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Animal Models of Respiratory Syncytial Virus Pathogenesis and Vaccine Development: Opportunities and Future Directions

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1. Introduction

Human respiratory syncytial virus (HRSV) is the leading cause of respiratory failure and viral death in infants (Thompson et al., 2003). In HRSV bronchiolitis, plugs of mucus, epithelial cell debris, and innate inflammatory cells obstruct the airways leading to pulmonary obstruction (Aherne et al., 1970; Lugo and Nahata, 1993). In autopsy lung tissues from fatal HRSV disease cases, epithelial damage and mechanical airway obstruction are implicated as key features of HRSV pathogenesis (Johnson et al., 2007; Welliver et al., 2007). Although laboratory animal models of HRSV pathogenesis have been widely used to determine pathogenic mechanisms of HRSV infection, the commonly used lab strains of HRSV do not cause airway epithelial cell desquamation, airway mucus production, or lung dysfunction in mice (Moore et al., 2009a; Peebles et al., 2001). However, some strains of HRSV (e.g. line 19 and clinical isolate A2001/2-20) were shown to cause airway mucus expression and lung dysfunction in mice, and clinical isolates can cause airway epithelial cell desquamation in mice (Moore et al., 2009a; Stokes et al., 2011). The fusion (F) glycoprotein of HRSV strain line 19 was implicated in the mechanism of line 19-induced airway mucus (Moore et al., 2009a). Variation in HRSV glycoprotein sequence may contribute to disease variation observed clinically. The pathogenesis of HRSV is likely due to a combination of host and viral genetic determinants. HRSV strain-specific virulence in mice facilitates mechanistic studies of HRSV pathogenesis and may provide more robust challenge models to test whether vaccines have potential to prevent HRSV disease.

Currently, there are no vaccines for prevention of HRSV disease. There are several obstacles to developing successful vaccines for HRSV, including immunological immaturity of infants, interference of immunogenicity by maternal Abs, and relatively poor immune responses to HRSV. In the 1960s, a formalin-inactivated HRSV (FI-HRSV) vaccine tragically resulted in enhanced disease in infants upon natural HRSV infection (Kapikian et al., 1969; Kim et al., 1969). Reasons for this may be formation of carbonyl groups on vaccine antigens

due to formaldehyde treatment and/or poor Toll-like receptor stimulation by inactivated HRSV (Delgado et al., 2009; Moghaddam et al., 2006). Another significant obstacle in vaccine development is the lack of stringent challenge animal models. Mice and cotton rats are semi-permissive for HRSV infection, and species other than the chimpanzee are not particularly advantageous over mice and cotton rats (Belshe et al., 1977; Coates and Chanock, 1962; Prince et al., 1978; Prince and Porter, 1976). The lack of robust HRSV replication and disease in rodents has led to false hopes of vaccine efficacy.

Bovine RSV (BRSV) is closely related to HRSV and causes a similar disease in young calves. The pathogenesis and biology of BRSV are highly similar to HRSV. Successful BRSV vaccines are in use, and the vaccinology of BRSV has relevance for HRSV vaccine strategies. Another model of HRSV pathogenesis in the natural host is pneumonia virus of mice (PVM), a pneumovirus that is a natural pathogen of murids. PVM causes a dose-dependent lethal infection in mice with pathologic features resembling severe HRSV bronchiolitis. The caveat of using BRSV and PVM models for vaccine advancement is that the antigens are not specifically HRSV. Future studies testing HRSV vaccine efficacy would benefit greatly from HRSV-BRSV and HRSV-PVM chimeric viruses. Chimeric HRSV-BRSV viruses have been generated by RSV reverse genetics, and additional chimeras could be useful as robust challenge strains (Buchholz et al., 2000). Such a chimeric approach is taken in the HIV and influenza vaccine fields to overcome limits on host restriction.

2. Pathologic features of HRSV, BRSV, and PVM in their natural hosts

2.1 Pathologic features of natural HRSV infection in infants

Pathologic features of HRSV infection have not been extensively characterized because few reports have described autopsy findings in immunocompetent humans infected with HRSV. Furthermore, HRSV-infected patients who die often do so after days to weeks of intensive therapy which may include mechanical ventilation, oxygen therapy, and other interventions which can superimpose non-HRSV induced pathology (DeVincenzo, 2007). However, an informative report described lesions in an HRSV-infected child who died in an automobile accident, along with lesions from 3 archived cases from 1931-1949, prior to the initiation of modern intensive care practices (Johnson et al., 2007). Immunohistochemical (IHC) staining revealed viral antigen in bronchiolar epithelial cells, including ciliated cells. Intrabronchiolar syncytia were present in one patient. Viral antigen was also identified in type I and type II alveolar epithelial cells. Debris in airways consisted of fibrin and mucus along with sloughed epithelial cells and macrophages that stained positive for HRSV. Peribronchiolar and periarteriolar inflammation was characterized by mononuclear and lymphocytic infiltration. Organized lymphoid aggregates were often present, and bronchial arteries were congested. Neutrophils could be seen migrating into bronchioles and along the bronchiolar epithelium. Eosinophils were occasionally present in the peribronchiolar infiltrate. In the pulmonary parenchyma, interstitial inflammation was present, with marked vascular congestion. Three patients had intraalveolar leakage of fibrin with infiltration of macrophages and occasional neutrophils. One patient had severe pneumonitis with rare focal necrosis and hemorrhage. In these children with natural HRSV infection, bronchiolar-associated lymphoid tissue (BALT) was prominent and often hyperplastic (Johnson et al., 2007).

Immunohistochemical staining to characterize the phenotype of infiltrating cells was undertaken in one case. In this patient, the BALT consisted largely of CD20⁺ B cells with some CD3⁺ T cells and CD68⁺ monocytes. In contrast, CD3⁺ T cells were most prominent in the spaces between pulmonary arterioles and small distal airways; they were also found in the bronchiolar epithelium and in the alveolar interstitium. Despite the presence of T and B cells, the prominent pathologic changes of fatal HRSV infection were widespread viral antigen in airway and alveolar epithelia and mechanical obstruction of airways.

A second report describing histopathologic changes in infants with fatal HRSV-induced disease who were not subjected to prolonged mechanical ventilation or antiviral or anti-inflammatory therapy revealed similar findings to those in of Johnson et al. (Welliver et al., 2007). In tissues from nine patients who died due to severe lower respiratory tract infection and which were positive on IHC staining for HRSV, the terminal bronchioles were plugged with sloughed epithelial cells with abundant viral antigen identified by IHC. Bronchiolar walls were infiltrated with neutrophils and macrophages. As compared to tissues from children infected with influenza, HRSV-induced bronchiolar epithelial damage seemed much greater, and the amount of viral antigen present was increased. In contrast to the single case in which T cell subsets were evaluated by Johnson et al., CD4⁺ or CD8⁺ cells were infrequently identified by IHC staining of bronchioles and alveoli (Welliver et al., 2007). Staining for CD16 was used to identify neutrophils and macrophages and found to be extensive in HRSV-infected children. Staining for the apoptosis marker caspase 3 was strongly positive in bronchiolar epithelial cells. The conclusion of Welliver et al. was that children dying of severe acute HRSV infection suffered from bronchiolar obstruction due to widespread death and sloughing of epithelial cells. An excessive immune response mediated by T cells did not seem to be involved because of relatively few T cells found in these tissues. Although immune responses play important roles in HRSV pathogenesis, these two pathology studies taken together show that severe, unchecked HRSV disease in infants is a pulmonary disease associated with virus-induced epithelial damage leading to airway obstruction and *not* associated with immunopathology per se, e.g. excessive T cells or cytokine storm (DeVincenzo, 2007; Johnson et al., 2007; Welliver et al., 2007).

2.2 Pathologic features of BRSV infection in calves

Pathologic changes in the lungs of calves that die or are euthanized due to natural BRSV infection share many similarities with those described for children with fatal HRSV infection. Bronchitis and bronchiolitis with necrosis and sometimes hyperplasia of the bronchiolar epithelium is present, with lumina of bronchioles containing sloughed epithelial cells, neutrophils, mononuclear cells, and proteinaceous debris (Bryson et al., 1983; Elazhary et al., 1982; Pirie et al., 1981). Viral antigen is identified in bronchiolar and alveolar epithelial cells by IHC. Mononuclear cells are present in the bronchiolar lamina propria and surrounding bronchioles (Bryson et al., 1983; Pirie et al., 1981). Alveolar inflammation is also present, with neutrophils, monocytes, and macrophages in alveolar lumina and in the interstitium. Edema is present in alveolar lumina as well as interstitially, sometimes with hemorrhage and emphysema. Syncytia in bronchiolar and alveolar lumina are frequent (Bryson et al., 1983; Pirie et al., 1981). From these reports it

appears that neutrophils play a larger role in the response to BRSV, although this may be in part due to frequent co-infection with mycoplasmas or other bacterial respiratory pathogens in calves in some reports (Bryson et al., 1983; Pirie et al., 1981). However, experimentally induced cases of BRSV infection where bacterial co-infection was not present were also characterized by bronchiolar epithelial necrosis and sloughing, with infiltration of neutrophils, lymphocytes, and mononuclear cells into bronchiolar and alveolar lumina (Bryson et al., 1983; McNulty et al., 1983). As noted for fatal HRSV, death of BRSV-infected bronchiolar and alveolar epithelial cells is associated with apoptosis (Viuff et al., 2002; Welliver et al., 2007).

As for HRSV, there is evidence that immune responses to BRSV infection can contribute to disease severity in at least some individuals. Although there are relatively few detailed studies of pathogenesis of naturally occurring disease due to BRSV infection, research by two different groups indicated a role for mast cell degranulation and mediator release in disease severity (Jolly et al., 2004; Kimman et al., 1989b). Unfortunately neither of these groups measured BRSV-specific IgE in affected calves so it is not clear if mast cell degranulation was triggered by virus-specific IgE or through other mechanisms. Other research has shown that BRSV-specific IgE concentration in blood or pulmonary efferent lymph can be strongly correlated with disease severity in BRSV infection, and that transcription of IL-4 mRNA is associated with IgE production (Gershwin et al., 2000).

2.3 Pathologic features of experimental PVM infection of mice

Like HRSV and BRSV, PVM is a member of the genus *Pneumovirus* within the family *Paramyxoviridae*. The PVM genome has 29-62% nucleotide identity to HRSV and BRSV, which are closely related to each other (Krempl et al., 2005). PVM is a natural respiratory pathogen of murids, and laboratory mouse strains are highly susceptible to experimental PVM infection (Rosenberg et al., 2005). PVM challenge of mice showed that changes in bronchial epithelial cells were evident as early as day 1 post-infection and were characterized by granular changes in the cytoplasm with lifting and stripping of the bronchial epithelium and shedding of cellular debris into bronchial lumina (Carthew and Sparrow, 1980). Plugs of cellular debris and neutrophils were evident by day 4 post-infection, as were severe alveolar congestion and edema, with infiltration of neutrophils and macrophages into alveolar spaces and the interstitium. In contrast to HRSV and BRSV, syncytial cells were not described in airways or alveoli. By day 7 post-infection alveolar spaces were filled with neutrophils, macrophages, and lymphocytes. IHC staining for PVM revealed virus in bronchial epithelial cells by day 2 post-infection, and virus in alveolar epithelial cells by day 4 post-infection. Virus was no longer evident by IHC staining after day 7 post-infection. As has been noted for BRSV (Gershwin et al., 1998) and HRSV (Stokes et al., 2011), pathologic changes in mice infected with PVM depend on the virulence of the challenge isolate; challenge of mice with the virulent strain J3666 induces more severe pathology than does challenge with strain 15 (Domachowske et al., 2002). Overall, the histopathology of severe HRSV, BRSV, and PVM infection in the natural host show striking similarities in 1) viral tropism for airway and alveolar epithelial cells, 2) airway epithelial desquamation, 3) obstruction of airways with mixtures of mucus, fibrin, and cells (epithelial debris and inflammatory cells), and 4) prominent neutrophil and macrophage inflammation in bronchiolar, alveolar, and interstitial spaces.

3. Animal models of HRSV pathogenesis

3.1 Mouse models of HRSV infection: Different HRSV strains

3.1.1 HRSV A2 strain mouse model

Mice are semi-permissive for HRSV replication, and BALB/c mice are one of the more susceptible strains (Prince et al., 1979). The A2 and Long strains are antigenic subgroup A reference strains. A2 was isolated in Australia in 1961, and Long was isolated in Baltimore in 1956. A hallmark of experimental pulmonary HRSV infection of mice is mononuclear cell lung infiltrates. The A2 strain has been used to elucidate roles T cells play in HRSV clearance, immunopathology, and immune modulation. Intranasal A2 strain infection of BALB/c mice induces IFN- γ and T cell-mediated clearance and immunopathology. Viral titers peak approximately 4 days post-infection and lymphocytic inflammation peaks one week post-infection. The peak lung T cell response of BALB/c mice to A2 infection is 6-12 days post-infection, and IFN- γ -expressing CD8⁺ T cells peak before HRSV-specific CD8⁺ T cells (Chang and Braciale, 2002; Chang et al., 2001; Hussell and Openshaw, 1998). CD4⁺ and CD8⁺ T cells exacerbate A2-induced illness and mediate virus clearance (Graham et al., 1991). Foxp3⁺ CD4 T cells (Tregs) coordinate CD8⁺ T cell recruitment, downregulate CD8⁺ T cell TNF- α production, and limit disease severity (ruffled fur) in HRSV A2-infected BALB/c mice (Fulton et al., 2010). The A2 strain has been used to define CD8⁺ T cell responses to specific HRSV epitopes (Lee et al., 2007; Ruckwardt et al., 2010). Although A2 infection of mice does not replicate human disease, the strain is important in the field as a reference strain, the basis of current reverse genetics systems, for HRSV protein structure studies, and for vaccine development (Collins et al., 1995; Jin et al., 1998; McLellan et al., 2011).

A2 HRSV inhibits T cell function in mice. During the CTL response to HRSV, approximately half of HRSV-specific CD8⁺ T cells in the lungs of BALB/c mice produce IFN- γ , compared to influenza virus infection where nearly all influenza-specific CD8⁺ T cells in the lung are IFN- γ ⁺ (Chang and Braciale, 2002). These data are in agreement with HRSV suppression of T cell proliferation *in vitro* (Preston et al., 1992, 1995; Roberts et al., 1986). HRSV inhibition of T cell responses may be an important reason why HRSV does not confer adequate immunity.

3.1.2 HRSV strain line 19 infection of mice

RSV strain line 19 is an antigenic subgroup A strain derived from a RSV clinical isolate obtained at the University of Michigan in 1967 (Herlocher et al., 1999). "Line 19" has been used as the name of a HRSV strain used by the Lukacs lab at the University of Michigan and other groups (Lukacs et al., 2006). The genome sequence of this line 19 was reported, and it is highly similar to the Long strain of HRSV (Moore et al., 2009a). The lack of nucleotide differences between line 19 and Long, particularly in the hypervariable regions of the G gene and in intergenic regions, raises the question of whether this line 19 is a passage variant of the Long strain. It may be that line 19 and Long derive from the same clinical isolate or nearly identical isolates circulating in Baltimore in 1956 (Long) and Ann Arbor in 1967 (line 19). However, it is interesting that the Long strain was adapted to mice by serial passage in the brains of suckling mice in the 1960s at the University of Michigan, and mouse passage number 19 in this study was noted as virulent (Cavallaro and Maassab, 1966). Whether currently used line 19 represents the 1967 Michigan clinical isolate (Herlocher et al., 1999) or

mouse-adapted Long (Cavallaro and Maassab, 1966) is not as important as the fact that line 19 is a good tool to study HRSV-induced pulmonary pathophysiology in mouse models.

The pathogenesis of HRSV line 19 differs from the pathogenesis of HRSV A2 (Lukacs et al., 2006). Similar to A2, line 19 induces high levels of IFN- γ in the lung (Lukacs et al., 2006; Tekkanat et al., 2001). A2 replicates in HEp-2 cells and in the lungs of BALB/c mice to higher titers than line 19 (Lukacs et al., 2006). In contrast to A2, line 19 infection of BALB/c mice increases IL-13 levels in the lungs (Lukacs et al., 2006; Tekkanat et al., 2001). Line 19 infection causes mucus production and airway hyperresponsiveness (AHR) in BALB/c mice, whereas A2 does not (Lukacs et al., 2006; Tekkanat et al., 2001). Line 19 infection-induced mucus and AHR are IL-13-dependent and STAT6-dependent (Lukacs et al., 2006; Tekkanat et al., 2001). Line 19-induced AHR is ameliorated by neutralization of the chemokine CCL5 (RANTES), and CCL5 production in the lung was shown to be IL-13-dependent in line 19-infected mice (Tekkanat et al., 2002).

More recently, the Lukacs group has identified a role for the cytokine IL-17 in RSV pathogenesis. IL-17 is produced by CD4⁺ TH₁₇ cells and has been associated with bacterial infections, neutrophilic inflammation, and pro-allergic airway inflammation (Alcorn et al., 2010). Mice deficient in toll-like receptor 7 (TLR7), an innate immune system molecule recognizing single stranded RNA in endosomes (a common feature of virus infection), had greater lung IL-17 levels than wild-type mice after HRSV line 19 infection, and neutralization of IL-17 in line 19-infected TLR7-deficient mice reduced airway mucin expression (Lukacs et al., 2010). Bone marrow-derived dendritic cells (DCs) from TLR7-deficient mice expressed higher levels of IL-23 (an IL-17-promoting cytokine) in response to line 19 infection in vitro than wild-type DCs (Lukacs et al., 2010). These data suggest that the innate TLR response to HRSV restricts a pathogenic IL-17 response. IL-17 was found in tracheal aspirates of infants with severe RSV illness, and line 19-infected IL-17-deficient mice had less airway mucin expression than control mice (Mukherjee et al., 2011). These studies indicate that TH₁₇-type inflammation contributes to pulmonary mucus in the pathogenesis of HRSV. Previous reports implicated IL-4 and TH₂-type lung inflammation in severe HRSV disease, although this association has not been consistent (Brandenburg et al., 2000; Garofalo et al., 2001; Legg et al., 2003; Roman et al., 1997). The overall picture from the literature is that the balance of canonical TH₁ (IFN- γ) and TH₂ (IL-4) cytokines does not correlate with HRSV disease severity. The findings from the Lukacs group suggest that IL-17 (TH₁₇) should be considered in assessing the role of host immune responses in HRSV pathogenesis.

3.1.3 HRSV clinical isolate infection of mice

HRSV is an RNA virus with a mutation rate of 3.3×10^{-3} substitutions/site/year, equivalent to human influenza A virus (Jenkins et al., 2002). The A2 and Long reference HRSV strains have been passaged extensively in vitro and do not fully represent circulating HRSV. Viral disease depends exquisitely on viral strains. Small changes in viral gene sequences can have a large impact on pathogenesis. For example, elevated virulence of 1918 and avian influenza strains hinges on few amino acids differences (Hatta et al., 2001; Tumpey et al., 2005; Yu et al., 2008). Virulence in animal models of virus infection invariably depends on viral strains, across virus families (Ahmed and Oldstone, 1988; Borisevich et al., 2006). Relatively minor differences between viral strains also play important roles in complex human diseases. Examples include papillomavirus types (e.g. HPV-16) strongly associated with cervical

cancer and hepatitis c virus genotypes associated with acute or persistent infection (Howley et al., 1989; Lehmann et al., 2004). Recently, a group of rhinoviruses (RV group C) was associated with half of all RV hospitalizations in young children, especially for asthma exacerbation (Miller et al., 2009).

HRSV clinical isolate strain 13018-8 was used to study the effects of HRSV on mouse DCs (Gonzalez et al., 2008). This HRSV caused maturation of mouse DCs, but the DCs were then unable to activate antigen-specific T cells in vitro, regardless of the antigen specificity (Gonzalez et al., 2008). The authors did not compare strain 13018-08 to a laboratory HRSV strain. The findings advance a mechanism (inhibition of DC-T cells immunological synapse formation) for HRSV suppression of T cell function (Gonzalez et al., 2008). HRSV clinical isolates exhibit varied pathogenesis phenotypes in BALB/c mice (Stokes et al., 2011). Out of six antigenic subgroup A isolates tested, three increased IL-13 levels in the lungs of BALB/c mice, and two (A2001/2-20 and A1997/12-35) induced greater IL-13 levels and weight loss than HRSV line 19 (Stokes et al., 2011). The clinical isolates had higher lung viral load than A2 and line 19 one day post-infection, viral antigen at this time point was localized to the bronchiolar epithelium, and there was corresponding histologic evidence of damage to the bronchiolar epithelium in mice infected with clinical isolates (Stokes et al., 2011). HRSV A2001/2-20 increased airway mucus expression and increased breathing effort in BALB/c mice whereas A2001/3-12 did not despite these two isolates exhibiting equivalent lung viral loads over a time course (Stokes et al., 2011). As with line 19, airway mucus induced in BALB/c mice by A2001/2-20 was IL-13-dependent (Stokes et al., 2011; Tekkanat et al., 2001).

3.2 HRSV infection of cotton rats

The cotton rat *Sigmodon hispidus* is one of the best small animal models of HRSV infection. Whereas infant but not adult ferrets are susceptible to HRSV, cotton rats are susceptible to HRSV infection throughout life (Prince et al., 1978; Prince and Porter, 1976). The virtues of cotton rats for HRSV research were recently reviewed and include approximately 100-fold more permissiveness to HRSV infection than mice (Boukhvalova et al., 2009). A disadvantage is that there are few reagents available for the cotton rats relative to mice. Cotton rats are susceptible to upper and lower respiratory tract infection with HRSV (Prince et al., 1978). HRSV replicated to relatively high titers in the nasal turbinates and lungs of naïve cotton rats (Prince et al., 1990; Prince et al., 1986; Prince et al., 1978). HRSV antigen was detected in nasal, bronchial, and bronchiolar epithelial cells (Prince et al., 1978). HRSV infection caused proliferative rhinitis, bronchiolitis, and the pulmonary infiltration of lymphocytes and neutrophils (Prince et al., 1986; Prince et al., 1978). The cotton rat model was used to study the Ab-mediated clearance of HRSV, and the data indicate that IgG-mediated HRSV clearance does not require antibody-dependent cellular cytotoxicity or complement (Prince et al., 1990). HRSV infection increased cotton rat lung mRNA steady state levels of RANTES, IP-10, MIP-1 β , GRO, IFN- α , IFN- γ , IL-6, IL-10, and TNF- α (Blanco et al., 2002). GRO is a homolog of the human IL-8 chemokine and functions to recruit neutrophils. IP-10 is a chemokine that is known to attract T cells and NK cells. In cotton rat treated with a TLR3 agonist then infected with HRSV, it was shown that type I IFN responses can augment lung inflammation in this model (Boukhvalova et al., 2010).

An interesting recent study performed with HRSV Long strain showed that HRSV treatment of mouse peritoneal and alveolar macrophages induces these macrophages to become

alternatively activated to express TH₂ cytokines IL-4 and IL-13, and infection of cotton rats with HRSV Long increased markers of alternatively activated macrophages (Shirey et al., 2010). Although the role of TH₂ cytokines in HRSV pathogenesis has been controversial, most studies have focused on a T cell source of these cytokines, e.g. by stimulation of patient peripheral blood mononuclear cells (PBMCs). Basophils can produce IL-4 in HRSV-infected mice, but these cells are rare (Moore et al., 2009b). The finding that macrophages can express TH₂ cytokines in response to HRSV (Shirey et al., 2010) is potentially very important because TH₂ cytokines (especially mucus-associated IL-13) are thought to contribute to HRSV disease and, as noted above, HRSV, BRSV, and PVM lung pathology in the natural host is characterized by prominent macrophage accumulation in bronchiolar and alveolar spaces.

3.3 Neonatal lamb model of HRSV infection

Neonatal (2-3 days of age) lambs were given a high dose of intrabronchial HRSV A2 infection (Olivier et al., 2009). Clinically, the lambs exhibited fever and, and some had a moderate cough (Olivier et al., 2009). Histopathology showed suppurative bronchiolitis with epithelial desquamation and cellular debris and neutrophils in airway lumina. HRSV antigen was detected by IHC, and it was fairly extensive in the bronchiolar epithelium and in alveolar regions where syncytia were evident (Olivier et al., 2009). In a second study from the Ackermann group, immune responses of newborn lambs infected with HRSV A2 were examined (Sow et al., 2011). A2 infection caused lung accumulation of neutrophils, macrophages, and CD4⁺ and CD8⁺ cells (Sow et al., 2011). Interestingly, IFN- α was not detected by RT-PCR, and lung IFN- β levels were actually lower in infected animals than in mock-infected animals, suggesting that HRSV suppressed type I IFN responses in this model (Sow et al., 2011). Cytokines and chemokines increased by HRSV A2 infection in the lungs of newborn lambs were TNF- α (day 3 post-infection), IL-10 (day 3 post-infection), IFN- γ (day 6 post-infection), IL-8 (day 6 post-infection), and MCP-1 (in macrophage-enriched laser capture microdissection regions day 6 post-infection). RT-PCR assays for IL-4, IL-13, and IL-17 mRNA levels gave results below the limit of detection (Sow et al., 2011). As in the pathology study of severe HRSV disease in infants noted above, there was evidence of widespread apoptosis in lungs of HRSV A2-infected newborn lambs (Sow et al., 2011; Welliver et al., 2007). New lambs appear to model HRSV disease closely, although PAS staining was not reported nor mucus evident in the airways in the histology micrographs.

3.4 Non-human primate models of HRSV infection

HRSV was originally isolated from chimpanzees, and this species is the only non-human primate model for HRSV that provides significantly greater permissivity than cotton rats. Young chimpanzees are productively infected with HRSV and exhibit upper respiratory tract illness, but adult squirrel monkeys, newborn rhesus monkeys, and infant cebus monkeys shed low virus levels and do not show clinical disease (Belshe et al., 1977). Bonnet monkeys can be infected with HRSV. However, a high HRSV dose resulted in viral titers similar to those seen in cotton rats, and the infected monkeys did not have clinical disease (Simoes et al., 1999). Chimpanzees can be valuable for HRSV vaccine evaluation (Teng et al., 2000). However high cost, regulatory issues, and genetic variability are major constraints.

3.5 Primary cell culture models of HRSV infection

Although cell and tissue culture systems lack an intact immune system, they can provide information about virus-host interactions, including immune pathways. Human airway epithelial (HAE) cells derived from surgery can be cultured at an air-liquid interface to produce a differentiated, polarized mucociliary epithelium. In this system, HRSV specifically infects ciliated epithelial cells via the apical membrane (Zhang et al., 2002). HRSV was shed from the apical surface, and there was no cytopathic effect (CPE) (Zhang *et al.*, 2002). However, it is possible that loosely adherent syncytia were washed off the apical surface in collection of supernatants for viral titration. Sendai virus (a paramyxovirus) readily causes syncytia in an elegant HAE system of well-differentiated pediatric bronchial epithelial cells (WD-PBEC) (Villenave et al., 2010). In non-polarized monolayers of primary pediatric bronchial epithelial cells, HRSV A2 replicated to higher titers and caused greater CPE than HRSV clinical isolates (Villenave et al., 2011). HRSV infection of primary epithelial cells results in the secretion of the chemokine IL-8 that is involved in neutrophil recruitment (Mellow et al., 2004; Villenave et al., 2011). In addition to epithelial cells, primary immune cells provide information about HRSV-host interactions. HRSV infects human monocyte-derived immature DCs inefficiently (Le Nouen et al., 2009). In a co-culture system of human DCs and T cells, it was shown that the HRSV NS1 protein (a type I IFN antagonist) suppressed CD8 T cell activation and proliferation, skews the CD4 T cell phenotype to T_H2 -type responses, and suppresses markers of DC maturation (Munir et al., 2011). Also in this DC-T cell co-culture system, comparison of influenza A virus, human parainfluenzavirus type 3 (HPIV3), HRSV A2, and human metapneumovirus (HMPV) revealed that HRSV and HMPV uniquely fail to stimulate CCR7 expression by DC, resulted in impaired DC migration (Le Nouen et al., 2011). DCs are key for initiating adaptive immune responses in lymph nodes. Therefore, HRSV suppression of DC migration may underlie the relatively (compared to influenza viruses) poor immunity elicited by HRSV infection.

4. Challenges to HRSV vaccine development

4.1 Inhibition of pediatric vaccines by maternal antibodies

Since hospitalization rates for HRSV infected children peak at 2 to 6 months of age, immunization for this age will be required for the prevention of severe HRSV disease (Boyce et al., 2000; Shay et al., 1999). However, there are several hurdles to develop HRSV vaccine in infants. One of the major obstacles is the inhibitory influence of maternal antibody (MatAb) on neonatal immunization (Crowe, 1998; Siegrist, 2003). Evidence of MatAb-mediated immune suppression in human studies has been well described in other infections such as measles, parainfluenza viruses (PIV) and influenza viruses (Albrecht et al., 1977; Crowe, 1998). Although underlying mechanisms by which MatAb suppresses infant responses to vaccination are not fully defined, possible hypothesized mechanisms are 1) Fc dependent phagocytosis, 2) neutralization of live viral vaccines, and 3) epitope masking by MatAb. First, inhibitory influence by MatAb could be mediated by Fc-dependent phagocytosis. The internalization of MatAb and antigen complex by antigen-presenting cells (APC) results in the inhibition of infant B cells in both human and mice. However, a study using FcγR knockout mice demonstrated that the existence of FcγR-independent inhibitory mechanisms (Karlsson et al., 1999). Next, the hypothesis that MatAb neutralizes the live

viral vaccine has been widely believed (Albrecht et al., 1977). MatAb can inhibit viral replication of live attenuated HRSV in mice (Crowe, 2001a). Notably, MatAb does not affect the induction of T cell responses to HRSV or measles virus (Crowe et al., 2001; Gans et al., 1999). Lastly, as mentioned above, the hypothesis of B cell epitope specific masking has been postulated based on the possibility of the existence of an Fc-independent mechanism. Evidence of MatAb epitope masking is that MatAb to the V3 loop of HIV gp120 inhibited induction of Ab responses only to V3 but not to other epitopes (Jelonek et al., 1996). Similar results were observed in human infants (Kurikka et al., 1996; Nohynek et al., 1999). Several strategies should be considered to overcome the obstacles mediated by MatAb to effective vaccination of infants. First, novel immunization routes can be tested to determine whether vaccination route can modulate susceptibility to inhibition by MatAb. Second, infant T cell vaccines can be considered. According to previous observations, MatAb inhibits infant Ab responses but not infant T cell responses (Gans et al., 1999; Pabst et al., 1999).

4.2 Non-durable immunity induced by natural HRSV infection

RSV elicits innate and adaptive immune responses that fail to establish long-lasting immunity because reinfection occurs throughout life (Collins et al., 2006). HRSV neutralizing Ab (nAb) can prevent RSV infection. Serum nAb titer is the best immune correlate of protection in humans, and a serum nAb titer of 1:300 is a benchmark for protection (Collins et al., 2006; Group, 1998; Prince et al., 1985). The RSV G and F proteins are virion surface glycoprotein spikes and the only known targets of RSV nAbs (Collins et al., 2006). Local secretory IgA also correlates with protection from RSV disease (Collins et al., 2006; Walsh, 1993). However, natural RSV infection is poorly immunogenic in children < 6 months old. Rates of detectable neutralizing Ab responses to natural infection in this age group are 50-75% (Brandenburg et al., 1997; Murphy et al., 1986a; Wagner et al., 1989; Welliver et al., 1980). In infants, immunological immaturity and suppression of immune responses by matAbs contribute to poor immunogenicity (Crowe, 2001c; Crowe and Williams, 2003). Seronegative infants and children can mount strong anti-G and anti-F Ab responses, although neonates produce poor Ab responses perhaps due to fewer somatic hypermutations in Ab genes (Shinoff et al., 2008; Weitkamp et al., 2005; Williams et al., 2009; Wright et al., 2000). Immunity to HRSV in adults is also non-durable. A striking example is a study in which HRSV nAbs were titrated in 457 cord blood samples in the Danish National Birth Cohort (DNBC) from 1998 to 2003 in order to study the temporal relationship between maternally-derived nAb and the seasonality of HRSV infant hospitalizations (Stensballe et al., 2009). The titer of nAb in cord blood oscillated. NAb titers were highest a few months after the annual HRSV winter epidemic and lowest just before the HRSV season, suggesting a cyclic model in which nAb titers in mothers (which are transferred to infants) wane after six months contributing to the next epidemic which boosts nAb titers for another six months. (Stensballe et al., 2009). In addition to low and non-durable Ab responses, the cellular immune response to HRSV infection is inhibited. As discussed above, HRSV inhibits T cell function in mice and in human DC-T cell co-cultures, and the HRSV NS1 protein (IFN antagonist) was implicated in suppression of T cell activation (Chang and Braciale, 2002; Le Nouen et al., 2011; Munir et al., 2011). Suboptimal immunity to HRSV infection is important for vaccine development because it implies that intranasal delivery of a live attenuated HRSV will not be effective. The degrees to which 1) matAb in infants, 2) immature immune

system in infants, 3) immune modulation by HRSV, and 4) lack of optimal assays to measure HRSV immune responses and protection in patients contribute to the interpretation of poor immunogenicity in the vaccine target population needs to be elucidated.

4.3 Instability of live attenuated HRSV vaccines

Live HRSV vaccine candidates with attenuating mutations have been created by traditional methods of cold-adaptation (cold passage, *cp*) and chemical mutagenesis to yield temperature-sensitive (*ts*) HRSV mutants that replicate at temperatures similar to the upper respiratory tract but not at higher temperatures (Collins and Murphy, 2005). These mutations were defined by sequencing, and mutant HRSV strains that harbor *cpts* mutations have been generated by reverse genetics and tested as vaccine candidates. Leading live attenuated HRSV vaccine candidates and HRSV vaccine development have been reviewed (Collins and Murphy, 2005; Crowe, 2001b; Graham, 2011). Attenuating mutations are not necessarily additive, but addition of attenuating mutations into an already attenuated RSV vaccine candidate can decrease the rate of reversion to less attenuated genotypes, thereby increasing “genetic stability” (Collins and Murphy, 2005). A leading vaccine candidate (rA2cp248/404/1030ΔSH) was adequately attenuated in infants, but one third of isolates from nasal washes of vaccinees contained HRSV with loss of attenuation, evidence of reversion to wild-type, confirmed by sequence analysis (Karron et al., 2005). A strategy was explored to stabilize the “248” point mutation in the viral polymerase gene by mutagenizing the codon to encode an attenuating mutation with more than one nucleotide difference from the wild type (Luongo et al., 2009), as more nucleotide differences per degree of attenuation will be less likely to revert to wild-type. However, in the case of the 248 point mutation, this was not possible due to sequence constraints (Luongo et al., 2009). Instability of live vaccine strains has been an issue with poliovirus. Codon “de-optimization” of poliovirus by titration of CpG dinucleotides into the genome has produced viruses with tunable fitness, a strategy that may work for HRSV (Burns et al., 2009).

4.4 Formalin-inactivated HRSV vaccination

HRSV was discovered in 1956 (Chanock et al., 1957; Chanock, 1956). Once HRSV was recognized to be a common cause of upper and lower respiratory tract disease in infants and children, efforts were undertaken to develop a vaccine. In 1966, a formalin-inactivated whole virus RSV vaccine adsorbed to alum adjuvant (FI-HRSV) was administered to children (Kapikian et al., 1969; Kim et al., 1969). The following winter, compared to unvaccinated children, those who received FI-HRSV had a higher incidence of hospitalization and greater LRI severity due to HRSV (Kapikian et al., 1969; Kim et al., 1969). Two FI-HRSV-immunized infants died, and HRSV was recovered from lung sections (Kim et al., 1969). FI-HRSV enhanced respiratory disease (ERD) can be recapitulated using mice, cotton rats, calves, and non-human primates by FI-HRSV vaccination and HRSV challenge (Delgado et al., 2009; Gershwin et al., 1998; Graham et al., 1993; Johnson et al., 2004a; Moghaddam et al., 2006; Prince et al., 1986). Key features of FI-HRSV ERD are 1) failure to protect against HRSV replication, 2) an Ab response lacking in virus-neutralizing Abs, 3) a poor HRSV-specific CD8⁺ T cell response, 4) an immunopathologic T_H2 immune response, and 5) immune complex deposition (Graham et al., 1993; Murphy and Walsh, 1988; Olson and Varga, 2007; Polack et al., 2002). HRSV G protein is implicated in ERD because priming

with HRSV G-expressing vaccinia virus (vac-G) followed by HRSV challenge results in ERD whereas vac-F primes for protection (Srikiatkachorn and Braciale, 1997). Vac-G-mediated ERD and FI-HRSV ERD act through different immune mechanisms, and G is not required for FI-RSV ERD (Johnson et al., 2004a; Johnson et al., 2004b). Nevertheless, vac-G studies revealed the potential for G to cause ERD. What causes FI-HRSV ERD? One hypothesis is that formalin disrupts antigenicity. Formalin causes formation of reactive carbonyl groups on HRSV proteins, and chemical reduction of these groups reduces FI-HRSV ERD (Moghaddam et al., 2006). Another mechanism invokes nonfunctional Ab responses in the absence of appropriate adjuvant, particularly TLR agonists. Co-formulation of ERD-causing inactivated HRSV vaccines with CpG ODN (a TLR9 agonist), MPL (a TLR4 agonist), and other adjuvants ameliorates ERD (Boukhvalova et al., 2006; Delgado et al., 2009; Mapletoft et al., 2008; Oumouna et al., 2005).

It appears that vaccination with FI-HRSV induced excessive activation of T_H2 cells, as measured by increased expression of cytokines including IL-4, IL-5, and IL-10, and relatively decreased expression of cytokines including IFN- γ and IL-2, by circulating or tissue lymphocytes following HRSV infection. The strong T_H2 response to FI-HRSV may suppress the anti-viral T_H1 response and development of cytotoxic T lymphocytes (CTL), mechanisms that limit viral replication in the normal response to HRSV infection. Immune pathways activated by T_H2 cells likely also contributed to the influx of eosinophils seen in the lungs of the two children who died as a result of FI-HRSV enhanced disease, which may have contributed to the excessive inflammatory response. However, utilization of eosinophil-deficient mice revealed that eosinophils are not required for ERD in mice induced by either vac-G and HRSV challenge or FI-PVM antigens and PVM challenge (Castilow et al., 2008; Percopo et al., 2009).

4.5 Extrapolation of vaccine efficacy from animal models

One of the challenges in determining how FI-HRSV caused ERD, and in determining how to safely and effectively vaccinate children with any type of HRSV vaccine, is limitations of animal models of HRSV infection. The strengths and weaknesses of the different models have been reviewed (Bem et al., 2011; Openshaw and Tregoning, 2005). Mouse models of HRSV infection and vaccination have been used widely, and they offer unparalleled opportunities to manipulate and dissect the immune response. However, because mice are not natural hosts of HRSV and are not very permissive to infection, relatively large amounts of virus must be administered to cause even mild disease. This may account for the fact that vaccination strategies that have appeared promising in mice have often not held up in subsequent research using non-human primate models or in human clinical trials. At this time it appears that no animal model will perfectly predict safety and efficacy of candidate HRSV vaccines. Thus candidate vaccines will always require careful and deliberate stepwise testing beginning with immunocompetent adults, then moving to seropositive children, then to seronegative children, to seropositive infants, and then to seronegative infants (Polack and Karron, 2004; Schickli et al., 2009). This pathway is time consuming, expensive, and fraught with the possibility of profound disappointment. Because of this, increased use of animal models of natural pneumovirus infection could provide a more efficient and fruitful testing ground for screening candidate vaccines and novel vaccination strategies before they are tested in non-human primates and in human clinical trials.

5. Vaccines for BRSV

Bovine HRSV and HRSV share many clinical and pathologic similarities in their respective hosts. Although no vaccine for human HRSV has yet been approved for use, vaccines for BRSV have been marketed since the 1980's. Because of the similarities between HRSV and BRSV, it should be possible to gain insight regarding factors that impact safety, efficacy, and duration of immunity in individuals vaccinated either in the presence or absence of circulating neutralizing antibodies using the BRSV model. Many published studies have shown that commercially available BRSV vaccines can induce protection against experimentally induced or naturally occurring BRSV disease (Durham and Hassard, 1990; Ellis et al., 2001; Van Donkersgoed et al., 1990b; Vangeel et al., 2007b; Verhoeff and van Nieuwstadt, 1984; West et al., 1999b; Xue et al., 2010).

Like HRSV vaccine ERD, BRSV vaccine ERD following natural BRSV infection has been identified; the problem has been associated with both live (Kimman et al., 1989a) and inactivated (Schreiber et al., 2000) BRSV vaccines. Kimman et al described a field outbreak of BRSV where disease appeared to be enhanced in calves that were vaccinated intramuscularly with commercially available modified-live BRSV vaccine while they were in an early stage of BRSV infection, as indicated by the retrospective identification of serum BRSV-specific IgM in calves at the time they were vaccinated. Disease was clinically evident at 1 to 2 weeks post vaccination and was more severe in vaccinated calves than in other calves on the farm that were not vaccinated. Schreiber et al described an episode of apparent vaccine ERD in calves given a commercially available vaccine inactivated with beta propiolactone and adjuvanted with alum and saponin and given by intramuscular injection. In an episode of natural BRSV infection that occurred 4 months after the last BRSV vaccination was given on this farm, older calves that had not been vaccinated and that had lower neutralizing Ab titers had less severe disease than younger calves that had been vaccinated and had higher neutralizing Ab titers. Notably, 11 of 35 vaccinated calves died during the outbreak, as compared to none of 24 unvaccinated calves. Histopathologic evaluation of lung tissue of vaccinated calves that died revealed changes consistent with BRSV infection but with infiltration of eosinophils, which are not frequently found in lung tissue of calves with severe BRSV disease that is not related to vaccination (Bryson et al., 1979; Pirie et al., 1981). The identification of eosinophils in lung tissue of calves evaluated in the study by Schreiber et al supported the concept that vaccination induced immune pathways supporting eosinophil recruitment, possibly related to production T_H2 cytokines such as IL-5 or release of chemokines such as eotaxin. The incident of vaccine-mediated ERD described by Schreiber et al was somewhat unusual in that some of the calves with vaccine ERD had higher serum neutralizing Ab titers than unvaccinated calves that had less severe disease. This is in contrast to the vaccine ERD induced in children given FI-HRSV in the 1960's (Murphy et al., 1986b), and indicates that it is possible for vaccine-induced immunopathologic mechanisms to be strong enough to overwhelm protective mechanisms. In most studies, inactivated BRSV vaccines induce low levels of neutralizing antibodies relative to total BRSV-specific antibody (Ellis et al., 1995; Gershwin et al., 1998).

The responses of calves vaccinated with FI-BRSV to BRSV challenge have been evaluated by at least 4 different groups (Antonis et al., 2003; Gershwin et al., 1998; Mohanty et al., 1981; West et al., 1999a). ERD followed challenge in some (Antonis et al., 2003; Gershwin et al., 1998) but not other (Mohanty et al., 1981; West et al., 1999a) studies. Gershwin's group

showed that the amount of viral protein in the FI-BRSV vaccine was associated with induction of vaccine ERD, with vaccine containing relatively smaller amounts of viral protein being associated with ERD (Kalina et al., 2005). When FI-BRSV vaccine enhanced disease has been identified in cattle, it has been associated with production of high concentrations of non-neutralizing antibodies in serum (Gershwin et al., 1998), low levels of virus-specific IFN- γ production by PBMCs (Woolums et al., 1999), high concentrations of BRSV-specific serum IgE and low concentrations of BRSV-specific IgG1 in lung washes, and influx of eosinophils into lung tissue (Antonis et al., 2003; Kalina et al., 2004). These findings are consistent with what has been deduced regarding immunopathogenesis of FI-HRSV vaccination from materials available from children vaccinated in the clinical trials in the 1960's, as well as from non-human primate and rodent models of FI-HRSV enhanced disease. All together, the extensive literature on BRSV vaccine ERD and similarities to HRSV vaccine ERD suggests that the BRSV model is relevant to HRSV vaccine safety. In the bovine model, BRSV vaccine ERD and efficacy can be studied in the context of natural infection.

Although BRSV vaccine ERD has been well-studied, extensive research and clinical experience indicate that BRSV vaccination can be safe and effective (Ellis et al., 1995; Ellis et al., 2005; Van Donkersgoed et al., 1990a; Vangeel et al., 2007a; Vangeel et al., 2009; Verhoeff and van Nieuwstadt, 1984; West et al., 1999b; Xue et al., 2010). In studies of resistance to experimental challenge, vaccination of calves with low or absent concentrations of serum neutralizing antibodies can protect them from virulent challenge. In a thorough evaluation of humoral and cellular immune responses following BRSV challenge of seronegative calves vaccinated with commercially available modified live vaccines given by intramuscular injection, serum BRSV-specific IgG concentration on days 4 – 7 post challenge and BRSV-specific IFN- γ production by PBMCs on the day of challenge best predicted protection against lung pathology (West et al., 1999b). Nasal fluid concentrations of BRSV-specific IgA at day 8 post-challenge were also significantly associated with protection against lung pathology, but inclusion of this outcome in the final statistical model did not improve prediction of protection. Vaccination with some inactivated vaccines has also provided good protection from virulent challenge without disease enhancement, proving that it is possible to use inactivated BRSV vaccines safely and effectively (Ellis et al., 1995; Ellis et al., 2005). Given the identification of both protection and enhanced disease in calves vaccinated with inactivated BRSV vaccines, it is likely that the formulation of the vaccine (as related to method of viral inactivation, concentration of viral protein, and nature of adjuvants and other components included) is critical to the outcome following BRSV infection of calves vaccinated with inactivated vaccines. Other factors such as host genetics and relative severity of viral challenge may also be involved.

Because disease due to BRSV is usually most severe in calves under 6 months of age, vaccines administered in the field need to be effective in calves with circulating antibodies of maternal origin. This is also true for HRSV vaccines. More research is needed to define how calves with maternal antibodies can be most effectively and safely vaccinated against BRSV. Work to date indicates that intramuscular (Harmeyer et al., 2006) or intranasal (Vangeel et al., 2007a) vaccination with modified live vaccines given to calves with moderate to high levels of serum maternal antibodies can be protective as measured by decreased severity of clinical signs and/or decreased duration of viral shedding post challenge. A small amount of information suggests that modified live but not inactivated vaccine given

intranasally may be superior to intramuscular or subcutaneous vaccination in calves with maternal antibody (Kimman et al., 1989c). However, modified live intranasal vaccination of calves with circulating maternal antibodies did not protect them from disease associated with virulent BRSV challenge 4.5 months after vaccination in one study (Ellis et al., 2010). The efficacy of vaccination in individuals with maternal antibody likely depends on a combination of factors including the nature of vaccine administered, the route of administration, the timing and number of doses administered prior to challenge, the concentration of serum neutralizing antibodies circulating at the time of vaccination, and the severity of challenge, including the titer and virulence of the challenge isolate.

Clinical trials testing BRSV vaccination efficacy have been described since the 1980's. Many evaluated the impact of BRSV vaccination on all (undifferentiated) respiratory disease; fewer have evaluated protection against disease following BRSV infection specifically. In trials evaluating the impact of BRSV vaccination on undifferentiated respiratory disease, BRSV vaccine included in a panel of vaccines given to at-risk cattle decreased disease in some but not all groups of cattle (Van Donkersgoed et al., 1990a). Trials evaluating the impact of BRSV vaccination on disease after BRSV infection are more informative about the risks and benefits of BRSV vaccination. Most such trials have been carried out in Europe; in one excellent example involving 530 calves on 27 farms, calves on farms where all calves were vaccinated with a modified live BRSV vaccine by intramuscular route had significantly decreased rates of BRSV infection and disease, compared to farms where calves were not vaccinated. Farms where half the calves were vaccinated had significantly decreased rates of disease but not infection (Verhoeff and van Nieuwstadt, 1984).

6. Candidate HRSV vaccines

HRSV vaccine candidates and development have been reviewed thoroughly, so here we selectively point out novel strategies (Anderson et al., 2010; Collins and Murphy, 2005; Crowe, 2001b; Kneyber and Kimpen, 2004; Moore and Peebles, 2006). Live attenuated vaccines have not successfully found a balance of immunogenicity and safety. Adjuvants have potential to achieve immunogenicity. CpG oligodeoxynucleotides (CpG ODN, a TLR 9 agonist) and monophosphoryl lipid (MPL, a TLR 4 agonist) adjuvants enhance immune responses to experimental HRSV vaccines (Boukhvalova et al., 2006; Mapletoft et al., 2008; Neuzil et al., 1997; Oumouna et al., 2005). A novel oil-in-water nanoemulsion adjuvanted inactivated HRSV vaccine showed promise in mice (Lindell et al., 2011). Virus-like particles (VLPs) are supramolecular assemblages of antigenic proteins in a repetitive, particulate structure (Jennings and Bachmann, 2008). Licensures of two VLP vaccines (papilloma virus and hepatitis B virus) reflect the potential of VLP vaccines. Viral proteins presented as VLPs are highly immunogenic and induce protective humoral, cellular, and mucosal immune responses (Kang et al., 2009; Quan et al., 2007). Recent studies with Venezuelan equine encephalitis virus replicon particles (VRPs) expressing HRSV G or F as well as HRSV G-expressing Newcastle disease VLPs suggest that HRSV nonreplicating VLP-like vaccines, even without adjuvants, are immunogenic, effective, and do not cause ERD (Mok et al., 2007; Murawski et al., 2009). These G- and/or F-expressing VLPs lack immunomodulatory NS proteins, so they may less interfere with host immune responses than live attenuated vaccines that do express NS proteins. Unfortunately, live attenuated viruses lacking NS proteins were over-attenuated (Jin et al., 2003).

7. Molecular epidemiology of HRSV

A better understanding of HRSV molecular epidemiology will be informative for vaccine development. HRSV has one serotype, within which there are two antigenic subgroups, A and B, defined by reactivity to monoclonal Abs (Collins et al., 2006). Amino acid changes in variable regions of the HRSV attachment glycoprotein (G) occur in response to immune pressure (Botosso et al., 2009; Cane and Pringle, 1992, 1995; Gaunt et al., 2011; Zlateva et al., 2005). Within antigenic subgroups, HRSV strains can be further classified into clades, and clades can be divided into subtypes based on nt sequence of a hypervariable region of the G gene (Cane, 2001; Peret et al., 2000; Peret et al., 1998). The classification of HRSV strains is *antigenic subgroup > clade > subtype > isolate/strain*. Phylogenetic analysis of a hypervariable region of the G gene revealed that subgroup A strains can be divided into seven clades (GA1-GA7) and subgroup B strains can be divided into four clades (GB1-GB4) (Peret et al., 2000; Peret et al., 1998). These clades have held up as distinct clusters of circulating HRSV in communities around the world, and new clades are being described (Agenbach et al., 2005; Botosso et al., 2009; Cane, 2001; Matheson et al., 2006; Parveen et al., 2006; Peret et al., 2000; Peret et al., 1998; Scott et al., 2006). HRSV subtypes exhibit >96% nt similarity within hypervariable G and thus represent closely related isolates (Peret et al., 1998). Annual HRSV epidemics consist of one dominant subtype accounting for approximately 50% of isolates, and a variable number of less prevalent subtypes in a small number of clades (Cane, 2001; Peret et al., 2000; Peret et al., 1998; Sullender, 2000). The Dominant HRSV subtype in a given location is replaced in one or two HRSV seasons (Cane, 2001; Peret et al., 2000; Peret et al., 1998). Although immune selection appears to drive mutation in the HRSV G protein by positive selection, these mutations are not progressive antigenic drift as in influenza (Botosso et al., 2009; Cane and Pringle, 1995; Hay et al., 2001). Rather, it appears that positively selected sites flip-flop over time (Botosso et al., 2009), suggesting an antigenic toggling in G as immunity in the population rises and falls to circulating strains.

NAbs to HRSV bind either the viral G or F protein (Collins et al., 2006). Responses to HRSV F protein are generally cross-neutralizing to diverse HRSV strains whereas Abs to G are generally more subgroup- and clade-specific. HRSV induces Abs in humans and mice that bind epitopes conserved between all HRSV strains, and these Abs cross-neutralize strains. Palivizumab, a monoclonal nAb given prophylactically to humans, binds to the F protein and binds all HRSV strains (Meissner and Long, 2003). Despite nAbs that neutralize all HRSV strains tested, strain-specific Abs likely contribute to protection. It has been clearly demonstrated that the antigenic subgroup (A or B) is important for Ab responses. Ab responses to the G protein are largely subgroup-specific whereas Ab responses to the F protein are cross-reactive between subgroups A and B (Sullender, 2000; Sullender et al., 1998). Sera from HRSV antigenic subgroup A strain-infected individuals neutralizes subgroup A strains *in vitro* better than subgroup B strains (Cane, 2001; Sullender, 2000). For this reason, bivalent vaccines containing G and/or F proteins from representative subgroup A and B strains have been developed and tested in animals (Cheng et al., 2001; Whitehead et al., 1999). In addition to subgroup-specific Abs to the G protein, HRSV induces clade- or subtype-specific Abs (Beeler and van Wyke, 1989; Johnson et al., 1987; Scott et al., 2007). The neutralizing titer (reciprocal of dilution required to inhibit infectivity) of pooled adult human HRSV anti-serum varies between 320 and 2,560 for different subgroup A strains (Beeler and van Wyke, 1989). This suggests that (G-expressing e.g. live attenuated) vaccines

based on one subgroup A strain will induce varied and suboptimal immunity to circulating subgroup A strains. Studies have shown that HRSV induces poor nAb titers in infants, and < 50% of young infants with culture-documented HRSV infection have detectable nAbs (Brandenburg et al., 1997; Wright et al., 2002; Wright et al., 2007). However, these studies used one subgroup A strain (A2) as the challenge strain for *in vitro* neutralization assays.

8. Conclusion and future directions

A major goal of studying RSV pathogenesis is to facilitate vaccines by elucidating virus-host interactions at the level of immunity and by providing challenge models to test candidate vaccines. The pathology of HRSV in infants resembles that of BRSV in calves and PVM in mice. The major features of this pathogenesis are 1) viral tropism for airway and alveolar epithelial cells, 2) airway epithelial desquamation, 3) obstruction of airways with mixtures of mucus, fibrin, and cells (epithelial debris and inflammatory cells), and 4) prominent neutrophil and macrophage inflammation in bronchiolar, alveolar, and interstitial spaces. Despite limitations, there are tractable non-chimpanzee animal models of HRSV pathogenesis that recapitulate key features of this pathogenesis. These include HRSV strain line 19 and HRSV clinical isolate infection of BALB/c mice, infection of cotton rats, and infection of newborn lambs. Each has advantages and caveats. Robust pathology and pulmonary dysfunction are endpoints that could prove useful for vaccine evaluation. Experimental BRSV and PVM infection in the natural host provide fully permissive comparative models of HRSV pathogenesis. In addition to comparative virology and pathogenesis, the BRSV field parallels HRSV with an extensive literature of vaccinology that includes inactivated virus ERD as well as numerous successful vaccines. Collaboration in these fields could facilitate insights into HRSV vaccines. Challenges such as MatAb, poor immunogenicity, immune modulation, and immature neonatal immunity exist in both infants and calves. One difference is that the molecular epidemiology of BRSV shows less virus variation than HRSV. Similarly, swine influenza exhibits low comparative variation, owing to the fact that these animals are culled, so the dynamics of immunity in the population differ from humans. One way to take advantage of the BRSV system would be to test HRSV vaccines in calves by challenging with chimeric BRSV viruses expressing the HRSV antigen(s).

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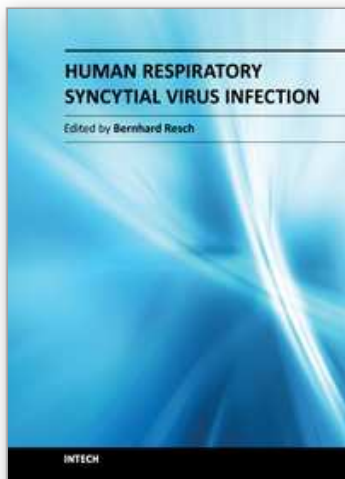
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In this online Open Access book on "Human RSV Infections", several distinguished authors contribute their experience in respiratory syncytial virology. A major focus lies on the fascinating pathophysiology of RSV and represents recent and actual work on different mechanisms involved in the complex pathogenesis of the virus. The second section elucidates epidemiologic and diagnostic aspects of RSV infection covering a more clinical view of RSV disease. At last, treatment modalities including the search for a vaccine that is still not in sight are discussed and conclude this book, thus building up a circle that runs from experimental models of RSV related lung disease over clinical aspects of disease to the latest news of therapeutic and prophylactic approaches to human RSV infection.

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