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Bluetongue virus infection activates bovine monocyte-derived macrophages and pulmonary artery endothelial cells

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Abstract

Bluetongue virus (BTV) is the cause of bluetongue (BT), an emerging, arthropod-transmitted disease of ungulates. The cellular tropism of BTV in ruminants includes macrophages, dendritic cells and endothelial cells (EC), and fulminant infection is characterized by lesions consistent with those of so-called viral hemorrhagic fevers. Specifically, BT is characterized by vascular injury with hemorrhage, tissue infarction and widespread edema. To further investigate the pathogenesis of vascular injury in BT, we evaluated the responses of cultured bovine pulmonary artery EC (bPAEC) and monocyte - derived macrophages (bMDM) to BTV infection by measuring transcript levels of genes encoding molecules important in mediating EC activation and/or endothelial barrier dysregulation. The data confirm that BTV infection of bPAEC resulted in increased transcription of genes encoding chemokine ligand 2 (CCL2) and E-selectin, and BTV-infection of bMDM resulted in increased transcription of genes encoding TNF- α , IL-1 β , IL-8, and inducible nitric oxide synthase (iNOS). The data from these *in vitro* studies provide further evidence that cytokines and other vasoactive substances produced in macrophages potentially contribute to vascular injury in BTV-infected ruminants, along with direct effects of the virus itself on ECs.

Keywords

Bluetongue; Virus; Macrophage; Endothelium

1. Introduction

Bluetongue virus ([BTV]; genus *Orbivirus*, family *Reoviridae*) is the etiologic agent of bluetongue (BT), an important and re-emerging arboviral disease of ruminants (Backx *et al.*, 2007; Darpel *et al.*, 2007; MacLachlan *et al.*, 2009; Verwoerd & Erasmus, 2004). BTV is transmitted by *Culicoides* insects, thus the global distribution of BTV infection coincides

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closely with that of competent vector species (Gibbs & Greiner, 1994; Pritchard *et al.*, 2004; Tabachnick, 2004). BT is most commonly described in sheep, although many species of domestic and wild ungulates are susceptible including cattle, but with lower morbidity and mortality (Jauniaux *et al.*, 2008; MacLachlan *et al.*, 2009; Meyer *et al.*, 2009; Verwoerd & Erasmus, 2004). The pathogenesis of BTV infection is apparently similar in all ruminant species (MacLachlan *et al.*, 2009).

Natural BTV infection of ruminants begins with intradermal introduction of the virus during the blood feeding of an infected *Culicoides* insect. The inoculated virus first interacts with mononuclear inflammatory cells, including dendritic cells, and it then drains to the local lymph node where further replication occurs prior to dissemination to secondary sites of replication such as the lungs, lymph nodes, and spleen (Barratt-Boyes & MacLachlan, 1995; Barratt-Boyes *et al.*, 1992; Hemati *et al.*, 2009; Pini, 1976). Virus replication occurs in mononuclear phagocytic, dendritic, and endothelial cells (EC), thus the infection and response of these cells is critical to the pathogenesis of BT (DeMaula *et al.*, 2002a; Hemati *et al.*, 2009; McLaughlin *et al.*, 2003; Whetter *et al.*, 1989). Whereas BTV infection of the ECs lining small caliber vessels is likely responsible for the thrombosis and subsequent infarction that produce oral ulcers and myonecrosis, the mechanism(s) responsible for the widespread edema that occurs in severely affected animals is less defined (MacLachlan *et al.*, 2008; MacLachlan *et al.*, 2009; Mahrt & Osburn, 1986). It is proposed that inflammatory and vasoactive mediators released by BTV-infected host cells contribute to the increased vascular permeability that characterizes severe BT; specifically, that vasoactive mediators released from BTV-infected cells exert paracrine effects that increase the permeability of adjacent blood vessels (MacLachlan *et al.*, 2009). Consistent with this hypothesis, dendritic cells in lymph draining from the site of BTV infection in sheep express higher levels of genes encoding pro-inflammatory molecules such as interleukin 12 (IL-12), IL-1 β and IL-6 (Hemati *et al.*, 2009).

To further characterize the potential role of BTV-induced host cell-derived mediators and interferon inducible proteins in the pathogenesis of BT, we evaluated the activational response of bovine monocyte derived macrophages (bMDM) and bovine pulmonary artery EC (bPAEC) to BTV infection. The data indicate that BTV infection causes transcriptional activation of both bMDM and bPAEC, suggesting that these responses may be important to the pathogenesis of BT in ruminants.

2. Materials and methods

2.1 Virus

The U.S. prototype strain of BTV serotype 10 (BTV-10) (ATCC, VR-1231) was used for all experiments. Stock virus was prepared by infecting baby hamster kidney (BHK-21) cells (ATCC, CCL-10). The infected cells were pelleted and then sonicated to release cell associated virus after cytopathic effect was advanced. Virus containing cell lysates were centrifuged at 400 g to remove the cellular debris from the virus suspension, prior to ultracentrifugation at 69,000 g through a 5 % sucrose cushion to remove virus from soluble, cell - derived inflammatory mediators. The partially purified virus was re-suspended in phenol red free Dulbeccos minimal essential media (DMEM; Gibco), aliquoted and frozen at -80°C . Virus titers (TCID₅₀) were determined as previously described (Barratt-Boyes *et al.*, 1992; MacLachlan *et al.*, 1984).

2.2 Bovine pulmonary artery endothelial cells (bPAEC) and monocyte-derived macrophages (bMDM)

The isolation and purification of bPAEC were described previously (DeMaula *et al.*, 2001). Cells were maintained in complete medium (DMEM, 10 % fetal bovine serum, MEM vitamins, non essential amino acids, L- glutamine, sodium pyruvate). Confluent monolayers of bPAEC of similar passage number (9 or 10) were used in all experiments.

The bMDM were also derived and characterized essentially as previously described (Weiss *et al.*, 2002). Briefly, 300 ml of peripheral blood was collected in citrated buffer from the jugular vein of each of five different adult Holstein cows. The blood was centrifuged at 600×g for 20 minutes in 50 ml conical tubes and the buffy coat collected and diluted with an equal volume of PBS. The cell suspension was placed on top of an equal volume of Histopaque 1.083 (Sigma, 10831) and centrifuged at 400 g for 30 minutes. Peripheral blood mononuclear cells (PBMCs) were washed twice in PBS and resuspended in freezing media (90% fetal bovine serum, 10% DMSO) and stored at –80°C. Prior to each experiment PBMCs were thawed and washed twice with cold PBS and their viability was determined using trypan blue staining prior to dilution at 6×10^6 cells ml⁻¹ in media containing 7% fetal calf serum, and plating on 24 – well plates. Non-adherent cells were gently removed at 2 and 24 hrs after incubation at 37 °C. Morphology of the adherent cells as macrophages was confirmed by light microscopy, and all studies were performed on bMDM that had been maintained in culture for 7 days.

2.3 Replication of BTV in bPAEC and bMDM

The growth kinetics of BTV in bPAEC and bMDM were determined using both one-step growth curve analysis and sequential immunofluorescence staining for the presence of BTV core protein VP7. Specifically, bPAEC and bMDM were infected with BTV-10 at a multiplicity of infection (MOI) of 1, and individual cultures were harvested at regular intervals thereafter. At each time point the cells were scraped from the culture plate or flask and combined with the supernatant prior to sonication (DeMaula *et al.*, 2001). The cell debris was removed by centrifugation (400 g) and the titer (log₁₀ TCID₅₀) of the supernatant determined by serial dilution as described (Barratt-Boyes *et al.*, 1992; MacLachlan *et al.*, 1984).

Immunofluorescence staining of BTV-infected bPAEC and bMDM was done at intervals after BTV infection using cells grown on sterile 12 mm coverslips. Individual coverslip cultures were fixed with 4 % paraformaldehyde, permeabilized with 0.01 % Triton X-100, and incubated with 10% bovine serum albumin (BSA) prior to addition of the primary antibody (MAb 290; Whetter *et al.*, 1989) diluted in 1.5 % BSA/PBS. After 1 hour, the coverslips were washed and incubated with the secondary antibody (goat anti-mouse Alexa594) prior to mounting in Prolong gold with DAPI (Invitrogen, P36935). Immunofluorescence staining was visualized with an Olympus BX61 microscope fitted with a xenon lamp and appropriate filters.

2.4 RNA extraction and cDNA preparation

Total RNA was collected and purified from BTV-infected and uninfected (control) bPAEC and bMDM using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and cDNA was synthesized using a reverse transcription kit (Qiagen, Valencia, CA).

2.5 Quantitation of cytokine transcripts and analysis of gene expression in bMDM

Gene expression quantification of the cDNA was carried out using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol and with the

primer pair sets listed in Table 1 for IL-1 β , TNF- α , IL-8, iNOS, MX-1, and ribosomal protein S9 (reference gene). Melting curve analysis was performed at the conclusion of the PCR assay. All assays were performed using the ABI 7500 instrument, and quantification of gene expression was performed using the $2^{-\Delta\Delta C_t}$ method as described by the manufacturer (“Guide to performing relative quantitation of gene expression using real-time quantitative PCR”).

2.6 Quantitation of cytokine transcripts and analysis of gene expression in bPAEC

Gene expression in BTV-infected bPAEC was quantitated using the TaqMan® Gene Expression Assay system (ABI) with the following primer and probe sets: Beta-2-microglobulin (Bt03251628_m1), chemokine ligand 2 (CCL2) (Bt03212322_m1), intercellular adhesion molecule 1 (ICAM1) (Bt03213907_m1), IL- 8 (Bt03211906), E-selectin (Bt03213082_m1), and vascular cell adhesion molecule 1 (VCAM1) (Bt03279189_m1). Assays were validated and expression of individual genes quantitated using the $2^{-\Delta\Delta C_t}$ method, as described by the manufacturer.

2.7 Statistical analysis

Results are presented as mean \pm SD. Repeated measures ANOVA, followed by the Bonferonni *post hoc* was used for analysis of differences between infected and uninfected samples. A p-value of < 0.05 was considered significant.

3. Results and discussion

3.1 Replication of BTV in bMDM and bPAEC

One-step growth curve analysis and immunofluorescence staining for the presence of BTV core protein VP7 indicate that productive replication of BTV occurs in both bPAEC and bMDM, consistent with our previous findings (DeMaula *et al.*, 2001; DeMaula *et al.*, 2002a; Whetter *et al.*, 1989). Specifically, there was an approximately ten-fold increase in virus titer after BTV infection of bMDM, and an approximately thousand-fold (10^3) increase in bPAEC (data not shown). Similarly, there was intense cytoplasmic immunofluorescence staining of VP7 in both BTV-infected bMDM and bPAEC at 8 hours after infection (Fig. 1).

3.2 BTV-infection results in transcriptional activation of bPAEC and bMDM

To evaluate the ability of BTV to induce activation of bPAEC, cellular transcript levels (mRNA) of genes encoding CCL-2, IL-8, VCAM1, ICAM1, and E-selectin were quantified following infection. Only levels of mRNA encoding CCL2 and E-selectin were significantly increased after BTV-infection (Fig. 2A, B; $p < 0.05$). BTV infection of bPAEC has previously been shown to causes increased levels of IL-1, IL-6, and cyclooxygenase-2, which were not evaluated in the current study (DeMaula *et al.*, 2002a). BTV infection of bovine microvascular EC also induced surface expression of E-selectin (DeMaula *et al.*, 2002b). Together these data confirm that BTV infection results in transcriptional activation of bovine EC, both those derived from the pulmonary microvasculature as well as the pulmonary artery, with increased expression of genes encoding cytokines and adhesion molecules. CCL-2 is a potent chemoattractant for macrophages and monocytes, causes firm adhesion of monocytes to vascular endothelium, and is involved in monocyte extravasation (Melgarejo *et al.*, 2009), consistent with the influx of mononuclear inflammatory cells to sites of BTV infection within the skin (Mahrt & Osburn, 1986). Similarly, increased expression of E-selectin on ECs would be expected in the inflammatory response in BTV infection by facilitating and promoting the margination, rolling, and eventual transmigration of leukocytes, including monocytes (Kumar *et al.*, 2005).

To determine the potential role of host cells other than ECs in the pathogenesis of BT, and macrophages in particular, transcript levels (mRNA) of genes encoding several key proinflammatory mediators, and a single interferon inducible protein, were quantified in BTV infected bMDM. Transcripts encoding TNF- α , IL-1 β , IL-8, iNOS, and MX1 were all significantly increased in BTV-infected bMDM, as compared to levels in uninfected cells (Fig. 3A, B; $p < 0.05$). Maximal expression of mRNA encoding TNF- α , IL-1 β , and IL-8, iNOS, and MX1 occurred at 4 – 8 hrs. BTV is a potent inducer of interferon- α , and there is a close temporal relationship between onset of interferon- α production and viremia in BTV-infected ruminants (Foster *et al.*, 1991; Fulton *et al.*, 1982; Jameson & Grossberg, 1978; MacLachlan & Thompson, 1985). MX proteins are interferon inducible, have antiviral activity and are expressed in a variety of cells, including peripheral blood mononuclear cells (Melgarejo *et al.*, 2009). Our data suggests that interferon inducible proteins may be involved in modulating viral replication *in vitro*, and, by extension, perhaps *in vivo*. Similarly, production of TNF, IL-1 β , and IL-8 by macrophages has been shown to contribute to the pathogenesis of the human viral hemorrhagic fevers (Basu & Chaturvedi, 2008; Bray, 2005; Marty *et al.*, 2006). These cytokines exert a profound effect on ECs leading to increased vascular permeability with subsequent tissue edema and vascular collapse (shock), either by direct injury or by receptor mediated increases in paracellular permeability (Lentsch & Ward, 2000). In particular, IL-8 can be a useful indicator of severe outcome with Dengue virus infection (Basu & Chaturvedi, 2008). Nitric oxide causes vasodilation, increased leukocyte adhesion, and thrombus formation (Tefamariam & DeFelice, 2007). It is logical to speculate, therefore, that production of these potent vasoactive mediators in BTV-infected macrophages, EC and dendritic cells contributes to the edema and vascular collapse observed in fulminant cases of BT in ruminants. However, *in vivo* studies will clearly be required to precisely characterize the pathogenesis of BT, specifically the relative roles and importance of direct virus injury and indirect paracrine effects of proinflammatory and chemokine mediators therein. Similarly, although the pathogenesis of BTV infection is apparently similar in all species of ruminants and vascular injury occurs in both BTV-infected sheep and cattle, the change is more pronounced in sheep (DeMaula *et al.*, 2002a; MacLachlan *et al.*, 2009). Thus, to confirm the data we obtained using bMDM and bPAEC, additional *in vitro* studies should be undertaken in the future utilizing macrophages and ECs derived from sheep and perhaps other species that are highly susceptible to BTV infection.

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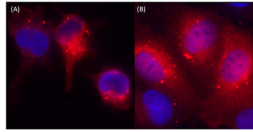


Fig. 1. Immunofluorescence staining of VP7 in BTV infected (8hr) bMDM and bPAEC. Granular, cytoplasmic staining in (A) bMDM and (B) bPAEC. (400X).

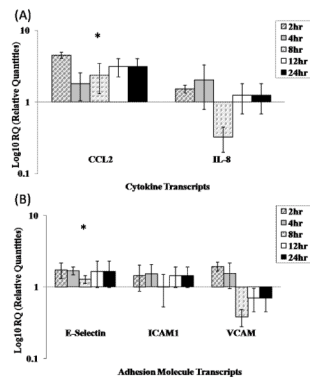


Fig. 2. Relative quantification of endothelial cell mRNA of genes encoding (A) proinflammatory mediators CCL2 and IL-8 and (B) adhesion molecules E-selectin, VCAM1 and ICAM1 from BTV infected bPAEC, as measured by real time quantitative PCR. Values are the means \pm SD of three replicate experiments performed in duplicate. Expression of genes encoding CCL-2 and E-selectin was significantly increased ($p < 0.05$). (*) indicates genes with significant increase in mRNA expression.

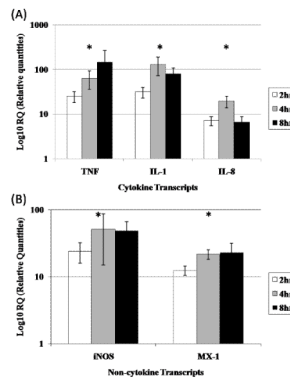


Fig. 3. Relative quantification of macrophage mRNA of genes encoding (A) proinflammatory and chemokine mediators TNF- α , IL-1 and IL-8 and (B) immune related and anti-viral molecules (iNOS and MX1) from BTV infected bMDM, as measured by real time quantitative PCR. Values are the means \pm SD of 5 replicate experiments (5 individual cows) performed in duplicate. Expression of genes encoding TNF- α , IL-1 β , IL-8, iNOS, and MX-1 was significantly increased ($p < 0.05$). (*) indicates genes with significant increase in mRNA expression.

Table 1

Nucleotide identity of primers used to amplify mRNA by quantitative RT-PC

Target	Sense primer	Antisense primer
IL-1b	5 - ggc tta cta cag tga cga gaa tga g - 3	5 - aac cga ggt cca ggt gtt g - 3
IL-8	5 - gaa gag agc tga gaa gca aga tcc - 3	5 - acc cac aca gaa cat gag gc - 3
TNF - α	5 - ctt ctg cct gct gca ctt cg - 3	5 - gag ttg atg tcg gct aca ac - 3
iNOS	5 - gca gcg gag tga ctt tcc aa - 3	5 - gga tgc cag gca aga ctt g - 3
MX1	5 - atc ttt caa cac ctg acc gcg - 3	5 - gga gca cga aca acg gga tga t - 3
S9	5 - gaa gct gat cgg cga gta tg - 3	5 - cgc aac agg gca tta cct tc - 3