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The immune response of ruminant livestock to bluetongue virus: From type I interferon to antibody



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ABSTRACT

Infection of ruminants with most (but not all) serotypes of bluetongue virus (BTV) leads to a highly blood cell-associated viremia that may be prolonged but not persistent. Furthermore, recovered animals are resistant to reinfection with the homologous virus serotype, which is the basis for vaccination strategies to prevent BTV infection and the clinical disease (bluetongue) that it causes in domestic livestock. BTV infection is initiated at the site of virus inoculation and the associated draining lymph node, from where the virus is then spread in lymph cells to the systemic circulation and secondary sites of replication. Replication of BTV in target cells, notably mononuclear phagocytic cells (dendritic cells and macrophages) and endothelium, leads to the generation of the innate and adaptive immune responses that mediate both initial virus clearance and subsequent resistance to infection with the homologous virus serotype. The goal of this review is to summarize current understanding of these innate and adaptive immune responses of animals to BTV infection.

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1. Introduction

Bluetongue (BT) is an arboviral disease of domestic and wild ruminants characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema (Hutcheon, 1902; Maclachlan et al., 2009; Spreull, 1905; Verwoerd and Erasmus, 2004). The causative agent of BT is bluetongue virus (BTV), the prototype member of the genus *Orbivirus*, family *Reoviridae* (Borden et al., 1971; Matsuo and Roy, 2013; Murphy et al., 1971; Schwartz-Cornil et al., 2008). The BTV virion consists of a diffuse outer protein coat and an inner icosahedral core that encloses the transcriptase complex (Stewart et al., 2012; Verwoerd et al., 1972). The genome includes 10 distinct genome segments of double-stranded RNA (dsRNA). Each gene segment encodes at least 1 protein, specifically 7 structural (VP1–7) and 5 nonstructural (NS1–3/3A, 4) proteins (Grubman et al., 1983; Mertens et al., 1984; Ratniner et al., 2011). Some 26 BTV serotypes are now recognized globally (Hofmann et al., 2008; Maan et al., 2012).

BTV infection occurs throughout temperate and tropical regions of the world coincident with the distribution of specific species of vector *Culicoides* biting midges, however the global distribution

of BTV has recently altered markedly, particularly in the northern hemisphere (Gibbs and Greiner, 1994; Maclachlan, 2011; Maclachlan and Mayo, 2013; Saegerman et al., 2008; Tabachnick, 2010). Thus, BT is viewed currently as a globally emerging disease (Gibbs et al., 2008; Guis et al., 2012; Jimenez-Clavero, 2012; Maclachlan and Guthrie, 2010; Purse et al., 2008; Weaver and Reisen, 2010). Outbreaks of BT can be economically devastating to livestock production and the presence of BTV in a country can adversely impact the trade and movement of livestock (Maclachlan and Osburn, 2006; Velthuis et al., 2010). Control of BTV infection and BT disease can be difficult, and prevention of the disease is typically reliant on vaccination of susceptible livestock (reviewed: Maclachlan and Mayo, 2013). Sound vaccination strategies should logically be based on a thorough understanding of the ruminant immune response to BTV, which is the focus of this review.

2. Innate immunity

The innate immune response constitutes the first line of defense of animals against viral infections. Innate immune mechanisms are also critical to transition to an effective adaptive immune response (humoral and cellular). Natural BTV infection of ruminants typically occurs following the bite of any one of the multiple species of hematophagous *Culicoides* midges that serve as biological vectors of the virus (Barratt-Boyes and Maclachlan, 1995; Gibbs and

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Greiner, 1994; Mellor et al., 2000; Tabachnick, 2010; Verwoerd and Erasmus, 2004). After cutaneous instillation of BTV following the bite of an infected midge, the virus is quickly translocated in dendritic cells to the regional lymph node where initial replication occurs (Barratt-Boyes and Maclachlan, 1994; Ellis et al., 1991; Hemati et al., 2009; Pini, 1976). Conventional dendritic cells are recruited in large numbers to the skin following virus infection, facilitating further replication of the virus at the site of initial infection in the skin (Hemati et al., 2009). Interruption of lymphatic flow to the regional lymph node adjacent to the site of virus inoculation markedly delays the onset of viremia (Barratt-Boyes and Maclachlan, 1994).

Initial replication of BTV in the regional lymph node likely occurs in dendritic cells, macrophages, endothelium and possibly lymphocytes, as these are major cellular targets of BTV (Maclachlan et al., 2009; Darpel et al., 2012). *In vitro* BTV infection of ovine conventional dendritic cells from skin lymph leads to the production of proinflammatory and immune cytokines (interleukins [IL-1 β , IL-6, IL-12]) as well as to expression of co-stimulatory molecules that promote the proliferation of BTV-specific CD4⁺ and CD8⁺ T cells (Hemati et al., 2009). Similarly, *in vitro* BTV infection of bovine monocyte-derived macrophages leads to their activation with production of tissue necrosis factor (TNF- α), interleukins (e.g. IL-1 β , IL-8) and inducible nitric oxide synthetase (Drew et al., 2010a). Replication of BTV in the regional lymph node stimulates a marked cellular response characterized by increased numbers of B cells within the node itself and the appearance of virus-specific antibodies and activated CD8⁺ T cells in the draining efferent lymph (Barratt-Boyes et al., 1995).

Following initial replication of BTV at the site of inoculation and within the adjacent draining lymph node, the virus is then disseminated to a variety of tissues throughout the body where secondary replication occurs in mononuclear phagocytic cells (macrophages and dendritic cells), endothelial cells, lymphocytes including $\gamma\delta$ T cells, and perhaps other cell types (Barratt-Boyes et al., 1992; Barratt-Boyes and Maclachlan, 1994, 1995; Darpel et al., 2012; DeMaula et al., 2001, 2002; Ellis et al., 1991; Hemati et al., 2009; Lee et al., 2011; Maclachlan et al., 1990, 2009; Mahrt and Osburn, 1986; Umeshappa et al., 2012; Whetter et al., 1989). Viral RNA (especially dsRNA) is detected by pattern recognition receptors (PRR) in the cytoplasmic and endosomal compartments of host cells, notably toll-like receptors (TLRs) or members of the retinoic acid inducible gene (RIG-1)-like family of cytoplasmic receptors (Levy et al., 2011). Different PRR utilize distinct signaling pathways to trigger the production of Type I and Type III IFNs and other proinflammatory cytokines.

It has long been recognized that BTV infection is a strong inducer of Type I IFN, both *in vivo* and *in vitro* (Foster et al., 1991; Fulton and Pearson, 1982; Huismans, 1969; Maclachlan et al., 1984; Maclachlan and Thompson, 1985; Russell et al., 1996). Whereas adult mice are resistant to productive BTV infection, Type I (α/β) IFN receptor knockout mice (IFNAR [–/–]) are highly susceptible to lethal infection with the virus (Calvo-Pinilla et al., 2009a, 2010; Franceschi et al., 2011). It recently has been shown that BTV induces Type I IFN production in plasmacytoid dendritic cells via a MYD88-dependent TLR7/8-independent sensing and signaling pathway, whereas cytoplasmic helicases (RIG-1, MDA5) mediate sensing and signaling in BTV-infected epithelial cells (Chauveau et al., 2012; Ruscanu et al., 2012). The BTV NS3 protein specifically interferes with IFN production in epithelial cells by inhibiting the RIG1-like receptor mediated signaling pathway (Chauveau et al., 2013). Plasmacytoid dendritic cells and Type I IFN may exert a central role in triggering B cell responses and promoting BTV-specific antibody synthesis (humoral immunity), as has recently been shown for rotavirus, which is another dsRNA virus of the family *Reoviridae* (Deal et al., 2013).

Infection of dendritic cells, macrophages, endothelial cells and others during BTV infection of ruminants leads to the production of a variety of proinflammatory and vasoactive mediators including Type I IFNs, TNF- α , and various interleukins and prostanoids, but the response can vary depending on the cell types that are infected as well as their tissue location (Channappanavar et al., 2012; DeMaula et al., 2001, 2002; Drew et al., 2010a; Hemati et al., 2009; Ruscanu et al., 2012, 2013; Umeshappa et al., 2012). Furthermore, although these virus-induced cytokine and chemokine mediators may serve to limit/control the infection during its initial stages and to promote the development of a strong acquired immune response, they may also contribute to the pathogenesis of the capillary leakage syndrome and coagulopathy that characterizes clinical BT in ruminants—the so-called “cytokine storm” of hemorrhagic fevers (Fig. 1) (Drew et al., 2010b; Maclachlan et al., 2008, 2009; Umeshappa et al., 2012). Thus, the virulence to sheep of different strains/serotypes of BTV is correlated with the severity of the vascular lesions they induce and to serum concentrations of acute phase proteins, and not to viral loads (Sanchez-Cordon et al., 2013). Similarly, DeMaula et al. (2002) showed that vascular injury and enhanced coagulation, as assessed by the ratio of plasma thromboxane to prostacyclin, were significantly greater in sheep than cattle inoculated with the same strain of BTV, consistent with the sensitivity of sheep and relative resistance of cattle to expression of clinical BT.

3. Cellular immunity

Cell-mediated immunity (CMI) typically limits viral spread during the initial stages of acute viral infections by the destruction of virus-infected cells (virus factories). Although BTV infection of ruminants clearly results in changes in lymphocyte populations and dynamics both locally (adjacent to the site of infection) and systemically (Barratt-Boyes et al., 1995; Ellis et al., 1990, 1991; Hemati et al., 2009; Umeshappa et al., 2012), the CMI response of ruminants to BTV infection remains poorly characterized. CD8⁺ cytotoxic T lymphocytes (CTLs) have been identified following BTV infection of both mice and sheep, although their activity is apparently transient and their precise role in mediating virus clearance is poorly defined (Andrew et al., 1995; Calvo-Pinilla et al., 2012; Jeggo and Wardley, 1982a,b; Jeggo et al., 1984, 1986). Adoptive transfer of CTLs harvested from the thoracic duct of a sheep previously infected with BTV partially protected recipient sheep against challenge infection with either homologous or heterologous serotypes of BTV (Jeggo et al., 1984, 1986).

The BTV NS1 and VP2 proteins have been proposed to be major targets of the ovine CMI response to BTV, although the viral protein-specific CTL response of individual sheep can vary markedly (Andrew et al., 1995; Janardhana et al., 1999; Takamatsu and Jeggo, 1989). Ovine CTLs specific for the non-structural NS1 protein are cross reactive amongst different BTV serotypes. Similarly, Calvo-Pinilla et al. (2012) described generation of NS1-specific CD8⁺ T cells and heterotypic immunity in IFNAR mice that were immunized with recombinant BTV proteins, and Jones et al. (1996, 1997) earlier showed that the BTV nonstructural proteins, especially NS2, can induce cross-reactive CTLs in mice. Together these findings offer some promise that a polyvalent vaccine strategy to prevent BTV infection is potentially feasible, as the NS1 and NS2 proteins are relatively conserved among field strains of BTV regardless of serotype. However, neither homologous nor heterologous protection was obtained with a recombinant adenovirus expressing only the NS1 protein of BTV-2 despite strong induction of protein-specific CD8⁺ T cells in the vaccinated sheep (unpublished data). Similarly, although BTV core-like particles that include only VP3 and VP7 have been reported to induce partial protection against

Role of Mononuclear Phagocytes in BTV Infection

1. Viral dissemination

Lymph and blood



2. Innate response

Virus control/
inflammation/
bleeding/coagulation



3. Acquired immunity

Viral clearance and
protection against
homologous reinfection

Fig. 1. Potentially critical interactions of BTV with mononuclear phagocytes (dendritic cells, macrophages) during infection of ruminants. Specifically, these cells may: (1) contribute to dissemination of BTV in afferent lymph and blood, (2) produce antiviral and inflammatory cytokines/chemokines that initially control BTV infection and possibly trigger the cytokine storm and associated coagulopathy and hemorrhage and (3) elicit acquired immune responses that lead to viral clearance and subsequent protection against re-infection with the homologous BTV serotype.

both homologous and heterologous challenge in sheep, complete protection requires immunization with the VP2 of the respective BTV serotype (Roy et al., 1994; Stewart et al., 2012). Thus, despite the potential promise of a polyvalent BTV vaccination strategy based on CMI responses to different BTV proteins, little progress has been made in confirming the viability of such an approach and the precise role of CMI in either mediating virus clearance during primary infection or conferring resistance to reinfection with homologous or heterologous BTV serotypes awaits thorough clarification.

4. Antibody (humoral) immunity

BTV infection of ruminants results in the prompt production of antibodies to both structural and nonstructural viral proteins (Huisman and Erasmus, 1981; Maclachlan et al., 1987; Richards et al., 1988). Immune competence of fetal ruminants to BTV infection arises prior to mid-gestation (Osburn et al., 1982; Maclachlan et al., 1984). Antibodies to core protein VP7 are broadly serogroup reactive as this protein is conserved amongst most BTV serotypes and strains, thus detection of antibodies to VP7 is the basis of the competitive ELISA (cELISA) assays that are now widely used for serological diagnosis of BTV infection of livestock (Afshar, 1994; Anderson, 1984; Maclachlan and Mayo, 2013). BTV-specific neutralizing antibodies can confer long-lasting resistance to reinfection with the homologous BTV serotype, which is the basis of vaccination strategies to prevent BT disease (Huisman et al., 1987; Noad and Roy, 2009; Savini et al., 2008; Spreull, 1905; Stewart et al., 2012; Verwoerd and Erasmus, 2004). Interestingly, although neutralizing antibodies confer serotype-specific resistance to reinfection, viremia can continue for extended intervals in the course of primary infection despite the presence of such antibodies (Barratt-Boyes and Maclachlan, 1994, 1995; Bonneau et al., 2002; Foster et al., 1991; Di Galleonardo et al., 2011; Luedke et al., 1969; Martinelle et al., 2011; Richards et al., 1988). This ability of BTV to avoid neutralization during primary infection is attributed to its intimate association with the cell membranes of circulating blood cells, erythrocytes in particular, which also leads to an extended duration (several months) of reverse-transcriptase-polymerase chain reaction (RT-PCR) positive status of ruminants following BTV infection (Barratt-Boyes and Maclachlan, 1994; Brewer and Maclachlan,

1992, 1994; Di Galleonardo et al., 2011; Luedke et al., 1969; Maclachlan et al., 1990, 1994, 2009; Martinelle et al., 2011).

The neutralizing epitopes of BTV are localized to specific interactive regions of the VP2 outer capsid protein. Sheep inoculated with VP2 that is either isolated from viral particles or generated by *in vitro* expression produce virus neutralizing antibodies and are resistant to challenge infection with the homologous BTV serotype (Huisman and Erasmus, 1981; Huisman et al., 1987; Roy et al., 1990, 1992, 1994; Stewart et al., 2012). At least some neutralization epitopes are highly conformationally dependent, reflecting not only folding of the VP2 protein on itself but also its conformational interaction with the other outer capsid protein, VP5 (Appleton and Letchworth, 1983; Cowley and Gorman, 1987; DeMaula et al., 1993, 2000; Gould et al., 1988; Gould and Eaton, 1990; Heidner et al., 1990; Mertens et al., 1989). DeMaula et al. (2000) showed that whereas most variants that were resistant to neutralization with individual neutralizing monoclonal antibodies (MAbs) had amino acid substitutions in key regions of VP2, the VP2 of one MAb-neutralization resistant variant virus was unchanged but this virus had a substitution in VP5, confirming unambiguously the importance of the conformational interaction between VP2 and VP5 in BTV neutralization. Individual neutralizing epitopes are sometimes conserved between serotypes in either a neutralizing or non-neutralizing conformation (Maclachlan et al., 1992; Pierce et al., 1995; Ristow et al., 1988; Rossitto and Maclachlan, 1992; White and Eaton, 1990). The presence of common neutralization epitopes between BTV serotypes is further confirmed by serological cross reactivity of certain serotypes of the virus, and by the fact that animals inoculated or immunized with one or more BTV serotypes can develop neutralizing antibodies against serotypes to which they were never exposed (Dungu et al., 2004; Rossitto and Maclachlan, 1992; Thomas, 1985).

Studies with BTV proteins expressed from recombinant pox, herpes and baculoviruses confirm that induction of neutralizing antibodies and protection of sheep against challenge infection with the homologous BTV serotype is best achieved by co-expression of both the VP2 and VP5 outer capsid proteins, including their co-expression as virus-like particles or in combination with BTV core proteins (Lobato et al., 1997; Ma et al., 2012; Roy et al., 1990, 1992, 1994). Thus, we have utilized the canarypox (CP) virus expression vector to evaluate induction of BTV-neutralizing antibodies in sheep using a CP construct co-expressing both VP2

Table 1
Effect of immunization of sheep with different doses of recombinant canarypox virus co-expressing the VP2 and VP5 genes of bluetongue virus (BTV) prior to challenge infection with BTV serotype 17.

TREATMENT	SN*		cELISA**		qRT PCR***	Temp. (>103.5° F)
	Pre ⁺	Post	Pre	Post		
Control	0/4	3/3	0/4	3/3	4/4	4/4
Vaccine-low	0/4	4/4	0/4	4/4	4/4	1/4
Vaccine-med	3/4	4/4	0/4	1/4	1/4	0/4
Vaccine-high	4/4	4/4	0/4	0/4	0/4	0/4

* SN = serum neutralization test to BTV-17; titer < 10 considered negative.

** cELISA = competitive ELISA to BTV core protein VP7 (VMRD).

*** qRT-PCR = quantitative PCR based on detection of BTV S10 gene after challenge infection, as previously described (Boone et al., 2007).

⁺ Serological status immediately before and 14 days after (Post) BTV challenge infection.

** 1 sheep died in the course of the study.

and VP5 of a strain of BTV-17 (CP-BTV) (Boone et al., 2007). This recombinant CP-BTV construct confers complete resistance of vaccinated sheep to challenge infection with the homologous virus serotype. In an effort to better establish the protective immunity induced by the recombinant CP-BTV construct we undertook a dose titration/challenge infection study using 16 seronegative sheep housed in insect-secure isolation facilities and methods essentially as previously described (Boone et al., 2007). The sheep were assigned individually to one of four treatment groups: four control sheep were immunized with a CP-vectored vaccine against West Nile virus (Recombitek®, Merial, GA); four high dose vaccinated sheep were inoculated subcutaneously and intramuscularly (SC/IM) with 2×10^8 particles of the recombinant CP-BTV construct; four medium dose vaccinated sheep were inoculated SC/IM with 2×10^7 particles of CP-BTV; and four low dose vaccinated sheep were inoculated SC/IM with 2×10^6 particles of CP-BTV. All sheep were immunized twice with their respective inocula at a 3 week interval and then challenged at 3 weeks after booster immunization by SC inoculation of approximately $10^{4.9}$ tissue culture infectious doses of a field strain of BTV-17, as previously described (Boone et al., 2007). All sheep were seronegative by group-specific cELISA prior to challenge (Table 1), confirming that sheep vaccinated with the CP-BTV can be distinguished from those previously infected with BTV (DIVA). Whereas all control and low dose vaccinates, and one of the four medium dose vaccinated sheep seroconverted by cELISA at 14 days after challenge infection, the three other medium dose and all of the high dose vaccinated sheep remained seronegative by cELISA following challenge. Furthermore, whereas the four control and one low dose vaccinated sheep all developed marked fever (>103.5° F) by day 6–7 after challenge infection, three of the four low dose vaccinates and all sheep immunized with either medium or high doses of the CP-BTV construct were resistant to challenge infection as determined by the absence of fever. Viral RNA was not detected by quantitative RT-PCR (qRT-PCR) in the blood any of the high dose vaccinated sheep, and in only one of the four medium dose vaccinated sheep. In contrast, all of the control and low dose vaccinated became infected after challenge as confirmed by qRT-PCR analysis of their blood (Table 1).

In summary, these studies confirm that induction of protective immunity in sheep can be accomplished by immunization with only the BTV outer capsid proteins, and that there is a titratable response to vaccination such that immunization with relatively large quantities of the CP-BTV construct can induce sterilizing immunity whereas lower doses protect against development of clinical signs following challenge but not infection. This immunity is somewhat correlated to induction of neutralizing antibody in serum, however the data further suggest that animals with a

minimal neutralizing antibody response following vaccination may be primed to more quickly and effectively respond to challenge infection. Similar observations of immune “priming” have been made with other recombinant vaccine constructs that utilize other expression vectors such as capripox and myxoma viruses (Perrin et al., 2007; Top et al., 2012), and with other CP-vectored animal vaccines such as that to West Nile virus (Gardner et al., 2007).

5. Vaccinology

A thorough understand of the immune response to animals to BTV infection is central to the logical development of efficacious vaccines, which is important because at-risk countries typically use vaccination of livestock to control any incursion or outbreak of BT (Maclachlan and Mayo, 2013). Early investigators recognized that livestock that recovered from BT were often resistant to the disease, which spurred initial efforts to develop vaccines (Spreull, 1905). However, it was later recognized that vaccination can be problematic given the plurality of BTV serotypes (currently 26) coupled with serotype-specific immunity in livestock, meaning that vaccines potentially have to be developed to all 26 currently recognized BTV serotypes (reviewed: Noad and Roy, 2009; Oya Alpar et al., 2009; Savini et al., 2008; Stewart et al., 2012; Zientara et al., 2010). Furthermore, there are few available commercial vaccine products for BTV other than the live-attenuated (modified live virus [MLV]) vaccines that continue to be used to prevent BT among commercial sheep in several areas of the world (e.g. South Africa, the United States of America). MLV vaccines are relatively inexpensive to produce and they are effective as they can induce protective immunity similar to that which follows natural infection. Despite these attributes, the use of inactivated vaccines is increasingly preferred as inactivated vaccines enjoy several inherent advantages over MLV vaccines. Specifically, as compared to MLV vaccines, inactivated vaccines cannot cause disease or revert to virulence, reassort genes with field or MLV viruses, nor cross the placenta to cause reproductive losses (reviewed: Maclachlan and Mayo, 2013; Noad and Roy, 2009; Savini et al., 2008; Stewart et al., 2012; Zientara et al., 2010). However, inactivated vaccines suffer from their relative slow onset of immunity as compared to MLV vaccines, and the lack of commercial products for most BTV serotypes.

Although not yet available commercially, replication defective vaccine viruses produced using reverse genetics technology offer the potential for vaccine constructs that combine the benefits of MLV vaccines with the safety of inactivated ones (Celma et al., 2013; Matsuo et al., 2011). Other “next generation” products such as baculovirus-expressed virus like particles (VLPs) and viral-vectored recombinant vaccines, including the CP-BTV recombinant described earlier, have been shown to be effective experimentally but their inherent cost and limited market potential have prevented their commercial use to date (Boone et al., 2007; Celma et al., 2013; Noad and Roy, 2009; Stewart et al., 2010).

The recent availability of IFNAR mice as an experimental model system has further facilitated the study of immunity to BTV infection, and to the development of potentially efficacious vaccine constructs (Calvo-Pinilla et al., 2009a, 2010). IFNAR mice can be protected against lethal BTV challenge by neutralizing antibodies induced by immunization with recombinant VP2 alone or in combination with other viral proteins (Calvo-Pinilla et al., 2009b, 2012; Franceschi et al., 2011; Jabbar et al., 2013; Ma et al., 2012). Immunization of IFNAR mice with individual or cocktails of recombinant BTV proteins also can induce varying degrees of heterotypic immunity associated with the production of protein-specific CD8+ T cells (Calvo-Pinilla et al., 2012). It is to be stressed, however, that although the IFNAR murine model provides a useful model for initial screening of potential vaccines, validation in ruminant livestock

is clearly required and particularly in situations where the Type I IFN response may be critical to the stimulation of critical acquired immune responses.

In summary, although ongoing studies have repeatedly confirmed that a recombinant subunit vaccination strategy is viable for protection of livestock against BTV infection, the expression of immunogenic VP2 can be challenging given the conformational nature of individual epitopes and their stability during storage, and because of the apparently serotype-specific nature of immunity to BTV infection of livestock (DeMaula et al., 2000; Savini et al., 2008; Stewart et al., 2012; Zientara et al., 2010). Thus, current vaccination strategies to prevent BTV infection of livestock continue to be reliant on historic technologies, with all of their attendant potential disadvantages (Maclachlan and Mayo, 2013; Savini et al., 2008).

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