The Role of Endothelial Cell-Derived Inflammatory and Vasoactive Mediators in the Pathogenesis of Bluetongue

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Bluetongue is an insect-transmitted disease of sheep and wild ruminants that is caused by bluetongue virus (BTV). Cattle are asymptomatic reservoir hosts of BTV. Infection of lung microvascular endothelial cells (ECs) is central to the pathogenesis of BTV infection of both sheep and cattle, but it is uncertain as to why sheep are highly susceptible to BTV-induced microvascular injury, whereas cattle are not. Thus, to better characterize the pathogenesis of bluetongue, the transcription of genes encoding a variety of vasoactive and inflammatory mediators was quantitated in primary ovine lung microvascular ECs (OLmVECs) exposed to BTV and/or inflammatory mediators. BTV infection of OLmVECs increased the transcription of genes encoding interleukin (IL) 1 and IL-8, but less so IL-6, cyclooxygenase-2, and inducible nitric oxide synthase. In contrast, we previously have shown that transcription of genes encoding all of these same mediators is markedly increased in BTV-infected bovine lung microvascular ECs and that BTV-infected bovine ECs produce substantially greater quantities of prostacyclin than do sheep ECs. Thus, sheep and cattle were experimentally infected with BTV to further investigate the role of EC-derived vasoactive mediators in the pathogenesis of bluetongue. The ratio of thromboxane to prostacyclin increased during BTV infection of both sheep and cattle, but was significantly greater in sheep (P = 0.001). Increases in the ratio of thromboxane to prostacyclin, indicative of enhanced coagulation, coincided with the occurrence of clinical manifestations of bluetongue in BTV-infected sheep. The data suggest that inherent species-specific differences in the production and activities of EC-derived mediators contribute to the sensitivity of sheep to BTV-induced microvascular injury.

Key Words: bluetongue virus; endothelium; cytokines; cattle; sheep.

INTRODUCTION

Bluetongue is an insect-transmitted virus disease of sheep and wild ruminants that is characterized by manifestations of microvascular injury, including pulmonary edema, disseminated intravascular coagulation (DIC), and vascular thrombosis with tissue infarction (MacLachlan, 1994; Mahrt and Osburn, 1986; Moulton, 1961; Pini, 1976; Spruell, 1905). In contrast, bluetongue virus (BTV) infection of cattle is typically asymptomatic, and cattle function as reservoir hosts of BTV (Barratt-Boyes and MacLachlan, 1995; MacLachlan, 1994). Sporadic occurrence of disease in BTV-infected cattle has been attributed to an IgE-mediated hypersensitivity reaction (Anderson et al., 1987). It is uncertain as to why BTV infection causes extensive microvascular injury in sheep but not cattle, as the cell and tissue tropism of BTV is similar in the two species. Specifically, BTV replicates principally in endothelial cells (ECs) and mononuclear phagocytic cells in the lungs and lymphoid tissues of both sheep and cattle (Barratt-Boyes and MacLachlan, 1994, 1995; Barratt-Boyes et al., 1995; MacLachlan et al., 1990; Pini, 1976).

Coen et al. first proposed that species-specific differences in EC infection and cytokine production are responsible for the different clinical outcomes of BTV infection of cattle and sheep (Coen et al., 1991). Subsequent studies confirmed that cytolysis and interferon production were different in BTV-infected ovine and bovine umbilical vein ECs (Russell et al., 1996), and we recently demonstrated that the mechanism of cell death (apoptosis or necrosis), infection kinetics, and the production of prostacyclin all differed between BTV-infected ovine and bovine pulmonary artery and lung microvascular ECs (DeMaula et al., 2001). A variety of inflammatory mediators are produced in response to virus infections, and those released from activated ECs that modulate vasomotor tone, thrombosis, and/or inflammation include nitric oxide, prostacyclin, interferons, interleukin 1 (IL-1), IL-6, and chemokines such as IL-8 (Cotran et al., 1999; Mantovani et al., 1992; Moncada et al., 1990). Prostacyclin may have an especially important role in determining the outcome of BTV infection of ruminants because it is a potent vasodilator and inhibitor of platelet aggregation that is produced primarily by ECs (DeMaula et al., 2001; Moncada and Amelzca, 1979).
To further characterize the central role of inflammatory mediators in the pathogenesis of BTV-induced microvascular injury, we evaluated the responses of pure cultures of primary ovine lung microvascular ECs (OLmVECs) to BTV infection and/or treatment with inflammatory mediators and compared the responses of OLmVECs to those of bovine lung microvascular ECs (DeMaula et al., 2002). We also compared levels of prostacyclin and thromboxane in the plasma of experimentally infected sheep and cattle to investigate the potential role of these potent vasoactive mediators in the pathogenesis of bluetongue.

RESULTS

BTV-induced transcription of genes encoding inflammatory and vasoactive mediators in OLmVECs

OLmVECs were highly susceptible to infection with either purified BTV or a lysate of BTV-infected OLmVECs (BTV/OLmVEC lysate) that contained both BTV and virus-induced EC-derived mediators, and cytopathic effect was complete (>95%) within 36 h after infection of the cultures (data not shown). High titers of BTV were present at 24 h after infection of OLmVECs with either purified BTV or the BTV/OLmVEC lysate (Fig. 1). The concentration of IL-1 mRNA was significantly increased (P < 0.024) in OLmVECs infected with purified BTV, whereas infection of OLmVECs with the BTV/OLmVEC lysate did not increase the transcription of IL-1 mRNA (Fig. 1). The concentration of IL-8 mRNA was significantly increased in OLmVECs infected with either purified BTV (P < 0.05) or the BTV/OLmVEC lysate (P < 0.047), although IL-8 mRNA concentrations were lower in OLmVECs infected with the BTV/OLmVEC lysate than in OLmVECs infected with purified BTV. Infection with either purified virus or the BTV/OLmVEC lysate induced only minimal increases in IL-6 mRNA in OLmVECs. Treatment of OLmVECs with a mixture of BTV-induced-EC-derived inflammatory mediators (virus-free) did not significantly increase transcription of mRNAs encoding any of the cytokines measured, and only IL-8 mRNA was significantly increased in OLmVECs treated with the cocktail of recombinant cytokines (P < 0.035).

BTV infection did not significantly increase the transcription of genes encoding either COX-2 or iNOS, enzymes that respectively function in the biosynthetic pathways of prostacyclin and nitric oxide. Similarly, treatment of OLmVECs with a mixture of BTV-induced-EC-derived inflammatory mediators (virus-free) did not significantly increase the concentration of COX-2 and iNOS mRNAs, and the cocktail of recombinant cytokines (positive control) induced only a very transient increase in COX-2 mRNA (P = 0.036) at 6 h after infection.

Mock-infected OLmVECs expressed only low levels of mRNAs encoding inflammatory and vasoactive mediators, confirming the specificity of the responses of the OLmVECs to BTV infection and/or exposure to inflammatory mediators.

The ratio of plasma thromboxane to prostacyclin is higher in BTV-infected sheep than in cattle

Sheep infected with BTV by the bites of Culicoides sonorensis developed pyrexia, lethargy, and tachypnea and one had an intermittent pleural friction rub between 7 and 11 days postinfection (d.p.i.), whereas infected cattle developed only transient and mild pyrexia (Table 1). BTV was first isolated from the blood of infected sheep at 5 d.p.i. and from the blood of cattle at 7 to 14 d.p.i. All animals seroconverted to BTV by 14 d.p.i. as determined by competitive enzyme-linked immunosorbent assay (cELISA; data not shown). Plasma thromboxane concentrations were significantly increased in sheep between 7 and 11 d.p.i. (P < 0.007), and in cattle at 9 and 11 d.p.i. (P < 0.009), as compared to levels at 0 d.p.i. (Fig. 2). Plasma prostacyclin concentrations progressively increased through 21 d.p.i. in cattle, whereas the concentration of prostacyclin decreased significantly after 7 d.p.i. in sheep (P < 0.002) and did not return to baseline levels by 21 d.p.i. The ratio of thromboxane to prostacyclin increased during BTV infection of both sheep and cattle, but was significantly greater (P = 0.001) in sheep at 9 d.p.i. The increased ratios of thromboxane to prostacyclin also coincided with the occurrence of pyrexia and maximal viremia in both BTV-infected sheep and cattle (Table 1). Uninfected control animals remained seronegative to BTV, and the concentration of plasma prostacyclin and thromboxane was constant over the 21-day period (data not shown).

Five additional sheep were infected with BTV by direct subcutaneous and intravenous inoculation to confirm the influence of BTV infection on prostacyclin production in sheep. These sheep developed relatively mild clinical signs of blue tongue that included fever, nasal discharge, inappetence, depression, tachypnea, and intermittent lameness between 10 and 21 d.p.i., and all animals seroconverted to BTV between 7 and 14 d.p.i. as determined by cELISA (data not shown). Plasma prostacyclin concentrations of these sheep also decreased significantly between 11 and 21 d.p.i. (P < 0.011), although the decline occurred 4 days later than in sheep infected with BTV by the bites of C. sonorensis insects (data not shown).

DISCUSSION

The highly purified cultures of sheep ECs used in this study provide a convenient, reproducible, and relevant in vitro model with which to evaluate the pathogenesis of bluetongue. We previously described differences in the response of ovine and bovine lung microvascular ECs to BTV infection (DeMaula et al., 2001), and data from the present study further confirm that there are marked spe-
cies-specific differences in the production of inflammatory (IL-1, IL-6, and IL-8) and vasoactive (COX-2 and iNOS) mediators by BTV-infected ruminant ECs. Specifically, although infection of OLMVECs with purified BTV resulted in markedly increased transcription of IL-1 mRNA, responses typically induced by IL-1, such as production of IL-6 and vasoactive mediators, were minimal in BTV-infected OLMVECs as compared to those we previously described in BTV-infected bovine lung microvascular ECs (DeMaula et al., 2002). In further contrast to bovine lung microvascular ECs, IL-1 mRNA was not increased in OLMVECs infected with the BTV/OLMVEC lysate, indicating that BTV-induced EC-derived cytokines likely suppress the production of some proinflammatory mediators in OLMVECs. We also previously reported that bovine ECs produced significantly higher levels of prostacyclin after BTV infection than did OLMVECs (DeMaula et al., 2001), which is consistent with the minimal increase in COX-2 mRNA that occurred in BTV-infected OLMVECs in the present study. Prostacyclin and nitric
oxide are potent vasodilators and antithrombotic agents that may counter the prothrombotic effects of IL-1 and subendothelial extracellular matrix exposed by cytolysis of ECs, as likely occurs during BTV infection (DeMaula et al., 2001; Mantovani et al., 1992; Cotran et al., 1999). Thus, these in vitro data are consistent with the sensitivity of sheep and resistance of cattle to BTV-induced microvascular injury and thrombosis, DIC, and hemorrhagic diathesis.

Prostanoids are central to the pathogenesis of a variety of vascular diseases. Continuous infusion of prostacyclin normalizes plasma markers of EC injury and platelet aggregation in primary pulmonary hypertension of humans for example (Friedman et al., 1997), and plasma prostacyclin and thromboxane levels are increased in human patients with acute myocardial infarction (Gurbel et al., 1999). Similarly, prostacyclin modulates vaso- spasm of bovine coronary arteries (Zou and Bachschmid, 1999; Zou et al., 1999a,b), and sheep with chronic lung injury that were treated with interferon-α developed pulmonary hypertension that was correlated with increases in plasma levels of thromboxane. Treatment of these sheep with a specific thromboxane synthase inhibitor blocked the hypertensive effects of interferon-α (Hanaoka et al., 1999). Data from the present study strongly suggest that the relative concentrations of plasma prostanoids also may determine the outcome of BTV infection of ruminants. Specifically, the decrease in plasma concentrations of prostacyclin in sheep is consistent with the minimal transcription of COX-2 mRNA in OLMVECs, and the most pronounced changes in plasma prostanoid concentrations coincided with the occurrence of clinical signs and maximal viremia in BTV-infected sheep. The significantly greater increase in the ratio of thromboxane to prostacyclin in the blood of BTV-infected sheep as compared to cattle is also consistent with the occurrence of DIC that characterizes bluetongue.

In summary, differences in the production of vasoactive and inflammatory mediators in BTV-infected ovine and bovine lung microvascular ECs parallel the different clinical consequences of BTV infection of sheep and cattle. Furthermore, the ratio of plasma thromboxane to prostacyclin, an indicator of enhanced coagulation, is significantly greater in BTV-infected sheep than in cattle. Inherent species-specific differences in BTV-induced production of EC-derived inflammatory and vasoactive mediators may, therefore, determine the susceptibility of sheep and resistance of cattle to expression of bluetongue disease.

**MATERIALS AND METHODS**

**Cells**

The isolation, cultivation, and purification of OLMVECs previously have been described in detail (DeMaula et al., 2001). Briefly, primary cultures of OLMVECs were derived from yearling sheep by enzymatic digestion of lung tissue followed by fluorescence-activated cell sorting until completely pure cultures were obtained. The identity and

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*Note. All animals seroconverted by 14 days postinoculation (DPI); nd = not done.*
FIG. 2. Concentrations of thromboxane and prostacyclin in the plasma of sheep and cattle infected with bluetongue virus by the bites of Culicoides sonorensis.
purity of the cultures were confirmed by immunohistochemical staining with a panel of antibodies to EC-specific markers. OLMVECs maintain their phenotype in culture (Meyrick et al., 1991), thus low passage stocks were cryopreserved and used between passages 7 and 15 in all experiments.

Virus

A strain of BTV serotype 17 in the blood of a sheep that died of naturally acquired bluetongue was passaged twice in seronegative cattle. The virus was then isolated from cattle blood in primary bovine lung microvascular ECs and purified as previously described (DeMaula et al., 2001). OLMVECs then were infected with partially purified BTV to produce the virus stock that was used in this study. The BTV-infected OLMVECs were frozen at –70°C, thawed, homogenized, and sonicated, and the titer (TCID<sub>50</sub>) of the virus stock was then determined by microtitration of BHK-21 cells as previously described (Barratt-Boyes et al., 1992; MacLachlan et al., 1984). This lysate (BTV/OLmVEC lysate) contained both BTV- and EC-derived inflammatory mediators that were induced by virus infection. A partially purified BTV inoculum was also derived from this BTV/OLmVEC lysate, but virus was separated from the mediators contained in the lysate by ultracentrifugation through sucrose as described previously (DeMaula et al., 2001).

BTV infection of OLMVECs

OLmVEC cultures of similar passage and cell density were infected at 6-h intervals with either partially purified BTV or the BTV/OLmVEC lysate (virus and mediators) at a multiplicity of infection (m.o.i.) of 0.5 to 1. Control monolayers were inoculated (mock infected) with culture medium or a lysate of uninfected OLMVECs also at 6-h intervals. The cytopathic effect in the various OLMVEC cultures was estimated at 6-h intervals as a percentage of the monolayer, and the cells in each culture were then prepared simultaneously for virus titration and TaqMan real-time polymerase chain reaction (PCR) assay for quantitation of mRNAs encoding a variety of mediators. Culture media from each flask was clarified by centrifugation at 400 g. Cells remaining in each flask were removed nonenzymatically with 0.05 M EGTA in Hank’s balanced salt solution, pooled with those previously pelleted from the media, and washed once with phosphate-buffered saline (PBS). Portions of the clarified media and cells collected from the various cultures were combined and the titer of BTV was determined by microtitration in BHK-21 cells as previously described (Barratt-Boyes et al., 1992; MacLachlan et al., 1984).

Treatment of OLMVECs with inflammatory mediators

BTV-induced EC-derived inflammatory mediators were harvested from the lysate of BTV-infected OLMVECs as the supernatant after virus was removed by ultracentrifugation. Uninfected OLMVEC cultures of similar passage and cell density were treated at 6-h intervals with the mixture of BTV-induced OLMVEC-derived inflammatory mediators or with a cocktail of recombinant cytokines that included murine IL-1β (10 ng/ml; Sigma), murine tumor necrosis factor α (TNFα, 1.64 ng/ml; Sigma), and platelet activating factor (PAF, 1 mM; Sigma). Murine IL-1β and TNFα activate human and ruminant ECs (Bargatz et al., 1994; DeMaula et al., 2002; Jutila et al., 1994; Meyrick et al., 1991) and synergistically interact with PAF (DeMaula et al., 2001; Sterner-Kock et al., 1996).

Quantitation of cytokine, COX-2, and iNOS mRNAs in OLMVECs

The TaqMan real-time PCR procedures for the quantitation of bovine IL-1β, IL-6, and IL-8; cyclooxygenase-2 (COX-2); and inducible nitric oxide synthase (iNOS) mRNAs have been previously described (Collins et al., 1999; DeMaula et al., 2002; Leutenegger et al., 2000; Leutenegger et al., 1999a,b). The TaqMan real-time PCR assays used for the quantitation of ovine IL-1β, IL-6, IL-8; iNOS; and COX-2 mRNAs were identical to those described for the equivalent bovine molecules with the exception of the IL-8 assay, which was optimized using the same procedures (Table 2). Primers and probes were designed with the PrimerExpress software package (Applied Biosystems) such that the probe spanned the junction of two exons covered by the primers, ensuring discrimination between genomic DNA and cDNA generated from mRNAs. Approximately 2 × 10<sup>6</sup> ECs were collected at each 6-h treatment interval, pelleted from the PBS wash, and stored at –70°C. The frozen cells were lysed in lysis buffer and total RNA was extracted using the RNaseasy Kit (Qiagen) according to the manufacturer’s instructions. The extracted RNA was treated with DNase I and reverse transcribed with SuperScript II reverse transcriptase (Life Technologies). The primers, probe, PCR master-mix, and AmpErase UNG were combined with the cDNA derived from each 6-h sample and amplified in an automated fluorometer (ABI Prism 7700 sequence detection system; Applied Biosystems). Cytokines, COX-2, and iNOS mRNAs were quantitated by the comparative cycle threshold (C<sub>T</sub>) method. Mock-infected samples were used as the reference sample (calibrator) for each me-
静脉，混合了抗凝剂和血小板氧合酶抑制剂，并立即放在冰上。血浆在-20°C保温1小时后收集。血浆样本被按照反相-相色谱图进行提取一个样品的抗原性成分和浓度。正常样品和抗凝剂从1ml牛血或血液浆被提取。通过免疫/荧光染色技术和流式细胞术，这些研究被支持。这些研究是通过农业部/竞争性研究资金支持96-35204-3849和99-35204-7863;和NIH比较医学科学训练项目支持G32-RR07038;资金由中心提供给食品动物健康。这项研究在动植物健康法第95-113条款下进行。作者感谢Bridget McLaughlin, Carol Oxford, and Drs. Udeni Balasuriya, Laurel DeMaula, Jodi Hedges, and Brian Moore的协助。

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