

Tumor necrosis factor-alpha expression in white-tailed deer (*Odocoileus virginianus*) infected with Epizootic haemorrhagic disease virus

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Summary

Epizootic haemorrhagic disease (EHD) is the most important infectious disease of white-tailed deer (WTD), however little is known about the role of inflammatory mediators in the pathogenesis. We characterized the expression of tumor necrosis factor-alpha (TNF- α) *ex vivo* in tissues of WTD experimentally or naturally infected with EHD virus serotype 2 and in WTD peripheral blood mononuclear cells (PBMC) infected with EHD virus serotype 2 *in vitro*. Circulating levels of TNF- α were evaluated in serum from experimentally infected deer via cytotoxicity assay. The expression of TNF- α in tissues was evaluated via immunohistochemistry (IHC) in both experimentally and naturally infected deer. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess the level of TNF- α mRNA in tissues from experimentally infected deer and WTD's PBMC. Circulating levels of TNF- α were not increased in infected animals and TNF- α was not detected in tissues of infected deer. Increased transcription of TNF- α was detected neither in infected WTD nor in the PBMC. Tumor necrosis factor-alpha may not play a significant role in the pathogenesis of EHD virus infection in WTD.

Ruolo del fattore di necrosi tumorale-alpha nel cervo dalla coda bianca (*Odocoileus virginianus*) infetto dal virus della Malattia emorragica epizootica

Parole chiave

Bluetongue,
Cervo dalla coda bianca
(*Odocoileus virginianus*),
Fattore di necrosi
tumorale-alpha,
Malattia emorragica
epizootica,
Virus della Malattia
emorragica epizootica.

Riassunto

La Malattia emorragica epizootica (EHD) è la più importante malattia infettiva del cervo dalla coda bianca (WTD) (*Odocoileus virginianus*), è tuttavia ancora poco conosciuto il ruolo giocato dai mediatori dell'infiammazione nella patogenesi. In questo studio, l'espressione del fattore di necrosi tumorale-alpha (TNF- α) è stata valutata sia *in vivo* nei tessuti sia *in vitro* nelle cellule mononucleari del sangue periferico (PBMC) di cervi infettati sperimentalmente o naturalmente dal sierotipo 2 del virus dell'EHD. I livelli ematici di TNF- α nel siero dei cervi infettati sperimentalmente sono stati misurati attraverso il saggio di citotossicità. L'espressione tissutale è stata misurata nei tessuti degli animali infettati sperimentalmente o naturalmente mediante immunistochimica (IHC). La reazione a catena della polimerasi trascrittasi inversa (RT-PCR) semi-quantitativa è stata impiegata per valutare l'espressione dell'RNA messaggero del TNF- α nei tessuti e nelle PBMC derivanti dagli animali infettati sperimentalmente. Non sono stati evidenziati aumenti di TNF- α nei tessuti e nel sangue dei cervi infetti così come non è stato rilevato un aumento dell'RNA messaggero del TNF- α nei tessuti o nelle PBMC. È probabile che la TNF- α non giochi un ruolo significativo nella patogenesi del virus EHD nei cervi dalla coda bianca.

Epizootic haemorrhagic disease (EHDV) and Bluetongue viruses (BTV) are important causes of morbidity and mortality in white-tailed deer (WTD) with variable clinical consequences, ranging from non-apparent to depression, fever, respiratory distress, and death (Nettles *et al.* 1992). However, little is known about the reasons for this variation in susceptibility. In WTD, clinical symptoms and mortality are primarily associated with virus replication in endothelial cells and subsequent microvascular damage and complications of intravascular coagulation (Howerth and Tyler 1988, Howerth *et al.* 1988, Tsai and Karstad 1973).

However, the severity of vascular damages is often out of proportion to the relatively small amount of virus detected in endothelial cells by immunohistochemistry (IHC), *in situ* hybridization or electron microscopy (Howerth and Tyler 1988). The EHDV can also replicate in endothelial cells from species relatively resistant to EHDV infection, e.g. cattle (McLaughlin *et al.* 2003). Thus, endothelial damage does not appear to correlate directly with viral replication in the endothelium and other factors, such as inflammatory and vasoactive mediators, may be involved. Yet, viral replication can probably potentiate endothelial damage or contribute to manifestations of disease and susceptibility, as observed with BTV infection in sheep and cattle (DeMaule *et al.* 2001).

Tumor necrosis factor (TNF), primarily derived from mononuclear phagocytes stimulated with a variety of exogenous and endogenous factors, e.g. viruses (Rink and Kirchner, 1996), is an important agonist in a number of inflammatory and immunologically mediated responses (Larrick and Kunkel 1988). The TNF orchestrates the inflammatory response and initiates a cascade of mediators directly responsible

for 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 overproduction of TNF may result in pathophysiologic events and generation of symptoms associated with different diseases (Strieter *et al.* 1993). An uncontrolled and an over-exuberant production of TNF has been associated with high morbidity and mortality rates in various diseases (Strieter *et al.* 1993, Nagaki *et al.* 2000, Rautenschlein and Sharma 2000, Atrasheuskaya *et al.* 2003).

We hypothesised that TNF production in mononuclear cells, induced by EHDV replication, accentuates endothelial damage and plays a role in the pathogenesis of Epizootic haemorrhagic disease (EHD) in WTD. We aim to characterize the expression of tumor necrosis factor- α (TNF- α) *ex vivo* in tissues of WTD infected with EHDV and in WTD peripheral blood mononuclear cells (PBMC) infected with EHDV. All the animals used in our study were managed in accordance with the applicable USDA Animal Welfare Regulations and the Guide for the Care and Use of Laboratory Animals.

To measure circulating TNF- α levels in serum from deer infected with EHDV serotype 2 (EHDV-2), a cytotoxicity assay was developed in L929 cells (ATCC). This assay was then used to evaluate TNF- α in serum samples from WTD from two previously reported experimental trials (Quist *et al.* 1997). In each trial, 8 WTD were infected with EHDV-2 and 2 sham-inoculated deer were used as controls. The circulating TNF- α level was compared between infected and uninfected deer in serum samples collected every 2 days during the course of infection [post inoculation (PI) days 0-18]. L929 cells were seeded at 2.22×10^5 cells/ml in 96-well Flat-bottom

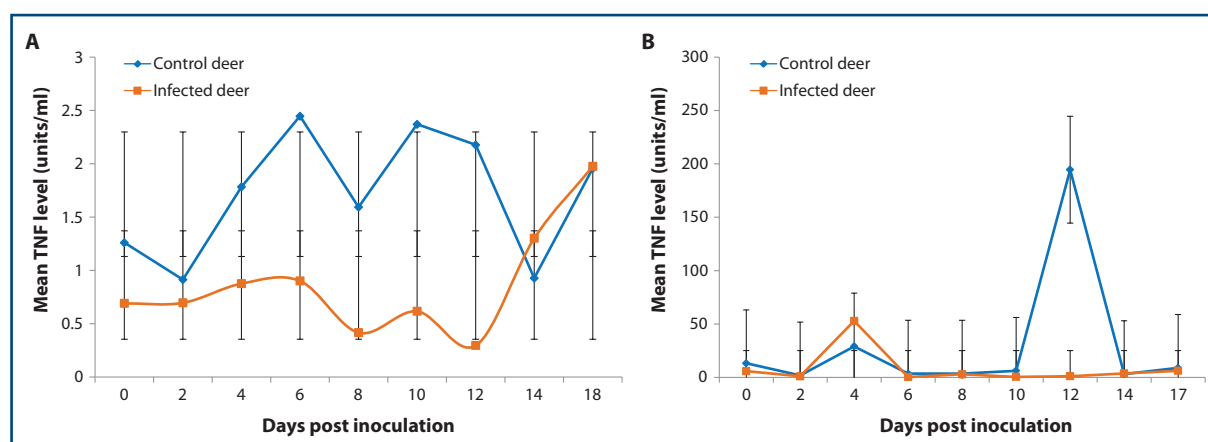


Figure 1. Cytotoxic assay for circulating tumor necrosis factor- α (TNF- α) in serum from white-tailed deer inoculated with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV) in 2 trials. (A) Means and standard deviations of circulating TNF- α for trial 1 for control and infected groups. Control $n = 2$; infected $n = 8$. (B) Means and standard deviations of TNF- α for trial 2 for control and infected groups. Control $n = 2$; infected $n = 8$. In both trials, EHDV inoculated deer developed mild to severe clinical disease, including death, but a significant increase in circulating TNF- α was not detected.

plates with RPMI-1640 medium containing 5% fetal bovine serum. Cells were treated with 15 μ l of MTT dye (Promega, Madison, Wisconsin, USA) followed by a stop solution (Promega stabilization solution, Madison, Wisconsin, USA) and the cell viability was quantified by using an automated microplate reader at a wavelength of 570 nm. Cells treated with medium or 5×10^{-3} μ g of TNF from mouse (Roche, Indianapolis, IN, USA) were used as negative and positive controls, respectively. The TNF titer was estimated from the cytotoxicity curve, as the reciprocal of the dilution that caused a 50% reduction in cell survival. Titers were converted to TNF units/ml by comparing them with the activity of the TNF- α standard in the assay system. Statistical analysis was performed using SAS PROC MIXED procedure. A p-value of < 0.05 was considered statistically significant. Circulating TNF level was not increased in the WTD infected with EHDV-2, even though all of them developed mild or severe clinical signs, including death (Figure 1, A and B).

To study the association between TNF- α levels in tissues and EHDV infection in WTD, fresh and formalin-fixed tissues were collected from deer either experimentally or naturally infected with EHDV-2 for reverse transcriptase polymerase chain reaction (RT-PCR) or IHC assays. The samples included:

1. biopsies from buccal mucosa on PI day 0 and 8;
2. biopsies from brain, skin, buccal mucosa, lymph nodes, gastrointestinal tract, lung, heart, liver, and spleen on PI infection 8;

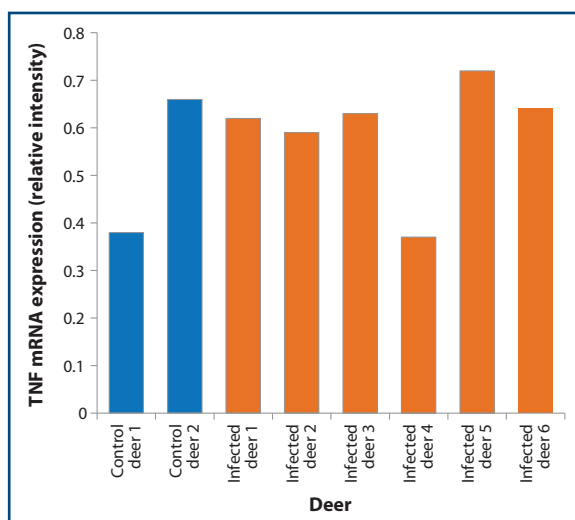


Figure 2. Semiquantitative determination of tumor necrosis factor- α (TNF- α) mRNA expression in right prescapular lymph nodes (side of inoculation in neck) from mock-infected control deer ($n = 2$) and deer experimentally infected with serotype 2 of the Epizootic haemorrhagic disease virus ($n = 6$) on days 7 or 8 post inoculation. Relative intensity was determined by optical density of bands on electrophoretic gels. Infected deer developed clinical disease, but up-regulation of TNF- α was not detected in lymph node.

3. biopsies from gastrointestinal tract, lymph node, buccal mucosa, liver, heart, and lung from 3 WTD after natural infection death. Only formalin fixed tissues were available from the naturally infected deer.

Semi-quantitative RT-PCR (Titan One Tube RT-PCR System; Roche, St. Louis, Missouri, USA) was performed to evaluate levels of TNF- α mRNA in right pre-scapular lymph node (side of experimental inoculation) and buccal mucosal biopsies using 0.1 μ g RNA (RNA extracted via RNA-Bee, Amsbio, Cambridge, Massachusetts, USA). The primers used were: TNF-forward primer: 5'-CCCATCTACCAGGAGGAGT and TNF-reverse primer: 5'-GGCGATGATCCCAAAGTAGA. Actin was used as a housekeeping gene (Actin-forward primer: 5'-CGCACTACTGGTATTGT and Actin-reverse primer: 5'-AGAGCTTCTCCTTGATGTC). Mean values and standard deviations were calculated for the assay and a student's t-test was applied (Graph Pad software, San Diego, CA, US) to determine differences between mean values of the data, with $p < 0.05$ considered significant. Tumor necrosis factor- α mRNA was detected in draining lymph nodes on the site of inoculation for the mock and all the 6 EHDV-2 infected deer from post inoculation samples at day 7 or day 8. There was no significant difference in the level of expression between control and infected deer ($P = 0.5216$) (Figure 2). The expression of TNF- α mRNA was also detected in buccal mucosa biopsies

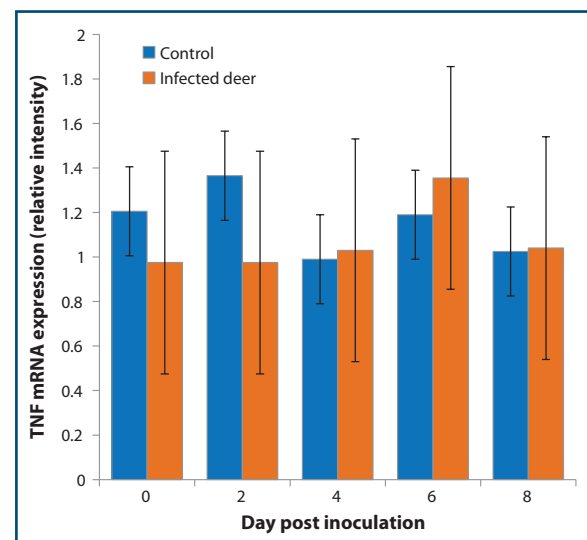


Figure 3. Semiquantitative determination of tumor necrosis factor- α (TNF- α) mRNA expression in buccal mucosa from sham-infected ($n = 2$) and deer experimentally infected with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV) ($n = 2$). Punch biopsies were obtained from buccal mucosa on days 0, 2, 4, 6, and 8 post inoculation. Relative intensity was determined by optical density of bands on electrophoretic gels. Infected deer developed clinical disease, but up-regulation of TNF- α was not detected in buccal mucosa, a common site of lesion development in EHDV infected animals.

from both control and infected deer in all the sampled time points; but there was no observable increase in levels of expression between control and infected deer (Figure 3).

A mouse anti-TNF- α antibody was used for TNF- α IHC (Clone 52B83, HyCult Biotechnology; 1:400). Detection was with a streptavidin-biotin technique using diaminobenzidine-tetrahydrochloride (DAB; Vector) as the chromogen. Tissue slides were counterstained with Mayers hematoxylin. Lung from a bovine pneumonia was used as a positive control. A WTD lymph node draining an area of bacterial infection was used to confirm cross-reaction of the primary antibody with deer TNF- α ; macrophages in this lymph node showed intense cytoplasmic staining for TNF- α protein. Neither natural nor experimental cases of EHD in WTD showed tissue expression of TNF- α protein by IHC.

We also evaluated *in vitro* production of TNF- α in WTD PBMCs infected with EHDV-2. Cells were isolated using Fico/Lite LymphoH 1.077 (Atlanta Biologicals, Atlanta, Georgia, USA), inoculated with EHDV-2 (multiplicity of 0.02 TCID₅₀/cell) and sampled at 24 and 48 hours PI. Cells inoculated with bacterial lipopolysaccharide (LPS) (20 ng/ml) or medium were used as positive and negative controls, respectively. Semiquantitative RT-PCR for TNF- α was done as described above and the results were analysed by a student's t-test with $p < 0.05$ considered significant. The TNF- α mRNA was detected in WTD PBMCs at 0 hours PI and in untreated, LPS treated, and EHDV-2 inoculated groups at 24 and 48 hours PI, but no significant upregulation was observed with EHDV-2 infection ($P = 0.9284$) or LPS stimulation (Figure 4).

Although TNF- α has been shown to be involved in disorders of inflammatory and non-inflammatory origin (Strieter *et al.* 1993) and also in the pathogenesis of viral diseases, like nephropathia epidemica-a European form of hantavirus-induced haemorrhagic fever with renal syndrome (Linderholm *et al.* 1996)-and dengue (Hober *et al.* 1993), the results of our *ex vivo* and *in vitro* studies suggest that TNF- α does not play a major role in the pathogenesis of EHD.

Biologically active circulating TNF was not detected in serum from of WTD infected with EHDV-2 in 2 trials. There was 100% morbidity and 31% mortality in EHDV-2 infected deer in these trials (Quist *et al.* 1997) so lack of circulating TNF cannot be attributed to the lack of the disease. Because samples were collected every 2 days, 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 like dengue and nephropathia epidemica in humans (Hober *et al.* 1993, Linderholm *et al.* 1996).

There was no detectable up-regulation of either TNF- α protein or the genes transcribing TNF- α in tissues of EHDV infected WTD. One deer that died on day 7 PI had decreased expression of TNF- α mRNA in tissue (lymph node) (relative intensity = 0.37 compared to 0.38-0.72 in other animals), suggesting that EHDV infection may actually depress gene expression of TNF- α or that viral infection may degrade the mRNA. Because TNF- α mRNA appears to be constitutively expressed in WTD tissues, a rapid production of TNF- α protein upon stimulation would be expected. Whether the EHDV does not stimulate the translation of the TNF- α mRNA or whether the translation is blocked is still unclear.

As EHDV replicates in WTD monocytes (Stallknecht *et al.* 1997) and because other monocytotropic viruses, such as African swine fever virus (Gomez *et al.* 1999), are known to upregulate TNF production in PBMCs *in vitro*, it was surprising to see that this was not the case with EHDV. The viral dose used to infect PBMC cultures in this study was low at a multiplicity of 0.02 TCID₅₀/cell, so it is possible that a higher viral dose would have induced better TNF- α expression. Nonetheless, the viral dose used in this *in vitro* study was previously shown to increase the expression of interleukin-2 receptor in bovine monocytes infected with BTV (Barratt-Boyes *et al.* 1992). In general, it is difficult to upregulate TNF- α in WTD PBMCs even in response to strong inducers such as endotoxin (Morris *et al.* 1990). In our studies, LPS failed to upregulate TNF- α in WTD PBMCs. However, the same dose has been shown to upregulate interleukin-1 beta, TNF- α and interleukin-6 in bovine alveolar macrophages

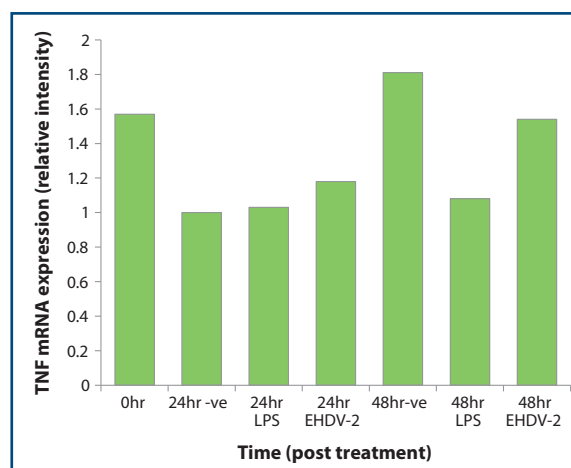


Figure 4. Semiquantitative determination of tumor necrosis factor-alpha (TNF- α) mRNA expression in white-tailed deer peripheral blood mononuclear cells inoculated with mock-inoculum (-ve), bacterial lipopolysaccharide (LPS), or serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2) at 0, 24, and 48 hours post inoculation. Relative intensity was determined by optical density of bands on electrophoretic gels. Tumor necrosis factor-alpha mRNA levels were not increased over the one of the control.

and in total PBMCs (Ito and Kodama 1996). Likewise only a small amount of circulating TNF- α was detectable in WTD serum in our cytotoxicity assay following an experimental injection of LPS. The dose of LPS used was determined on the basis of studies in other species-i.e. cattle, which are closely

related to WTD (Gerros *et al.* 1993) in which similar or lesser amounts had caused a significant increase in circulating TNF- α (MacKay *et al.* 1991, Allen *et al.* 1993, Perkowski *et al.* 1996). However, we conclude that that TNF- α might not be the major player in the pathology caused by EHDV.

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