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Bovine respiratory syncytial virus infection

Jean-Francois VALARCHER^a, Geraldine TAYLOR^{b*}

^a IVI-Animal Health, Lärkbacken, 740 20 Vänge, Uppsala, Sweden

^b Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN, United Kingdom

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Abstract – Bovine respiratory syncytial virus (BRSV) belongs to the pneumovirus genus within the family *Paramyxoviridae* and is a major cause of respiratory disease in young calves. BRSV is enveloped and contains a negative sense, single-stranded RNA genome encoding 11 proteins. The virus replicates predominantly in ciliated respiratory epithelial cells but also in type II pneumocytes. It appears to cause little or no cytopathology in ciliated epithelial cell cultures in vitro, suggesting that much of the pathology is due to the host's response to virus infection. RSV infection induces an array of pro-inflammatory chemokines and cytokines that recruit neutrophils, macrophages and lymphocytes to the respiratory tract resulting in respiratory disease. Although the mechanisms responsible for induction of these chemokines and cytokines are unclear, studies on the closely related human (H)RSV suggest that activation of NF- κ B via TLR4 and TLR3 signalling pathways is involved. An understanding of the mechanisms by which BRSV is able to establish infection and induce an inflammatory response has been facilitated by advances in reverse genetics, which have enabled manipulation of the virus genome. These studies have demonstrated an important role for the non-structural proteins in anti-interferon activity, a role for a virokinin, released during proteolytic cleavage of the fusion protein, in the inflammatory response and a role for the SH and the secreted form of the G protein in establishing pulmonary infection. Knowledge gained from these studies has also provided the opportunity to develop safe, stable, live attenuated virus vaccine candidates.

BRSV / pathogenesis / respiratory disease / cattle / vaccines

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* Corresponding author: geraldine.taylor@bbsrc.ac.uk

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1. INTRODUCTION

Bovine respiratory syncytial virus (BRSV) is an enveloped, non-segmented, negative-stranded RNA virus and is a major cause of respiratory disease in young calves [128]. BRSV is closely related to human (H)RSV, which is a major cause of respiratory disease in young children, and the epidemiology and pathogenesis of infection with these viruses are similar [156]. These features make BRSV infection in calves a good model for the study of HRSV. In return, findings obtained by studying HRSV *in vivo* in humans, in small animal models or *in vitro* have allowed a better understanding of some virological and pathogenic characteristics of its counterpart in bovines. Although these viruses are very similar in many aspects, there are differences, such as their level of replication *in vitro* and *in vivo* in their respective hosts, differences in the peptide released as a result of cleavage at the two furin cleavage sites of the F protein and the relative functions of NS1 and NS2 in the inhibition of type I interferon induction.

HRSV and BRSV are members of the *Pneumovirus* genus within the subfamily *Pneumovirinae*, family *Paramyxoviri-*

dae of the order *Mononegavirales* [26]. Other pneumoviruses include pneumonia virus of mice (PVM), ovine (O)RSV and caprine (C)RSV. Although the RS viruses are structurally and antigenically related, BRSV and CRSV are the most closely related [2, 82, 123, 147].

By electron microscopy, the morphology of the RSV virions appears to be either very pleomorphic, with a shape roughly rounded and a diameter between 150 and 35 nm, or filamentous with a length that can reach 5 μm and a diameter between 60 and 100 nm [147]. Regardless of the structure, each infectious particle contains a single functional copy of the genome. In contrast to HRSV, but similar to 80% of caprine virions, BRSV virions are organized in a network and viral particles are linked to each other by bridges of 12 ± 3 nm [10]. The roles of these structures remain unexplained.

In contrast to HRSV, several vaccines against BRSV are available on the market. However, the development of a second generation of BRSV vaccines with greater efficacy in the presence of maternal antibodies and which induce more durable protection would be facilitated by a greater understanding of the pathogenesis of the

virus. In this paper, after describing the characteristics of the virus and the disease induced by BRSV, we will review some recent findings on host-pathogen interactions that could provide a basis for the future development of safe, stable, live attenuated virus vaccines.

2. THE VIRION

The BRSV virion consists of a lipid envelope, derived from the host plasma membrane, containing three virally encoded transmembrane surface glycoproteins, which are organised separately into spikes on the surface of the virion (Fig. 1). These glycoproteins are the large glycoprotein (G), the fusion protein (F) and the small hydrophobic protein (SH) [33]. The envelope encloses a helical nucleocapsid, which consists of the nucleoprotein (N), phosphoprotein (P), the viral RNA-dependent polymerase protein (L) and a genomic RNA of around 15000 nucleotides. In addition, there is a matrix M protein that is thought to form a layer on the inner face of the envelope and a transcriptional anti-termination factor M2-1. The genome also encodes an RNA regulatory protein M2-2 and two non-structural proteins, NS1 and NS2 [33] (Fig. 1). In addition, the viral particle contains cellular proteins, such as actin, which has been demonstrated on the surface of HRSV [148], caveolin-1 [18] and MHC class I molecules¹. Thus, BRSV propagated in bovine cells can be neutralised by monoclonal antibodies specific for bovine MHC class I.

The genomic RNA is the template for replication and transcription. The genomic RNA, which is transcribed in a sequential fashion from the 3' end, encodes ten mRNA. There is a polar transcription gradient such that 3'-terminal genes are transcribed more frequently than those at the 5'

end (Fig. 2). The 10 mRNA are then translated into 11 viral proteins. Some properties of the BRSV proteins and their amino acid (AA) identity with HRSV proteins are shown in Table I. The *Pneumovirus* genome is characterised by the existence of two non-structural proteins, NS1 and NS2, and a transcriptional overlap between M2 and L that lead to the synthesis of M2-1 and M2-2 proteins.

2.1. BRSV proteins

2.1.1. Non-structural proteins NS1 and NS2

One of the major differences between the pneumoviruses and the other *Paramyxoviridae* is the presence of two non-structural (NS) proteins, NS1 and NS2, which have 136 AA and 124 AA, respectively [102]. The genes encoding these two proteins are abundantly transcribed in virus-infected cells, however, the proteins are detected only in trace amounts in purified virions. There is evidence that the HRSV NS1 protein coprecipitates with the M protein [42], and is a strong inhibitor of viral RNA transcription and replication [7]. The NS2 protein also appears to be a transcriptional inhibitor but at a lower level than the NS1 protein [7]. The NS2 protein colocalizes with the P and N proteins in infected cells [164] but does not coprecipitate with any viral protein [42]. These proteins are not essential for virus replication in vitro, although the growth of recombinant HRSV and BRSV lacking one or other of these proteins is attenuated in cell culture [20, 115, 137]. The NS1 and NS2 proteins play an important role in regulating IFN α/β and their role in the pathogenesis and host-range restriction of BRSV is discussed below.

2.1.2. Small hydrophobic SH protein

The SH protein is a short integral membrane protein and is not essential for virus

¹ Taylor G., unpublished observations.

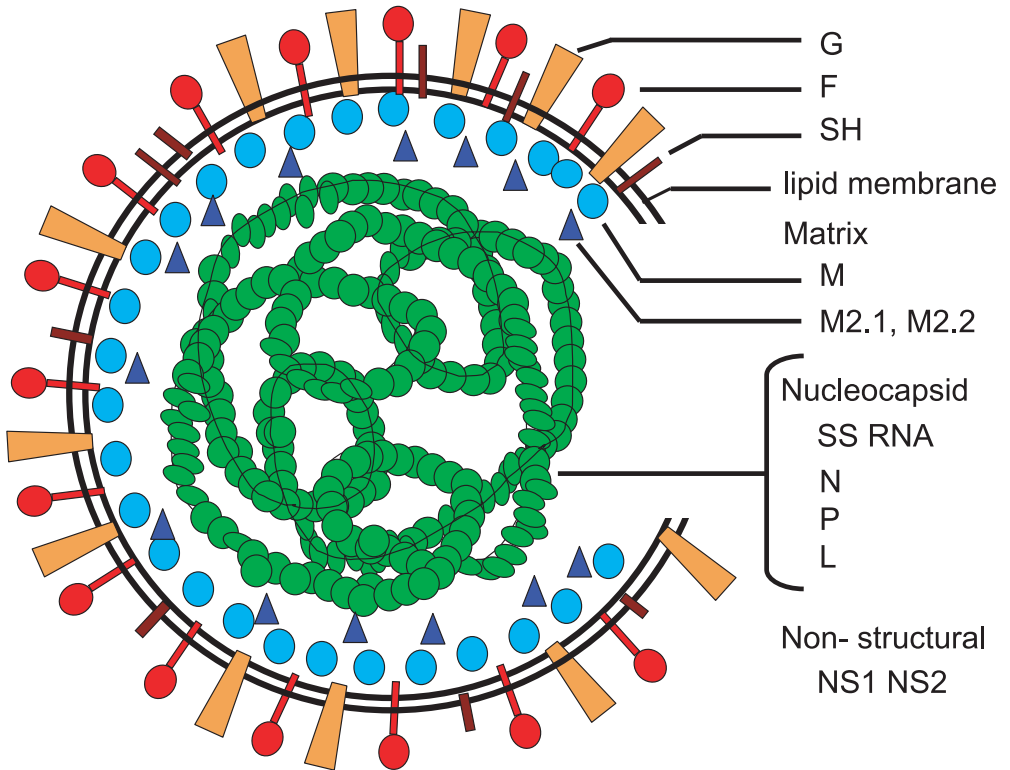


Figure 1. Diagram of bovine respiratory syncytial virus.

replication in vitro or in vivo and its function is not well defined [65]. This protein is 81 AA [111] and varies by up to 13% between different BRSV isolates [109]. The SH protein of HRSV exists as different forms, SHg (13–15 kDa), SHp (21–30 kDa), non-glycosylated SH0 (7.5 kDa) and a nonglycosylated form SHt (4.8 kDa) which is derived from initiation at a second methionine in the open reading frame (ORF) [4, 111]. There is evidence that the SH protein may play a role in virus-mediated cell fusion by interacting with the F protein [43, 59].

2.1.3. Glycoprotein G

The G glycoprotein is 257AA or 263AA depending upon the BRSV isolate [92] and

it is a type II glycoprotein with a signal/anchor domain between AA residues 38 and 66. The G protein was identified as the major attachment protein because antibodies specific to the G protein blocked the binding of virus to cells [86] and it is a major protective antigen of BRSV [132, 133]. However, the G protein is structurally different from its counterparts in other paramyxoviruses (HN and H proteins) [78, 79, 165] and is a heavily glycosylated nonglobular protein similar to cellular mucins. The G protein is synthesised as two forms, a membrane-anchored form and a secreted form [60, 61], which arises from translational initiation at a second AUG in the ORF [108]. Around 80% of the G protein is produced as the secreted form 24 h after infection [61].

Table I. BRSV proteins.

Protein	Protein name	Size (AA)	%AA identity with HRSV	M _r (kd)	Functions
NS1	Non-structural protein 1	136	69 [102]	13.8	Inhibition of and resistance to IFN α/β
NS2	Non- structural protein 2	124	84 [102]	14.5	Inhibition of and resistance to IFN α/β
F	Fusion protein	574	81 [84]	70	Membrane fusion
G	Glycoprotein	257	30 [83]	90	Attachment
SH	Small hydrophobic protein	73	38–44 [4]	7.5–60	Unknown function
M	Matrix protein	256	89 [111]	25	Virion assembly
M2-1	Matrix protein 2-1	186	80 [172]	22	Trancription anti-termination factor
M2-2	Matrix protein 2-2	95	43 [172]	11	RNA regulatory protein
N	Nucleoprotein	391	93 [112]	45	Binds to genomic and anti-genomic RNA to form RNase-resistant nucleocapsid
P	Phosphoprotein	241	81 [91]	33	Chaperonin and polymerase co-factor
L	Polymerase protein	2161	84 [171]	250	RNA dependent RNA polymerase

The BRSV G protein appears as a trimer at the surface of the virion and contains several independently folded regions, in which the ectodomain consists of a conserved central hydrophobic region located between two polymeric mucin-like regions [31, 77–80, 166]. This conserved central hydrophobic region contains four conserved cysteine residues, which form two disulphide bridges. However, in some field isolates of BRSV, one or several of the cysteines is mutated to either an alanine or an asparagine [126, 150]. The major epitope of this region is located at the tip of a loop, overlapping a relatively flat surface formed by the double disulphide-bonded cysteine noose and lined by highly conserved residues [78, 79]. This epitope appears to be immunodominant [79] and the effect of the loss of one or more cysteines on antibody recognition has not been determined.

The G protein of HRSV and BRSV probably favours the attachment of the virion at the cell surface by the in-

teraction of its heparin-binding domains with glycosaminoglycans on cell membranes [138]. In addition, the G is suspected to have other roles by interacting with the immune system. It has been proposed that the secreted form might act as a decoy by binding to neutralising antibodies. Furthermore, the conserved cysteine rich region has homology with the CX3C chemokine motif, which may facilitate infection by interacting with the CX3C chemokine receptor, CX3CR1 [146]. The G protein may also interact with L-selectin (CD62L), annexin II [90], and surfactant proteins [48, 62]. The role of the G protein in the pathogenesis of BRSV infection will be discussed below.

2.1.4. Fusion F protein

The F protein mediates binding of virus to cells and is responsible for virus penetration by fusion between the viral and host cell membranes, delivering the nucleocapsid to the cytoplasm. It is also

bovine CD8⁺ T cells [46], protection induced by these proteins may be mediated by cytotoxic T cells. The N protein is present in large amounts in the virion and in infected cells and has several functions. In combination with P, L and possibly M2-2, the N protein is a major element of the nucleocapsid and protects the viral genome RNA from RNase [93, 112]. The N protein seems to play a role in the transition between the transcription and the replication phases of the viral RNA [93]. The P protein has 241 AA [2] and appears to act as a chaperone for soluble N and is implicated as a regulation factor for viral transcription and replication. The polymerase L of BRSV has a size of 2162 AA [171] and is an RNA-dependant RNA polymerase. This protein is responsible for the viral transcription and replication [171].

2.1.6. Matrix proteins

In contrast to the *Paramyxoviridae*, RSV has 3 matrix proteins. The M protein is 256 AA in length and has little sequence relatedness with other paramyxovirus M proteins. This protein is located on the inner surface of the viral envelope and plays an important role in the formation of virus-like particles [136]. The M2-1 and M2-2 proteins are both encoded by the M2 mRNA, which contains two overlapping translational ORF [30]. The upstream ORF1 encodes the M2-1 protein (194 AA) and the downstream ORF2 encodes the M2-2 protein (83-90 AA) [1, 14]. The M2-1 protein is an anti-termination factor that promotes transcriptional chain elongation and increases the frequency of readthrough at gene junctions [57]. The M2-2 protein mediates a regulatory switch from transcription to RNA replication [14].

2.2. Virus replication

Following fusion of the viral envelope and the cell membrane, the ribonucleopro-

tein (RNP) complex is released into the cytoplasm and transcription of the viral RNA by the polymerase begins. Transcription involves a sequential start-stop mechanism that produces subgenomic RNA. It is guided by short conserved signals that flank each mRNA coding unit, namely a transcription gene start (GS) signal and a termination/polyadenylation gene end (GE) signal [73, 74]. There is a polar transcription gradient with promoter-proximal genes being transcribed more frequently than downstream genes. RNA replication occurs when the polymerase switches to a readthrough mode resulting in the synthesis of a positive-sense replicative intermediate which acts as a template for replicating the negative strand genomic RNA (Fig. 2). Both genomic and antigenomic RNA are packaged. The nucleocapsids are assembled in the cytoplasm and then migrate with the M protein toward the cellular membrane in which viral glycoproteins are present. The viral budding might occur directly at the surface of the cellular membrane or into cytoplasmic vesicles [6]. In polarised airway epithelial cells, budding of HRSV occurs at the apical surface [173] and this is also true for BRSV infection of polarised bovine airway epithelial cells, *in vitro*².

2.3. Antigenic and genetic subgroups of BRSV

Antigenic and genetic subtypes have been defined for BRSV through several studies [44, 81, 98, 120, 150]. Using monoclonal antibodies (mAb) directed against the G protein, a classification into four antigenic subgroups, A, B, AB and untyped, has been established for BRSV [44, 120]. Until recently, isolates of subgroup B BRSV had not been detected since 1976 [126]. The existence of six genetic

² Valarcher J.F., Sadler R., Taylor G., unpublished observations.

subgroups based on G and of five based on F or N has also been established [150]. This classification showed a spatial clustering of BRSV isolates that has been confirmed by studies including isolates collected in many countries [126, 154, 170]. The degree of genetic variability of BRSV is limited being less than 15%, which is less than that observed within one subgroup of HRSV [105]. The evolution of BRSV appears to be continuous and it has been proposed that evolution may be driven by selective pressure as a result of the immune response induced by vaccination [150].

The biological significance of these subgroups is not known. However, polyclonal sera obtained from calves vaccinated with the BRSV G protein from subgroup A virus recognised a different subgroup A BRSV but not a subgroup B or an untyped isolate [45]. Furthermore, recognition of a subgroup AB virus was less than that of the subgroup A isolate. Thus, mutations in the immunodominant region (AA 174–188) of the G protein may contribute to the lack of cross-protection between vaccine and field isolates. This might be relevant for the development of subunit vaccines.

3. EPIDEMIOLOGY AND CLINICAL SIGNS OF DISEASE

3.1. Epidemiology

Although cattle are the natural host of BRSV, it is possible that other species such as ovine, caprine, bison, chamoix or camelids may play an epidemiological role in certain circumstances [29, 40, 107, 113, 157]. The distribution of BRSV is worldwide and the virus has been isolated from cattle in Europe, America and Asia [63, 100, 122]. The virus causes regular winter outbreaks of respiratory disease in cattle [127]. A seroprevalence of 30–70% have been detected in cattle [3, 41, 54]. The frequency of BRSV infections is very

high and the virus might be responsible for more than 60% of the epizootic respiratory diseases observed in dairy herds [9, 41, 149] and up to 70% in beef herds [27, 114, 127]. The frequency of BRSV infections is correlated to the density of the cattle population in an area [41] and the age of the animal. Indeed more than 70% of beef calves were infected with BRSV by the age of nine months in England [127] and in cattle less than one year old in The Netherlands [70]. BRSV antibodies in calves between 5 and 11 months of age were detected in 35% of dairy herds ($n = 118$) in a Swedish study [54]. The frequency of infection in adults is difficult to assess because of the high BRSV seroprevalence in this category of animals.

Severe clinical signs are mainly observed in calves [70, 127, 160], but might also be observed in adult cattle [41]. The higher frequency of clinical signs induced by BRSV in young calves compared with adults can be explained by the level of specific immunity following frequent exposure to the virus. Indeed clinical signs are usually observed in cattle of all ages when BRSV is introduced in herds where most of the animals are naïve to the virus and are observed only in calves when the virus circulates regularly in the herd [156]. Maternally derived antibodies provide at least partial protection against clinical signs after natural and experimental BRSV infection [11, 68–70]. Although virus shedding has occasionally been detected upon experimental BRSV re-infection, little or no clinical disease is observed in reinfected animals [69, 109, 134, 145]. Similar to observations made for HRSV [67], exacerbated clinical signs have been observed following a natural BRSV infection in animals immunised with inactivated vaccines [5, 47, 119].

BRSV infection is associated with a high morbidity (60 to 80%) and mortality can reach up to 20% in some outbreaks. Clinical disease caused by BRSV is mainly

diagnosed in the autumn and winter in temperate climate zones [127]. Although BRSV infection occurs mainly in these seasons [155], it might also occur in the summer [41]. BRSV is mainly transmitted by direct contact between infected animals or by aerosol [94] but it cannot be excluded that it might also be spread by humans acting as a passive vector as observed for HRSV [55]. Some data indicate that BRSV may persist in infected animals [39, 140, 151]. However attempts to demonstrate re-excretion of BRSV from previously infected animals by treatment with 3-methyl indol, BVDV, BHV1 or dexamethasone have failed [158] and transmission of virus from carriers to susceptible animals has not been proven.

3.2. Clinical signs of disease and pathology

The incubation period for BRSV is estimated to be between 2 and 5 days. BRSV-infection may either be asymptomatic, limited to the upper airways or involve both the upper (URT) and lower respiratory tracts (LRT). URT disease is characterised by a cough with a seromucoid nasal and ocular discharge. In more severe infections, there is slight depression and anorexia, a decrease in milk yield in lactating cows, hyperthermia, polypnea (respiratory rate ≥ 60 movements per min) and an abdominal dyspnea. On auscultation of the lung abnormal breathing sounds caused by bronchopneumonia or bronchiolitis might be detected [159]. Animals may develop severe respiratory distress with a grunting expiration and breathing through an open mouth with the neck stretched and the head down, with saliva poring on the floor and with the tongue out. In these animals, pulmonary emphysema and oedema with some crackles and wheezes may be detected [12] and in some cases subcutaneous emphysema might occur [12, 19].

At necropsy, a broncho-interstitial pneumonia may be observed [19, 161]. Areas of the cranio-ventral parts of the lung are consolidated and a mucopurulent discharge may be seen from the bronchus and small bronchi. The caudo-dorsal parts of the lungs are often distended because of interlobular, lobular and sub-pleural emphysematic lesions [19]. Tracheobronchial and mediastinal lymph nodes may be enlarged, oedematous and sometimes haemorrhagic. If bacterial super-infections occur, the lung parenchyma is more swollen and consolidated and fibrin or suppurative bronchopneumonia may be observed.

Microscopic lesions are characterised by a proliferative and exudative bronchiolitis with accompanying alveolar collapse and a peribronchiolar infiltration by mononuclear cells [141]. Necrosis of the epithelium and apoptotic epithelial cells, which may be phagocytosed by neighbouring cells, can be seen [162]. Giant cells or syncytia may be present, either free in the bronchi lumen, in the bronchiolar epithelium or in the alveolar walls and lumina [162]. The lumen of bronchi, bronchioles and the alveoli are often obstructed by cellular debris consisting mostly of neutrophils, desquamated epithelial cells, macrophages and sometimes eosinophils [162] and may be aggravated by bronchiolar repair and reorganisation [71]. Eosinophils and lymphocytes (CD4⁺, CD8⁺ and WC1⁺ γ/δ T cells) are also observed in the lamina propria [141, 143, 162]. Alveolar changes are marked by an interstitial pneumonia and atelectasis in the consolidated areas and there may be severe emphysema and oedema with a rupture of alveolar walls in the caudo-dorsal area of the lung. The presence of microscopic changes in the caudo-dorsal area are rarely associated with the presence of BRSV antigen, syncytia or bronchiolitis [71]. An alveolar epithelisation with a pneumocyte hyperplasia

contributes to the enlargement of the alveolar septa with the cell infiltration. Hyaline membranes may be present in the alveoli following inflammation and pneumocyte necrosis [19].

4. PATHOGENESIS OF BRSV

BRSV replicates primarily in the superficial layer of the respiratory ciliated epithelium and replication can also be detected in type II pneumocytes [161, 162]. Although BRSV is cytopathic in tissue culture, little or no cytopathic effects are seen following infection of differentiated bovine airway epithelial cell cultures, *in vitro*³. A similar lack of obvious cytopathology has been observed in human airway epithelial cell cultures infected with HRSV [173], suggesting that the host response to virus infection plays a major role in RSV pathogenesis.

HRSV infection of human airway epithelial cells and alveolar macrophages results in activation of NF- κ B which leads to the induction of inflammatory chemokines and cytokines, such as RANTES (CCL5), MIP-1 α (CCL3), MCP-1 (CCL2), eotaxin (CCL11), IL-8 (CXCL8), TNF- α , interleukin (IL)-6, IL-1 etc. [15, 52, 53, 58, 95, 99], which contribute to inflammation by recruiting neutrophils, macrophages and lymphocytes to the airways. Although less well studied, BRSV infection induces a similar up-regulation of pro-inflammatory chemokines and cytokines in the bovine lung. Thus, increased levels of mRNA for IL-12, IFN γ , TNF α , IL-6, IL-18, IL-8, RANTES, MCP-1, MIP-1 α , IFN α and IFN β have been detected in pneumonic lesions from BRSV-infected gnotobiotic calves⁴. The molecular mecha-

nisms involved in RSV-induced activation of NF- κ B and initiation of the innate response are complex and appear to be mediated, at least in part by the interaction of the F protein with Toll-like receptor 4 (TLR4) [75] and by the interaction of dsRNA with TLR3 [110] (Fig. 3). Although TLR4 is expressed at high levels by macrophages and dendritic cells (DC) it is expressed only at very low levels on airway epithelial cells. However, HRSV infection increases TLR4 expression on human airway epithelial cells and increases their responsiveness to LPS [96]. Studies in BALB/c mice infected with HRSV demonstrated two peaks of NF- κ B activation. The immediate response following RSV inoculation was TLR4-dependent [52] and the second peak, which required RSV replication was mediated via TLR3 signalling pathways [110]. It is likely, but has not been formally demonstrated, that BRSV infection induces activation of NF- κ B and induction of pro-inflammatory cytokines by pathways similar to those demonstrated for HRSV.

An understanding of the mechanisms by which BRSV is able to establish infection in the bovine respiratory infection, induce an inflammatory response and respiratory disease has been greatly facilitated by advances in reverse genetics. This involves the production of infectious virus from cloned cDNA [32, 37]. The recovery of RSV from cDNA requires co-expression in cell culture of a complete copy of the viral RNA genome and the N, P, M2-1 and L proteins engineered to be expressed by bacteriophage T7 RNA polymerase. As described above, these are the constituents of the nucleocapsid and polymerase complex, which is the minimum unit of infectivity for mononegaviruses. The expressed viral components assemble and result in a productive infection. The recombinant virus produced in this way is identical to the biologically-derived virus except for whatever mutations have been introduced into

³ Sadler R., Valarcher J.-F., Hibbert L., Taylor G., unpublished observations.

⁴ Hibbert L., Valarcher J.-F., Taylor G., unpublished observations.

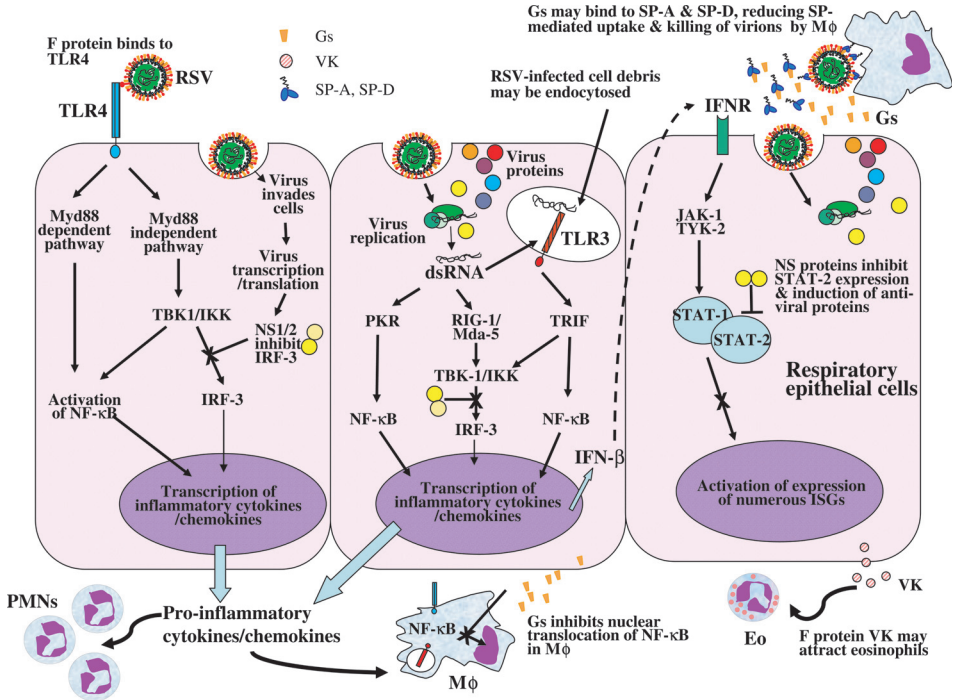


Figure 3. Molecular mechanisms involved in the initiation of the innate response to RSV. BRSV attaches to respiratory epithelial cells via glycosaminoglycans and possibly other receptors. The interaction of the F protein with TLR4 leads to activation of NF- κ B via the Myd88-dependent pathway. Double-stranded (ds)RNA, a by-product of virus replication, leads to activation of the transcription factors NF- κ B, IRF-3 and AP-1 (not shown), which act cooperatively to fully activate the IFN β promoter. The BRSV NS proteins block activation of IRF-3, inhibiting the induction of IFN β . Any IFN produced as a result of BRSV infection binds to the type I interferon receptor (IFNR) and activates the expression of numerous interferon stimulated genes (ISG) via the JAK/STAT pathway, which establishes an antiviral state and the activation of IRF-7 which amplifies the IFN response. The NS proteins decrease STAT2 expression and inhibit IFN signalling. Activation of NF- κ B via TLR4 and TLR3 leads to the induction of pro-inflammatory cytokines and chemokines and recruitment of polymorphonuclear neutrophils (PMN), macrophages (M ϕ) and NK cells to the sites of infection. The soluble form of the G protein (Gs) may compete with the virus for binding to surfactant proteins (SP-A and SP-D) and may act to inhibit the early innate response by suppressing the activation of NF- κ B. The virokinin (VK) released following cleavage of the BRSV F protein may contribute to eosinophil (Eo) recruitment or to bronchoconstriction.

the cDNA. The role of different proteins in the pathogenesis of BRSV has been investigated by analysing the effects of deleting genes or introducing mutations in the cDNA copy of the virus genome. Some of these studies are described below.

4.1. The role of the NS proteins in the pathogenesis of BRSV

Interferon (IFN) α/β are produced by most eukaryotic cells in response to virus infection and establish a first line of

defence. Transcription of IFN α/β is mediated by the transcription factors IRF-3, NF- κ B and AP-1. Once secreted, IFN α/β bind to cell surface receptors and activate the JAK/STAT signalling pathway which induces further production of IFN and an array of IFN-stimulated genes, including ones that establish an antiviral state [50] (Fig. 3). In order to establish infection, viruses have evolved a variety of mechanisms to counteract the IFN α/β response. BRSV and HRSV are poor inducers of IFN α/β and are resistant to the antiviral effects of IFN α/β [8, 115]. The ability to regulate the IFN α/β response is mediated by the NS proteins of BRSV and HRSV [16, 17, 115, 124, 152]. The BRSV NS2 protein appears to have a greater inhibitory effect on IFN α/β than the NS1 protein, which is the converse of that described for HRSV [124, 152]. The BRSV and HRSV NS proteins prevent induction of IFN α/β and the establishment of an antiviral state by interfering with the activation of IRF-3 [17, 125] (Fig. 3). In addition, the HRSV NS proteins inhibit IFN α/β signalling by inducing a decrease in Stat2 expression [87, 106] (Fig. 3). Further studies have demonstrated that the NS genes may also play a role in activation of NF- κ B. Thus, activation of NF- κ B in Vero cells, which lack IFN α/β structural genes, infected with rHRSV lacking NS2 (Δ NS2) or lacking both NS1 and NS2 (Δ NS1/2) was significantly lower than that in cells infected with wild-type HRSV [125]. The role of the BRSV NS proteins in NF- κ B activation is not known.

Although the NS proteins are not essential for virus replication *in vitro*, growth of recombinant BRSV lacking one or the other of these proteins is attenuated in cell culture [20, 115, 152]. Furthermore, replication of NS deletion mutants of BRSV in young calves is highly attenuated [152]. Thus, following intranasal (i.n.) and intratracheal (i.t.) inoculation of 2 week-old, gnotobiotic calves with rBRSV Δ NS1 or

rBRSV Δ NS2, only low titres of virus could be isolated from the nasopharynx for only 1 to 2 days and virus could not be detected in the lungs at post-mortem, 6 to 7 days after infection. BRSV lacking both NS1 and NS2 was even more attenuated and virus could not be recovered from calves at any time post-infection. In contrast to calves infected with wild-type rBRSV, neither macroscopic nor microscopic lung lesions could be detected in any of the calves inoculated with any of the NS deletion mutants. These observations highlight the critical role of IFN α/β in the innate response of the bovine respiratory tract against BRSV infection.

4.2. The role of the F protein in the pathogenesis of BRSV

Cleavage of the BRSV F0 protein by a furin endoprotease occurs at two sites, FCS-1 (RKRR¹³⁶) and FCS-2 (RAR/KR¹⁰⁹), and results in the formation of F1 and F2 subunits linked by a disulphide bridge and in the release of an N-glycosylated peptide of 27 amino-acids (pep27) [49, 174]. Cleavage at both sites is required for efficient syncytium formation. In BRSV-infected cells, pep27 is further subjected to post-translational modifications and is converted into virokinin, a member of the tachykinin family, which includes substance P, neurokinins A and B, hemokinin and endokinin A and B [176]. Virokinin induces smooth muscle contraction, *in vitro*, and may therefore contribute to bronchoconstriction *in vivo*. Using recombinant (r)BRSV with mutations in FCS-2 (K108N/K109N) that abolished cleavage at this site or in which pep27 was deleted (Δ p27), neither FCS-2 nor pep27 was found to be essential for virus replication *in vitro* [72, 176]. However, mutant BRSV in which cleavage was abolished at FCS-2 did not grow as efficiently as the parental wild-type virus during early replication cycles. Furthermore,

both rBRSV (108/109) and Δ p27 showed reduced syncytium formation in cell culture [176].

Despite the differences seen *in vitro* with these viruses, they replicated to similar levels as the wild-type rBRSV in the bovine upper and lower respiratory tract [153]. However, both the FCS-2 and Δ p27 mutants induced significantly less pulmonary inflammation compared with the parental wild-type rBRSV [153]. Furthermore, calves infected with the F mutant viruses showed a marked reduction in the numbers of eosinophils in the lamina propria of the large bronchioles suggesting that the virokinin may play a role in eosinophil recruitment (Fig. 3). Nevertheless, the virokinin did not have any direct chemoattractant properties on inflammatory cells, *in vitro* [153]. The chemokines RANTES (CCL5) and MIP-1 α (CCL3) are potent attractants for human eosinophils and there is a correlation between levels of MIP-1 α and eosinophil cationic protein in the lower airways of infants with severe HRSV disease [58]. However, there were no significant differences in mRNA for either RANTES or MIP-1 α in the lungs of calves infected with the F mutant viruses, suggesting that these chemokines may not be involved in eosinophil recruitment in the calf [153].

The primary sequence of virokinin is conserved in all BRSV isolates studied to date [176], which suggests that it provides some selective advantage to the virus. Eosinophil products might be expected to have a detrimental effect on the virus, however these products might damage respiratory mucosa and induce ciliostasis, which could favour viral replication. In contrast to BRSV, the 27-mer peptide produced during the maturation of HRSV F does not contain a tachykinin motif [176] suggesting that virokinin may be an additional factor to chemokines involved in eosinophil recruitment in BRSV infection and/or may contribute to species specificity.

BRSV infection is associated with a reduction in mitogen-induced lymphocyte proliferation in both calves and lambs [66, 121, 168]. BRSV-infected cells are able to inhibit mitogen-induced lymphocyte proliferation, *in vitro*, and this effect was found to be mediated by direct contact of the lymphocytes with the F protein [116]. The precise mechanisms responsible for this inhibition are not known, however, contact with the F protein resulted in a defect or delay in the transit of lymphocytes from G₀/G₁ to S-phase. Although the implications of this suppressive effect for the pathogenesis of BRSV infection are not clear, it may contribute to a decrease in effector function of CD8⁺ T cells in the respiratory tract, which has been reported in HRSV-infected mice [28] and/or may influence the generation of BRSV-specific memory T cells.

4.3. The role of the G protein in the pathogenesis of BRSV

As mentioned above, the G protein was thought to be the major attachment protein of RSV. However, recombinant BRSV and HRSV lacking the G protein (Δ G), and or the SH protein, which is the other surface glycoprotein, can be rescued and such viruses replicate efficiently in cell culture [65, 135]. Thus, the F protein alone is sufficient to mediate attachment and fusion in the absence of G and SH. Whereas there is evidence that for HRSV, expression of the G protein enhances binding of virus to tissue culture cells, cell-to-cell fusion and virion assembly and release [135], this was dependent upon the tissue culture cells [138] and a similar effect on BRSV replication in MDBK cells could not be demonstrated [65]. Analysis of the replication of rBRSV Δ G in differentiated, bovine ciliated airway epithelial cell cultures has shown that although the virus can infect these cells, replication is slightly

attenuated when compared with that of wild-type rBRSV⁵.

In contrast to the limited effects of the G protein on virus replication *in vitro*, expression of the G protein is essential for significant replication of BRSV and HRSV *in vivo* [118, 138]. Thus, following *i.n.* inoculation of calves within the first week of life with Δ G rBRSV, virus could not be re-isolated from the nasopharynx, although some virus replication was detected by RT-PCR, whereas the parental wild-type virus reached peak titres of approximately 10^4 pfu/mL [118]. In these studies, the effects of Δ G virus on lower respiratory tract infection were not investigated. However in mice infected with Δ G HRSV, although virus could not be recovered from the nasal turbinates, it could be isolated from the lungs of about 60% of the mice at titres that were 1 000-fold less than that from mice infected with wild-type HRSV [138].

In order to understand the role of the G protein in the pathogenesis of RSV infection, further recombinant viruses expressing only the membrane-anchored (Gm) or only the secreted form of the G (Gs) protein have been studied. Viruses expressing Gm were produced by introducing a point mutation in the second ATG of the viral G ORF encoding Met-48 to ATC encoding Ile-48 and viruses expressing Gs were made by deleting the first 141 nucleotide segment encoding the cytoplasmic and part of the transmembrane domains of the G protein [138]. Recombinant viruses expressing only Gm or only Gs replicated as efficiently as the wild-type virus in cell culture⁶ [138]. As shown for HRSVGs in

mice [138], studies in gnotobiotic calves have demonstrated that rBRSVGs is highly attenuated in the lungs and moderately attenuated in the nasopharynx, with peak titres in the nasopharynx approximately 100-fold less than that of the wild-type virus. Therefore BRSV only expressing Gs does not appear to be as attenuated as Δ G virus suggesting that the function of Gm can be supplied, at least in part, by the secreted form of G. In studies investigating the replication of HRSVGm in the murine respiratory tract, HRSVGm replicated in nasal turbinates and lungs as efficiently as the wild-type virus [138], whereas in another study, the replication of this virus in the lungs was reduced 10-fold [89]. In cattle, although rBRSVGm replicated as efficiently as the wild-type virus in the upper airways, titres in the bronchoalveolar lavage (BAL) were 10-fold lower and there was little or no replication of the Gm virus in the lung parenchyma. Furthermore, in contrast to calves infected with wild-type rBRSV, animals infected with the Gm virus did not develop gross pneumonic lesions and microscopic lesions were minimal. These studies suggest that the secreted form of the G protein is important in establishing infection in the lower respiratory tract.

The mechanisms by which Gs mediates the establishment of LRT infection are not known, but it may be that this form of G binds to surfactant proteins in the lower respiratory tract, reducing their effects on the virion itself (Fig. 3). Support for this suggestion comes from the observations that susceptibility to severe HRSV infection in infants is linked to polymorphisms in SP-A and SP-D genes [76, 88] and SP-A deficient mice have more severe HRSV infection than their wild-type littermates [85]. Furthermore, there is evidence that the HRSV G protein can suppress TLR4-mediated cytokine production by monocytes and macrophages by inhibiting nuclear translocation of NF- κ B [104]

⁵ Sadler R., Valarcher J.-F., Taylor G., unpublished observations.

⁶ Sadler R., Buchholz U., Taylor G., The secreted form of the G protein is a virulence determinant of bovine respiratory syncytial virus (BRSV), in: Abstracts of the 156th meeting of the Society for General Microbiology, 2005, pp. 80.

(Fig. 3). The mechanisms by which the G protein mediates this effect are not known. However, the conserved cysteine-rich region of the G protein has homology with the fourth domain of the TNF receptor [80] and may inhibit components of the innate response by binding to TNF α or an unknown TNF homologue. Studies with rHRSV lacking the cysteine-rich region of the G protein suggest that although this region is not required for efficient viral replication in mice [139], it may play a role in suppressing the anti-viral T-cell response [56]. As mentioned previously, field isolates of BRSV have been identified that lack one or more of the cysteines in the central conserved region (see Sect. 2.1.3), but there is no information on the virulence of these isolates. Studies are in progress to determine the role of the central conserved cysteine-rich region in the pathogenesis of BRSV in calves.

4.4. The role of the SH protein

The role played by the SH protein during RSV replication is unclear. BRSV lacking the SH protein replicates as efficiently as the wild-type virus in cell culture [65]. However, there is some evidence from studies on HRSV that the SH protein may have a negative effect on virus fusion in cell culture [135]. When inoculated into mice, Δ SH HRSV resembled the parental wild-type virus in the efficiency of its replication in the lungs, whereas it replicated 10-fold less efficiently in the nasal turbinates [22]. These observations contrast with those of rBRSV Δ SH in chimpanzees where virus replication was similar to that of the wild-type virus in the nose but was reduced 40-fold in tracheal lavage [167]. Preliminary studies in calves infected with rBRSV Δ SH indicate that although the SH protein does not influence virus replication in the nasopharynx, it is important in establishing lower respiratory

tract infection⁷. Thus like Gs, the SH protein may suppress some component(s) of the innate response important in mediating resistance of the lung to BRSV.

4.5. The role of viral proteins in determining host-range specificity

Although closely related, BRSV and HRSV display a highly restricted host range *in vivo*. Thus, there are no reports of BRSV infection in humans and there is little or no replication of BRSV in chimpanzees following experimental infection [21]. In contrast, although HRSV does not replicate very efficiently in the bovine nasopharynx, it replicates moderately well in the lungs and induces some pneumonic lesions following simultaneous *i.n.* and *i.t.* inoculation of young gnotobiotic calves [142]. Studies on the role of different viral proteins in determining host-range restriction have demonstrated that the F and G proteins contribute to host range restriction but are not the major determinants [21]. Thus, whereas HRSV and BRSV replicated more efficiently in human and bovine cells respectively, rBRSV in which the F and G proteins had been replaced with those from HRSV exhibited intermediate growth characteristics in a human cell line and grew better than either parent in a bovine cell line. Furthermore, the chimaeric virus was more competent than BRSV for replication in chimpanzees, but remained highly restricted compared with HRSV.

Studies of HRSV and BRSV infection of differentiated respiratory epithelial cells, peripheral blood lymphocytes and macrophages also showed a pronounced host-range restriction [117]. Using recombinant HRSV and BRSV expressing chimaeric F proteins assembled from BRSV or HRSV F1 and F2 subunits, the species specificity correlated with the origin of the

⁷ Taylor G., unpublished observations.

F2 subunit [117]. Although the HRSV and BRSV G proteins have only 30% amino acid identity [83], the G protein did not appear to contribute to host-range restriction.

The NS proteins also appear to contribute to the host-range restriction of HRSV and BRSV. In a recombinant BRSV in which the NS genes were replaced with those from HRSV, the exchanged genes could fully substitute for BRSV NS1/NS2 in IFN α / β -negative cells. However, in IFN-competent bovine cells, replication of rBRSV expressing HRSV NS1 and NS2 was attenuated [16]. Taken together, these studies indicate that host-range restriction of HRSV and BRSV is dependent upon the actions of several proteins, including the F and NS proteins.

5. PREVENTION, CONTROL AND VACCINATION

Since the peak incidence of severe BRSV disease is between 2 and 6 months, an effective BRSV vaccine must be capable of stimulating an effective immune response within the first months of life. The presence of maternally-derived, RSV-neutralising, serum antibodies poses a major obstacle to successful vaccination at this time. Furthermore, there is evidence from studies in man that vaccination can exacerbate RSV disease. Thus, a formalin-inactivated (FI)-HRSV vaccine not only failed to protect infants against HRSV infection but increased the severity of respiratory disease when they became infected [64,67]. Vaccine-augmented BRSV respiratory disease has been reproduced experimentally in calves [5,47] and severe BRSV disease has been reported in calves vaccinated with a β -propiolactone-inactivated virus [119]. It is likely that a parenterally-administered, inactivated virus vaccine would not be effective in inducing a mucosal IgA antibody response, which would help to limit infection of the respiratory tract, and would not be

effective in priming BRSV-specific CD8⁺ T cells, which are important in eliminating virus [131]. Furthermore, there is evidence that the FI-HRSV induced antibodies were poorly neutralising [97]. Therefore, the absence of a strong mucosal and systemic protective immune response left the vaccinees susceptible to natural HRSV infection, whereupon the expression of viral antigens initiated an immunopathogenic response. It has been suggested that this immunopathogenic immune response may have been mediated by the deposition of immune complexes and complement activation in the lungs and/or the induction of a strong Th-2 biased immune response which resulted in the exaggerated recruitment of other inflammatory cells into the lungs [35,36,51,103,163].

Whatever the mechanisms of vaccine-augmented disease may be, it has been proposed that since natural infection with RSV does not predispose to severe disease upon subsequent exposure to the virus, a live attenuated virus vaccine would induce the most appropriate protective immune response. Furthermore, the mucosal route of vaccination is more resistant to the immunosuppressive effects of maternal antibodies than the parenteral route of administration [13,38]. Whilst it is possible to generate live, attenuated viruses by passage in cell culture, it has been difficult to produce a genetically stable HRSV with an appropriate balance between attenuation and immunogenicity [34,169]. Furthermore, we have increased the virulence of a BRSV isolate by sequential passage in gnotobiotic calves⁸. The ability to recover infectious recombinant BRSV from cDNA has greatly facilitated the production of live, attenuated, genetically stable vaccine candidates. Deletion of non-essential genes represents an attractive option for production of a live, attenuated virus vaccine, since they should be particularly refractory

⁸ Taylor G., unpublished observations.

to reversion and may be suitable as marker vaccines.

5.1. NS deletion mutants as live vaccines

As described above, the replication of NS deletion mutants of BRSV in the bovine respiratory tract is highly attenuated and does not result in the development of a pulmonary inflammatory response. Despite the poor replication of the NS deletion mutants, infection with either the Δ NS1 or the Δ NS2 mutant induced a BRSV-specific antibody response, primed BRSV-specific CD4⁺ T cells and induced protection against a subsequent challenge with a virulent strain of BRSV [152]. Although there were no detectable differences in the ability of the Δ NS1 or the Δ NS2 mutants to replicate in the bovine respiratory tract, the Δ NS2 mutant induced higher titres of neutralising serum antibodies, higher titres of BRSV-specific IgG2 antibodies, greater priming of BRSV-specific IFN γ -producing CD4⁺ T cells and greater protection against a subsequent BRSV infection than the Δ NS1 mutant. Since IFN α/β have profound immunomodulatory effects and can enhance the adaptive immune response, it has been suggested that the greater immunogenicity of the Δ NS2 mutant is related to the greater ability of this virus to induce IFN α/β compared with the Δ NS1 virus. Although the Δ NS2 mutant was highly attenuated and immunogenic, it has not yet been evaluated in calves with maternal antibodies and it is possible that it will be too attenuated to induce an effective immune response in such animals.

5.2. FCS-2 cleavage mutants or Δ p27 mutants as live vaccines

Although disruption of furin-mediated cleavage at FCS-2 or deletion of pep27 did not affect virus replication in the bovine

respiratory tract, viruses with these mutations induced little or no pulmonary inflammation, suggesting that they may be ideal live vaccine candidates. However, tachykinins are potent immunomodulators and it is possible that loss of expression of the virokinin may affect the induction of immunity. Studies in calves inoculated i.n. and i.t. demonstrated that neither disruption of furin-mediated cleavage at FCS-2 nor the loss of pep27 influenced the induction of BRSV-specific serum antibodies, as detected by ELISA, priming of BRSV-specific T cells, nor the induction of a protective immune response in young calves, 6 weeks after mucosal vaccination [153]. However, disruption of furin-mediated cleavage at FCS-2, did appear to influence the induction of BRSV-specific neutralising antibodies, which were 10-fold lower than those induced by either Δ p27 or wild-type rBRSV. Thus, incomplete cleavage of the BRSV F protein appears to influence both the magnitude and the duration of neutralising antibodies.

5.3. G protein mutants as live vaccines

Recombinant BRSV lacking the G protein (Δ G) appears to be highly attenuated in calves inoculated via the i.n. route. Nevertheless, mucosal immunisation with the Δ G virus induced serum neutralising antibodies, although the titres were 4 to 32-fold lower than those induced by the parental wild-type virus [118]. Following challenge with a virulent strain of BRSV, there was a significant reduction in virus titres in both the nasopharynx and the lungs of calves previously infected with the Δ G virus. However, protection against challenge was not as great as that induced by the parental wild-type virus. These studies suggest that the Δ G virus may be too attenuated to induce a fully protective immune response. Furthermore, the G protein is a major protective antigen and should ideally be present in a BRSV vaccine.

In contrast to the ΔG rBRSV, virus expressing only the membrane-anchored form of the G protein replicated as efficiently as the wild-type virus in the nasopharynx of calves but was attenuated in the lungs. Inoculation of calves by the i.n. and i.t. routes with rBRSVGm induced a serum antibody response and T-cell response indistinguishable from that induced by wild-type rBRSV⁶. Furthermore, the Gm virus induced complete protection against subsequent challenge with a virulent strain of BRSV. These findings suggest that the rBRSVGm has promise as a live, attenuated virus vaccine candidate. However, it differs from wild-type BRSV by a single point mutation and the ability of this virus to revert to virulence on repeated passages in calves is not known.

5.4. SH deletion mutants as live vaccines

As described previously, rBRSV ΔSH replicates as efficiently as wild-type virus in the bovine nasopharynx but is attenuated in the lungs making this virus a suitable vaccine candidate. Although the immunogenic potential of ΔSH virus has not yet been evaluated in calves, inoculation of chimpanzees by the i.n. and i.t. routes with HRSV ΔSH induced serum neutralising antibodies comparable to those induced by wild-type HRSV [167]. However, in this study the chimpanzees were not challenged with virulent HRSV.

5.5. Other strategies for the development of live attenuated BRSV vaccine candidates

Deletion of M2-2 in HRSV produced a virus that was attenuated and immunogenic in chimpanzees and a similar deletion mutant of rBRSV may also be a suitable vaccine candidate for calves. Apart from deleting non-essential genes, it is

also possible to target a specific protein and replace charged amino acids with non-charged ones. This has been done with the HRSV L protein and a number of the mutations were attenuating [129]. Another strategy is to alter the order of the viral genes. Gene transcription in RSV, has a polar gradient such that genes proximal to the 3' promoter are expressed more efficiently than downstream genes (see Fig. 2). Rearrangement of the gene order might yield sub-optimal ratios of proteins and attenuate the virus. This method has been used for BRSV and we have demonstrated that altering the positions of the BRSV F and G proteins to positions 3 and 4 in the genome instead of positions 7 and 8 (see Fig. 2) resulted in increased expression of the F and G proteins in vitro, attenuation of the virus in young calves and induced protection against subsequent challenge with virulent BRSV⁹. It may also be possible to introduce a gene encoding for a cytokine such as IL-2, IFN γ or GMCSF into the BRSV genome. This has been done for HRSV and although such viruses were attenuated in mice, their ability to induce antibodies and/or prime T cells was similar to that of wild-type HRSV [23–25]. Similarly, introduction of an extra gene such as green fluorescent protein into the viral genome has been shown to attenuate BRSV in young calves (unpublished observations). Recombinant BRSV expressing bovine IL-2 or IL-4 have been produced by replacing the F protein peptide pep27 coding sequence with that of the bovine cytokine [72]. However, these recombinants have not been tested in cattle.

6. CONCLUSIONS

The ability to manipulate the genome of BRSV has increased our understanding of the role of different proteins in the

⁹Taylor G., Valarcher J.-F., Buchholz U., manuscript in preparation.

pathogenesis of this virus in calves and has provided opportunities for the development of stable, live attenuated virus vaccines, administered by the mucosal route. This approach to vaccination may be more effective at inducing both mucosal and systemic immunity of longer duration compared to those that are on the market. All of the studies described in this review have used mutant viruses derived from BRSV strain ATue51908 [20, 115], which appears to be attenuated when compared with more virulent BRSV strains. Thus, although the parental rBRSV strain ATue51908 replicates in both the nasopharynx and lungs and induces pneumonic lesions, it does not induce clinical signs of disease. Therefore, using this virus, it has not been possible to determine the role of various viral proteins in the development of clinical respiratory disease. For example, it was not possible to determine the effects of virokinin, produced as a result of cleavage of the F protein, on the development of bronchoconstriction in calves. Nevertheless, studies using mutants of this strain of BRSV have demonstrated the role of the NS proteins in inhibiting the IFN α / β response, a potential role for virokinin in eosinophil recruitment and a role for the SH protein and the secreted form of the G protein in establishing lower respiratory tract infections. In addition, a number of these mutants induced an immune response and protection against experimental BRSV infection comparable to that induced by wild-type BRSV and are therefore suitable vaccine candidates for the control of BRSV respiratory disease in the field.

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