

Cytokine release and endothelial dysfunction: a perfect storm in orbivirus pathogenesis

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Summary

Although Bluetongue viruses (BTV) and Epizootic haemorrhagic disease viruses (EHDV) are closely related, there are differences in susceptibility to these viruses both between and within a species. White-tailed deer (*Odocoileus virginianus*) are susceptible to disease by both BTV and EHDV, sheep are susceptible to BTV, but resistant to EHDV, and cattle can be infected with both viruses but disease is usually subclinical. Host genetics probably play a role in the disease outcome, but cytokine and endothelial responses are likely to determine if subclinical or clinical disease develops. Dendritic macrophages deliver virus to lymph nodes following the bite of an infected *Culicoides*. The virus then disseminates to many organs replicating in mononuclear phagocytes and endothelium. Initially, an interferon-1 response probably determines if the disease develops. Replication in mononuclear cells and endothelium results in the release of cytokines and vasoactive mediators, and may result in endothelial cell death leading to the clinical features of fever, hyperaemia, exudation of fluid, and haemorrhage. Disease outcome may also be linked to virus binding Toll-like receptor-3 and upregulation of endothelial surface receptors potentiating cytokine release and allowing transmigration of inflammatory cells, respectively. Despite a wealth of information, host genetics involved in resistance to BTV and EHDV and how variations in cytokines and endothelial responses determine clinical outcome still need further elucidation.

Rilascio di citochine e disfunzione endoteliale nel determinismo della patogenesi degli orbivirus

Parole chiave

Bovino,
Cervo dalla coda bianca,
Citochine,
Endotelio,
Ovini,
Virus della Bluetongue,
Virus della Malattia
emorragica epizootica.

Riassunto

Nonostante i virus della Bluetongue (BTV) e della Malattia emorragica epizootica (EHDV) siano strettamente correlati, la suscettibilità a questi virus varia nella stessa specie e tra specie differenti. Il cervo dalla coda bianca (*Odocoileus virginianus*) è suscettibile a BTV e EHDV, gli ovini lo sono al BTV ma risultano resistenti all'EHDV, i bovini lo sono a entrambi i virus ma raramente si ammalano. I fattori genetici contribuiscono a determinare l'esito della malattia ma sono con ogni probabilità il rilascio delle citochine e le risposte endoteliali che fanno sì che un'infezione si manifesti in forma clinica o subclinica. In seguito alla puntura di un culicoide infetto, il virus viene trasmesso ai linfonodi attraverso le cellule dendritiche. Successivamente, il virus raggiunge diversi organi replicandosi nei fagociti mononucleati e nell'endotelio. È probabile che la risposta iniziale dell'interferone-1 determini l'andamento, clinico o subclinico, della malattia. Il replicarsi del virus nelle cellule mononucleate e nell'endotelio determina il rilascio di citochine e mediatori vasoattivi che possono portare a morte le cellule endoteliali e, quindi, determinare manifestazioni cliniche come febbre, iperemia, essudazione di liquidi ed emorragia. L'esito della malattia può essere collegato anche al recettore Toll-like-3 e all'aumento dei recettori superficiali endoteliali che potenzia il rilascio di citochine, consentendo la tras migrazione delle cellule infiammatorie. Nonostante i numerosi studi, ulteriori approfondimenti sono necessari per capire il ruolo giocato dai fattori genetici dell'ospite nel determinare la resistenza a BTV e EHDV, o come le variazioni dei livelli di citochine e della risposta endoteliale influenzano l'andamento di un'infezione.

Introduction

The Bluetongue viruses (BTV) and Epizootic haemorrhagic disease viruses (EHDV) are closely related orbiviruses that infect a wide range of ruminants, both domestic and wild (Barnard 1997, Howerth *et al.* 2001), camelids (Ortega *et al.* 2010), and sometimes even carnivores, such as dogs (Akita *et al.* 1994) and wild cats (Alexander *et al.* 1994). However, there are differences in susceptibility to disease induced by these viruses both between and within a species. For example, both BTV and EHDV are major causes of mortality in white-tailed deer (*Odocoileus virginianus*, WTD) in the United States (Howerth *et al.* 2001), but susceptibility to disease has been shown to vary with subspecies (Gaydos *et al.* 2002). At the same time, sheep are susceptible to BTV with death often ensuing, but are resistant to EHDV-induced disease. At the other end of the spectrum, cattle can be infected with both viruses, but the disease is usually subclinical, with only occasional morbidity. Host genetics and host and population immunity probably play a role in disease outcome, but as these viruses replicate in mononuclear phagocytic cells and endothelium, cytokine and endothelial responses to infection are likely to ultimately determine whether an animal develops subclinical or clinical disease. In this article, we briefly explore host differences that might explain variable disease outcomes, particularly the cytokine and endothelial responses, and disease outcome at the level of the host using WTD, sheep, and cattle as examples.

Host genetics and resistance

Historical, experimental, and field study data provide evidence that host genetics play a role in resistance to BTV and/or EHDV-induced disease in both deer and sheep, but the specifics have not been determined. Variation in susceptibility to these viruses has been detected in WTD at the subspecies level in both experimental and field studies. When fawns of 2 WTD subspecies, one from epizootic areas of the Eastern US – where seroprevalence is low and WTD EHDV/BTV-induced mortality is common (*Odocoileus virginianus borealis*) – and 1 from enzootic areas of the South-Western US – where seroprevalence is high and WTD BTV/EHDV-induced mortality is uncommon (*O. virginianus texanus*) – were experimentally infected with either EHDV serotype 1 or serotype 2 similar viral titers and humoral immune responses developed in both subspecies (Gaydos *et al.* 2002). Clinical disease and mortality only occurred in fawns of the *O. virginianus borealis* subspecies suggesting innate disease resistance in *O. virginianus texanus*. Results from field studies in the US states of Texas (Stallknecht *et al.*

1996), Oklahoma (Kocan *et al.* 1987), and Kansas (Flacke *et al.* 2004), areas inhabited by WTD of the *O. virginianus texanus* subspecies, also support the hypothesis that this subspecies is highly resistant to Epizootic haemorrhagic disease (EHD). In these areas, seroprevalence to a number of EHDV and BTV serotypes is high, but clinical disease does not typically occur. The apparent innate resistance of *O. virginianus texanus* from endemic areas is speculated to have developed as a result of constant selective pressure related to annual or year round transmission of these viruses (Nettles and Stallknecht 1992), and may only be indirectly or partially related to subspecies because clinical disease does occur in WTD of the *O. virginianus texanus* subspecies in more northerly portions, where EHDV/BTV-associated WTD mortality is commonly reported.

Although sheep are considered extremely susceptible to Bluetongue (BT), certain breeds of sheep appear resistant to disease, although within the same breed or within a flock there may be variable severity of disease (Verwoerd and Erasmus 2004). European breeds, such as Dorset and Merino, are considered very susceptible to BTV-induced disease, whereas African and Asiatic breeds are described as resistant (Verwoerd and Erasmus 2004, Sreenivasulu *et al.* 2004). Bluetongue was first seen when European sheep were imported into South Africa; these animals had a higher susceptibility to BT than indigenous animals (Coetzee *et al.* 2012). While host genetics and the long-term selection of genetically resistant animals in indigenous breeds could explain the difference in susceptibility, other factors such as herd immunity may also play a role. It is possible that elucidation of the genetic basis behind BTV resistance in certain breeds may help explain why sheep, although susceptible to BTV, are resistant to EHDV. However, more recently, no major differences in susceptibility to BTV disease were observed between Mediterranean area and Northern European breeds using a standardized experimental design (Caporale *et al.* 2014). Thus, variations in breed susceptibility may not be as marked as originally thought, underscoring the need for more standardized testing of genetic susceptibility.

Cattle are typically resistant to BTV and EHDV-induced disease and are often considered reservoirs due to the prolonged viraemia that occurs in this species (MacLachlan *et al.* 1990). Nonetheless, there are multiple instances where infection, either with BTV or EHDV, has caused cattle morbidity. Two of the more recent examples are the 2006 EHDV-7 outbreak in Israel, where clinical signs, reduced milk production and a mild increase in mortality, occurred in dairy cattle (Kemi *et al.* 2010), and the 2008 BTV-8 outbreak throughout Europe where clinical illness occurred in cattle (Dal Pozzo *et al.* 2009). Factors

involved in the development of disease associated with these viruses in cattle are currently unknown and attempts at experimental infection, even with viruses known to cause natural disease in cattle, have typically been unrewarding (e.g., Ruder *et al.* 2015, forthcoming), although they are worthy of additional study as this might give insight on species or individual susceptibility to these viruses.

Host infection and variable clinical outcome

Following the bite of a virus infected *Culicoides*, dendritic macrophages are infected and deliver the virus to the regional lymph node where primary replication occurs. It is then disseminated to many organs with replication primarily in mononuclear phagocytes and endothelium (Figure 1). Initially, there is an interferon-1 response to viral infection, which probably plays a large role in determining if disease develops. In WTD, following infection with EHDV-2, peak viraemia and interferon-1 levels correspond (day 6 post infection) after which both rapidly decline (Quist *et al.* 1997), which is similar to the viraemia and interferon response in BTV infected sheep (Foster *et al.* 1991). This is in contrast to the response in cattle, which are more resistant to disease, where interferon production occurs prior

to day 4 post infection unassociated with viraemia (MacLachlan and Thompson 1985). The importance of interferon is exemplified by the fact that type 1 interferon receptor deficient mice are susceptible to disease caused by both BTV and EHDV, whereas, conventional mice are not (Calvo-Pinilla *et al.* 2009, Eschbaumer *et al.* 2011). The interferon response is understudied, but recent work (Doceul *et al.* 2014, Vitour *et al.* 2014) identified a number of cellular sensors and pathways involved in the induction and control of the interferon response to BTV.

Replication in mononuclear cells and endothelium results in the release of other inflammatory cytokines, e.g. interleukin-1 (IL-1), and vasoactive mediators, e.g. nitric oxide. This stimulates production of acute phase reactants, causes vasodilation, and increases vascular permeability leading to fever, hyperaemia, and exudation of fluid into soft tissues and body cavities, typical clinical features of BT and EHD. Eventually endothelial cells die and detach leading to haemorrhage and thrombosis, another clinical manifestation of disease. However, clinical disease does vary within and between species.

For example, in WTD, infection with either BTV or EHDV results in a rapid onset of clinical signs with fever (Howerth *et al.* 1988, Ruder *et al.* 2012) and, often, with rapid weight loss (Howerth personal experience), possibly associated with cytokinemia,

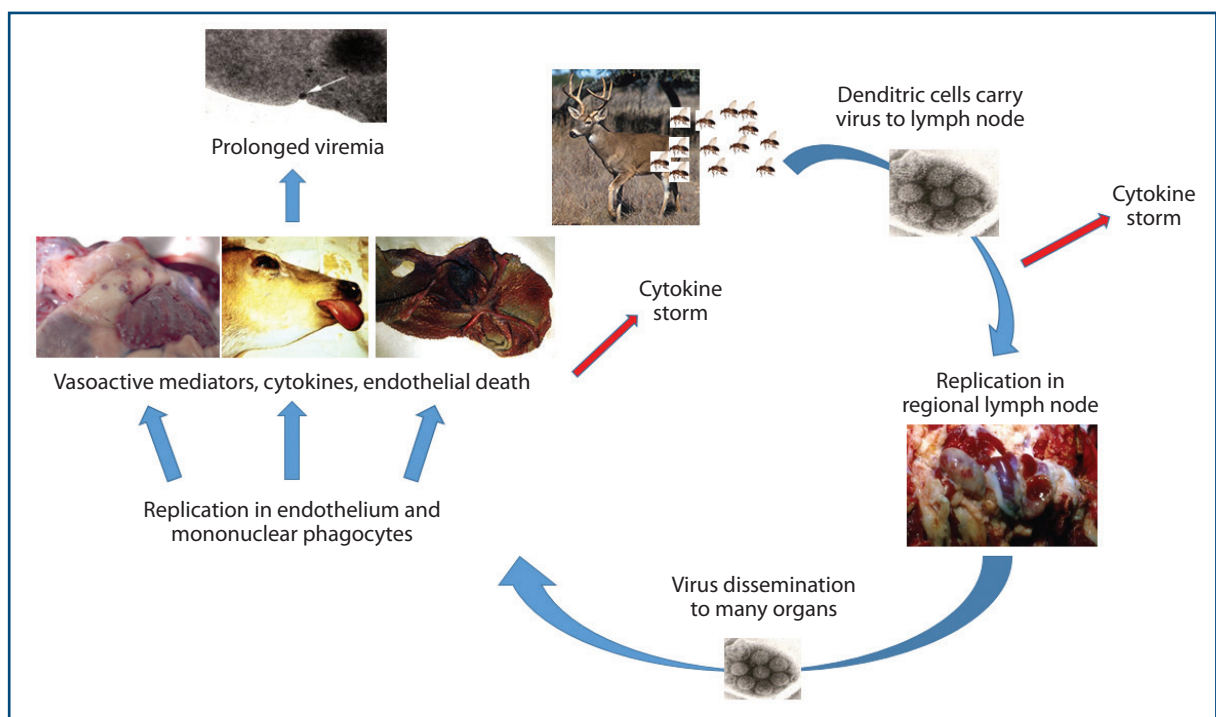


Figure 1. Bluetongue and Epizootic haemorrhagic disease are transmitted by the bite of an infected *Culicoides* sp. Dendritic cells then carry the virus to the regional lymph node where replication occurs and may trigger a cytokine storm. Virus is disseminated from the regional lymph node to many organs where the virus replicates in endothelium and mononuclear phagocytes. Elaboration of vasoactive mediators and cytokines along with endothelial damage and death occurs resulting in clinical features of haemorrhage and fluid exudation. A cytokine storm could potentially occur at this phase too. Animals that survive have a prolonged viraemia associated with viral particles sequestered in pits in the red blood cell plasma membrane.

e.g. circulating IL-1 and tumor necrosis factor (TNF) which can alter hypothalamic function causing food aversion and anorexia (Ackermann 2012). Variable clinical outcomes in individual WTD (Figure 2) range from peracute disease (usually seen within 4 days of infection) with sudden death due to the development of pulmonary oedema, potentially secondary to a cytokine storm, to an acute form where death associated with extensive haemorrhage is the predominant manifestation (from days 5-12 post infection), due to vascular damage and a coagulopathy. Clinical outcomes in individual WTD also include a sub-acute form characterized by the development of ulcers in the oral cavity and gastrointestinal tract [usually greater than 12 days post infection (Howerth *et al.* 2001)]. A similar spectrum of disease occurs in other susceptible species too (Caporale *et al.* 2014). However, haemorrhage is more pronounced in certain species, such as WTD, where the disease is called 'haemorrhagic disease' (Howerth *et al.* 2001).

The ultimate expression of disease in individual animals is probably related to the response of the 2 cell types that these virus replicate in, macrophages and endothelium. Much of the information on the response of mononuclear phagocytes/

macrophages to BTV and EHDV infection has been gathered through *in vitro* studies in peripheral blood mononuclear cells (PBMC). *In vitro* infection results in the production of a variety of inflammatory cytokines, e.g. interleukins and vasoactive mediators (nitric oxide). Studies have used peripheral blood mononuclear cells (PBMC) from a number of species, such as bovine (Drew *et al.* 2010), sheep and goats (Dhanasekaran *et al.* 2013), and WTD (Sharma *et al.* 2015 a, c, forthcoming) to measure a variety of interleukins (typically IL 1, 6, 8, and 10), gamma-interferon, tumor necrosis factor (TNF), and iNOS. Unfortunately, not all studies used similar experimental designs or viruses, and parameters measured varied, making correlations among and between species difficult.

More recently, attempts have been made to elucidate the response of specific types of monocytic cells, *i.e.* dendritic cells, to infection. Bluetongue virus has been shown to replicate in and activate conventional DCs (cDCs) and plasmacytoid DCs (pDCs) *in vitro* releasing proinflammatory molecules, such as IL 1, 6 and 12 (Hemati *et al.* 2009, Ruscanu *et al.* 2012) At the same time, genome-wide expression profiling (Ruscanu *et al.* 2013) has been used to demonstrate that in sheep BTV infection modifies



Figure 2. Clinical outcomes in white-tailed deer infected with epizootic haemorrhagic disease virus. **A.** Animals rapidly lose weight and condition within days of infection. This animal on day 4 post infection is in poor body condition with a rough hair coat. Note hyperaemia around the eyes. **B.** Disease may be peracute with death within 4 days of infection, usually as a result of severe pulmonary oedema. **C.** Animals that survive longer develop the more typical acute form of disease characterized by severe haemorrhage as seen in the forestomachs of this white-tailed deer on day 8 post infection. **D.** Vascular damage results in ulceration of the oral cavity and gastrointestinal tract in the subacute form of the disease.

gene expression of cDCs and pDCs depending on the lymphoid organ and that the gene signature of blood derived pDCs suggests their involvement in systemic inflammation and vasopermeability in the host. As such, but not as yet proven, dendritic cells may participate in cytokine storm-associated peracute death in BTV and EHDV infected animals.

In BTV or EHDV infected animals, variable response of the endothelium to circulating cytokines and vasoactive mediators produced by other cells types, in this case virus infected macrophages, and variable response to viral infection in these cells may help explain species and individual variation in clinical outcome. However, most of what we know about the endothelial response to BTV and EHDV infection is from *in vitro* studies on endothelium derived from a variety of sources, including ovine and bovine umbilical endothelium (Russell *et al.* 1996), bovine carotid artery (Sharma *et al.* 2015b, forthcoming), pulmonary artery (Drew *et al.* 2010), and sheep and bovine microvascular endothelium (DeMaula *et al.* 2001), using a variety of viruses uncharacterized except by serotype. These studies have typically measured mRNA upregulation or protein expression of a limited number of different cytokines and vasoactive substances or evaluated mechanisms of cell death. Because of the differences in experimental design (different endothelium, viruses, variable cytokines and vasoactive mediators evaluated, etc.) results are sometimes conflicting making translation to clinical outcome in the host difficult. For example, BTV infected ovine umbilical cell cultures bound more virus and produced more virus than bovine umbilical cell cultures (Russell *et al.* 1996), while the reverse was true for BTV infected ovine and bovine microvasculature (DeMaula *et al.* 2001). The reason for disparate results is unknown, but type of endothelium and/or differences in infecting viruses are potential factors.

Other potential causes for variable clinical outcomes in the host are also poorly studied,

such as regulation of surface receptors, *e.g.* P- and E-selectins, in viral infected endothelium, which could alter the transmigration of inflammatory cells and help explain the paucity of inflammation in these diseases. Initial studies (Dhanasekaran *et al.* 2013) have also shown that disease outcome may be linked to virus binding of Toll-like receptor-3 (TLR3), which binds dsRNA initiating interferon-1 and proinflammatory cytokines, and the role of TLR3 in innate resistance to BTV and EHDV deserves further attention. Viral replication in endothelium can also result in cell death with endothelial denudation, but this only leads to extensive thrombosis, severe haemorrhage, and sometimes disseminated intravascular coagulation in certain species, such as WTD (Howerth and Tyler 1988, Howerth *et al.* 1988). The factors contributing to this variant in clinical picture are as yet unexplained.

Concluding thoughts

Host genetic factors that determine resistance and how variations in cytokine expression and endothelial cell response to cytokines and infection determine species and individual susceptibility needs to be further elucidated. This article had the goal of drawing attention to the fact that there is already a wealth of information available on the host response to BTV and EHDV infection and a meta-analysis needs to be done to capture this and help drive future research. In addition, there needs to be standardization of both *in vitro* and *in vivo* studies, including the use of genetically defined viruses and host animals, so that studies can be better compared. Much of the research developed to date has been *in vitro*. This needs to be translated into large animal models to more accurately predict clinical outcome. New techniques such as genome-wide and protein expression profiling will better help us unravel pertinent pathways and proteins involved in host resistance and clinical outcome of disease.

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