated by exposing to ultra-violet light (999,900 uJ/cm² for 12 hours) in an Electronic Ultraviolet Crosslinker (Ultralum, Inc.). The viral inoculum was tested for inactivation on monolayers of cow pulmonary artery endothelial cells and the cells were observed for one week for cytopathic effect; no cytopathic effects were observed. For the mock inoculum uninfected BHK cells were prepared similar to the unpurified EHDV-2 inoculum; aliquots were frozen at -70°C .

At 72 hours post seeding monolayers were infected with either $10^{5.08}$ TCID₅₀/ml of unpurified or partially purified EHDV-2, similar doses of the inactivated inoculums, or TNF (0.1 µg/ml; TNF- α , mouse; recombinant, Sigma) as a positive control. Monolayers inoculated with mock-inoculum served as negative control. At 0, 18, 24 and 42 hours post inoculation the monolayers in the flasks were scraped and cells and supernatant harvested for virus titration, RT-PCR, TUNEL and immunohistochemistry for caspase-3 (cleaved), p53 and EHDV-2. 500 µl of fluid containing scraped endothelial cells and media was sonicated for virus titration. The remaining scraped cells and media were pelleted by centrifugation (14000 rpm for 10 minutes at room temperature). The pellet was resuspended in media and divided into two parts. One part was used for RNA extraction and the other was used for preparing samples for TUNEL and immunohistochemistry for caspase-3 (cleaved), p53, and EHDV-2. The cell suspension for immunohistochemistry and TUNEL was pelleted by centrifugation and the pellet fixed in 10% buffered formalin (4 hours at room temperature). The fixed cells were pelleted by centrifugation and the cell pellet resuspended in 2% agarose and embedded in paraffin. Sections of paraffin-embedded tissue (3 µm) were used for TUNEL and immunohistochemical staining.

Bovine carotid artery endothelial cells

Bovine carotid artery endothelial cell (CPAE) were isolated from a 9-month-old steer as previously described (Howerth & Stallknecht, 1995) and characterized as endothelium by morphology and immunohistochemistry for Factor-VIII-related antigen. Cells were propagated in CPAE media (modified Eagle medium with nonessential amino acids containing 10% fetal calf serum, L-glutamine (final concentration 2mM), and antibiotics/antimycotics (50 units penicillin/ml, 5 µg streptomycin/ml, 12 µg amphotericin B/ml)) and passaged using trypsin/EDTA. Cells at passage level 4-9 were used for all experiments.

FragEL DNA fragmentation detection assays

A Klenow-FragEL kit (Oncogene Research Products) was used to detect DNA fragmentation in trial one; chamber slides were stained as per the manufacturer's instructions.

Agarose gel electrophoresis of whole DNA

Genomic DNA was extracted using a Puregene kit (Gentra Systems). Briefly, 3 ml DNA lysis solution (Puregene™ was added to cell pellets and DNA was extracted as per manufacturer's instructions. 350 ng of DNA was separated on a 2.5% agarose gel by electrophoresis and the gel was stained with ethidium bromide (0.5 µg/ml).

Annexin V-binding assay

Annexin V binding to exposed phosphatidyl serine was detected using an Annexin V-biotin apoptosis detection kit (Oncogene Research Products). Cells were adjusted to a concentration of 1×10^6 cells/ml and the assay was performed the manufacturer's instructions using fluorescein conjugated streptavidin (Jackson Laboratory) for detection of binding. Briefly, 0.5 ml of cells were incubated with Annexin V-biotin for 15 min at room temperature, pelleted, resuspended in binding buffer, and then fluorescein conjugated streptavidin (at 15 µg/ml) and propidium iodide were added to the cell suspension. 25-50 µl of stained cells were placed on a glass slide, cover slipped, and examined with a fluorescent microscope equipped with a FITC and rhodamine filters. For flow cytometry, additional 0.5 ml aliquots of cells were incubated with annexin and fluorescein conjugated streptavidin only, propidium iodide only, fluorescein conjugated streptavidin only, or nothing. Samples were kept on ice until analysis via flow cytometry using an Epics-Elite flow counter (Coulter Electronics).

In situ end-labeling by terminal transferase (TUNEL) assays

DNA nick-end labeling was performed using Apotag Plus In situ Apoptosis Detection kit (Chemicon international, Inc.) according to the instructions of the manufacturer. Briefly, deparaffinized sections were rehydrated and treated with Proteinase K (Qiagen; 20ug/ml) in phosphate buffer saline (PBS) for 15 minutes at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 minutes at room temperature and equilibration buffer (1X) was applied directly on the specimens and incubated for 15 seconds at room temperature. The sections were then incubated with TdT enzyme in reaction buffer at 37°C for 1 hour, agitated briefly in stop buffer, and then incubated with it for 10 minutes at room temperature. Negative control samples were incubated with water mixed with reaction buffer. Sheep anti-digoxigenin-AP (750 U/ml; Roche) diluted 1:500 in antibody diluent (Dako) was applied to the sections for 30 minutes at room temperature. Fast Red was used as a chromogen (Dako). The sections were counterstained with hematoxylin and cover-slipped.

Immunohistochemistry

The paraffin-embedded sections were heated at 63°C for 30 minutes, deparaffinized in xylene and rehydrated through a series of graded ethanols to distilled water. Antigen retrieval for caspase was done by steaming the sections in BORG Decloaker (Biocare Medical) for 60 minutes and cooled for 10 minutes. For p53 the slides were steamed in citrate buffer (0.01M, pH 6.0) for 20 minutes and then allowed to cool for 10 minutes and for EHDV the slides were treated with 1X Universal decloaker (Biocare Medical) in distilled water, at 120°C for 3 minutes, 85°C for 10 seconds and then allowed to cool for 5 minutes. Protein block (Dako X0909) was used for blocking (7 minutes at room temperature). For Caspase-3 (cleaved)

detection rabbit anti- Caspase-3 (cleaved) (Biocare Medical REF CP229B) diluted 1:100 in antibody diluent (Dako S0809) was applied for one hour at room temperature. Mach 3 rabbit probe (Biocare Medical) was then applied on the sections for 20 minutes at room temperature. Mach 3 R-polymer ALP (Biocare Medical) was applied for 20 minutes at room temperature. Vulcan fast Red was used as chromogen (Biocare Medical). For p53 detection mouse monoclonal anti-human p53 (PharMingen) diluted 1:300 in antibody diluent (Dako) was applied and the specimens were incubated for 1 hour at room temperature. Negative control samples were incubated with negative control for supersensitive mouse antibodies (Biogenex). Biotinylated anti-mouse antibody (Vector), at 5 μ g/ml was applied for 25 minutes at room temperature followed by the avidin-biotin peroxidase complex (ABC, Vectastain kit). Diaminobenzidine-tetrahydrochloride (DAB; Vector) was used as the chromogen. A bovine squamous cell carcinoma with p53 expression was used as a positive control. For EHDV-2 detection sections were incubated with rabbit anti-EHDV-2 antibody dilute 1:300 in antibody diluent (Dako) for 1 hour at room temperature and then treated with Supersensitive Link biotinylated antirabbit immunoglobulins (Biogenex) for 20 minutes at room temperature. The sections were then treated with alkaline phosphatase conjugated streptavidin (Biogenex) for 20 minutes at room temperature. Fast Red (Dako) was used as chromogen. The specimens were double-labeled for p53 and EHDV-2 by doing immunohistochemistry for p53 followed by EHDV-2 as mentioned previously, in the immunohistochemistry procedures for p53 and EHDV-2 alone. P53 was detected using ABC peroxidase method with DAB chromogen. EHDV-2 was detected using a streptavidin-biotin alkaline phosphatase method with fast red chromogen. The slides were counterstained with Mayers hematoxylin, dehydrated and cover-slipped.

RNA extraction

The cell suspension in the media was centrifuged at 14000 rpm for 10 minutes at room temperature and pelleted. Supernatant was removed and 200µl RNA-Bee (TEL-TEST, Inc, CS-104B) was added to the cell pellet. The cell-pellet was resuspended in RNA-Bee and RNA was extracted as per manufacturer's instructions. The extracted RNA was used for semiquantitative RT-PCR.

RT-PCR for p53 gene upregulation

Semiquantitative RT-PCR was done using the Titan One Tube RT-PCR System (Roche). 0.1 µg RNA and was cycled as follows: 50°C for 60 minute, 94°C for 1 minute, 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute and 20 seconds (34 times to step 3), 68°C for 7 minutes. Primers that amplified bovine p53 were designed from sequences in GenBank (p53-forward primer-5'-CTCACCATCATCACAAGTGG; p53 reverse primer-5'-ACCCACGGATCTGAAGAGTG). Beta actin was used as a house-keeping gene (Keefe *et. al*, 1997, beta actin forward primer-5'-CCTTTTACAACGAGCTGCGTGTG; beta actin reverse primer-5'-ACGTAGCAGAGCTTCTCCTTGATG). Products were separated on 2% agarose gels and the location of a predicted PCR product was confirmed using a ladder (Promega) as a standard-size marker (p53, 252 bp; beta-actin, 390 bp). PCR products (p53) were extracted from 2% agarose gel using QIAquick Gel Extraction Kit (Qiagen) and sequenced for confirmation. The extracted p53 product matched 100% with bovine sequences for p53 in the GenBank. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired under ultraviolet illumination and scanned at 600 dots per inch (DPI) (Umax powerlook 1120). The mean luminosity of the bands was measured in Adobe photoshop 5.5 and the band intensity expressed as relative intensity units. The ratio between the mean luminosity of sample RNA (p53) and

beta-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency.

RESULTS

Documentation of EHDV-induced apoptosis

Agarose gel electrophoresis: DNA laddering typical of apoptosis was seen in bovine carotid artery endothelial cell cultures infected with EHDV-2. Laddering was seen as early as 24 hours post inoculation and became more prominent by 36 hours (Fig. 4.1).

Annexin binding assay: When examined by fluorescent microscopy many cells in the EHDV-2 infected and TNF treated bovine carotid artery endothelial cell cultures had green fluorescence on the cell membrane surface indicative of annexin staining and thus early apoptosis by 24 hours post inoculation; a few EHDV infected cells also had annexin staining plus propidium iodide (PI) staining nuclei (an indication of membrane leakage and late apoptosis or necrosis). By 48 hours, more cells with membrane staining for annexin also had nuclear staining by PI indicating that cells were progressing to late apoptosis. These findings were confirmed by flow cytometry. By 24 hours post inoculation many more cells stained for annexin in the EHDV-2 infected cultures than the control cultures; small numbers of the EHDV-2 infected cells stained with both annexin and PI. Overtime, there was a shift from most cells in EHDV-2 infected cultures staining with annexin alone to more cells staining with both annexin and PI. (Fig.4.2).

FragEL and TUNEL DNA fragmentation detection: This assay labeled numerous cells with morphologic features of apoptosis in EHDV infected and TNF treated endothelial cell cultures as early as 24 hrs post inoculation. The numbers of cells staining increased over time in the EHDV infected cultures (Fig. 4.3). Few or no cells stained in negative control cultures.

Correlation between EHDV-induced apoptosis and viral replication and p53 regulation

In situ nick end-labeling (TUNEL) assays: Only cells inoculated with live EHDV, both purified and unpurified inoculums, had appreciable numbers of cells staining positive by TUNEL (Table 4.1; Fig. 4.3). The number of TUNEL labeled cells increased with time along with increasing viral titer and percent CPE (Table 4.1; Fig. 4.3). Most cells staining positive by TUNEL also had nuclear condensation and pyknosis typical of apoptosis (Fig. 4.6). A few cells with normal morphology were also labeled by this method. Mitotic cells were not labeled.

Caspase-3 (cleaved) immunohistochemistry: Caspase 3 (cleaved) was detected in controls and all treatment groups (Table 4.1; Fig. 4.4). However, only in cultures inoculated with live virus or TNF (the positive control) were there appreciable numbers of caspase positive cells (Table 4.1; Fig. 4.4 & 4.6). At 18 hours post inoculation (PI), the number of caspase positive cells in the live partially purified virus and TNF treatment groups was 3 and 2.4 fold greater than the maximum number in the sham inoculated control (24 hours PI; 25 cells/100cells), but the number of caspase positive cells in these groups decreased rapidly with time. Caspase expression in cells inoculated with live unpurified virus was biphasic and increased over the maximum control count at 18 and 42 hours PI. There was no apparent correlation of caspase expression with virus titer, CPE, TUNEL staining or p53 expression (Fig. 4.4 & 4.5).

Immunohistochemistry for p53: No p53 expression was detected in controls and there was virtually no staining in cells treated with either live virus inoculum (Table 4.1; Fig. 4.6). Surprisingly, there was p53 expression in cultures inoculated with both killed virus inoculums (Table 4.1), however, the number of cells expressing p53 was never as high as the maximum number in the TNF treated group (18 hours PI; 13 cells/100 cells). No cells with positive staining for both p53 and EHDV-2 could be found in any slide, though cells with individual positive staining for p53 (nuclear staining) or EHDV-2 (cytoplasmic staining) were observed in the sections.

Virus titer, CPE, and EHD virus immunohistochemistry: The number of EHD virus immunopositive cells increased with time as the viral titer and percent CPE increased with both live viruses (Table 4.1; Fig. 4.6). However, the number of positive cells did not correlate well with the virus titer or percent CPE. For example, at 42 hours PI, there were 44 positive cells/100 with as a titer of $10^{5.1}$ TCID₅₀ and CPE of 60% in the partially purified group while the unpurified group had 86 positive cells/100 with only a titer of $10^{3.1}$ TCID₅₀ and CPE of 10%. Rare EHD virus immunopositive cells were seen in cultures inoculated with both killed viruses (Table 4.1).

RT-PCR for p53 gene regulation: p53 mRNA expression was detected in bovine endothelial cells at 0 hours PI and in controls and all treatment groups at all subsequent time periods. Visually, there did not appear to be upregulation of p53 mRNA (Fig. 4.7 A). The normalized relative intensity for each treatment group is presented in figure 4.7 B. At 18 hours PI, the unpurified virus had a slightly greater normalized mean relative intensity than the negative

control. Overall the normalized mean relative intensities were lowest, and below the negative control, for the partially purified virus (killed and live) groups. There was no correlation of increased p53 gene expression with number of cells positive for p53 by immunohistochemistry.

DISCUSSION

We have demonstrated by several methods, including end labeling of fragmented DNA via FragEL and TUNEL, annexin binding, and gel electrophoresis of genomic DNA, that EHDV induces apoptosis in bovine endothelial cells *in vitro*. The results of this study also indicate that EHDV-2 induced apoptosis *in vitro* is directly related to viral replication and most likely p53 independent.

Our study adds EHDV to the growing list of viruses that can induce apoptosis. Previously, bluetongue virus, an orbivirus closely related to EHDV, was shown, via annexin binding and flow cytometry, to induce apoptosis in bovine and sheep endothelial cells *in vitro* (DeMaula *et al.*, 2001). In that study, apoptosis was the major cause of cell death in ovine cells, as compared to the bovine cells which mainly exhibited necrosis suggesting that the difference in clinical signs of sheep and cattle in bluetongue could partly be due to enhanced susceptibility of ovine endothelium to apoptotic cell death *in vivo*. Results of our study show that EHDV also induces apoptotic cell death in bovine endothelial cells *in vitro*. But the exact mechanism, proteins involved and biological significance of this process need further investigation.

Increased TUNEL and caspase 3 (cleaved) staining in endothelium infected with partially purified virus confirmed that apoptosis can be viral induced and not dependent on cellular proteins or mediators released into the media from the cells used to produce the inoculum. Additionally, TUNEL staining in live virus inoculated cultures increased over time as did the

CPE and viral titer suggesting that apoptosis is an important mechanism for viral release. Apoptosis appeared to be dependent on the presence of live virus as the number of TUNEL staining cells in cultures inoculated with inactivated virus was negligible compared to those inoculated with live virus. In addition, even though uninfected cultures did show rare cells with morphologic features of apoptosis, the cultures inoculated with live virus had a remarkably increased number of apoptotic appearing cells.

In cells, extracellular or intracellular signals that are potentially apoptotic are transduced by adapter proteins and transmitted to specific cysteine proteases called "initiator caspases," primarily caspase-9. These initiator caspases subsequently cleave caspase 3, which initiates the execution stage of apoptosis (Thonberry & Lazebnik, 1998). Caspase 3 was clearly activated in the live virus inoculated cells as the number of caspase 3(cleaved) positive cells was much higher than in the control cultures or cultures receiving inactivated virus. Because caspase 3 activation was higher in cultures with replicating virus, its possible that its activation was mostly induced by intracellular signals. However, cultures inoculated with inactivated virus did express small amounts of caspase 3 (cleaved) suggesting that mere viral attachment resulted in some degree of receptor-mediated activation of apoptosis. Although inactivated virus appeared to activate caspase 3, this did not proceed to cell death, so virus replication appears to be needed for apoptosis to be fully executed.

Although p53 is an important protein in the regulation of apoptosis, viral induced apoptosis may be p53 dependent or independent (Devireddy & Jones, 1999; Danen-van Oorschot *et al.*, 2000). Viruses in which apoptosis is p53 dependent generally cause increased levels of p53 (Bates and Vousden, 1996). As no significant increase in p53 mRNA or protein expression was found in any of the virus treatment groups it would appear that EHDV-induced endothelial

apoptosis is not p53 dependent. It is possible that the final amount of RNA (0.1 µg) used in the RT-PCR reaction was too great to see a difference between the EHDV-infected and uninfected endothelial cell cultures. However, if this were the case one would expect band intensity on gel electrophoresis to be similar for all the treatments and our bands for p53 expression varied in intensity, so this seems unlikely. It seems that the failure to detect p53 protein by immunohistochemistry is real and not an error of antibody cross reactivity nor timing. Although the antibody used for immunohistochemistry was anti-human in origin, we believe it is capable of cross-reacting with both wild type and mutant bovine p53. We achieved excellent staining of mutant nuclear p53 in bovine squamous cell carcinomas (unpublished data) and we saw multiple stained nuclei in bovine endothelial cells treated with TNF, a known stimulator of p53 (Rokhlin et al., 2000). In normally growing cells, p53 half-life is limited to minutes (Maltzman & Czyzyk, 1984) and it is possible that, in our experiment, the cultures were examined too late to see this effect. However we think this is unlikely as infected cultures were examined at multiple time points and we did see staining in TNF treated cultures at the same timepoints.

The pathophysiological significance of EHDV-induced endothelial apoptosis is unknown. However, in preliminary work, via TUNEL staining, we have demonstrated apoptosis in buccal mucosal endothelium of a WTD with EHDV-2 infection suggesting that apoptosis might play a role *in vivo* (fig 4.6). Further studies are warranted to determine the exact mechanism of EHDV-induced apoptosis, which viral proteins are involved, and the biological significance to both host and virus.

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Figure legends.

Fig. 4.1. Gel electrophoresis of genomic DNA from bovine endothelium. Lane 1: 24 hour, untreated. Lane 2: 24 hour, EHDV-2 infected. Lane 3: 24 TNF treated. Lane 4: 36 hour, untreated. Lane 5: 36 hour, EHDV-2 infected. Lane 6: 36 hour, TNF treated. Slight laddering is seen in the 24 hour, EHDV-2 infected cells and is more obvious by 36 hours.

Fig. 4.2. Annexin V binding in bovine carotid artery endothelium analyzed by flow cytometry. (A). Untreated cell culture at 24 hours. Most cells are in quadrant 3 indicating that most cells are viable; very few cells are present in quadrant 4 or 2. (B). EHDV-2 infected culture at 24 hours. Most cells are in quadrant 4 indicating that most cells are apoptotic, but they are also increased in quadrant 2 indicating that cells are progressing to late apoptosis or necrosis (C) % apoptosis in control, TNF treated, and EHDV-2 infected cells at 24 and 48 hours as determined by flow cytometry showing increased apoptosis in EHDV-2 infected cells over time.

Interpretation: Early apoptotic endothelial cells (quadrant 4) bind annexin V but exclude propidium iodide; late apoptotic or necrotic cells (quadrant 2) bind annexin and stain with propidium iodide; viable cells (quadrant 3) do not bind annexin V and exclude propidium iodide.

Fig. 4.3. Comparison of viral titers, CPE, and TUNEL in bovine endothelium at 18, 36, and 42 hours post treatment. Only cultures infected with EHDV-2 have significant TUNEL staining and increasing numbers of TUNEL staining cells increases as CPE and viral titer increase. C = Mock inoculated. Killpp = inactivated partially purified EHDV-2. pp = partially purified EHDV-2. Killun = inactivated unpurified EHDV-2. Un = Unpurified EHDV-2.

Fig. 4.4. Comparison of caspase 3 (cleaved) with viral titer and CPE in bovine endothelium at 18, 36, and 42 hours post treatment. Caspase 3 (cleaved) staining is most appreciable in EHDV-2 infected and TNF treated cultures. Levels do not correspond well with viral titer or CPE. C = Mock inoculated. Killpp = inactivated partially purified EHDV-2. pp = partially purified EHDV-2. Killun = inactivated unpurified EHDV-2. Un = Unpurified EHDV-2.

Fig. 4.5. Comparison of caspase 3 (cleaved) with TUNEL and p53 in bovine endothelium at 18, 36, and 42 hours post treatment. Caspase 3 (cleaved) staining does not correspond well with TUNEL and p53 staining. C = Mock inoculated. Killpp = inactivated partially purified EHDV-2. pp = partially purified EHDV-2. Killun = inactivated unpurified EHDV-2. Un = Unpurified EHDV-2.

Fig. 4.6. A) TUNEL staining of buccal mucosa from a deer infected with EHDV. Endothelium lining small vessels in the submucosa (arrows) has positive staining indicative of apoptosis. Fast green counterstain. 200 X original magnification. B) Apoptosis of EHDV infected bovine carotid artery cells detected by fluorescence microscopy using annexin-V binding (FITC)/propidium iodide double staining. Area of cytopathic effect with abundant FITC staining of cell membranes is indicative of annexin-V binding to phosphatidyl serine externalized during apoptosis. Only a couple of nuclei have propidium iodide staining (arrows). 400 X original magnification. C) TUNEL stained bovine carotid artery endothelial cells infected with EHDV. Many cells morphologically consistent with apoptosis stain positive. Fast red chromagen. D) Bovine carotid artery endothelial cells infected with EHDV and stained for EHDV by immunohistochemistry. Numerous cells have positive cytoplasmic staining. Fast red

chromagen. E) Bovine carotid artery endothelial cells infected with EHDV and stained for p53 by immunohistochemistry. Very few cells have positive nuclear staining (arrows). DAB chromagen. F) Bovine carotid artery endothelial cells infected with EHDV and stained for caspase 3 (cleaved) by immunohistochemistry. Many cells morphologically consistent with apoptosis stain positive. Fast red chromagen.

Fig. 4.7. (A) RT-PCR for p53 regulation. Lane 1: PCR marker; lane 2: Water control; lane 3: 0 hour p.i.; lane 4: negative control, 18 hours p.i.; lane 5: positive control (TNF), 18 hours p.i.; lane 6: inactivated ppEHDV-2, 18 hours p.i.; lane 7: inactivated unpurified EHDV-2, 18 hours p.i.; lane 8: ppEHDV-2, 18 hours, p.i.; lane 9: unpurified EHDV-2, 18 hours p.i.; lane 10: negative control, 24 hours p.i.; lane 11: positive control (TNF), 24 hours p.i.; lane 12: inactivated ppEHDV-2, 24 hours p.i.; lane 13: inactivated unpurified EHDV-2, 24 hours p.i.; lane 14: ppEHDV-2, 24 hours, p.i.; lane 15: unpurified EHDV-2, 24 hours p.i.; lane 16: negative control, 42 hours p.i.; lane 17: positive control (TNF), 42 hours p.i.; lane 18: inactivated ppEHDV-2, 42 hours p.i.; lane 19: inactivated unpurified EHDV-2, 42 hours p.i.; lane 20: ppEHDV-2, 42 hours, p.i.; lane 21: unpurified EHDV-2, 42 hours p.i.. Specific PCR product size for beta-actin and p53 is 390 bp and 251 bp respectively. **(B)** Comparison of relative intensities for p53 expression in various treatments groups.

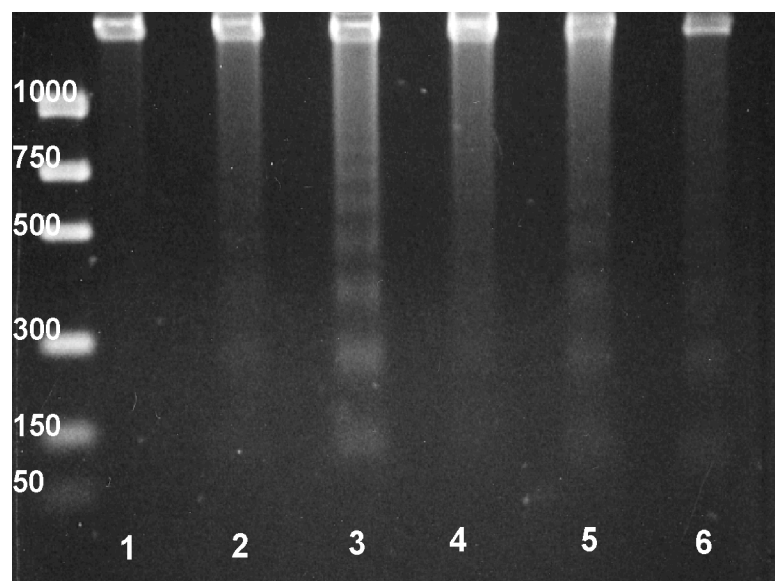
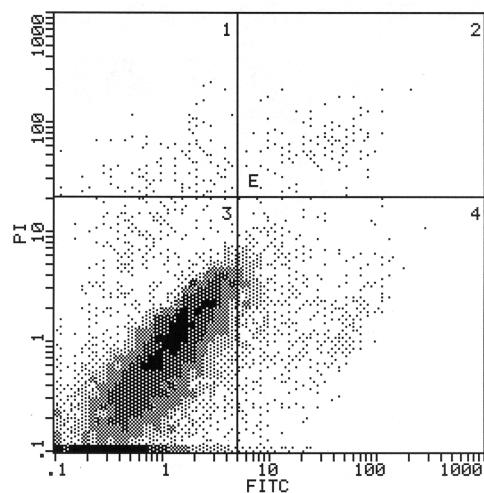
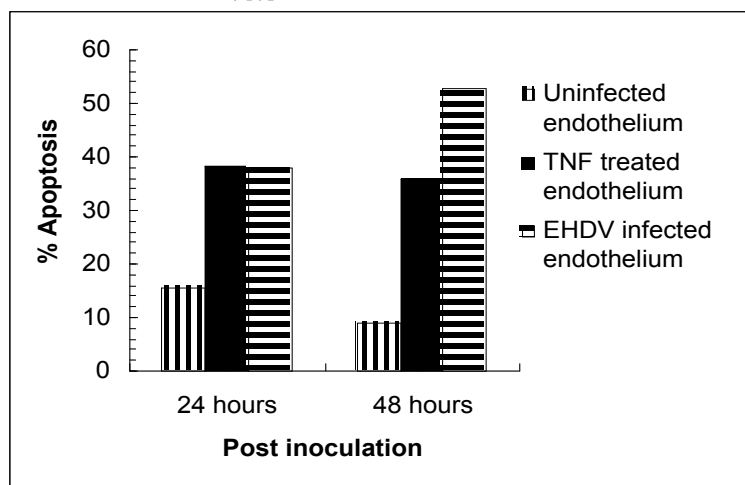
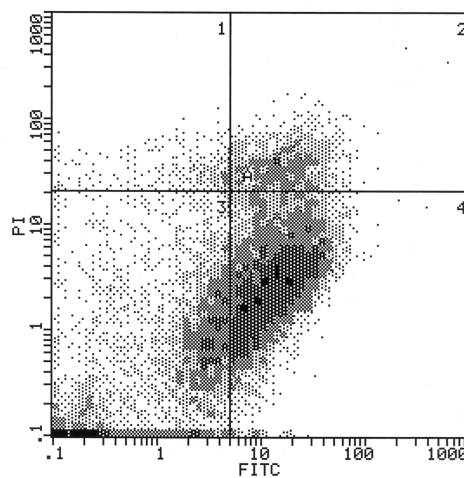
Figure 4.1.

Figure 4.2.

(A)

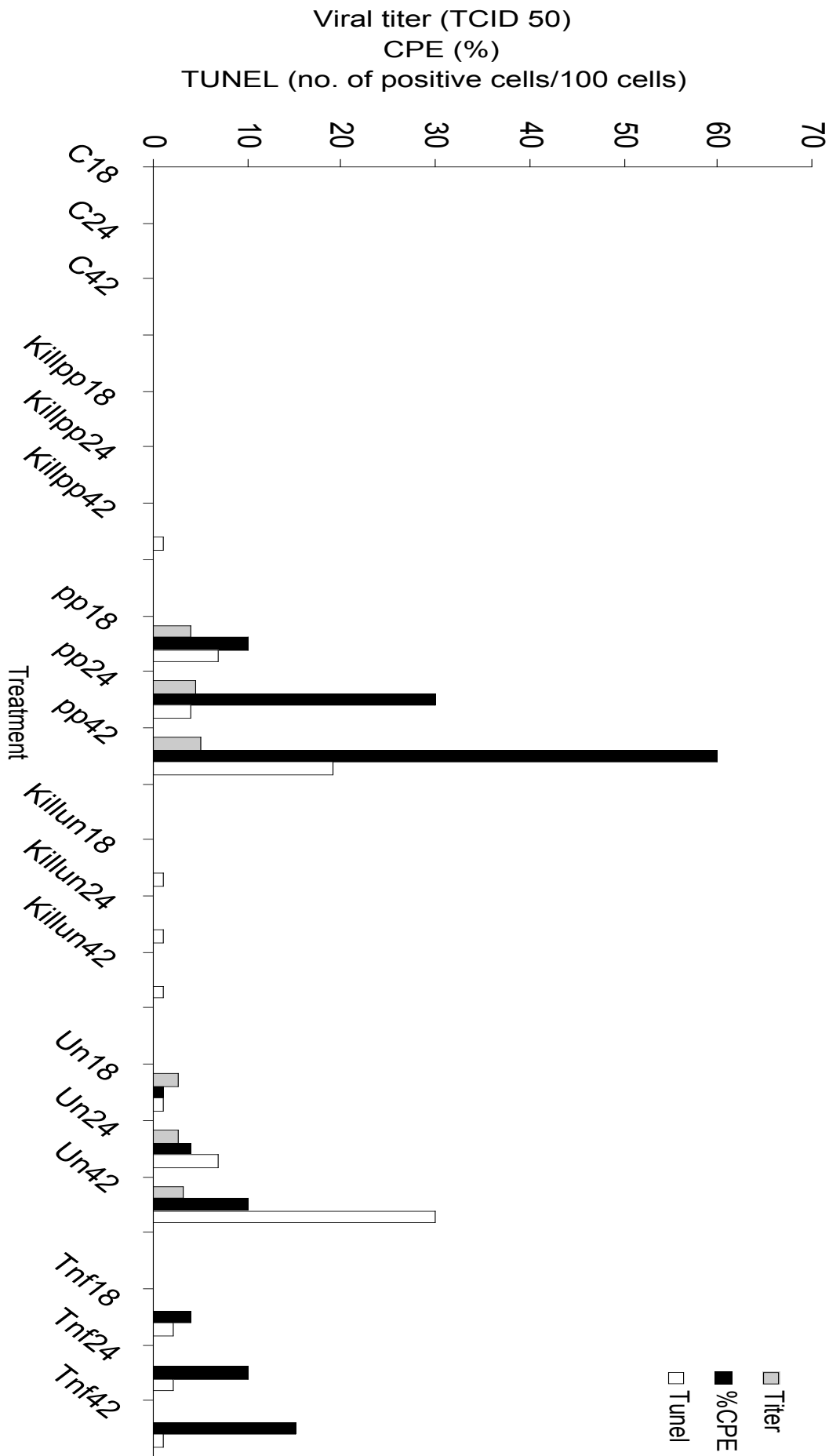


(B)



(C)

Figure 4.3.



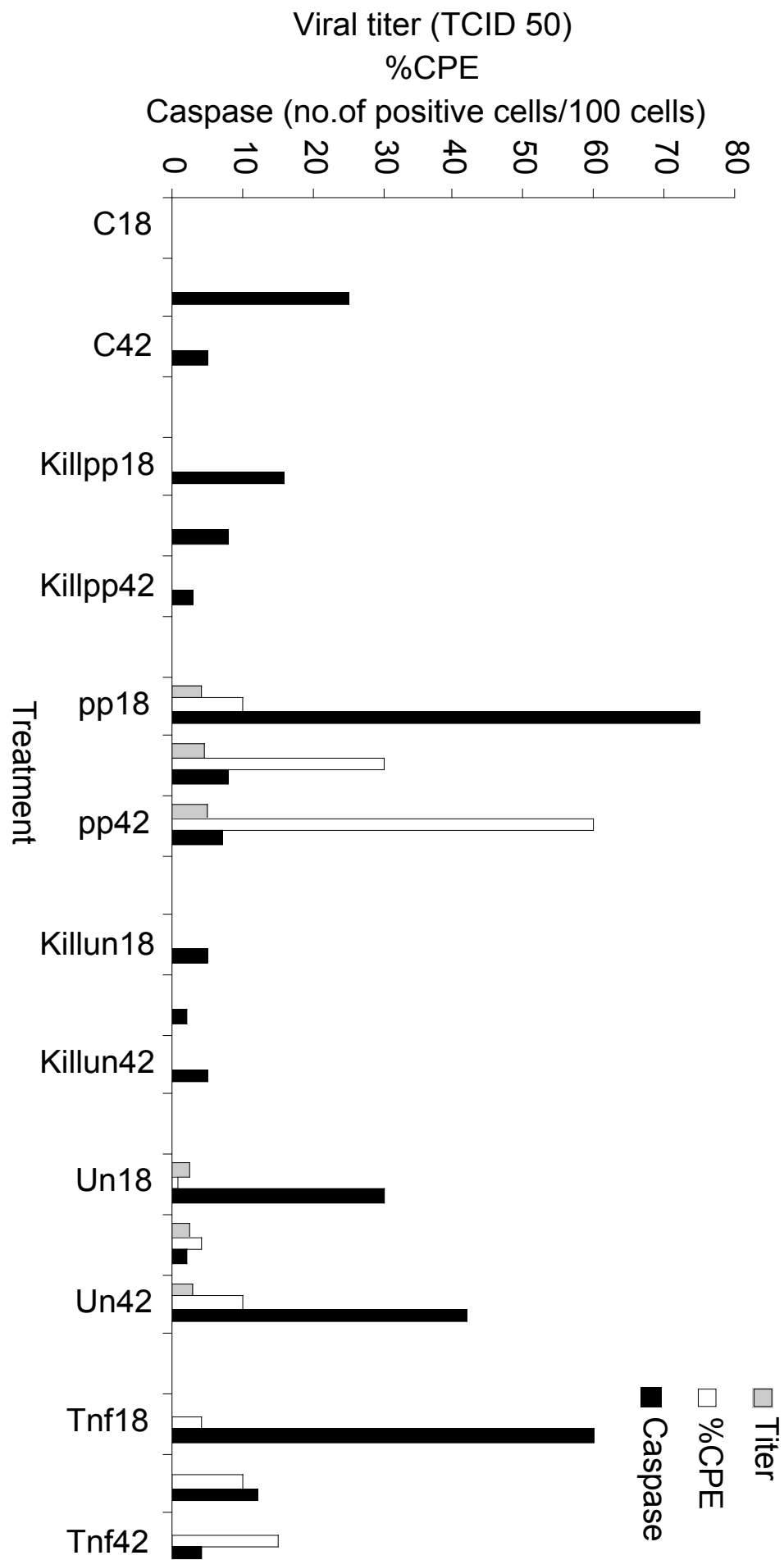


Figure 4.4

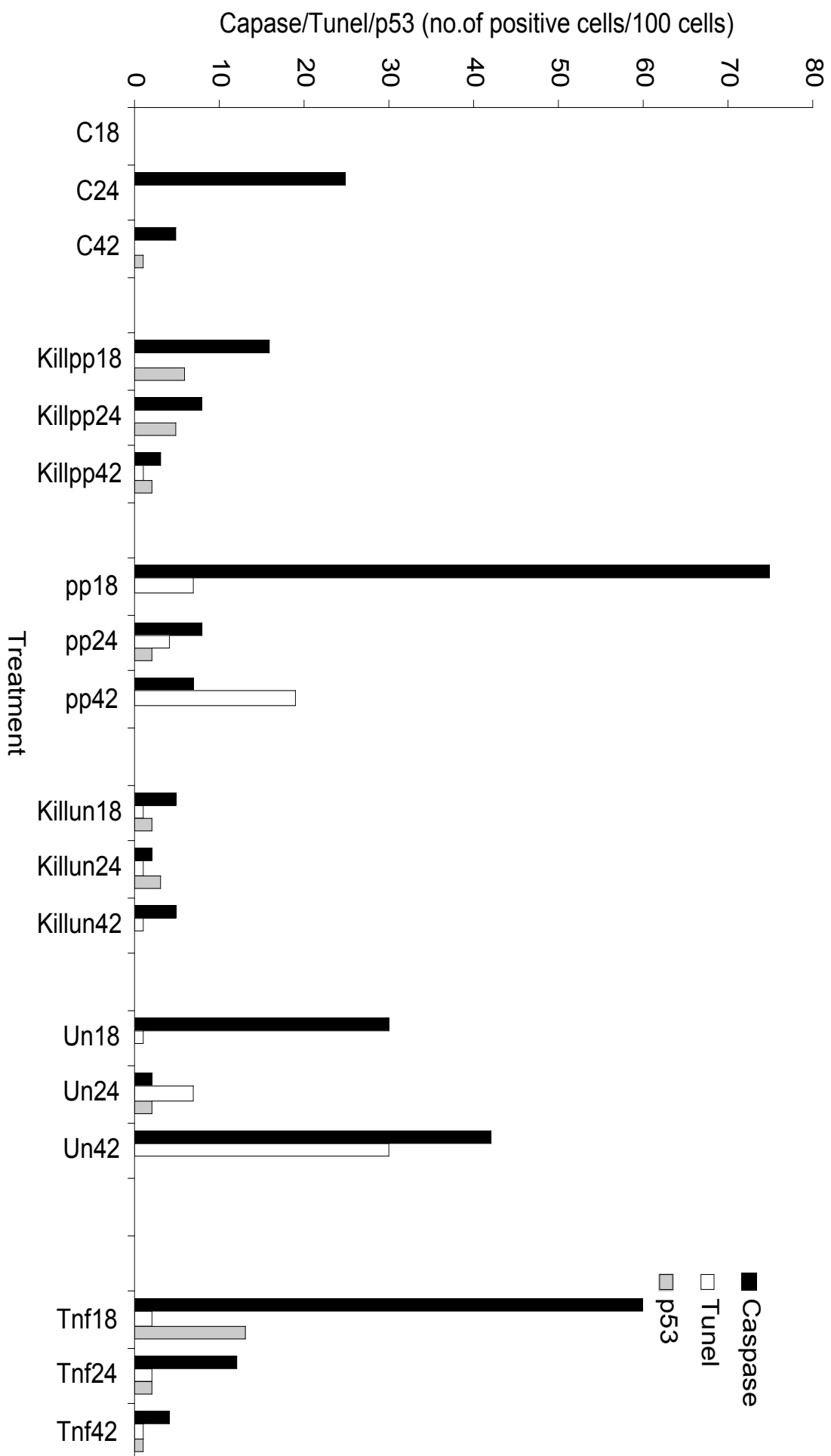


Figure 4.5.

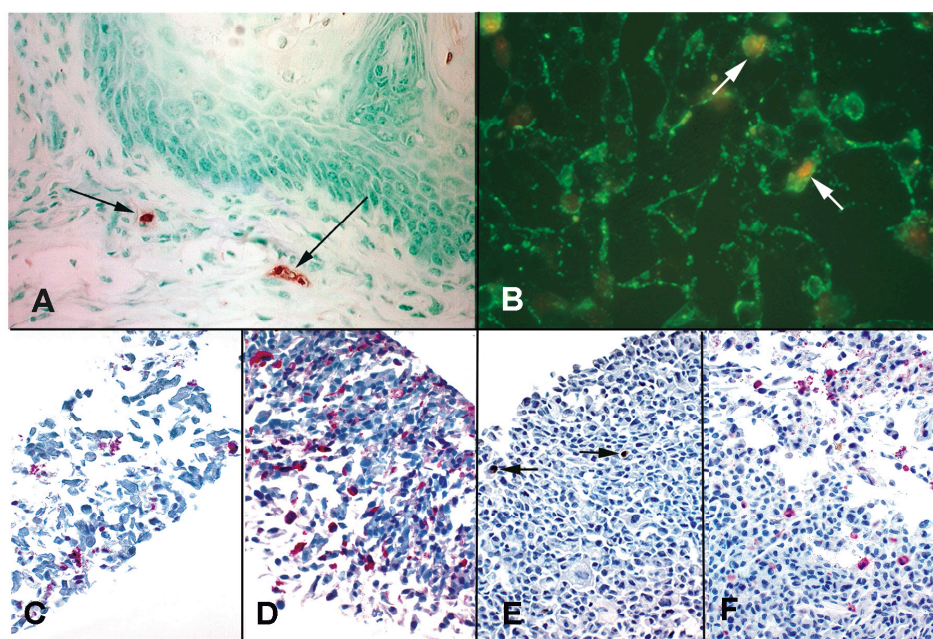
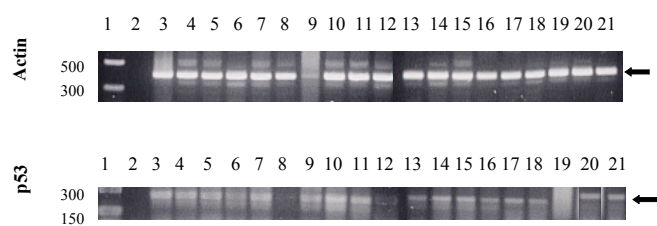
Figure 4.6

Figure 4.7.

(A)



(B)

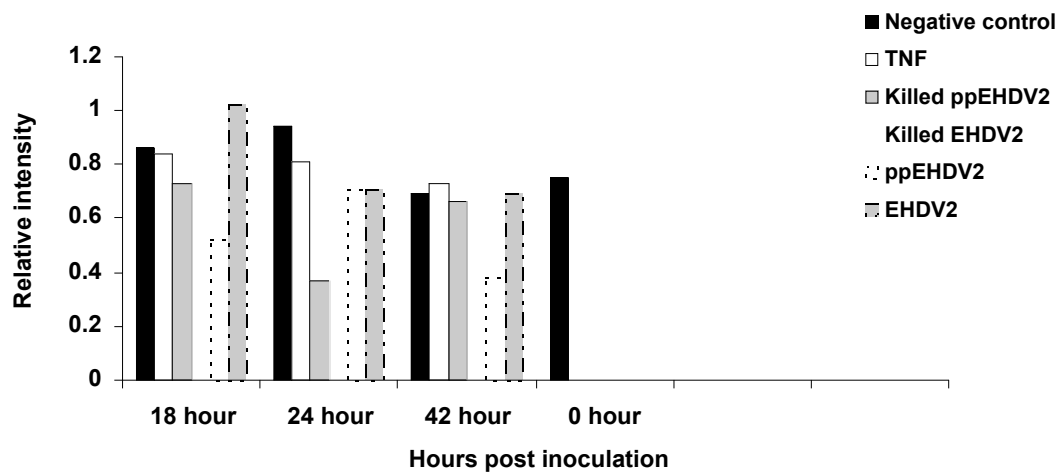


Table 4.1. Virus titers, % CPE, and EHDV, caspase 3 (cleaved), TUNEL and p53 staining cells in bovine endothelium at 18, 24, and 48 hours post treatment.

Treatment	<u>Virus titer*</u>			<u>% CPE⁺</u>			<u>EHD[#]</u>			<u>Caspase^{&}</u>			<u>TUNEL[^]</u>			<u>P53^{>}</u>		
	18	24	42	18	24	42	18	24	48	18	24	48	18	24	48	18	24	48
Control ¹	0	0	0	0	0	0	0	0	0	0	25	5	0	0	1	0	0	0
Killed partial purified ²	0	0	0	0	0	0	0	1	0	16	8	3	0	0	1	6	5	2
Live Partial Purified ³	4.1	4.6	5.1	10	30	60	29	35	44	75	8	7	7	4	19	0	2	0
Killed unpurified ⁴	0	0	0	0	0	0	0	1	0	5	2	5	1	1	1	2	3	0
Live Unpurified ⁵	2.6	2.6	3.1	1	4	10	11	70	86	30	2	42	1	7	30	0	2	0
TNF ⁶	0	0	0	4	10	15	0	0	0	60	12	4	2	2	1	13	2	1

*Virus titer log 10 TCID₅₀, ⁺% cytopathic effect, [#]Number of EHDV staining cells/100 cells, [&] Number of caspase 3 (cleaved) staining cells/100 cells, [^]Number of TUNEL staining cells/100 cells, [>]Number of p53 staining cells/100 cells. Treatments included: ¹mock inoculated control, ² Inactivated partially purified EHDV-2 at similar dose as 3. ³ Partially purified EHDV-2 at 105.8 TCID₅₀/m/, ⁴ Inactivated unpurified EHDV-2 at similar dose as 5, ⁵ Unpurified EHDV-2 at 105.8TCID₅₀/ml, ⁶TNF at 0.1 µg/ml.

CHAPTER 5:

EXPRESSION OF INTERLEUKIN-1 BETA AND INTERLEUKIN-6 IN WHITE-TAILED
DEER INFECTED WITH EPIZOOTIC HEMORRHAGIC DISEASE VIRUS¹

¹ Sharma, P., D.E. Stallknecht, M. Murphy and E.W. Howerth. To be submitted to the *Journal of Wildlife Diseases*.

ABSTRACT:

The pathogenesis of epizootic hemorrhagic disease (EHD) in white-tailed deer (*Odocoileus virginianus*) may be related to factors other than direct viral damage, specifically the cytokines interleukin-1 beta (IL-1 beta) and interleukin-6 (IL-6). To determine if circulating and/or tissue levels of IL-1 and IL-6 are upregulated in EHDV infection, WTD were experimentally infected with epizootic hemorrhagic disease virus 2 (EHDV-2, *Orbivirus*, *Reoviridae*). The circulatory level of IL-1 beta was assayed by enzyme linked immunosorbent assay (ELISA). Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were used to detect upregulation of IL-1 beta in tissues. Upregulation of IL-6 in tissues was determined by RT-PCR. RT-PCR also was used to determine if IL-1 and IL-6 were upregulated in WTD peripheral blood mononuclear cells (PBMNCs) infected with EHDV-2 *in vitro*. ELISA, RT-PCR and IHC showed an increase in the circulatory and tissue levels of IL-1 in the infected deer. A significant upregulation of IL-6 was observed in the lymph nodes of infected deer and EHDV infected deer monocytes, *in vitro*, as determined by RT-PCR. These findings suggest a role of IL-1 beta and IL-6 in the pathogenesis of EHD in WTD.

Key Words: Epizootic hemorrhagic disease, epizootic hemorrhagic disease viruses, white-tailed deer, interleukin-1 beta, interleukin-6.

INTRODUCTION

Epizootic hemorrhagic disease (EHD) is an important viral disease of white-tailed deer (WTD) (*Odocoileus virginianus*) caused by either *epizootic hemorrhagic disease virus* 1 (EHDV-1) or 2 (EHDV-2) (*Reoviridae: Orbivirus*). The EHDV are vectored by biting midges in the genus *Culicoides* (Sellers, 1991). In natural and experimental infections, clinical disease and mortality are primarily associated with microvascular damage (Howerth et al., 1988; Tsai and Karstad, 1973). As EHD viruses are both monocytotropic and endotheliotropic (Tsai and Karstad, 1973; Stallknecht et al., 1997), microvascular damage may represent individual or combined effects of viral infection of endothelial cells and inflammatory mediators, such as IL-1 and IL-6, released from infected cells. Likewise, many of these mediators have additional functions that could modulate the course of disease.

In this study, we were specifically interested in defining the roles of IL-1 and IL-6 in EHD. IL-1 is primarily produced by macrophages, but various other cells types including endothelium, keratinocytes, epithelium and cells of the CNS, also produce IL-1 (Dinarello, 1991). IL-1 can activate endothelium, increasing the procoagulant activity of vessels and causing vasodilation, or induce acute-phase responses (Dinarello, 1991), such as fever, that are typical of EHD (Quist et al., 1997). IL-1 also can induce IL-6, which is also produced by macrophages and endothelial cells in response to tissue injury and other inflammatory stimuli (Abbas et al., 2000). IL-6 may induce either a pro- or an inhibitory inflammatory response and is one of the principal mediators of the clinical manifestations of tissue injury, including fever, cachexia, leukocytosis, thrombocytosis, increased plasma levels of acute phase proteins, and decreased plasma levels of albumin (Wilder, 1998).

The potential role of IL-1 and IL-6 in the pathogenesis of the closely related bluetongue viruses (BTV) has been investigated. Transcription of genes encoding IL-1 and IL-6 has been shown to be upregulated in microvascular endothelial cell (MEC) cultures of both sheep and cattle infected with purified BTV (DeMaula et al., 2002 a and b). However, there were species differences in cytokine production in response to BTV infection and inflammatory mediators that could help explain species-specific differences in susceptibility to BTV. In cattle MEC cultures, transcription of both cytokines was greatly increased and expression potentiated by the addition of inflammatory mediators released from BTV infected MEC cultures, while in sheep MEC cultures, transcription of IL-1 was greatly increased while IL-6 was only minimally induced and the addition of inflammatory mediators suppressed the production of both. Variable induction of IL-1 and IL-6 and the response of endothelium to these cytokines could also help explain the variability in severity of EHD in “susceptible” and “resistant” deer populations.

Gaydos et al. (2002) found that deer from two populations of different subspecies, *Odocoileus virginianus texanus* from Texas and *Odocoileus virginianus borealis* from Pennsylvania, varied in their susceptibility to both EHDV-1 and EHDV-2. Although clinical response varied greatly with mortality restricted to the *O. v. borealis* group, deer in both groups developed similar levels of viremia and humoral immune responses suggesting that variable expression of disease was due to innate resistance. The potential to identify individuals or populations of varying susceptibility offers a unique opportunity to elucidate the potential role of cytokines in the pathogenesis of EHD. The goal of this study was to better characterize the pathogenesis of EHDV-2 in WTD, specifically looking at the expression of IL-1 beta and IL-6 in deer from potentially “resistant” and “susceptible” populations. Our first objective was to determine if tissue and circulating levels of IL-1 and IL-6 were upregulated in EHDV-2 infected WTD. Our

second objective was to determine if IL-1 and IL-6 upregulation occurs in WTD monocytes infected with EHDV-2 and BTV-10 *in vitro*.

MATERIALS AND METHODS

IN VIVO IL-1 BETA AND IL-6 PRODUCTION

Experimental infection

Animals- White-tailed deer fawns (7 males, 3 females) approximately four-to-eight-week-old were acquired from the Kansas State Conservation Commission and the New Jersey Division of Fish and Wildlife. These included two fawns from western Kansas, three fawns from eastern Kansas and three from New Jersey. They were moved to an indoor facility at the University of Georgia and hand-reared until they were approximately 5-6 months old. At the onset of the study fawns weighed between 35.2 kg and 74.8 kg (mean=23.1 kg) and were serologically negative for antibodies for EHD and BT viruses by agar gel immunodiffusion (AGID). Animals were housed indoors to prevent contact with *Culicoides* vectors. Because of the variation in clinical response reported by Gaydos et al. (2002), which may be related to WTD subspecies, the inclusion of deer originating from different geographic areas (KS and NJ) was intentional. These deer, based on location of origin and the reported range of WTD subspecies (Baker, 1984), represented three subspecies including *O. v. texanus* (Western KS, n=2), *O. v. macaourus* (Eastern KS, n=3) and *O. v. borealis* (NJ, n=3). Based on varying susceptibilities of these subspecies described in both experimental (Gaydos et al., 2002) and field studies (Flacke et al., 2004), the *O. v. macaourus* and *O. v. borealis* fawns were considered as the “susceptible” group and *O. v. texanus* fawns as the “resistant” group for data analysis.

Design-One deer (from eastern Kansas) was initially infected with EHDV-2 to verify infectivity of the viral inoculum; it developed severe clinical disease by post inoculation (p.i.) day 8. Subsequently, five deer (three from New Jersey and two from Western Kansas) were inoculated with EHDV-2 (day 0); two deer from Eastern Kansas received sham inoculation and served as controls. For all inoculations, deer were sedated with approximately 0.5mg/kg xylazine (Rompun^R, Miles, Inc., Shawnee Mission, Kansas, USA), and the hair on the right side of the neck was clipped. For the EHDV-2 inoculation, deer received $10^{7.03}$ median tissue culture infective doses (TCID₅₀) (1.3 ml) subcutaneously and $10^{7.03}$ TCID₅₀ (1.3 ml) intradermally in multiple sites on the right side of the neck. Sham-inoculated deer received 1.3 ml of sham inoculum subcutaneously and 1.3 ml intradermally in multiple sites on the right side of the neck. Post inoculation sedation was reversed with 2 to 5 mg yohimbine (Yobine, Lloyd Laboratories, Shenandoah, IO, USA) administered intramuscularly. During the study, the deer were manually restrained every other day up to p.i. day 8 for physical examination, body temperature evaluation, blood collection for hematology and coagulation assays, and to collect samples from skin and buccal mucosa. All blood collections were done via jugular venipuncture. Blood anticoagulated in K₃ EDTA was used for white blood cell counts and differentials, platelet counts, and plasma protein measurement. Sodium citrate anti-coagulated blood was used for viral isolation and plasma (frozen at -70 C until assayed) for activated partial thromboplastin time (APTT). Triplicate 4.0 mm punch-biopsies of skin and buccal mucosa were taken: one was fixed in 10 % buffered formalin, one placed on ice for RT-PCR, and one placed in viral transport media for virus isolation. One infected deer died on day 7 p.i.; the remaining surviving animals were euthanized on day 8 p.i, with an overdose of euthanasia solution (Beuthanasia-D Special, Schering-Plough Animal Health Corporation, Union, NJ, USA). Necropsies were performed on

all deer within one hour following euthanasia or immediately upon being found dead. Triplicate samples of brain, skin, buccal mucosa, right and left prescapular and prefemoral lymph nodes, gastrointestinal tract, lung, heart, liver and spleen were taken at necropsy on day 8 p.i. One was fixed in 10 % buffered formalin for histopathology and immunohistochemistry; one was placed on ice for RT-PCR and then snap frozen in liquid nitrogen, and stored at -80°C; and one placed in viral transport media for virus isolation. Clinical disease severity scores (CDSS) were calculated for each infected deer as previously described (Gaydos et al. 2002) using the following clinical and laboratory parameters: depression, erythema, salivation, plasma protein, activated partial thromboplastin time (APTT), platelet count, lymphocyte count, hematocrit and rectal temperature.

Viral and mock inoculum- The EHDV-2 used in this study was isolated during 2000 in baby hamster kidney (BHK) cells from spleen of a dead WTD from Kansas. To prepare the inoculum, 100µl infected tissue culture media (MEM with 3% FBS, 50 units penicillin/ml, 5 µg streptomycin/ml, 12 µg amphotericin B/ml) from passage 1 (original isolation) was inoculated into two T75 flasks containing confluent monolayers of BHK cells. Cells were incubated at 37°C until cytopathic effect was observed in approximately 50% or more of the cell monolayer (96 hour post inoculation). Cells were scraped and the contents were centrifuged at 1500xG for 15min. Media was removed and the cell pellet was resuspended in 5 ml of media and sonicated. The sonicated cells were centrifuged at 1500xG for 15 min and the supernatant was added to the previously removed media. Supernatant was frozen in aliquots at -70°C. The titer of the frozen stock was $10^{6.78}$ /ml. For the sham inoculum uninfected BHK cells were prepared similar to the EHDV-2 inoculum; aliquots were frozen at -70°C.

Virus isolation and titration- Blood samples collected in sodium citrate and tissue samples collected during the in vivo infection and at necropsy were used for virus isolation and titration. Virus isolation and titration was done in BHK and cattle pulmonary artery endothelial (CPAE) cells as previously described (Quist et al., 1997).

ELISA for IL-1 beta

Plasma was obtained from the blood samples collected from the two control deer (KS 890 and KS 962), four “susceptible” (KS 891, NJ1, NJ2 and NJ4) and two “resistant” EHDV-infected deer on day 0, 2, 4 6 and 8 post inoculation. IL-1 beta was assayed by ELISA using the human IL-1 beta Quantikine kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. The standards and plasma samples were assayed in duplicate and read at 450 nm in an automated plate reader (Bio-Rad) within 30 minutes, with a wavelength correction of 570 nm. A standard curve was generated for each set of samples assayed by the plate reader and concentration (pg/ml) of IL-1 beta was obtained.

RT-PCR for IL-1 beta

Semiquantitative RT-PCR was done on prescapular lymph nodes taken at necropsy from two control deer (KS 890 and KS 962), four infected “susceptible” deer (NJ1, NJ2, NJ4, KS 891) two infected “resistant” deer (KS 888, KS 961). RT-PCR was also done on buccal mucosa of two infected “susceptible” deer (NJ2 and NJ4) and two control deer (KS 890, KS 962) taken on post inoculation day 0, 2, 4, 6 and 8. Using the Titan One Tube RT-PCR System (Roche), 0.1 ug RNA from lymph nodes and 0.5 µg RNA from buccal mucosa was cycled as follows: 50°C for 60 minute, 94°C for 1 minute, 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute and

20 seconds (34 times to step 3), 68°C for 7 minutes. To ensure the integrity of RNA, the expression of housekeeping gene beta-actin was assessed for each sample. The nucleotide sequence of the primers (10µm) were IL-1 beta-forward primer: 5'-GCTGAGGAACAGTGCC and IL-1 beta-reverse primer: 5'-CCAGCACCAGGGATTT; actin-forward primer: 5'-CCGCACTACTGGTATTGT and actin-reverse primer: 5'-AGAGCTTCTCCTTGATGTC. These primers were designed based on deer nucleotide sequences present in Genbank (IL-1 beta, accession number-AY509603). Bovine beta actin primers (Keefe et al., 1997) were used to amplify deer actin and then specific primers were designed based on the deer nucleotide sequences. PCR products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide. Location of a predicted PCR product was confirmed using a DNA ladder (Promega) as a standard-size marker. Specific PCR products for IL-1 beta and beta-actin were at 181 bp and 212 bp, respectively. The PCR product (IL-1 beta) was extracted from a 2% agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), sequenced and verified as IL-1. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired under ultraviolet illumination and scanned at 600 DPI (dots per inch). The mean luminosity of the bands was measured in Adobe photoshop 5.5 and the mean band intensity was expressed as relative intensity units. The ratio between the mean band intensity of the sample IL-1 beta and beta-actin was calculated, with beta-actin coexpression considered 100%, to normalize for initial variations in sample concentration and as a control for reaction efficiency.

Immunohistochemistry for IL-1 beta- The following tissue, obtained at necropsy, were immunostained for IL-1 beta: right and left prescapular lymph nodes from a control deer (KS 962), four “susceptible” deer (NJ4, NJ2, NJ1, KS 891) and two “resistant” deer (KS 888, KS 961) and buccal mucosa, rumen, abomasum, lung, spleen and intestines from a control deer (KS 962), two infected “susceptible” deer (NJ4 and NJ2) and two infected “resistant” deer (KS 888 and KS 961). Samples from buccal mucosa from one infected “susceptible” deer (NJ4) and one control deer (KS 890) taken at p.i. days 0, 2, 4, 6 and 8 were also stained for expression of IL-1 beta. Paraffin-embedded tissue sections (3 µm) were heated at 63°C for 30 minutes and then deparaffinized in xylene, and rehydrated through a series of graded ethanol baths to distilled water. Antigen retrieval for IL-1 beta was done by steaming the slides in EDTA (1mM, pH=7.0) for 30 minutes and then cooling for 10 minutes. The specimens were blocked with Protein block (Dako, CA, USA) for 7 minutes at room temperature. Primary antibody-mouse anti-ovine IL-1 beta (Serotec, Raleigh, NC, USA) diluted 1:50 in antibody diluent (Dako) was applied for 1 hour at room temperature. Biotinylated anti-mouse antibody (Vector, Burlingame, CA, USA), at 5µg/ml, was then applied for 25 minutes at room temperature. The sections were quenched with 3% H₂O₂ in distilled water for 30 minutes at room temperature, followed by the avidin-biotin peroxidase complex (ABC, Vectastain kit). DAB was used as the chromogen for the peroxidase reaction. The slides were counterstained with Mayers hematoxylin, dehydrated and cover-slipped. Intestinal tissue sample from a cow with paratuberculosis was used as a positive control.

RT-PCR for IL-6

Semiquantitative RT-PCR for IL-6 was done on samples from prescapular lymph nodes and buccal mucosa as previously described for IL-1 beta. The nucleotide sequence of the primers (10µm) were IL-6-forward primer: 5'-ATTCAATCAGGAGACTTGCT and IL-6-reverse primer: 5'- CGGTTGGAGTGGTTACTAGA. These primers were designed based on deer nucleotide sequences present in Genbank (IL-6, accession number-AF495656). Specific PCR product for IL-6 was at 185 bp.

DETECTION OF APOPTOSIS IN LYMPH NODES AND ASSOCIATION WITH EHDV AND p53

In situ nick end-labeling (TUNEL) assay

To evaluate apoptosis in lymph nodes of the experimentally infected deer, DNA nick-end labeling was performed on paraffin embedded sections (3µm) from right prescapular lymph nodes from control (n=2; KS 890 and KS 962) and infected deer (n=6; KS 891, KS 888, KS 961; NJ1, NJ2 and NJ4) using an Apotag Plus In situ Apoptosis Detection kit (Chemicon international, Inc., Temecula, CA, USA) according to the instructions of the manufacturer. Briefly, the sections were heated at 63°C for 30 minutes, deparaffinized in xylene, rehydrated through a series of graded ethanols to distilled water and treated with Proteinase K (Qiagen, Valencia, CA, USA), 20ug/ml in phosphate buffer saline (PBS), for 15 minutes at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide in distilled water for 5 minutes at room temperature. Equilibration buffer (1X) was applied and incubated for 15 seconds at room temperature. The sections were incubated with TdT enzyme in reaction buffer, for 37°C in a humidified chamber for 1 hour, agitated briefly in stop buffer, and then

incubated with it for 10 minutes at room temperature. Negative control samples were incubated with water mixed with reaction buffer. Sheep anti-digoxigenin-AP (750 U/ml, Roche, Indianapolis, IN, USA) diluted 1:500 in antibody diluent (Dako) was applied to the sections and incubated in a humidified chamber for 30 minutes at room temperature. Fast Red was used as a chromogen (Dako). The sections were counterstained with hematoxylin and cover-slipped.

Immunohistochemistry for p53 and EHDV-To evaluate whether apoptosis detected above was directly related to viral replication and if p53 dependent, immunohistochemistry for p53 and EHDV was done on the same prescapular lymph nodes as used in the TUNEL assay. Sections of paraffin-embedded prescapular lymph nodes (3 μ m) were heated at 63°C for 30 minutes and then deparaffinized in xylene, and rehydrated through a series of graded ethanol baths to distilled water. Antigen retrieval for p53, was done by steaming the slides in citrate buffer (0.01M, pH 6.0) for 20 minutes and then allowed cooling for 10 minutes. Antigen retrieval for EHDV was done by treating the slides with 1X Universal decloaker (Biocare Medical) in distilled water at 120°C for 3 minutes, followed by 85°C for 10 seconds and then allowed to cool for 5 minutes. The specimens were blocked with Protein block (Dako, CA, USA) for 7 minutes at room temperature. Primary antibodies were purified mouse anti-human p53 (PharMingen, San Diego, CA, USA) diluted 1:300 in antibody diluent and rabbit anti-EHDV-2 antibody diluted 1:300 in antibody diluent. Each antibody was applied for 1 hour at room temperature. For p53 immunohistochemistry, biotinylated anti-mouse antibody (Vector, Burlingame, CA, USA), at 5 μ g/ml, was applied for 25 minutes at room temperature. The sections were quenched with 3% H₂O₂ in distilled water for 30 minutes at room temperature, followed by the avidin-biotin peroxidase complex (ABC, Vectastain kit). DAB was used as the chromogen for the peroxidase

reaction. For EHD immunohistochemistry, supersensitive biotinylated antirabbit immunoglobulins (Biogenex) was applied for 20 minutes at room temperature. The sections were then treated with alkaline phosphatase conjugated streptavidin (Biogenex) for 20 minutes at room temperature. Fast Red (Dako) was used as chromogen. The slides were counterstained with Mayers hematoxylin, dehydrated and cover-slipped. A bovine squamous cell carcinoma with p53 expression was used as positive control for p53. Bovine endothelial cells infected with EHDV-2 and harvested at 36 hours post inoculation were used as positive control for EHDV immunohistochemistry.

IN VITRO IL-1 BETA AND IL-6 PRODUCTION

Isolation and infections of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMNCs) were isolated from sodium citrate anticoagulated blood from a control WTD (n=1; KS 890) using Fico/Lite LymphoH 1.077 (Atlanta Biologicals) and approximately 2×10^6 cells were seeded per well in a 48-well plate with 1 ml RPMI-1640 media (89% RPMI-Sigma R6504; 1% 100X antibiotic/antimycotic and 10% fetal bovine serum). The plate was incubated for 48 hours at 37°C to facilitate the adherence of macrophages. After 48 hours the cells were inoculated with EHDV-2 (multiplicity of 0.02 TCID₅₀/cell). PBMNCs inoculated with bacterial lipopolysaccharide at 20 ng/ml (Sigma, St. Louis, MO, USA) were used as positive controls. Mock-infected PBMNCs from the same deer were used as negative controls. At 0 hr, 24 hr and 48 hr post inoculation, the adherent and non-adherent cells were harvested for RNA extraction. The supernatant from each well was removed and centrifuged to isolate non-adherent cells. The adherent cells were lysed directly by adding 200 µl RNA-Bee

(TEL-TEST, Inc, CS-104B). The cell pellet of non-adherent cells was resuspended with lysed adherent cells in RNA-bee and RNA was extracted for RT-PCR as per the manufacturer's instructions.

RT-PCR for IL-1 beta and IL-6

Semiquantitative RT-PCR was done using Titan One Tube RT-PCR System (Roche), to evaluate levels of IL-1 beta and IL-6 in PBMNCs peripheral blood mononuclear cells with 0.1 µg RNA, using the protocols described earlier.

Statistical analysis

The deer used for in vivo infection in our study were divided into groups: control deer (n=2) and “susceptible” (n=4) and “resistant” (n=2) EHDV infected deer. Components of the clinical disease severity scores (e.g. hematology) were analyzed by analysis of variance and significant differences were compared by t-test (Graph Pad software, San Diego, CA). ELISA and RT-PCR results from the control, “susceptible” and “resistant” EHDV infected deer were analyzed by analysis of variance and significant differences were further examined using student's t-test (Graph Pad software, San Diego, CA). $P < 0.05$ was considered to be statistically significant.

RESULTS

IN VIVO STUDY

Clinical findings and virus titers

Clinical disease severity scores in the deer from the potentially resistant population (n=2; $\mu=3$) were lower than those from potentially susceptible populations (n=4; $\mu=7.75$) (Table 5.1). One

of the “susceptible” deer (NJ4; *O. v. borealis*) died. Plasma protein concentrations in the infected deer decreased with time and were significantly lower than the controls on p.i. day 8 ($P=0.0063$), but the decrease was not significantly different between the “susceptible” and “resistant” deer ($P=0.7641$). Peak prolongation of APTT in infected deer was on p.i.day 8 (excluding the deer that died on p.i.day 7) at which time the APTT of “susceptible” deer was significantly prolonged over the control deer ($P=0.0135$). Platelet counts were decreased in all infected deer as compared to the controls, but never fell below $100 \times 10^3/\mu\text{l}$. All the “susceptible” deer except for one became severely lymphopenic ($<1000 \text{ cells}/\mu\text{l}$) beginning as early as p.i.day 2. The “susceptible” deer with the lowest lymphocyte count on p.i.day 6 died on p.i.day 7. Only one of the “resistant” deer was ever lymphopenic ($<1000 \text{ cells}/\mu\text{l}$) and this occurred on p.i.day 8.

At necropsy, lesions were severe in the “susceptible” deer but insignificant in the “resistant” deer. The “susceptible” deer had edema of the head, neck and lungs and multifocal hemorrhage and congestion was observed in the heart muscle, ruminal papillae, and serosa of abomasum and intestines. Multifocal ulceration, petechial hemorrhage and congestion accompanied by rare infiltrates of lymphocytes and few macrophages, were observed in the oral mucosa of the “susceptible” deer microscopically. Multifocal hemorrhage in the mucosa of rumen, abomasum, and intestines was also observed histologically in the “susceptible” deer. Negligible to minimal infiltrates of lymphocytes were observed microscopically in the oral mucosa of the “resistant” deer.

Deer became viremic by p.i.day 4 (fig. 5.1.). Blood viral titers were highest in “susceptible” and “resistant” deer on p.i.day 6, but peak titers were higher in “susceptible” than in “resistant” deer. Virus was first detected in buccal mucosa and skin on p.i.day 4 (fig. 5.1 B and C) and

titers were relatively low compared to blood and higher (fig 5.1 A) in “susceptible” deer than in “resistant” deer. Virus was isolated from feces of only one deer (KS 891) on one day (p.i.day 6). At necropsy, highest mean viral titers were observed in the spleen followed by the lung of “susceptible” deer. In general, higher viral titers were observed in the tissue samples of “susceptible” deer as compared to the “resistant” deer and deer with high CDSS had higher blood and tissue viral titers than those with low CDSS scores.

ELISA for IL-1 beta

The mean level of circulating IL-1 beta was higher in all infected deer on p.i.day 4 as compared to the uninfected deer (fig. 5.2) although not out of the reference range (1.15-4.44 pg/ml) established in the control deer. The trend of increased plasma IL-1 was more marked when deer were separated into “resistant” (n= 2 *O.v.t.* both with CDSS 3) and “susceptible” deer (*O.v.b.* and *O.v.m.* with CDSS 7 or 8) groups. “Susceptible” deer had greater mean IL-1 levels ($\mu=4.652 \pm 2.323$) than the resistant deer ($\mu=2.532 \pm 1.349$) and controls ($\mu=2.001 \pm 1.201$) on p.i.day 4. Mean IL-1 levels in “susceptible” deer decreased after p.i. day 4 whereas levels in “resistant” deer increased throughout the infection.

Immunohistochemistry for IL-1 beta

Intense, positive cytoplasmic staining was observed in the macrophages and lymphocytes in right and left prescapular lymph nodes of infected deer (Fig. 5.3). About 40% of the lymphocytes and macrophages, mostly in the sinuses and few in the follicles, of right prescapular lymph node and 20 % in the left node of infected “susceptible” deer had positive staining. About 15% cells in right prescapular lymph node and 10% in the left node of “resistant” deer expressed IL-1 beta.

Only about 5% lymphocytes and macrophages stained positively in right or left prescapular lymph nodes of the control deer. Infrequently, macrophages in the spleen and mucosa of small intestines in the infected deer and control had positive staining. No staining was observed in the lung, buccal mucosa, abomasum or rumen of any treatment group.

RT-PCR for IL-1 beta and IL-6

In right prescapular lymph node, mRNA expression of IL-1 was higher in the infected deer (mean=0.6099 standard deviation=0.1888) as compared to the control deer ($\mu=0.4785 \pm 0.0205$), but this was not statistically significant ($P=0.3870$). Likewise, IL-1mRNA expression in “susceptible” deer ($\mu=0.6464 \pm 0.2326$) was higher than “resistant” ($\mu=0.537 \pm 0.0056$) or control deer ($\mu=0.4785 \pm 0.0205$). Expression of IL-1 mRNA was especially high in two deer (KS 891 and NJ1) from the “susceptible” group.

The average relative intensities for IL-6 mRNA were higher in the infected deer ($\mu=0.524 \pm 0.2363$) as compared to the uninfected deer ($\mu=0.426 \pm 0.0749$) (fig. 5.4 A), and was significant ($P=0.0185$). Expression of IL-6 mRNA was greater in “susceptible” infected deer than in the “resistant” infected ($P<0.0001$) and control deer ($P=0.0058$) (Fig. 5.4 B). No significant difference of IL-6 mRNA expression was observed in the “resistant” deer as compared to the control deer ($P=0.3367$).

IL-1 and IL-6 gene expression was compared on right (draining site of inoculation) and left (not draining site of inoculation) prescapular lymph nodes from one control and one “susceptible” infected WTD. Gene expression of IL-1 and IL-6 appeared greater in the infected deer, as determined by the intensity of bands, in the right prescapular lymph node as compared to

the left prescapular lymph node (data not shown). Expression of the IL-1 and IL-6 genes appeared to be the same in right and left prescapular lymph nodes of the control deer.

Very little expression of IL-1 and IL-6 mRNA was present in buccal mucosa from both control and infected deer on p.i. day 0 and there was no change in expression following infection.

TUNEL assay, p53 and EHDV immunohistochemistry

By TUNEL, prescapular lymph nodes of infected deer had positive nuclear staining of about 25-30% macrophages and lymphocytes as compared to 2% of the cells in the control deer (Fig. 5.5 A). The number of positive cells was approximately the same in right and left prescapular lymph nodes of infected deer; in “resistant” and “susceptible” deer; in deer with high or low viral titers (in lymph node); and in deer with mild or marked lymphopenia. Nuclear staining for p53 was not observed in cells in lymph nodes from either control or infected deer. Cytoplasmic staining for p53 was observed in a few cells (macrophages) in both control (Fig. 5.5 B) and infected deer. Positive staining for EHDV was not observed in lymph nodes from either control or infected deer.

IN VITRO IL-1 BETA AND IL-6 PRODUCTION

RT-PCR for IL-1 and IL-6

Upregulation of both IL-1 and IL-6 mRNA was detected in PBMNCs inoculated with EHDV-2 at 24 hours p.i. and expression of both was also greater than controls at 48 hours p.i. (Fig. 5.6). PBMNCs inoculated with lipopolysaccharide (LPS) used as a positive control had detectable

upregulation of IL-1 but not IL-6 at 24 hours p.i. and decreased levels as compared to the controls at 48 hours p.i. (probably due to cell destruction by LPS).

DISCUSSION

Combined roles of IL-1 and IL-6 have been investigated in disease processes as indicators of outcome or as linked to severity of clinical signs in several viral infections. Results of our *in vivo* studies indicate that IL-6 and IL-1 beta may play a similar role. In our study, upregulation of IL-1 and IL-6 was greater in deer with high CDSS suggesting that these cytokines may contribute to the severity of clinical disease. IL-1 and IL-6 in tissue and circulation of infected WTD may also act as an indicator of outcome of EHD with increased levels signifying a poorer prognosis.

The deer used in this study were from 3 different populations and potentially different subspecies and could potentially be similar to the “resistant” and “susceptible” deer studied by Gaydos et al. (2002). Indeed, the results of our *in vivo* infection suggested that the severity of disease was greater in deer from the suspected “susceptible” subspecies as compared to the suspected “resistant” subspecies. Though a similar viral dose was given to all the infected deer, in general, higher viral titers were observed in blood and the tissue samples from the suspected “susceptible” deer as compared to the “resistant” deer suggesting that decreased (“resistant” deer) or increased (“susceptible” deer) viral replication may contribute to the variation in severity of disease associated with infection in these two subspecies.

In the current study, the circulatory levels of IL-1 in infected deer appeared to peak at p.i.day 4 in infected deer and were higher in the “susceptible” deer which had greater CDSS, but

statistical differences were not observed. It is possible that the IL-1 ELISA test we used was not very sensitive for deer IL-1 leading to our statistically insignificant differences. However, IL-1 shows remarkable conservation between diverse species (O'Neill and Greene, 1998), suggesting that the antibodies used in the kit would most likely cross-react with deer IL-1

Immunohistochemistry for IL-1 beta indicated that up-regulation of protein expression was primarily limited to lymph nodes and was greater in lymph nodes draining the site of inoculation than elsewhere in the body. Despite high viral titers in lymph nodes, no cells stained positive for EHDV by IHC suggesting that the upregulation of IL-1 may not be directly related to virus replication in these cytokine expressing cells. Although virus titers were high in other tissues examined by IHC, there was no indication that virus replication induced IL-1 production in these tissues. Thus, while local tissue production of IL-1 does not appear to play a role in lesion development, circulatory IL-1 from lymph node possibly could. There was greater protein expression in the lymph nodes of “susceptible” infected deer as compared to the “resistant” deer suggesting that IL-1 could play a role in disease severity.

Transcription of genes encoding for IL-1 and IL-6 was up-regulated in lymph nodes and, like protein expression mRNA expression of IL-1, was greater on the side of inoculation. Thus, at least for IL-1, these results corroborate the immunohistochemical findings. There was no apparent upregulation of transcription of genes encoding IL-1 and IL-6 in the buccal mucosa, a common tissue with lesions, during infection. Absence of IL-1 and IL-6 mRNA upregulation in buccal mucosa even in the presence of virus in the tissue indicates that upregulation of IL-1 and IL-6 at the tissue level is not totally dependent on viral replication. It is possible that the presence of lesser number of monocytes and lymphocytes in buccal mucosa as compared to the lymph node, could also have been a reason for lower levels of IL-1 and IL-6 in buccal mucosa,

but other cells can produce these cytokines. Upregulation of IL-1 and IL-6 mRNA was greatest in the deer of “susceptible” subspecies suggesting that levels of IL-1 and IL-6 may play some role in the variability of expression of EHD in the deer from these different subspecies.

Lymphopenia as seen in the deer of this study is a common finding in deer with EHD (Quist et al., 1997), though the mechanism of lymphopenia has not been elucidated. In our study, TUNEL assay and immunohistochemistry for EHDV were done to correlate apoptosis seen in the lymphocytes and macrophages of infected deer with viral-replication and the lymphopenia observed in the infected deer. The percentage of apoptotic cells was much higher in the lymph nodes from infected deer as compared to the control deer suggesting that apoptosis could be the mechanism of lymphopenia in EHD. The inability to detect virus in nodes by IHC and the presence of similar numbers of apoptotic cells in animals with high or low nodal viral titers suggests that apoptosis in lymph nodes is related to factors other than virus replication. No correlation of number of apoptotic cells was seen with nodal viral titer or degree of lymphopenia in the infected deer. The apoptotic pathway employed in the pathology of EHD is probably p53 independent as there was no detectable p53 expression by IHC.

Viral infection of monocytes has been known to trigger cytokine production, which in turn contributes to the pathology associated with the viral disease. A hemorrhagic disease in humans caused by Marburg and Ebola viruses, which is quite similar to EHD in WTD, results in the activation of monocytes, triggering the release of IL-1 beta and IL-6 and contributing to the clinical signs like coagulation disorders and variable degrees of hemorrhage (Ströher et al., 2001). In virulent classical swine fever, infection of monocytes triggers the release of IL-1 coinciding with the onset of fever and coagulation disorders (Knoetig et al., 1999). The results of our semiquantitative RT-PCR on infected PBMCs suggest that EHDV infection of

mononuclear cells could have triggered the release of IL-6 and possibly IL-1 beta.

Consequently, the release of IL-1 and IL-6 from mononuclear cells, after EHDV infection, could contribute to the severity of hemorrhagic disturbances associated with EHD in WTD.

The results of our in vivo and in vitro studies indicate that IL-6 and IL-1 may play a role in the pathology associated with epizootic hemorrhagic disease in WTD and could be used as indicators of severity or outcome of the disease. However, the number of animals used in this study was small, and future studies with more deer from potentially “susceptible” and “resistant” populations are needed to clarify the role of IL-1 and IL-6 in the pathogenesis of EHD in WTD, especially with regards to variation in expression of the disease.

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Figure legends.

Fig. 5.1. Mean virus titers for “susceptible” deer, “resistant” deer and over all range of viral titers in blood (fig. 5.1 A), buccal mucosa (fig. 5.1 B) and skin (fig. 5.1 C) samples done on day 2, 4, 6 and 8 post inoculation.

Fig. 5.2. ELISA for plasma levels of IL-1 on control and EHDV-2 infected white-tailed deer. **(A)** Average concentration of IL-1 beta (pg/ml) in control (n=2) and all infected deer (n=6) over time in the experiment. **(B)** Average concentration of IL-1 beta (pg/ml) in control (n=2) and EHDV-2 infected WTD of “susceptible” (n=4) and “resistant” (n=2) populations. **(C)** Immunohistochemistry for IL-1 on a lymph node from a WTD experimentally infected with EHDV-2 using secondary antibody from IL-1 beta ELISA kit stained cells similar to those stained using ovine anti-IL-1 beta confirm that the kit could detect deer IL-1 beta. Magnification x40.

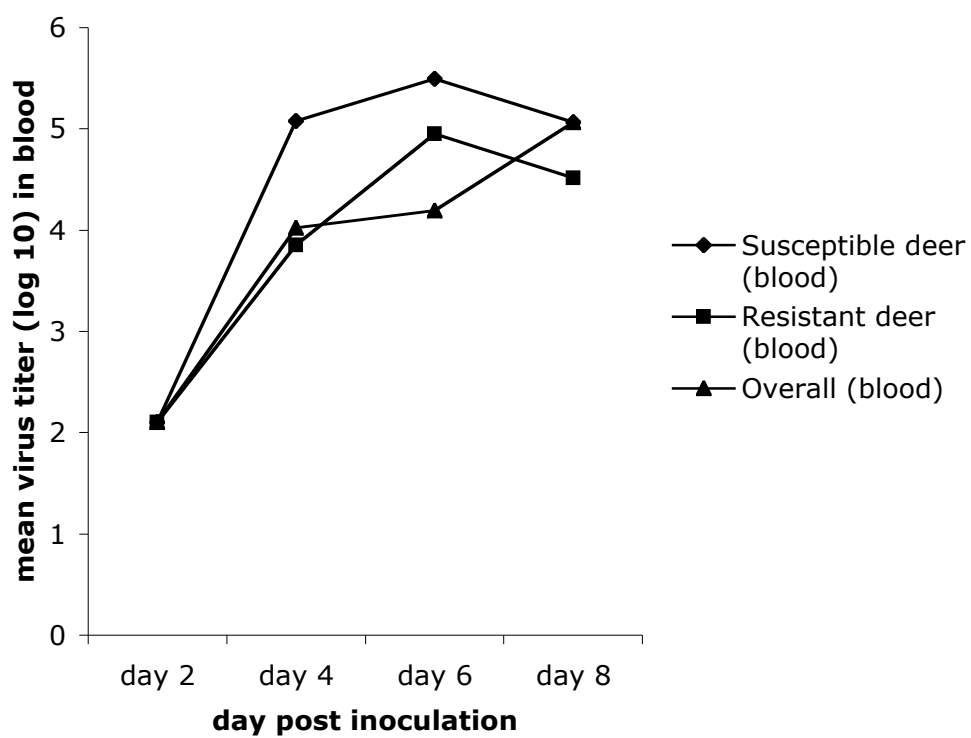
Fig. 5.3. Immunohistochemistry for IL-1 beta done on right prescapular lymph node from an EHDV-2-infected white-tailed deer, sampled at necropsy (day 8, p.i.). Magnification x40. Arrows point to few macrophages and lymphocytes expressing IL-1 beta protein.

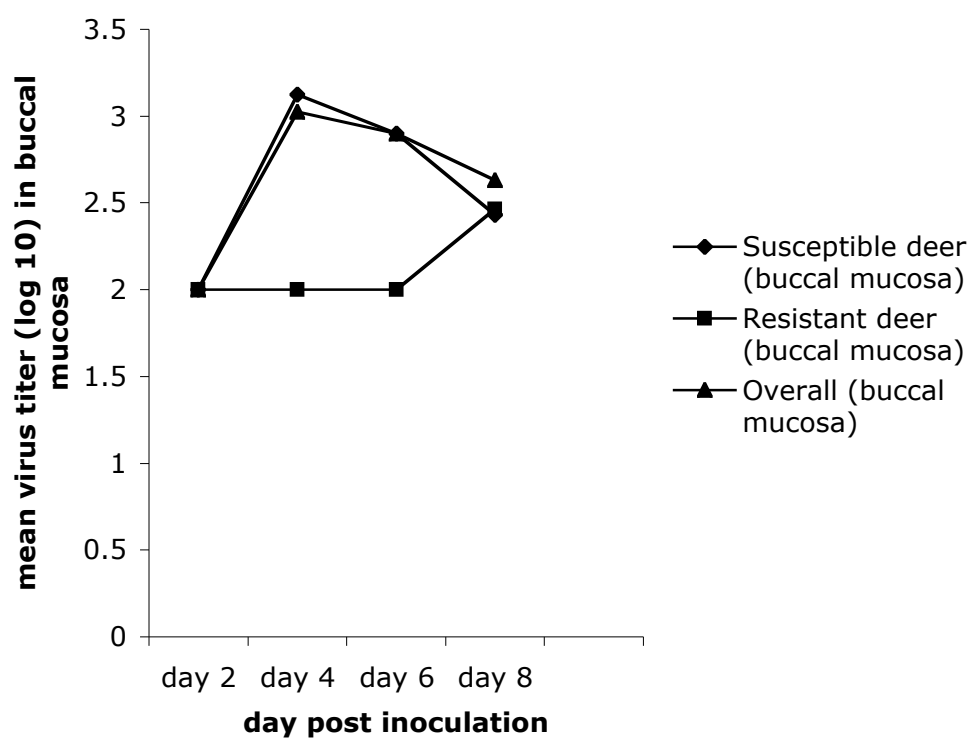
Fig. 5.4. Semiquantitative RT-PCR for IL-1 beta and IL-6 done on right prescapular lymph nodes taken at necropsy from mock-infected and EHDV-2-infected white-tailed deer **(A)** Relative intensities for IL-1 and IL-6 expression in control and EHDV-2-infected WTD (control deer n=2; infected deer n=6); and **(B)** control and EHDV-2-infected deer from potential resistant and susceptible populations (control deer n=2; resistant deer n=2; susceptible deer n=4).

Fig. 5.5. (A) TUNEL assay done on right prescapular lymph node of EHDV-2-infected white-tailed deer, sampled at necropsy (day 8, p.i.). Magnification of x 40. Arrows point to few TUNEL-positive cells or to nuclei that contain condensed chromatin. **(B)** Immunohistochemistry for p53 done on right prescapular lymph node of mock-infected white-tailed deer, sampled at necropsy (day 8, p.i.). Magnification x40. Arrows point to cells expressing p53 protein.

Fig. 5.6. (A) RT-PCR for IL-1 beta and IL-6, done on peripheral blood mononuclear cells isolated from a white-tailed deer in an in vitro study. Agarose gel electrophoresis of PCR products showing beta-actin and corresponding IL-6 and IL-1 beta production. Lane1: PCR marker; lane 2: water control; lane 3: 0 hour, p.i.; lane 4: negative control cells (mock-inoculated), 24 hours, p.i.; lane 5: negative control cells, 48 hours, p.i.; lane 6: LPS inoculated cells (positive control), 24 hours, p.i.; lane 7: LPS inoculated cells (positive control), 48 hours, p.i.; lane 8: EHDV-2 inoculated cells, 24 hours, p.i.; lane 9: EHDV-2 inoculated cells, 48 hours, p.i. Sizes of specific PCR products for beta actin, IL-1 beta and IL-6 are 212 bp, 181 bp and 185 bp respectively. Arrows point to some specific bands for beta-actin/ IL-6 and IL-1 beta. **(B)** Relative intensities for IL-1 and IL-6 expression (deer n=1).

Figure 5.1. (A)





(B)

(C)

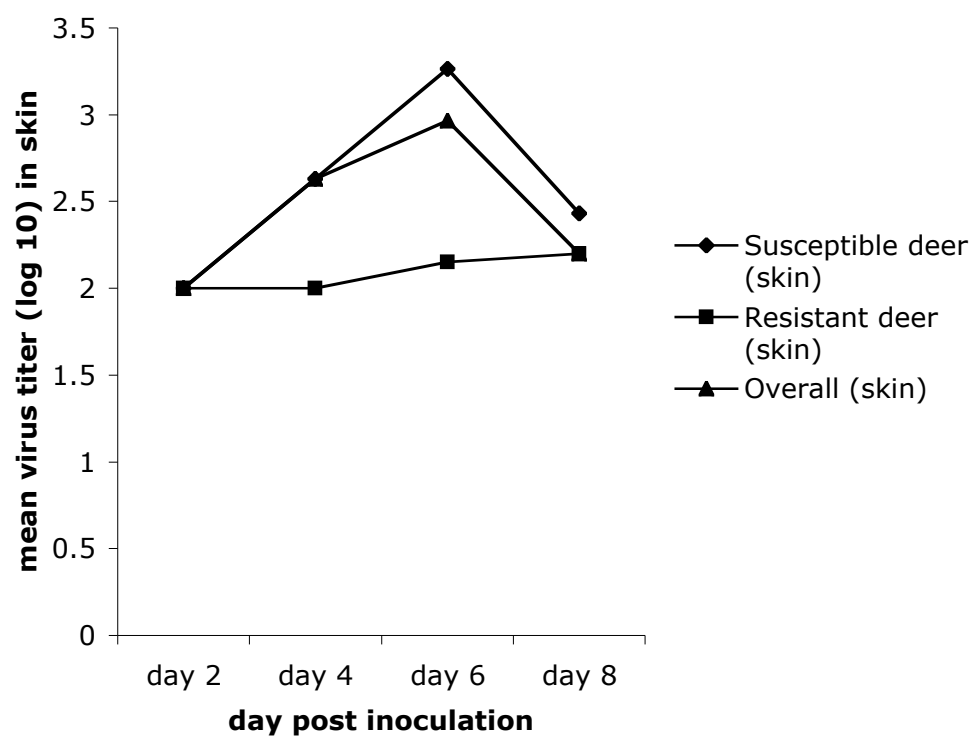


Figure 5.2.

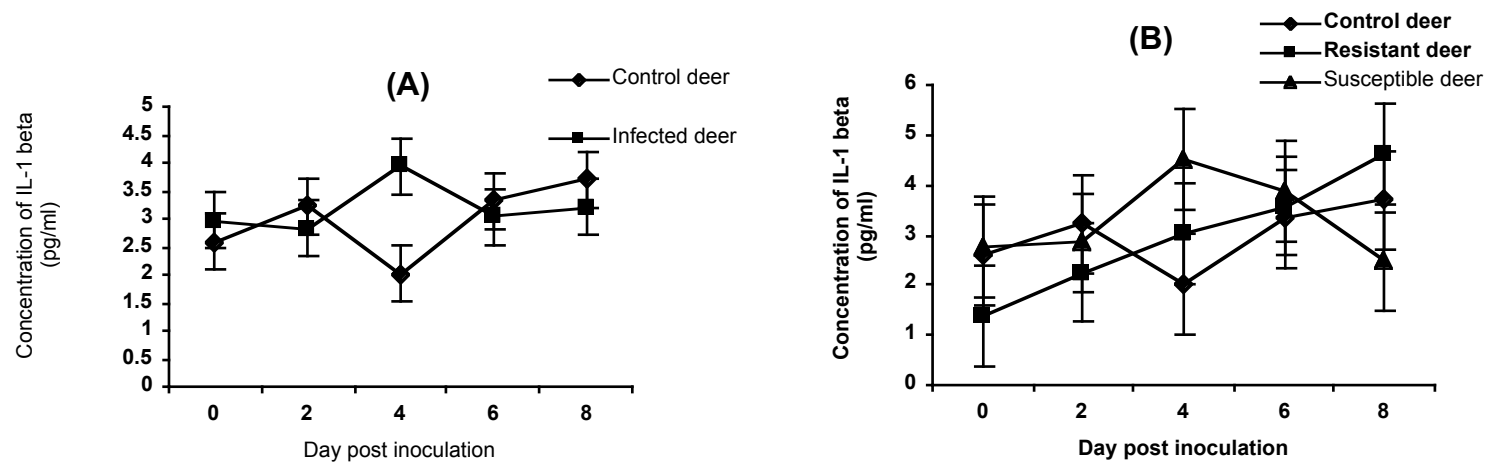


Figure 5.3.

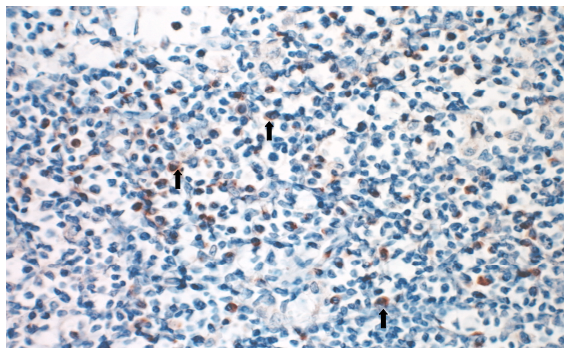


Figure 5.4.

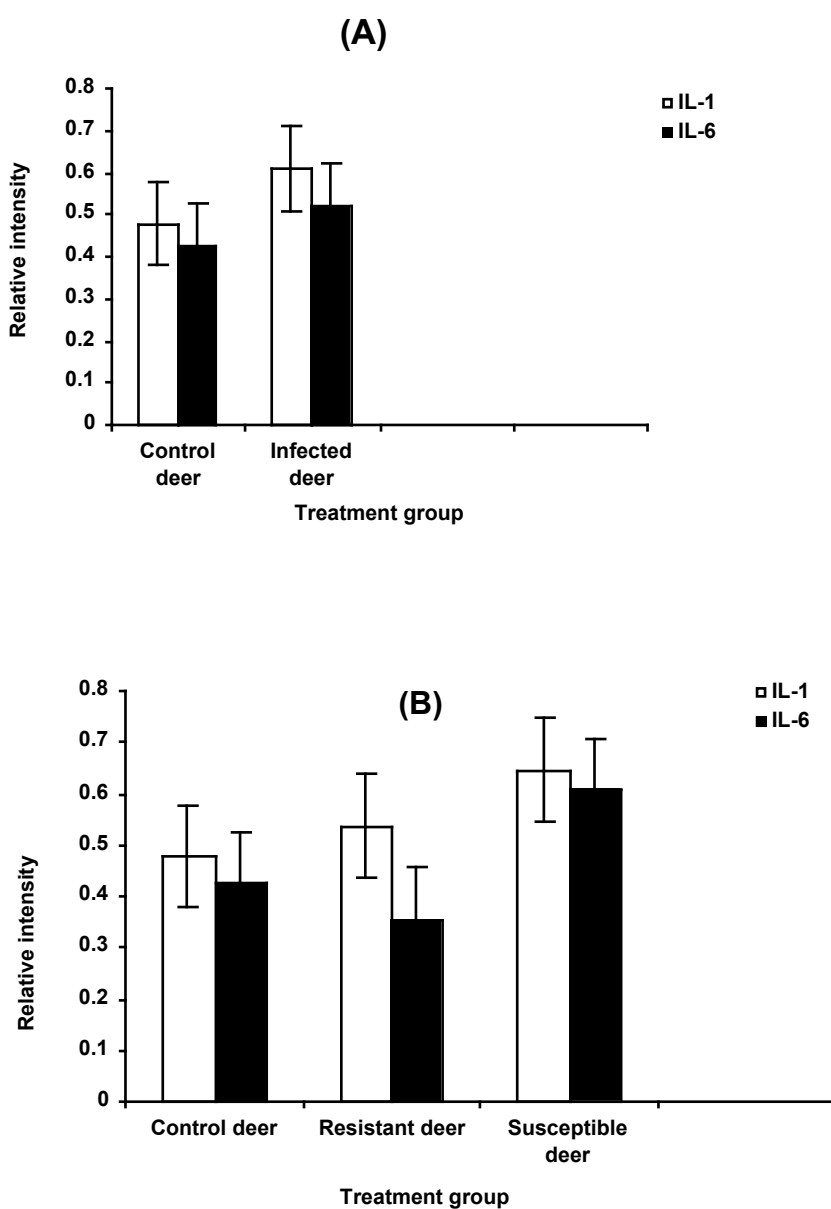


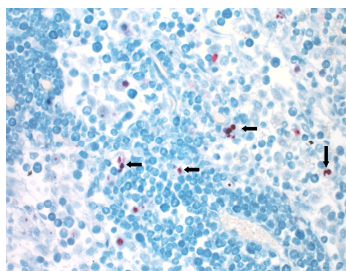
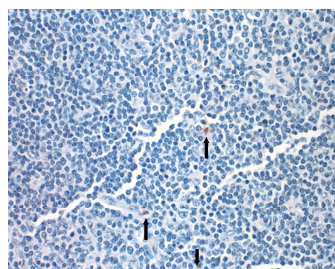
Figure 5.5. (A)**(B)**

Figure 5.6.

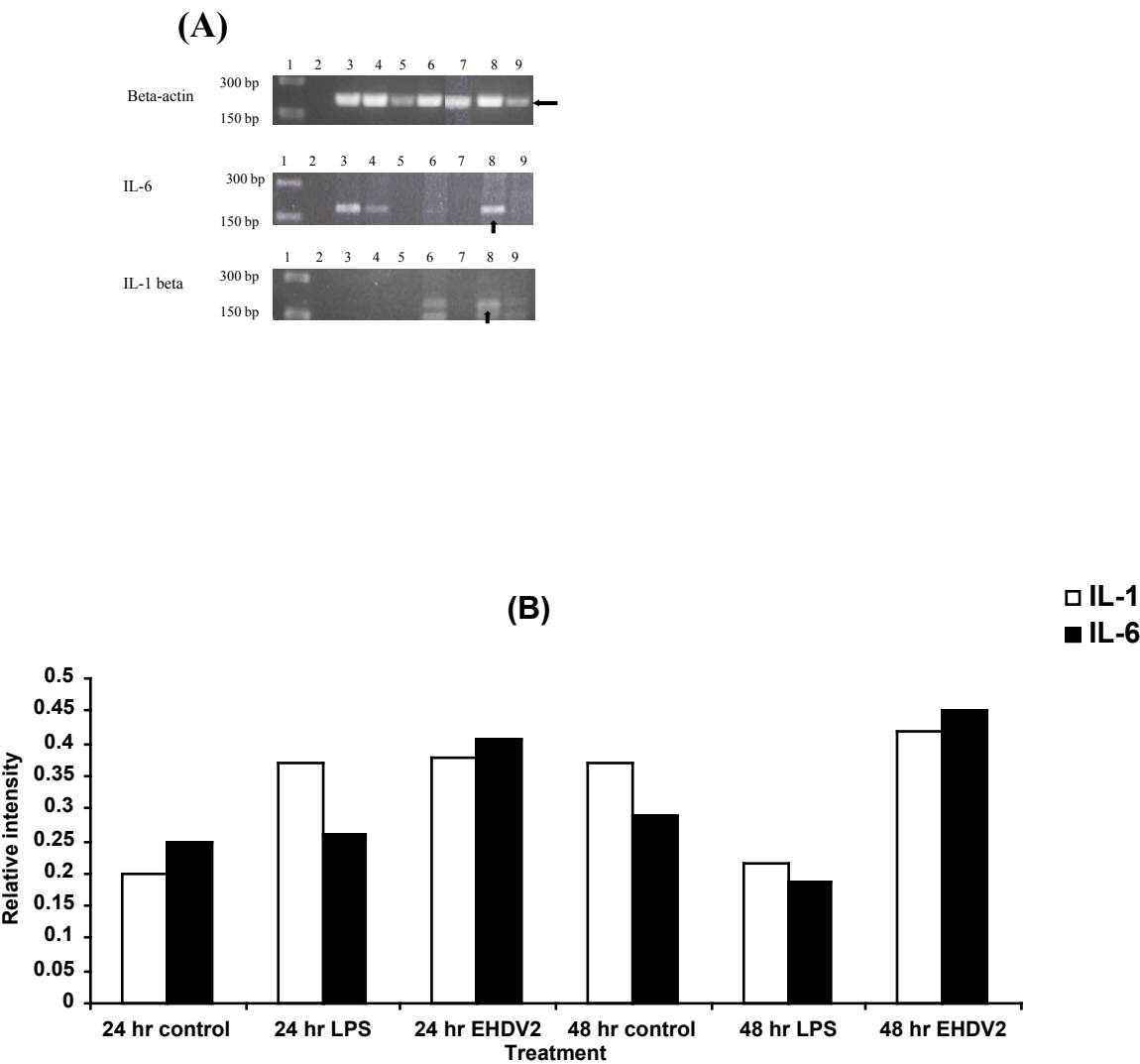


Table 5.1. Clinical disease severity scores for fawns infected with EHDV-2.

	KS 891¹	NJ 1¹	NJ 2¹	NJ 4¹	KS 888²	KS 961²	KS 890³	KS96 2³
depression	1	1	1	1	0	0	0	0
erythrema	1	1	1	1	0	0	0	0
salivation	1	1	1	1	0	0	0	0
decreased plasma protein	1	1	1	1	1	1	0	0
fever	1	1	0	1	0	1	0	0
Prolonged APTT	1	1	1	1	0	0	0	0
decreased HCT	0	0	1	0	1	1	0	0
decreased platelets	1	0	1	1	0	0	0	0
decreased lymphocytes	1	1	1	1	1	0	0	0
total score	8	7	8	8	3	3	0	0

1= “Susceptible” deer from New Jersey (NJ1, NJ2 and NJ4) and eastern Kansas (KS891)

2= “Resistant” deer from Western Kansas

3= Control deer (“susceptible” deer from eastern Kansas-KS 890, KS 962)

Table 5.2. Virus titers (log 10) for fawns at necropsy (day 7 or 8 p.i.).

<u>Tissue</u>	Susceptible deer range (Geometric mean) n=4	Resistant deer range (Geometric mean) n=2
Prescapular lymph node (right)	3.6-4.26 (3.93)	<2.2-2.6 (1.3)
Prescapular lymph node (left)	2.6-3.93 (3.265)	2.6-4.1 (3.35)
Prefemoral lymph node (right)	3.6-3.93 (3.765)	2.93-3.2 (3.065)
Prefemoral lymph node (left)	3.26-4.6 (3.93)	<2.2-2.26 (1.13)
Heart	2.26-4.1(3.18)	2.26-3.26 (2.76)
Lung	3.93-4.26 (4.095)	2.26-3.6 (2.93)
Ileum	3.1-4.6 (3.85)	2.26-2.6 (2.43)
Cerebrum	3.26-4.26 (3.76)	<2.2.-2.6 (1.3)
Mid brain	3.1-4.1 (3.6)	<2.2-2.93 (1.465)
Cerebellum	3.1-4.26 (3.68)	<2.2 (0)
Liver	3.1-4.26 (3.68)	2.6-3.1 (2.85)
Rumen	2.26-3.26 (2.76)	<2.2-3.1 (1.55)
Colon	2.6-4.1 (3.35)	2.6 (2.6)
Abomasum	2.26-3.93 (3.095)	2.26 (2.26)
Spleen	4.1-4.26 (4.18)	3.26-4.26 (3.76)

CHAPTER 6: CONCLUSIONS

Epizootic hemorrhagic disease caused by epizootic hemorrhagic disease (EHD) viruses is an important disease of white-tailed deer (WTD) (*Odocoileus virginianus*). EHD viruses cause disease in both domestic and wild ruminants, particularly WTD, that is associated with viral replication in endothelial cells and subsequent endothelial damage (Howerth *et al.*, 1988). For poorly understood reasons, clinical disease is variable both within and between species but factors other than direct viral damage are thought to be involved. We postulated that the production of inflammatory mediators released from infected monocytes and endothelium may contribute to expression of disease. We were particularly interested in the triad of cytokines TNF, IL-1, and IL-6 that are known to be involved in the pathogenesis of a variety of diseases or are, at least, markers of disease severity.

Apoptosis is an important method of cell death induced by many viruses, but the manner by which it is induced is variable among viruses. Some viruses induce apoptosis by a p53 dependent mechanism, e.g. bovine herpes virus 1 (Devireddy & Jones, 1999), while others activate apoptosis in a p53 independent system, e.g. chicken anemia virus (Danen-van Oorschot *et al.*, 2000). We postulated that cell death in EHDV infected endothelial cells is due to apoptosis and dependent on p53 expression.

Through this study we contributed to an understanding of the regulation of TNF- α , IL-1 β and IL-6 in EHD and their role in disease in deer of potentially “resistant” and “susceptible” populations. We also evaluated the mechanism of EHD virus-induced apoptosis. The work presented in this dissertation is summarized in the following paragraphs.

Chapter 3: Tumor necrosis factor alpha expression in white-tailed deer infected with epizootic hemorrhagic disease viruses.

This study characterized the expression of tumor necrosis factor-alpha (TNF- α), an important inflammatory mediator in a wide variety of diseases, in deer and their monocytes when infected with EHD virus (EHDV). Circulating levels of TNF- α were evaluated in deer experimentally infected with EHDV using a cytotoxicity assay, tissue expression of TNF- α in deer naturally and experimentally infected with EHDV was evaluated via immunohistochemistry, and upregulation of TNF messenger RNA in tissues from deer experimentally infected with EHDV and deer mononuclear cells infected with EHDV in vitro were assessed by semi-quantitative RT-PCR. Circulating levels of TNF were not significantly increased, TNF was not detected in tissues during EHDV infection, and EHDV failed to upregulate TNF mRNA in either EHDV infected WTD or their peripheral blood mononuclear cells. Overall, this study showed that it is unlikely that TNF- α plays a significant role in the pathogenesis of EHDV infection in WTD.

Chapter 4: Epizootic hemorrhagic disease virus induced apoptosis in bovine and deer endothelial cells.

This study examined whether EHD viruses can induce endothelial cell death by apoptosis and, if so, whether it is related to virus replication and is p53 associated. Using in situ nick end-labeling (TUNEL), caspase-3 (cleaved) immunohistochemistry, flow cytometry and annexin staining, and agarose electrophoresis of DNA we documented apoptosis as a mechanism of endothelial cell death in EHD virus infected bovine carotid artery endothelial cells and that apoptosis appeared to be directly related to viral replication. Assessment of p53 gene regulation and protein expression in infected endothelial cells suggested that apoptosis was p53

independent. This study adds EHDV to the growing list of viruses that can induce apoptosis. The significance of EHDV induced apoptosis is unknown, but we demonstrated TUNEL staining in endothelium (the target cell of EHDV) of a WTD with EHDV-2 infection suggesting that apoptosis may play a role in the development of the endothelial damage typical of this disease.

Chapter 5: Expression of interleukin-1 beta and interleukin-6 in white-tailed deer infected with epizootic hemorrhagic disease virus.

In this study, we postulated that IL-1 beta and IL-6 could be involved in the pathogenesis of EHD in WTD. To determine if circulating and/or tissue levels of IL-1 and IL-6 are upregulated in EHDV infection, WTD from potentially “susceptible” and “resistant” populations were infected with EHDV-2. An enzyme linked immunosorbent assay (ELISA) was used to assess the circulatory levels of IL-1 beta and reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were used to evaluate the upregulation of IL-1 beta in tissues. Upregulation of IL-6 was determined by RT-PCR. To determine if IL-1 and IL-6 are upregulated in monocytes infected with EHDV, peripheral blood mononuclear cells (PBMNCs) from WTD were inoculated with EHDV-2 in vitro and upregulation was assessed by RT-PCR.

Results of ELISA, RT-PCR and immunohistochemistry for IL-1 suggest that the circulatory and certain tissue levels of IL-1 beta were upregulated in EHDV infected deer. RT-PCR analysis showed a noticeable upregulation of IL-6 in the lymph nodes of infected “susceptible” deer. Noticeable upregulation of IL-6 and mild upregulation of IL-1 was also observed in the infected blood mononuclear cells, in vitro, at 24 and 48 hours post inoculation, as determined by RT-PCR, suggesting that EHDV infection of mononuclear cells could be the source of these cytokines in vivo. These observations suggest that IL-6 and probably IL-1 may be involved in

the pathogenesis of EHD in WTD or may act as markers of severity or clinical outcome of the disease.

Our in vivo studies also showed a variation in clinical severity of EHD in the deer from “resistant” and “susceptible” subspecies similar to the results of the study done by Gaydos et al., (2002) where EHDV infected *O. v. borealis* (“susceptible” deer) developed much more severe clinical disease as compared to *O. v. texanus* (“resistant” deer). Unlike the study by Gaydos et al. (2002), in this study, blood and tissue viral titers were in general higher in the “susceptible” deer as compared to the “resistant” deer and virus was rarely detected in feces.

The syndrome of hemorrhagic disease in white-tailed deer is complex and multifactorial, and no single clinical or laboratory parameter has been identified that is a reliable indicator of eventual outcome of infection. These studies have expanded the existing knowledge of the response of white-tailed deer to EHDV infection. They indicate that IL-6 and possibly IL-1 beta, but not TNF-alpha, may play a role in the pathogenesis of EHD in WTD; however, future studies are needed to more clearly define their role. They also suggest that p53-independent apoptosis is the mechanism of endothelial cell death in EHDV infection; however, its exact mechanism, proteins involved, and biological significance remain unknown.

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APPENDIX:
DATA FROM THE MANUSCRIPT
EXPRESSION OF INTERLEUKIN-1 BETA AND INTERLEUKIN-6 IN WHITE-TAILED
DEER INFECTED WITH EPIZOOTIC HEMORRHAGIC DISEASE VIRUS ¹

¹ Sharma P., Stallknecht D.E. and Howerth E.W. To be submitted to the Journal of Wildlife Diseases.

Figure 1. Clinical disease severity scores for fawns infected with EHDV-2.

EHDV-2 Clinical disease severity score

	KS 891	NJ 1	NJ 2	NJ 4	KS 888	KS 961	KS 890	KS962
depression	1	1	1	1	0	0	0	0
erythrema	1	1	1	1	0	0	0	0
salivation	1	1	1	1	0	0	0	0
decreased plasma protein	1	1	1	1	1	1	0	0
fever	1	1	0	1	0	1	0	0
increased APTT	1	1	1	1	0	0	0	0
decreased HCT	0	0	1	0	1	1	0	0
decreased platelets	1	0	1	1	0	0	0	0
decreased lymphocytes	1	1	1	1	1	0	0	0
total score	8	7	8	8	3	3	0	0

Figure 2. Virus titers (log 10) in blood for fawns during EHDV-2 infection.

EHDV-2 virus titers (log 10) in blood

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	0	Positive (not recorded)	6.05	6.39	ND	7.2
NJ 1	0	2.2	5.6	6.1	ND	6.6
NJ 2	0	<2.2	4.1	5.6	ND	6.93
NJ 4	0	<2.2	4.3	4.6	2.93	X
KS 888	0	2.2	4.6	5.6	ND	5.1
KS 961	0	<2.2	3.1	4.3	ND	3.93
KS 890	0	<2.2	<2.2	<2.2	ND	ND
KS 962	0	<2.2	<2.2	<2.2	ND	ND

ND = Not Done

X = Value not available because the animal died on day 7

Titration of purified virus:

In BHK cells: 4.33/25ul

In CPAE cells: 4.57/25ul

Figure 3. EHDV-2 virus titers (log 10) in buccal papillae

EHDV-2 virus titers (log 10) in buccal papillae

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	ND	<2.2	4.05	3.8	ND	3.26
NJ 1	0	<2.2	2.6	2.6	ND	2.93
NJ 2	0	<2.2	2.2	2.2	ND	3.26
NJ 4	0	<2.2	3.1	<2.2	2.26	X
KS 888	0	<2.2	<2.2	<2.2	ND	2.93
KS 961	0	<2.2	<2.2	<2.2	ND	<2.2
KS 890	0	<2.2	<2.2	<2.2	ND	ND
KS 962	0	<2.2	<2.2	<2.2	ND	ND

ND = Not Done

X = Value not available because the animal died on day 7

Figure 4. EHDV-2 virus titers (log 10) in feces

EHDV-2 virus titers (log 10) in feces

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	0	<2.2	<2.2	(Positive) 2.2	ND	<2.2
NJ 1	0	<2.2	<2.2	<2.2	ND	<2.2
NJ 2	0	<2.2	<2.2	<2.2	ND	<2.2
NJ 4	0	<2.2	<2.2	<2.2	<2.2	X
KS 888	0	<2.2	<2.2	<2.2	ND	<2.2
KS 961	0	<2.2	<2.2	<2.2	ND	<2.2
KS 890	0	<2.2	<2.2	<2.2	ND	ND
KS 962	0	<2.2	<2.2	<2.2	ND	ND

ND = Not Done

X = Value not available because the animal died on day 7

Figure 5. Rectal temperature of fawns during the EHDV-2 infection.

Rectal temperature of fawns during the EHDV-2 infection (°F)

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	103.2	102.7	103.8	104.2	ND	104.7
NJ 1	103.2	102.2	103.4	104.8	ND	101.9
NJ 2	103.2	102.8	100.5	99.9	ND	102.1
NJ 4	99	102.5	104	101.6	ND	X
KS 888	102.8	102.7	102.4	103.5	ND	103.2
KS 961	102.3	101.9	102.2	104.4	ND	104.3
KS 890	103.2	102.9	102.3	102.1	ND	102.9
KS 962	103	103.8	102.9	102.9	ND	102.9

ND = Not Done

X = Value not available because the animal died on day 7

Figure 6. Mean Activated Partial Thromboplastin Times (APTT) as measured for fawns during EHDV-2 during infection.

EHDV-2 Mean APTT times (seconds)

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	21.1	20.5	17.1	25.9	ND	36.9
NJ 1	23.4	21.5	23.9	29.7	ND	34
NJ 2	26	30.8	28.3	29.2	ND	40.3
NJ 4	28.9	26.9	27.7	33.8	ND	X
KS 888	21	23.9	22.7	26.5	ND	28.5
KS 961	22.4	19.4	20.9	23.6	ND	23.6
KS 890	28	24.9	24.4	20.6	ND	24.8
KS 962	22.1	24.4	23.1	24.9	ND	24.7

Normal deer APTT=22.7

ND = Not Done

X = Value not available because the animal died on day 7

Figure 7. Platelet Counts as measured for fawns during EHDV-2 infection.

EHDV-2 Platelet Counts ($\times 10^3/\text{ul}$)

	D 0	D 2	D 4	D 6	D 7	D 8
<u>KS 891</u>	538	614	346	389	ND	222
NJ 1	642	963	932	826	ND	648
NJ 2	469	443	546	637	ND	483
NJ 4	666	520	599	621	ND	X
KS 888	615	642	625	733	ND	797
KS 961	901	853	781	682	ND	989
KS 890	755	742	727	701	ND	1046
KS 962	1006	1048	957	949	ND	1203

ND = Not Done

X = Value not available because the animal died on day 7

Figure 8. Hematocrits as measured for fawns during EHDV-2 infection.

EHDV-2 Hematocrit (%)

	D 0	D 2	D 4	D 6	D 7	D 8
KS 891	46	40.5	36	45	ND	47
NJ 1	35	33	32.5	36	ND	40
NJ 2	34	35.5	35	35	ND	31.5
NJ 4	32	32.5	34.5	45	ND	X
KS 888	32	31	34	32	ND	35
KS 961	35	28.7	31	31	ND	31
KS 890	36	37.2	34	35	ND	34.5
KS 962	32.5	38	34	35	ND	34

ND = Not Done

X = Value not available because the animal died on day 7

Figure 9. Lymphocyte counts as measured for fawns during EHDV-2 infection.

EHDV-2 Lymphocytes counts (x 10³/ul)

	D 0	D 2	D 4	D 6	D 7	D 8
KS 891	*	1.68	0.756	0.52	ND	0.3
NJ 1	1.68	1.938	0.84	0.792	ND	0.748
NJ 2	1.548	1.44	1.248	1.352	ND	0.638
NJ 4	2.684	1.9	0.686	0.322	ND	X
KS 888	1.056	0.72	0.49	0.378	ND	0.58
KS 961	1.368	1.612	1.462	1.1	ND	1.2
KS 890	1.764	1.496	1.232	1.276	ND	1.428
KS 962	1.786	1.11	1.12	1.24	ND	0.8

ND = Not Done

X = Value not available because the animal died on day 7

*Day 0 value not available

Figure 10. Virus titers (log 10) for skin of fawns during EHDV-2 infection.

EHDV-2 virus titers (log 10) for skin

	D 0	D 2	D 4	D 6	D 7	D 8
KS 891	ND	<2.2	3.26	3.93	ND	2.26
NJ 1	0	<2.2	2.2	2.6	ND	2.6
NJ 2	0	<2.2	<2.2	2.6	ND	2.26
NJ 4	0	<2.2	2.9	3.1	2.26	X
KS 888	0	<2.2	<2.2	2.3	ND	2.4
KS 961	0	<2.2	<2.2	<2.2	ND	<2.2
KS 890	0	<2.2	<2.2	<2.2	ND	ND
KS 962	0	<2.2	<2.2	<2.2	ND	ND

ND = Not Done

X = Value not available because the animal died on day 7

Figure 11. Plasma protein as measured for fawns during EHDV-2 infection.

EHDV-2 Plasma Protein (g/dl)

	D 0	D 2	D 4	D 6	D 7	D 8
KS 891	5.8	5.8	5.9	5	ND	4.8
NJ 1	5.6	5.7	5.4	4.5	ND	4.3
NJ 2	5.5	5.9	6	5.2	ND	4.5
NJ 4	5.5	5.7	5.7	4.3	ND	X
KS 888	5.5	5.8	5.6	5.1	ND	4.5
KS 961	5.9	5.7	5.7	5.2	ND	5
KS 890	6	5.8	5.8	5.9	ND	5.9
KS 962	5.9	6	6	5.9	ND	5.8

ND = Not Done

X = Value not available because the animal died on day 7

Figure 12. Virus titers (log 10) for fawns at necropsy (at the end of the experiment).

EHDV-2 virus titers (log 10) at necropsy

	KS891	NJ1	NJ2	NJ4	KS888	KS961
Prescapular lymph node (right)	3.6	4.1	4.26	4.26	<2.2	2.6
Prescapular lymph node (left)	3.6	3.93	3.6	2.6	4.1	2.6
Prefemoral lymph node (right)	3.6	3.93	3.26	3.6	3.2	2.93
Prefemoral lymph node (left)	3.26	4.6	3.93	3.93	2.26	<2.2
Heart	2.26	4.1	2.26	4.1	3.26	2.26
Lung	3.93	4.26	4.26	4.1	3.6	2.26
Ileum	3.93	4.1	3.1	4.6	2.6	2.26
Cerebrum	3.26	3.93	4.26	4.1	<2.2	2.6
Mid brain	3.1	4.1	3.93	3.1	2.93	<2.2
Cerebellum	3.1	3.26	3.26	4.26	<2.2	<2.2
Liver	4.1	4.1	4.26	3.1	3.1	2.6
Rumen	>2.26	3.26	3.26	3.26	3.1	<2.2
Colon	2.6	3.26	4.1	3.26	2.6	2.6
Abomasum	2.26	3.93	3.93	3.93	2.26	2.26
Spleen	4.26	4.26	4.1	4.1	4.26	3.26
Tongue	4.1	ND	ND	ND	ND	ND

ND = Not Done