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Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years

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Abstract

Human respiratory syncytial virus (RSV) is a ubiquitous pathogen that infects everyone worldwide early in life and is a leading cause of severe lower respiratory tract disease in the pediatric population as well as in the elderly and in profoundly immunosuppressed individuals. RSV is an enveloped, nonsegmented negative-sense RNA virus that is classified in Family *Paramyxoviridae* and is one of its more complex members. Although the replicative cycle of RSV follows the general pattern of the *Paramyxoviridae*, it encodes additional proteins. Two of these (NS1 and NS2) inhibit the host type I and type III interferon (IFN) responses, among other functions, and another gene encodes two novel RNA synthesis factors (M2-1 and M2-2). The attachment (G) glycoprotein also exhibits unusual features, such as high sequence variability, extensive glycosylation, cytokine mimicry, and a shed form that helps the virus evade neutralizing antibodies. RSV is notable for being able to efficiently infect early in life, with the peak of hospitalization at 2–3 months of age. It also is notable for the ability to reinfect symptomatically throughout life without need for significant antigenic change, although immunity from prior infection reduces disease. It is widely thought that re-infection is due to an ability of RSV to inhibit or subvert the host immune response. Mechanisms of viral pathogenesis remain controversial. RSV is notable for a historic, tragic pediatric vaccine failure involving a formalin-inactivated virus preparation that was evaluated in the 1960's and that was poorly protective and paradoxically primed for enhanced RSV disease. RSV also is notable for the development of a successful strategy for passive immunoprophylaxis of high-risk infants using RSV-neutralizing antibodies. Vaccines and new antiviral drugs are in pre-clinical and clinical development, but controlling RSV remains a formidable challenge.

1. History

Human respiratory syncytial virus (RSV) was first isolated in 1955 from a captive chimpanzee with upper respiratory tract illness (URI) (Morris et al., 1956). It was quickly identified as a human virus and shown to be a major pediatric respiratory pathogen (Chanock and Finberg, 1957; Chanock et al., 1957). RSV is now recognized as the most important viral agent of lower respiratory tract illness (LRI) in infants worldwide (Hall et al., 2009; Nair et al., 2010), as well as an important cause of LRI in older individuals with

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cardiopulmonary disease or in whom immune responses are impaired or reduced (Dowell et al., 1996; Falsey, 2007; Falsey et al., 2005; Hall et al., 1986; Whimbey and Ghosh, 2000). Infants and young children at high risk for severe RSV disease can be substantially protected by the passive administration of a commercially available RSV-neutralizing monoclonal antibody (MAb) (Mejias and Ramilo, 2008; Wu et al., 2008). The guanosine analog ribavirin is available for antiviral therapy; however, its efficacy is marginal and controversial and it is not recommended for routine use (2006). There remains a need for more effective antiviral drugs and for a vaccine for protection of the general population. This article will review notable features of the virus, new findings, and the prospects for control of this important pathogen.

2. Virus, RNAs, and proteins

RSV is an enveloped non-segmented negative-sense RNA virus. It replicates in the cytoplasm and buds at the plasma membrane (Fig. 1). RSV is classified in Family *Paramyxoviridae*, Order *Mononegavirales*. This family has two subfamilies (i) *Pneumovirinae*, which consists of RSV, human metapneumovirus (HMPV), and their animal relatives, and (ii) *Paramyxovirinae*, which includes Sendai virus, the human parainfluenza viruses (HPIVs), measles virus, and a number of other pathogens of humans and animals.

Some of the notable features of RSV molecular biology are summarized in Table 1. RSV grown in cell culture includes pleomorphic spherical particles but predominantly consists of long filaments. Progeny particles mostly remain cell-associated, suggestive of incomplete budding. Release requires sonication, vortexing, or some other means, increasing the contamination by cellular material. Titer is modest, approximately 10^7 infectious units per ml. Infectivity is unstable and can readily be lost during handling, although losses can be substantially reduced by inclusion of sucrose or other stabilizers. These factors pose substantial obstacles to research and vaccine development.

The RSV genome is 15.2 kb in length and has 10 genes in the order 3′ NS1-NS2-N-P-M-SH-F-G-M2-L (Fig. 1). These are transcribed into 10 separate mRNAs. Each encodes a single polypeptide except for the M2 mRNA, which has an upstream ORF encoding the M2-1 protein and a second, downstream ORF that briefly overlaps the upstream ORF and encodes the M2-2 protein. Translation of the M2-2 ORF depends on re-initiation by ribosomes as they exit the M2-1 ORF; surprisingly, re-initiation was found to be influenced by a RNA structure located ~150 nucleotides upstream of the M2-2 translational start site (Gould and Easton, 2005; Gould and Easton, 2007). With a total of 11 distinct viral proteins, RSV is one of the more complex members of *Paramyxoviridae*: compared to other members of the family, the RSV genome is similar in size but has several additional genes and proteins, namely NS1, NS2, SH, M2-1 and M2-2 (Fig. 1).

RSV transcription and RNA replication follow the general *Mononegavirales* model. The polymerase enters the genome at the 3′ end, which begins with a 44-nucleotide leader region (Fearns et al., 2002; McGivern et al., 2005). Genes are transcribed sequentially guided by short gene-start and gene-end signals to yield individual mRNAs (Kuo et al., 1996). During RNA replication, the polymerase ignores transcription signals and produces a complete positive sense replicative intermediate called the antigenome, which then serves as the template for producing progeny genomes. Somewhat unusually, two of the RSV genes (M2 and L) overlap by 68 nucleotides (Collins et al., 1987): studies with mini-replicons showed that, following transcription of the M2 gene, the viral polymerase scans in both the upstream and downstream directions to locate the L gene start site (Fearns and Collins, 1999a). Scanning may be a more general activity of the polymerase, and is speculated to occur at each gene junction during sequential transcription as well as during initiation of

transcription and RNA replication. Other recent mini-replicon studies mapped a cis-acting signal necessary for both transcription and RNA replication to the first 11 nucleotides of the genome and identified additional leader sequences necessary for optimal transcription or encapsidation (Fearns et al., 2002; McGivern et al., 2005). These studies confirmed that chain elongation of replicative RNA depends on encapsidation (McGivern et al., 2005), and made the somewhat unusual observation that the first nucleotide of nascent mRNA and antigenome can be chosen by the polymerase independent of the template (Kuo et al., 1997; Noton et al., 2010).

RNA synthesis is carried out by the large L polymerase protein, which also performs mRNA capping (Liuzzi et al., 2005) and polyadenylation. The actual template is the viral ribonucleoprotein (RNP) or nucleocapsid, a complex of viral genomic or antigenomic RNA tightly and completely bound by N protein. The tight encapsidation of the viral genome (and antigenome) is characteristic of *Mononegavirales*. This likely prevents degradation of these RNAs, which lack the stabilizing features of a 5′ cap and 3′ poly A. It also is thought that encapsidation minimizes detection of these RNAs by pattern recognition receptors in the host cell, in particular (i) the cytoplasmic helicases RIG-I and MDA-5, which detect cytoplasmic tri-phosphorylated RNA and dsRNA and initiate signal transduction to activate the cellular transcription factors IRF3 and NFκB involved in inducing type I interferon (IFN) and pro-inflammatory cytokines, and (ii) dsRNA-activated protein kinase R (PKR), which also activates NFκB as well as phosphorylating eukaryotic elongation factor 2a (eIF-2a) to inhibit protein synthesis as part of antiviral defense.

A crystal structure was recently described for the RSV N protein expressed in bacteria and isolated as a decamer ring in association with bacterial RNA (Tawar et al., 2009). This ring likely mimics one turn of the helical RNP. Each N monomer was organized as a core region containing two domains, N- and C-terminal, which were connected through a hinge region that was associated with seven nucleotides of the bound RNA. The arrangement of the nucleotides relative to the protein suggested that passage of the polymerase induces a transient local conformational change that allows access to the RNA template. The N-terminal domain of N has a long β hairpin projecting away from the core that is not present in the 3D structure of other *Mononegavirales* N proteins and may be a site of contact with the L polymerase. The structure also indicated that the C-terminus of the N protein extends above the plane of the ring, thus being available for interaction with the P protein during RNA synthesis.

The P protein is a homotetramer which interacts with N (Garcia-Barreno et al., 1996), L (Khattar et al., 2001), and M2-1 (Asenjo et al., 2006) and which is an essential co-factor of the polymerase. Although much shorter than other *Paramyxoviridae* counterparts, RSV P has similar activities. The C-terminus of P interacts with the C-terminus of N to open the RNP structure so that the polymerase, tethered by P, can reach the bases in the viral RNA. In addition, P interacts with newly synthesized N (N°) to prevent illegitimate assembly of the latter and to deliver it to the nascent chain during genome replication (Castagne et al., 2004; Curran et al., 1995). Promoter clearance and chain elongation by the viral polymerase during transcription appears to be dependent on the P protein (Dupuy et al., 1999) and on capping of the nascent transcript (Liuzzi et al., 2005).

M2-1 and M2-2 are novel RNA synthesis factors. M2-1 is a transcription processivity factor: in its absence, transcription terminates prematurely and non-specifically within several hundred nucleotides (Collins et al., 1996; Fearns and Collins, 1999b). M2-1 also enhances read-through transcription at gene junctions to generate polycistronic RNAs (Hardy and Wertz, 1998), which may reflect the same processivity activity. M2-1 is a homotetramer that binds to the P protein and RNA in a competitive manner, suggesting that P associates with

soluble M2-1 and delivers it to the RNA template (Tran et al., 2009). M2-1 contains a zinc finger motif that appears to be related to the cellular zinc finger protein tristetraprolin (Hardy and Wertz, 2000). Tristetraprolin binds cellular mRNAs and affects mRNA stability, but the significance of this similarity remains unclear. The other factor involved in RNA synthesis, the M2-2 protein, is a small, non-abundant species that accumulates during infection and appears to shift RNA synthesis from transcription to RNA replication (Bermingham and Collins, 1999).

The non-structural NS1 and NS2 accessory proteins also may affect RNA synthesis, since they inhibited transcription and RNA replication by a mini-replicon (Atreya et al., 1998). Other paramyxovirus accessory proteins also have been shown to down-regulate viral RNA synthesis, and recent studies with Sendai virus and HPIV1 indicate that preventing overly robust RNA synthesis avoids the accumulation of unencapsidated genomes and dsRNA that would otherwise be recognized by RIG-I, MDA-5, and PKR (Boonyaratanakornkit et al., 2011; Takeuchi et al., 2008). Therefore, part of the inhibition of IFN induction mediated by NS1 and NS2 may be related to this apparent capacity to down-regulate viral RNA synthesis.

The M protein is thought to line the inner surface of the viral envelope. M appears to play a central role in budding (Henderson et al., 2002; Teng and Collins, 1998) and may also silence viral RNA synthesis in preparation for packaging (Ghildyal et al., 2003). A crystal structure was recently determined for the RSV M protein and revealed a monomer that is organized into compact N-terminal and C-terminal domains joined by a short linker (Money et al., 2009). The surface of M contains a large positively charged region that extends across both of the domains and the linker and may mediate association with the plasma membrane and RNP.

Progress also has been made in further characterizing the RSV glycoproteins (Fig. 2). The RSV F protein is generally similar in overall structure and function to its counterparts in *Paramyxovirinae* despite extensive sequence divergence. Like these counterparts, RSV F mediates viral penetration as well as fusion between infected cells and their neighbors. The RSV F protein also activates toll-like receptor (TLR)4 on human leukocytes independent of virus replication to initiate innate immune responses (Kurt-Jones et al., 2000). The RSV F0 precursor is cleaved at two closely spaced sites (instead of one for other paramyxoviruses) by a furin-like enzyme to generate the F1 and F2 chains (F2 N-terminal to F1) and become fusion competent (Gonzalez-Reyes et al., 2001; Zimmer et al., 2001). In addition, the RSV F protein efficiently mediates fusion on its own, whereas fusion by members of *Paramyxovirinae* usually requires cooperative interaction between F and the cognate attachment protein. Recently, insertion of the two RSV cleavage sites into the F protein of recombinant Sendai virus resulted in a hyperfusogenic phenotype with reduced thermostability of the virus, decreased dependence on the attachment (HN) protein for fusion, and decreased dependence on sialic acid for attachment (Rawling et al., 2011; Rawling et al., 2008). Thus, several notable properties of RSV may be linked to the unusual double-cleavage sequence of its F protein.

As with other *Paramyxoviridae*, the RSV F protein is present in the virus particle in a metastable pre-fusion structure (Lamb, 1993). Binding with the target cell triggers a series of conformational changes in the F protein including the formation of a pre-hairpin intermediate, in which the hydrophobic fusion peptide at the N-terminus of the F1 chain is inserted into the target membrane. Refolding of this intermediate results in the assembly of a highly stable post-fusion structure dictated mostly by formation of a six-helix bundle (6HB) containing heptad repeats A and B (HRA and HRB) from each monomer (Zhao et al., 2000). Such an arrangement places the fusion peptide and the transmembrane region of the F1

chain at the same end of the F protein with the consequent merging and fusion of the viral and cell membranes. The free-energy released on 6HB formation drives the membrane fusion process (Russell et al., 2001).

Two different groups have solved recently the atomic structure of the RSV F protein ectodomain in its post-fusion conformation based on analysis of a version of the protein that was engineered to remove the fusion peptide and the transmembrane domain and cytoplasmic tail (McLellan et al., 2011; Swanson et al., 2011). As predicted, the structure is similar to those described for the HPIV3 (Yin et al., 2005) and Newcastle disease virus (Swanson et al., 2010) F proteins, with a cone shape that resembles the images obtained by electron microscopy of a soluble RSV F protein ectodomain preparation (Calder et al., 2000). Significantly, the local structures of epitopes recognized by neutralizing MAbs, such as have recently been visualized in complexes of Fab-peptides (McLellan et al., 2010a; McLellan et al., 2010b), are present in the post-fusion structure as well as in a predicted pre-fusion conformation that was modeled from the equivalent structure from PIV5 (Yin et al., 2006). In agreement with this hypothesis, it was recently shown that several MAbs could bind and immunoprecipitate a pre-fusion form of the RSV F protein (Chaiwatpongsakorn et al., 2011), including the antibody precursor of palivizumab that is also able to bind to post-fusion F (McLellan et al., 2011). Conservation of these epitopes in the two otherwise dissimilar structures can explain the recent finding that MAbs able to bind to the post-fusion form of RSV F can neutralize virus infectivity if pre-incubated with the virus before addition to cells; i.e., before the F protein is activated for fusion (Magro et al., 2010). It has been proposed that these MAbs bind to the pre-fusion conformation of RSV F and inhibit the subsequent conformational changes required for membrane fusion.

Recent findings indicate that the HRSV F protein also plays a major role in viral attachment, involving interaction with the cellular protein nucleolin (Tayyari et al., 2011). This interaction was shown to be necessary for efficient infection in cell culture and in the mouse model. Previously, the G protein had been identified as playing a major role in RSV attachment (Levine et al., 1987). G indeed is essential for efficient replication *in vivo*, although virus lacking the G gene can replicate efficiently in Vero cells *in vitro* (Karron et al., 1997a; Techaarpornkul et al., 2001; Techaarpornkul et al., 2002; Teng and Collins, 1998; Teng et al., 2001). Both G and F had been shown to bind to glycosaminoglycans (GAGs), which are long unbranched chains of repeating disaccharide subunits that are part of the host cell glycocalyx (Feldman et al., 1999), and binding to GAGs is necessary for efficient infection of cell lines by RSV. Taken together, these observations suggest that efficient attachment by RSV *in vivo* depends on both F and G, and may depend on two different binding events involving GAGs and nucleolin.

RSV G is an unusual protein. Its ectodomain consists mainly of two large “mucin-like” domains, so-named because, like cellular mucins, they are rich in proline, serine, and threonine residues, contain several N-linked and many O-linked sugar side chains, and may have extended, unfolded secondary structures (Johnson et al., 1987b; Wertz et al., 1985). The carbohydrate content of the G protein is considerable, shifting the apparent molecular weight of the polypeptide backbone from 32,000 to 90,000 (Wertz et al., 1989). More recently, analysis of G protein produced in an *in vitro* model of human airway epithelium (HAE) – a pseudostratified mucocilliary tissue that closely resembles the authentic epithelium (Zhang et al., 2002) – suggested that the carbohydrate content is much higher, yielding an apparent molecular weight of 180,000 (Kwilas et al., 2009). It is speculated that this sheath of host-specified carbohydrate helps shield the protein from immune recognition. Whether due to carbohydrate, or its unfolded structure, or some other reason, G is a less efficient neutralization and protective antigen compared to F (Olmsted et al., 1986), and most individual MAbs against G do not neutralize infectivity (Martinez et al., 1997).

However, mixtures of MAbs directed against non-overlapping epitopes of G have a synergistic effect on virus neutralization, suggesting that steric hindrance of binding to the cell surface may be the basic mechanism by which serum polyclonal anti-G antibodies inhibit RSV infectivity (Martinez and Melero, 1998). Recently, high-affinity anti-G antibodies were obtained by cloning mRNAs obtained from rare human B cells (Collarini et al., 2009). These antibodies were highly neutralizing *in vitro* and may use a still-uncharacterized mechanism to inhibit virus infectivity different from those of other more common anti-G antibodies.

The two mucin-like domains in G are highly variable and contain multiple epitopes that are poorly conserved between strains. These two domains are separated by a central region to which conserved epitopes have been mapped (Martinez et al., 1997). This conserved region includes a segment of 13 highly conserved amino acids that overlaps with a segment containing four closely spaced, invariant cysteine residues that are disulfide-bonded to form a cystine noose (Gorman et al., 1997; Johnson et al., 1987b). The downstream pair of cysteine residues conform to a CX3C motif that is embedded in a region of limited sequence relatedness to the CXC3 chemokine fractalkine, and a peptide containing this sequence mimicked the leukocyte attractant activity of fractalkine in an *in vitro* assay (Tripp et al., 2001). Studies in the mouse model comparing wild-type RSV with a mutant lacking the CX3C motif showed that fractalkine mimicry reduces the pulmonary influx of immune cells involved in innate and adaptive responses to infection (Harcourt et al., 2006). In addition, the cysteine-rich domain of G was shown to inhibit activation of TLR2, 4, and 9 in human monocytes, thus suppressing innate immune responses (Polack et al., 2005). G also is expressed in an abundant secreted form due to use of a secondary translational initiation codon (Hendricks et al., 1988; Roberts et al., 1994). Recent findings showed that this secreted form functions as an antigen decoy to help the virus escape neutralizing antibodies, and also acts to reduce antibody-mediated clearance by immune cells (Bukreyev et al., 2008). A comparable secreted antigen is not found in other common respiratory viruses and may contribute to the ability of RSV to infect unusually early in life and re-infect throughout life despite the presence of antibodies from maternal transfer or from previous infection.

RSV encodes a third, short transmembrane glycoprotein, SH, that (like G) is anchored into the membrane by a hydrophobic signal-anchor sequence near the N-terminus, with the C-terminus oriented extracellularly (Collins and Mottet, 1993). SH accumulates in multiple forms due to the use of two translational start sites and the variable presence of N-linked sugar and polylactosaminoglycan, although the significance of the multiple forms is unknown (Anderson et al., 1992; Olmsted and Collins, 1989). The SH protein shares structural features with a class of small hydrophobic proteins, the viroporins (Gonzalez and Carrasco, 2003), that insert into the membrane of infected cells and induce permeability to ions and small molecules. In agreement with this hypothesis, SH increases membrane permeability when expressed in bacteria (Perez et al., 1997), and when incorporated into artificial membranes it forms pentameric and hexameric pore-like structures with cation channel-like activity (Carter et al., 2010; Gan et al., 2008). The SH protein was reported to reduce apoptosis and to inhibit tumor necrosis factor (TNF)-α expression and signaling, but the effects seemed small (Fuentes et al., 2007). Deletion of SH resulted in a virus that was slightly attenuated in mice and chimpanzees (Bukreyev et al., 1997; Whitehead et al., 1999), but deletion of SH from an experimental vaccine candidate did not increase its attenuation in seronegative children (Karron et al., 2005).

The NS1 and NS2 proteins interfere with both the induction of IFN-β and subsequent signaling from the IFN receptor. The NS1 protein had the greater effect on inhibiting IFN induction, although inhibition was greatest with both proteins together (Spann et al., 2004). NS1 and NS2 may form functional heteromers. These proteins impede the signal

transduction pathway leading to up-regulation of IFN-β at multiple steps: NS2 binds to the cytoplasmic receptor RIG-I (Ling et al., 2009); either protein (and especially NS1) mediates a decrease in TRAF3 (Swedan et al., 2009); and NS1 mediates a decrease in IKKε (Swedan et al., 2009). The NS proteins also strongly suppress IFN-induced signal transduction from its receptor through the JAK/S TAT pathway (Ramaswamy et al., 2004). This suppression is mediated by proteosomal degradation of the STAT2 signal transduction factor (Ramaswamy et al., 2004). This is due primarily to NS2 (Lo et al., 2005; Ramaswamy et al., 2006; Swedan et al., 2009), although there also is evidence that NS1 can assemble ubiquitin ligase enzymes to target STAT2 for the proteosome (Elliott et al., 2007a). RSV also inhibits induction of type III IFN in human epithelial cells and macrophages (Spann et al., 2004), which is thought to involve the same signaling pathway (Donnelly and Kotenko, 2010). Type III IFN uses a different receptor, but the downstream signaling depends on STAT1 and STAT2 (Donnelly and Kotenko, 2010; Mordstein et al., 2010) and thus should similarly be suppressed by the RSV NS proteins. NS1 and NS2 also activate pro-survival pathways in the infected cell, prolonging its survival and increasing viral yield (Bitko et al., 2007).

Recent studies have revealed additional, novel ways that RSV uses to prevent activation of cellular defense in response to infection. For example, the RSV N protein was shown to bind to PKR, preventing it from phosphorylating eIF-2a, and presumably also from activating NFκB (Groskreutz et al., 2010). In another study, RSV was shown to block the formation of stress granules, which can otherwise restrict RSV replication, via expression of RNA derived from the trailer region (Hanley et al., 2010). RSV infection also was recently shown to result in degradation of the outer mitochondrial membrane-associated adaptor MAVS, which is in the signal transduction pathways leading to activation of IRF3 and NFκB (Yoboua et al., 2010). Thus, RSV has multiple means of inhibiting IFN induction and signaling, the induction of pro-inflammatory cytokines, the induction of apoptosis, and other aspects of cellular defense against infection. At least in the case of type I IFN, the inhibition exerted by RSV appears to be more effective than that of HMPV, HPIVs, and influenza virus (Guerrero-Plata et al., 2005a; Guerrero-Plata et al., 2005b; Hall et al., 1981; Hall et al., 1978; McIntosh, 1978). This likely contributes to the high infectivity of RSV, and may also reduce immune responses by precluding the adjuvant effects of IFN (Valarcher et al., 2003).

3. Epidemiology and Evolution

RSV is a highly contagious virus that can infect an individual multiple times throughout life. Human are the only natural host for RSV, although the virus can readily infect and sometimes cause severe disease in non-human primates (Kondgen et al., 2008; Morris et al., 1956). RSV infections occur mostly in yearly epidemic outbreaks during the winter months in temperature countries or during the rainy season in tropical countries. Most children are infected by RSV during the first year of life and virtually all are infected by the age of two (Glezen et al., 1986). Re-infection is frequent during the first few years of life: in one prospective study, of the children who had been infected during the first year of life, 47% and 45% were re-infected during the second and third years of life, respectively (Glezen et al., 1986). Re-infection does not depend on antigenic differences. LRI can occur during the first or second infections, but there was a considerable reduction in disease severity in subsequent infections that presumably reflects increasing protective immunity (Henderson et al., 1979). Hospitalization for severe HRSV disease is most frequent between 6 weeks and 6 months of life, with a peak incidence at 2–3 months of life.

A recently published large prospective study in the United States provided a detailed evaluation of the pediatric impact of RSV in an affluent country. This study estimated that 2.1 million children under the age of five require medical attention each year due to RSV, resulting annually in 1,534,064 (1 of 13 for that age group) office visits, 517,747 (1 of 38)

visits to the emergency room, and 57,527 hospitalizations (Hall et al., 2009). Sixty-one percent of the office visits were in children of 2 to 5 years of age: this showed that, while the impact of RSV disease is greatest during the first year of life, RSV also causes a substantial disease burden in children beyond this first year (Hall et al., 2009).

Another new study estimated that, globally, RSV caused almost 34 million LRI in children under 5 years of age in 2005, ten percent of them requiring hospitalization (Nair et al., 2010). This resulted in an estimated 66,000–199,000 deaths, with 99% of these deaths occurring in developing countries, reflecting both a greater population of infants and young children and the unjust consequences of inadequate resources. These values for morbidity and mortality probably are under-estimates, since RSV testing was incomplete and community LRI undercounted.

In affluent countries, deaths due to RSV appear to have declined in recent years. A survey from the United Kingdom in the mid 1970s estimated the fatality rate at 0.5% to 2.5% of hospitalized children with RSV infection (Clarke, 1978), and in 1985 RSV was estimated to be responsible for 4,500 pediatric deaths annually in the United States (Meissner, 1994). In contrast, more recent estimates are as low as 0.3% of hospitalized children. In the United States, the pediatric mortality rate was recently estimated to be 5.3 and 0.9 per 100,000 per year for individuals < 1 year of age and 1–4 years of age, respectively, totaling approximately 300 deaths per year (Thompson et al., 2003). This likely reflects improvement in supportive care.

A number of factors can increase the risk for severe RSV disease early in life, including prematurity, low titers of maternal antibodies or lack of previous RSV infection (Berkovich, 1964; Cunningham et al., 1991), underlying cardiopulmonary disease (Groothuis et al., 1988; MacDonald et al., 1982), and immunosuppression or immunodeficiency disorders (Fishaut et al., 1980; McIntosh et al., 1984). In one study, the estimated number of RSV hospitalizations per 1000 during the first year of life was 388 for infants with chronic lung disease, 92 for those with congenital heart disease, 66 for those born at 29 to <33 weeks, and 30 for term infants with no underlying disease (Boyce et al., 2000). However, it is important to note that more than half of RSV hospitalizations occur in previously healthy, full term individuals. A number of recent studies have described associations between genetic polymorphisms involving host defense genes and severe pediatric RSV disease (Miyairi and DeVincenzo, 2008). Among other findings, these studies have implicated polymorphisms in pulmonary surfactant proteins, or associated with reduced activation of TLR4, or associated with increased expression of the IL-4 or IL-8 gene, or associated with other innate immune response factors (El Saleeby et al., 2010; Hacking et al., 2004; Janssen et al., 2007; Lofgren et al., 2010; Miyairi and DeVincenzo, 2008; Puthothu et al., 2007; Schuurhof et al., 2010; Siezen et al., 2009). However, findings have often been inconsistent, probably because the studies have been insufficiently large to validate associations.

RSV re-infects healthy adults at a rate of approximately 5–10% per year, a rate that increases with increased exposure to the virus, such as with health care personnel (Falsey, 2007; Falsey et al., 2005). Hospitalization due to RSV in otherwise healthy non-elderly adults is rare, but RSV is considered to be second only to seasonal influenza as a cause of medically significant respiratory tract disease in adulthood. Morbidity and mortality due to RSV is substantially increased in the elderly, presumably due in part to immune senescence. RSV is estimated to cause on average 17,358 deaths annually in the United States, with 78% of these deaths in adults over age 65 (Thompson et al., 2003). Thus, in more affluent countries, deaths due to RSV are much more frequent in the elderly than in the pediatric population, whereas in less affluent countries the pediatric burden is likely to be greater.

Morbidity and mortality due to RSV also is substantially increased in adults with underlying pulmonary or cardiac disease or who are severely immunosuppressed, especially with T cell deficiencies (Falsey, 2007; Falsey et al., 2005; Whimbey and Ghosh, 2000). In particular, the mortality rate associated with severe RSV infection in adults with profound immunosuppression due to leukemia or hematopoietic stem cell transplant can be as high as 80–100% (Whimbey and Ghosh, 2000).

Human RSV isolates are classified into two antigenic groups (A and B) (Mufson et al., 1985) that represent separate genetic lineages who divergence was recently calculated to have occurred approximately 350 years ago (Zlateva et al., 2005). The highest sequence divergence between the two groups is found in the G protein ectodomain and particularly in the two mucin-like regions mentioned before (Johnson et al., 1987a; Johnson et al., 1987b). Multiple genotypes within subgroups A and B have been identified that can co-circulate within the same season and community, with one or two dominant genotypes being replaced in successive years (Cane, 2001). In addition, there can be shifts in the predominance of subgroup A versus B occurring in 1- or 2-year cycles (Waris, 1991). This reflects a modest advantage of the heterologous strain in evading previously-induced immunity (White et al., 2005), but re-infection by the same subgroup also is very frequent.

Accumulation of genetic and antigenic changes with time within the same evolutionary branch, as well as identification of sites of positive selection which coincide partially with epitopes recognized by anti-G MAbs are suggestive of immune driven RSV evolution (Botosso et al., 2009). However, the RSV evolutionary pattern does not resemble that of influenza A virus, in which a strong immune selection by prevailing antibodies dictates the fast replacement worldwide of dominant strains in a linear manner. Instead, RSV evolution resembles that of influenza B viruses, in which a less strong immune selection favors slower co-evolution of several branches (Rota et al., 1992). This is exemplified by the recently described BA genotype within the antigenic group B of human RSV. These viruses that contain a 60-nucleotide duplication in the G gene were first identified in 1998. Although they spread very quickly worldwide, it was not until 2005 that they replaced other prevailing genotypes of RSV group B (Trento et al., 2010).

In apparent contradiction to the weak selection exerted by anti-G antibodies, the G protein is one of the most variable gene products found in any human virus. It is possible that this variability simply reflects the extreme plasticity of G to accommodate not only amino acid substitutions but in addition other more drastic sequence changes, such as insertions, deletions, frame-shift mutations and premature stop codons (Melero et al., 1997). The extensive variability of the G protein contrasts with the high level of sequence conservation of the F protein, including epitopes that are recognized by highly efficient neutralizing antibodies. It may be that structural restrictions limit the antigenic diversity of F; consistent with this, a number of escape mutants selected with F MAbs exhibited reduced fitness, although one actually had an apparent increase in fitness (Zhao et al., 2006; Zhu et al., 2011).

4. Protective immunity

A wealth of evidence indicates that protection against RSV infection is conferred mainly by neutralizing antibodies. The F and G glycoproteins are the only viral antigens able to induce neutralizing antibodies as well as relatively long-lived protection in animal models (Connors et al., 1991; Stott et al., 1987). Passive transfer of these antibodies (Graham et al., 1993; Prince et al., 1985a) or MAbs directed against F or G (Taylor et al., 1984; Walsh et al., 1984) protects mice and cotton rats against RSV challenge. Likewise, infants at high risk of severe RSV disease can be substantially protected by the prophylactic administration of

RSV-neutralizing polyclonal or monoclonal antibodies, as discussed later (see section 7. *Immunoprophylaxis with antibodies*). Furthermore, a positive correlation has been found between high titres of serum neutralizing antibodies and protection of human volunteers against RSV challenge (Hall et al., 1991), and there is an inverse correlation between the titres of RSV-neutralizing serum antibodies and the risk of infection in children (Glezen et al., 1986) and in the elderly (Falsey and Walsh, 1998). Local secretory antibodies are thought to be particularly efficient in restricting RSV replication but generally decrease with time. Serum antibody responses are more durable, at least outside of infancy. However, serum antibodies gain access to the respiratory tract primarily by the inefficient process of passive transudation. This results in a steep concentration gradient, especially in the upper respiratory tract. For example, in the cotton rat model, RSV-neutralizing serum antibody titers of 1:390 and 1:3500 were required for a 99% reduction in RSV replication in the lower and upper respiratory tract, respectively (Prince et al., 1985b; Siber et al., 1994). In humans, there was a 1:350 concentration gradient between nasal washes and sera for influenza A virus antibodies (Wagner et al., 1987), which likely is similar for RSV. As a consequence, relatively high titers of serum antibodies are needed to confer protection in the respiratory tract.

Following RSV infection, there is an initial influx of NK cells to the site of infection that produce IFNγ and are cytotoxic to virus-infected cells. This is followed by recruitment of helper $CD4^+$ and cytotoxic $CD8^+$ lymphocytes to the site of infection. IFNγ enhances the differentiation of $CD8^+$ lymphocytes and influences the differentiation of $CD4^+$ lymphocytes that contribute to the generation and amplification of the humoral and cellular immune responses. CD8+ T cells also help regulate T cell and inflammatory responses by mechanisms that initially were thought to involve IFNγ but now appear to be more complex and are incompletely understood (Olson et al., 2008; Olson and Varga, 2007; Stevens et al., 2009). Recent studies in mice showed that macrophages provide an immediate response of pro-inflammatory cytokines following RSV infection (Pribul et al., 2008), and are a major producer of type I IFN (Kumagai et al., 2007). Macrophages appear to be important both in restricting the virus and in clearing debris later in infection that otherwise can promote further damage and inflammation (Reed et al., 2008). Other features of innate immunity also may contribute to restricting RSV infection and replication, such as surfactant proteins (Glasser et al., 2009; LeVine et al., 2004) and potentially eosinophils (Phipps et al., 2007) and neutrophils (Lukens et al., 2010).

Although antibodies are important for resistance to infection, T cell responses are probably of greater importance for virus clearance. For example, individuals with compromised T cell immunity can shed virus for months (Hall et al., 1986), and prolonged virus shedding is also observed in nude or irradiated BALB/c mice (Cannon et al., 1987) and in mice depleted of both $CD4^+$ and $CD8^+$ cells (Graham et al., 1991b). In mice, RSV-specific CD8+ T cells also provide protection against infection, but the effect is short-lived (Connors et al., 1991; Kulkarni et al., 1995).

Immune responses usually are markedly reduced during infancy compared to older individuals, which is particularly relevant to RSV given its high rate of infection during this period. For example, the titers of RSV-specific serum antibodies induced by primary RSV infection were 8- to 10-fold lower in individuals of 4–8 months of age compared to those of 9–21 months of age (Murphy et al., 1986). Antibody responses in young infants often are poorly neutralizing and are short-lived compared to older individuals: post-infection increases in RSV-specific IgA and IgG responses in infants were found to be greatly reduced one year later (Welliver et al., 1980). One factor in the reduced immune responses early in life is immunologic immaturity, which limits innate, antibody, and cellular responses (Adkins et al., 2004; Levy, 2007). For example, the B cell response to RSV

infection of young infants <3 months of age was found to have a biased antibody gene repertoire and a greatly reduced frequency of somatic mutations compared to older individuals, which likely contributes to the poor neutralizing activity characteristic of responses in young infants (Williams et al., 2009). A second factor is immunosuppression by RSV-specific maternal serum antibodies that typically are present in young infants. In experimental animals, passive serum antibodies were shown to suppress both serum and secretory antibody responses to RSV infection, but did not suppress the cell-mediated response or priming for a secondary antibody response (Crowe et al., 2001; Murphy et al., 1988). A recent study examined a cohort of infants of <6 months of age with very low titers of RSV-specific maternal antibodies and found that the titers of RSV-neutralizing serum antibodies induced by primary RSV infection were indistinguishable from those of individuals of 6–24 months of age (Shinoff et al., 2008). This showed that, when the immunosuppressive effect of maternal antibodies is minimal, wild type RSV can induce a substantial antibody response even in young infants.

The observation that RSV can reinfect symptomatically without need for significant antigenic change is widely interpreted as evidence that there are long-term deficiences in protective immunity to RSV beyond the reduced responses that are characteristic of infancy. One possibility is that exposure to RSV antigen in the context of immunologic immaturity and maternal antibody-mediated immunosuppression may have long-term deleterious effects on protective responses. There indeed is evidence from the mouse model that exposure to RSV very early in life results in skewed responses during primary and secondary infection (Culley et al., 2002; Tasker et al., 2008). In addition, viral mechanisms might substantially inhibit the induction of protective immunity. For example, as already noted, the CX3C fractalkine motif in the G protein was shown to inhibit cellular responses to RSV infection in the mouse model (Harcourt et al., 2006), and the cysteine-rich domain of G was shown to reduce activation of TLR2, TLR4, and TLR9 in human cells (Polack et al., 2005). Other studies showed that the RSV NS1 protein partly suppresses human dendritic cell (DC) maturation and skews CD4+ and CD8+ T cell activation (Munir et al., 2011; Munir et al., 2008), and that RSV infection of human DC does not efficiently induce up-regulation of the chemokine receptor CCR7 necessary for DC migration to secondary lymphatic tissue (Le Nouen et al., 2011). RSV-infected DC also have been reported to direct reduced activation and altered polarization of CD4+ T lymphocytes *in vitro* (de Graaff et al., 2005; Guerrero-Plata et al., 2006; Rothoeft et al., 2007), although other results suggest that RSV does not differ significantly from HMPV, HPIV3, and influenza A virus in this regard (Le Nouen et al., 2010; Le Nouen et al., 2009). HRSV also can suppress T cell proliferative responses *in vitro* by direct contact mediated by the F protein (Schlender et al., 2002). Also, as noted, RSV appears to inhibit the host IFN system more efficiently than influenza and parainfluenza viruses.

However, these various mechanisms notwithstanding, it is not clear that protective immunity to RSV indeed is markedly deficient or inhibited – apart from the reduced responses in infancy as already noted. Primary infection of mice and cotton rats with HRSV induces robust antibody and cellular immune responses and long-lived protective immunity (Graham et al., 1991a; Prince et al., 1983). Infection of seronegative chimpanzees with wild-type HRSV or attenuated live vaccine candidates induced very robust serum antibody titers and protection (Crowe et al., 1994; Teng et al., 2000; Whitehead et al., 1999; Whitehead et al., 1998b). The titers of RSV-neutralizing antibodies in human adults also are quite high, giving no evidence of a deficient response (Falsey et al., 2006). As noted, RSV infection of young infants with very low maternal antibody titers resulted in very robust responses (Shinoff et al., 2008). Most importantly, re-infection with RSV usually is associated with substantially reduced disease, indicating that prior infections induce substantial protection.

Re-infection may be aided by features that help RSV evade, rather than directly inhibit, host defences. Several factors that allow RSV to evade host defense have already been noted, including the secreted form of the G protein (Bukreyev et al., 2008), the glycoprotein sheath of the G protein, and the presence of two antigenic subgroups (Waris, 1991). Other factors in evasion include the tropism of the virus for the superficial layer of the respiratory epithelium and its relatively non-invasive nature (described in the next section). This may delay and reduce the exposure of viral antigen to the host immune system. As noted, the lumen of the respiratory tract is poorly accessed by serum antibodies, sharply reducing their ability to restrict viral replication. In addition, recent studies in mice indicate that CD8+ cytotoxic T lymphocytes are functionally down-regulated in the lung, which may be a host mechanism to reduce tissue damage but would reduce immune protection (DiNapoli et al., 2008; Vallbracht et al., 2006). Thus, host and viral factors contribute to reduce the efficiency of immune control of RSV.

5. Pathogenesis

Disease manifestations during primary RSV infection vary widely among individuals, including URI, fever, otitis media, LRI that can vary widely from mild manifestations to life-threatening bronchiolitis and pneumonia, death in rare cases, post infection abnormalities in respiratory function that can persist through adolescence, and possible sensitization to asthma. Numerous host and viral factors have been suggested to be involved in RSV disease, but their roles remain controversial and likely vary in different individuals (Collins and Graham, 2008).

Some reports have suggested that RSV subgroup A or a particular genotype of subgroup A may be associated with greater clinical disease, but other studies have not found such a link (Brandenburg et al., 2000b; Martinello et al., 2002; Smyth et al., 2002). However, a subgroup A strain called line 19 recently was shown to induce enhanced production of IL-13 and mucus in mice compared to other strains, an effect that mapped to the F gene (Moore et al., 2009). It is possible that further studies will identify clinically relevant differences in RSV strains.

It is thought that RSV disease arises from both direct viral damage and the host immune response, but the relative contributions remain controversial. It generally is thought that there is a positive correlation between the level of virus replication and disease severity (DeVincenzo et al., 2010; DeVincenzo et al., 2005; Karron et al., 1997b; Martin et al., 2008), although this was not observed in some studies (Bennett et al., 2007; Hall et al., 1976; Wright et al., 2002). However, the extent to which this reflects increased viral damage versus an increased immune response is unclear.

Studies using *in vitro* models of adenoid or HAE epithelium showed that RSV infection is limited to the superficial ciliated cells and did not induce syncytia or invade underlying cells (Tristram et al., 1998; Wright et al., 2005; Zhang et al., 2002), which is consistent with histopathologic findings (Aherne et al., 1970; Johnson et al., 2007; Welliver et al., 2007). In the *in vitro* models, during an infection of several weeks, there was little visible damage to the tissue except that ciliary beating usually was impaired, in contrast to the rapid tissue destruction observed with influenza A virus (Wright et al., 2005; Zhang et al., 2002). This suggests that RSV is not inherently a highly cytopathic virus, although effects on ciliary function would facilitate the airway obstruction that is characteristic of RSV disease. In RSV-infected cotton rats, antiviral therapy that rapidly reduced virus replication had little effect on lung histopathology, whereas treatment with anti-inflammatory glucocorticoids reduced disease even though it interfered with clearance of the virus (Prince et al., 2000). This implied that disease was largely immune-mediated. However, the situation in humans

is less clear. Treatment of RSV-infected humans with antiviral antibodies or drugs that reduce virus replication provided no improvement or limited improvement (Lagos et al., 2009; Malley et al., 1998; Rodriguez et al., 1994). However, treatment with anti-inflammatory corticosteroids alone also does not significantly reduce RSV disease (Krilov, 2011; Patel et al., 2004).

There is abundant evidence of inflammatory cytokines and activated granulocytes in the airways of infants and children with severe RSV disease, with neutrophils being by far the most abundant immune cell (Abu-Harb et al., 1999; Everard et al., 1994; Garofalo et al., 1992; McNamara et al., 2004; McNamara et al., 2003; Rosenberg and Domachowske, 2001; Smyth et al., 2002). A recent study of infants hospitalized for severe RSV disease showed that the appearance of neutrophil precursors in the peripheral blood, which precedes their influx into the lungs, closely followed the peak of virus shedding and was coincident with clinical symptoms, implying possible roles both in protection and disease (Lukens et al., 2010). However, while RSV may indeed induce a strong inflammatory response, the extent to which this is pathogenic versus protective remains unclear (Bennett et al., 2007; Laham et al., 2004; Sheeran et al., 1999).

Depletion studies in the mouse model of RSV infection indicated that the CD4+ and CD8+ T lymphocyte subsets contribute individually to clearing the infection, but that both also contribute to disease, especially CD8+ T cells (Graham et al., 1991b). This suggested that RSV disease might involve an overly robust CD8+ T cell response. Infants and children indeed have increases in CD8+ T cells in response to RSV infection, and a greater proportion of CD8+ T cells compared to individuals infected with rhinovirus (Everard et al., 1994; Heidema et al., 2007; Heidema et al., 2008; McNamara et al., 2003). However, recent studies indicated that the timing the CD8+ T cell response in hospitalized RSV-infected infants occurs substantially after that of peak clinical symptoms (Heidema et al., 2007; Lukens et al., 2010), and that the magnitude of the CD8+ T cell response did not correlate with disease severity (Heidema et al., 2007). Thus, CD8+ T lymphocytes may contribute to disease in some individuals, but do not seem to be a major determinant.

Th2 biased stimulation of CD4+ T lymphocytes also has been suggested to mediate RSV disease. The excessive production of mucus, airway plugging, wheezing, and long-lasting effects on lung function that are common manifestations of RSV disease have some similarity with asthma, which involves a Th2-bias. Also, immune responses in young infants can have a Th2 bias remaining from the prenatal period (Adkins et al., 2004). A positive association was found between RSV disease and a genetic polymorphism in the IL-4 gene that increases gene expression (high IL-4 levels are normally associated with a Th2-bias) (Miyairi and DeVincenzo, 2008). However, while some studies provided evidence of a Th-2 biased response in infants with severe RSV disease (Bendelja et al., 2000; Roman et al., 1997), other studies documented Th1 responses (Brandenburg et al., 2000a), or identified infants with either Th1 or Th2 responses (Lee et al., 2007; Mobbs et al., 2002), or found Th2-biased responses primarily in individuals with a history of asthma or allergy (Kim et al., 2003; Legg et al., 2003). Another study documented Th2 responses in infants ≤3 months of age in response to RSV, influenza virus, and HPIVs, suggesting that this is specific to young age rather than RSV (Kristjansson et al., 2005). Early studies suggested that infants who developed wheezing in response to RSV infection had increases in RSV-specific IgE, which is promoted by the Th2 cytokines IL-4 and IL-13 and causes the release of inflammatory mediators from mast cells (Welliver et al., 1981), but this was not observed by others (De Alarcon et al., 2001). Thus, Th2 responses may contribute to RSV disease in some individuals, but may not be a predominant determinant.

The high rate of RSV infection and disease before six months of age implies that young age is important in pathogenesis. Reduced immune responses in young infants, as already noted, reduce the ability to control infection. The Th2 bias of young infants may play a role, as noted. The small diameter of bronchioles in infants makes them particularly susceptible to obstruction by edema, secretions, and immune and exfoliated cells (Hogg et al., 1970). Infancy is a time of lung growth and development, which may increase susceptibility to viral disease and possible long-term effects on lung function (Gern et al., 2005). For example, infants with severe RSV infection express increased levels of neutrotropic factors and receptors (Tortorolo et al., 2005), which may be associated with a higher index of obstructive sleep apnea (Snow et al., 2009). Thus, the ability of RSV to infect and cause disease somewhat earlier in life than other respiratory viruses may be a factor in its greater contribution to human disease.

A number of studies have shown that RSV bronchiolitis or pneumonia early in life is frequently followed by lingering abnormalities in airway function, including recurrent wheezing, reduced oxygenation, and deficits in pulmonary function tests suggestive of peripheral airway obstruction (Hall et al., 1984; Long et al., 1995; Noble et al., 1997; Sigurs et al., 2010; Stein et al., 1999). These effects are rarely severe but can persist through adolescence and early adulthood. It is not clear to what extent these sequelae are caused by the viral infection *per se* or reflect underlying anatomical or functional abnormalities that existed before the infection and indeed may have contributed to its severity. A recent study provided evidence that prevention of severe RSV disease by the use of RSV-neutralizing antibody substantially reduced the risk of recurrent wheezing in children who lacked a predisposition to asthma, suggesting that RSV infection plays a causal role (Simoes et al., 2010). Conversely, other studies provided evidence for a role of pre-existing vulnerability (Castro-Rodriguez et al., 1999; Thomsen et al., 2009). Finally, some studies have suggested that pediatric RSV infection can result in allergic sensitization and the development of asthma (Sigurs et al., 2010; Sigurs et al., 2005), but other studies have not observed this link and it remains controversial.

6. Antivirals

Ribavirin, a nucleoside analogue that interferes with the replication of a number of RNA and DNA viruses, was the first drug licensed for treatment of RSV infection in humans. Despite exhibiting potent activity against RSV in tissue culture and experimental animals (Hruska et al., 1982), ribavirin use in the clinic is now very limited due to the lack of proven efficacy (Rodriguez et al., 1994), the difficulty of administration (i.e. usually by aerosol), and concerns for toxicity. Currently, ribavirin is not recommended for the routine treatment of RSV infection (2006). It does find use in treating RSV infection in high-risk individuals, sometimes in combination with intravenous RSV-neutralizing immunoglobulin and corticosteroids, but efficacy remains controversial.

A number of small molecule a nti-RSV compounds have been described over the last 15–20 years. Most of them have been discovered by screening of chemical libraries of natural products using classic virologic assays, modified for high throughput testing. Time-of-addition experiments have been used to disclose the step of the replication cycle blocked by the drugs, and selection of escape mutants followed by sequencing has uncovered the viral targets of some of these compounds. Several drugs have been tested in animal models, and a few of them are in the early stages of clinical trials.

Remarkably, many of the small molecule RSV inhibitors turned out to target the F protein. One of the reasons for this predominance may be that fusion of the viral and cell membranes occurs outside the cell and therefore inhibitors do not need to cross the membrane barrier. A

series of benzimidazoles derivatives have been reported to interact with the F protein and, although dissimilar in structure, they all seem to bind to a hydrophobic pocket of the trimeric coiled-coil made by HRA (amino acids 153–209) of the F1 chain (Zhao et al., 2000). Binding to this pocket would interfere with the proper interaction of HRA with HRB (amino acids 482–520) and the formation of the 6HB that is required for completion of membrane fusion (Melikyan et al., 2000).

For example, it was shown that a radiolabelled analogue of the benzimidazole BMS-433771 containing a photoreactive diazirine group was covalently linked to Tyr198 of the F protein when photoactivated with UV light in the presence of virus (Cianci et al., 2004). Recently, the crystal structure of another benzimidazole derivative (TMC353121) bound to the 6HB formed by isolated sequences of HRA and HRB was solved (Roymans et al., 2010). TMC353121 was seen to interact with key residues of the hydrophobic cavity, including Tyr198, resulting in a stable but distorted 6HB. An important difference between TMC353121 and BMS-433771 was that the former required both HRA and HRB for binding whereas the latter could bind to peptides of HRA structured as trimeric coiled-coils in the absence of HRB. This difference may be due to the specific interactions that each drug makes with HRA and HRB residues.

A diverse array of other benzimidazoles (e.g., JNJ-2408068) and chemically unrelated compounds (e.g., VP14637) with subnanomolar activities have been described. Interestingly they also seem to target the hydrophobic pocket of the 6HB (Douglas et al., 2005; Lundin et al., 2010). Consequently, many of these compounds select escape mutants with similar or identical amino acid changes and which exhibit cross-resistance. Some of the selected mutations alter residues at or near the hydrophobic pocket that may interact with drugs. This has been shown directly for TMC353121. In other mutants, however the selected mutations lie outside the expected site in the F molecule, and the actual mechanism by which they mediate resistance is not understood at present.

Other RSV inhibitors have been described besides those targeting the F protein. For instance, NMSO3, a sulphated sialyl lipid, has been reported to inhibit virus binding, and mutants resistant to this compound had mutations in the G protein, identifying it as the presumptive target (Kimura et al., 2004). Many negatively charged polymers, including heparin and dextran sulphate, also interfere with RSV binding, but their inhibitory concentrations are too high to find application in the clinic.

Sudo et al. (Sudo et al., 2005) discovered a benzazepine derivative (YM-53404) that worked relatively late in the replication cycle and that selected mutations in the L gene. Another benzodiazepine compound (RSV604) that worked late in the replication cycle was shown to select mutations in the N gene, pointing to this molecule as the drug target (Chapman et al., 2007). Interestingly, these mutations were located in the long β hairpin of the RSV N structure noted above that may mediate interaction between N protein in the RNP and the polymerase complex (Tawar et al., 2009).

Besides chemical compounds, other types of molecules have been shown to inhibit RSV infectivity. For instance, HRB-derived peptides, analogous to those used to treat human immunodeficiency virus (HIV) infections, were demonstrated to block RSV induced membrane fusion (Lambert et al., 1996), but no further progress has been reported on these inhibitors. Small interfering RNAs (siRNAs) targeting the NS1 (Zhang et al., 2005) or P (Bitko et al., 2005) gene were shown to inhibit RSV replication in tissue culture and in the mouse model. More recently, a randomized clinical trial with intranasal siRNA targeting the N gene (ALN-RSV01) was reported to have protective activity in human volunteers challenged with RSV (DeVincenzo et al., 2010), and the same drug had some beneficial

effect on long-term allograft function in lung transplant recipients with RSV infection (Zamora et al., 2011).

As described in the following section (*7. Immunoprophylaxis with antibodies*), prophylaxis with RSV-neutralizing antibodies has been effective in reducing RSV disease in young infants; therefore, these preparations also have been evaluated as therapy of established RSV infection. For example, the RSV-neutralizing MAbs palivizumab or motavizumab have been administered intravenously to infants and children hospitalized for RSV disease. Treatment was associated with a ~10-fold reduction in shedding, but with no clear effect on clinical outcome (Lagos et al., 2009; Malley et al., 1998). A phase 2 study to evaluate motavizumab for treatment of children hospitalized with RSV disease has been completed (clinicaltrials.govNCT00421304), although results have not been reported. Antibodies also have been used on an individual basis in treatment of severely immunocompromised individuals, in whom shedding can be much more prolonged (Lazar et al., 2006). A preparation of purified serum antibodies from adults selected for high RSV-neutralizing activity (RI-001, ADMA Biologics) was recently evaluated in a phase 2 study for safety and the ability to prevent the development of LRI in RSV-infected immunocompromised adults (clinicaltrials.govNCT00632463) and also has been used in treatment of several immunocompromised patients (Falsey et al., 2009).

Several difficulties remain for the therapeutic use of antivirals against RSV. One of them is the narrow window for intervention. RSV infects and causes symptoms in the upper respiratory tract before reaching the lungs and there is a delay of ~ 4 days between the first symptoms and hospitalization. Rapid diagnostic tests are therefore vitally important for rapid identification and proper treatment of naturally occurring infections. Another problem is the idea that host immune responses contribute to viral disease, as noted. Therefore, combined anti-viral and anti-inflammatory therapy with more specific and less toxic drugs may be a promising alternative. Finally, the emergence of resistant viruses that are easily selected *in vitro* with various drugs challenges the widespread use of anti-RSV compounds. As with other viruses (e.g. HIV), combination therapies with drugs targeted to different RSV gene products should avoid selection of resistant viruses.

With the lack of available effective antiviral therapy, treatment of acute RSV LRI mainly involves supportive care, including administration of intravenous fluids, humidified oxygen, and mechanical ventilation in more severe cases. Systemic or inhaled corticosteroids – used on the premise that inflammation contributes to RSV disease - provide no clear improvement and are not recommended for routine use (2006; Krilov, 2011; Patel et al., 2004). The leukotriene receptor antagonist montelukast – another anti-inflammatory agent - was not effective in acute disease (Amirav et al., 2008), and whether it has an effect on post-RSV wheezing remains unclear (Kim et al., 2010; Krilov, 2011; Proesmans et al., 2009). In the United States, infants with severe RSV disease are often treated with inhaled bronchodilators in an attempt to relieve airway constriction, but this provides only modest short-term benefit (2006; Levin et al., 2008).

7. Immmunoprophylaxis with antibodies

Systemic immunoprophylaxis with RSV-neutralizing antibodies provides substantial protection against severe RSV disease in high-risk infants and children (2009; Mejias and Ramilo, 2008; Wu et al., 2008). The first product for human use, RSV Immune Globulin Intravenous (RS-IGIV, Respigam™, produced by MedImmune), consisted of purified serum antibodies from donors screened for high RSV-neutralizing activity and was licensed in 1996 (this product is similar to, and preceded, RI-001 mentioned above). Monthly administration during RSV season reduced the frequency of hospitalization for RSV disease

by 55% or more and days spent in intensive care by 97% (1997; Groothuis et al., 1993; Mejias and Ramilo, 2008). However, this product had the disadvantage of involving intravenous infusion, the theoretical risk of adventitious agents, and possible interference with live pediatric vaccines due to its polyvalent nature (Wu et al., 2008). RSV-IVIG was superseded by development of the MAb palivizumab and has not been commercially available since 2004.

Palivizumab (Synagis™; MedImmune) is an RSV-neutralizing, F-specific MAb that was developed by humanizing a murine MAb (Beeler and van Wyke Coelingh, 1989; Johnson et al., 1997; Wu et al., 2008). Licensed in 1998, this MAb is 50- to 100-fold more effective on a weight basis than RSV-IVIG, allowing administration in a much smaller volume by monthly intramuscular injection. Its clinical efficacy is similar to that of RSV-IGIV (1998). Whereas RSV-IVIG was not recommended for use in infants with cyanotic heart disease due to the large dose volume (Groothuis et al., 1993), palivizumab is safe and effective in this population (Feltes et al., 2003). Palivizumab is widely used for prophylaxis of infants and young children at high risk for severe RSV disease due to prematurity and underlying disease, and also can be used in hematopoietic stem cell recipients (Boeckh et al., 2001). Antibody prophylaxis thus represents the first and only means available to specifically reduce the incidence of severe RSV disease. These products do not necessarily prevent RSV infection, but can restrict replication sufficient to reduce disease. Antibody-resistant mutants have not been a significant problem: they are detected in ~5% of treated individuals who nonetheless developed RSV LRI, and they usually appear to have a modest reduction in growth fitness in vitro (Zhu et al., 2011).

Palivizumab was modified by *in vitro* affinity maturation to create a more potent derivative, called motavizumab (MEDI-524 or Numax, MedImmune)(Wu et al., 2008), This resulted in a 70-fold increase in antigen binding, a 20-fold increase in neutralization activity *in vitro*, and increased protection in cotton rats including in the upper respiratory tract (Mejias and Ramilo, 2008; Wu et al., 2007). In a clinical study involving 6635 preterm infants, the group that received motavizumab had 26% and 55% reductions in HRSV hospitalization and medically-attended HRSV LRI (MALRI), respectively, compared to palivizumab (Carbonell-Estrany et al., 2010; Gill and Welliver, 2009). However, this product was associated with a slight increase in the incidence of hypersensitivity reactions and anti-drug antibodies. In a June 2, 2010 meeting, the US FDA Antiviral Drugs Advisory Committee declined to support this drug application, and its further development for RSV prophylaxis is not being pursued, but this antibody might find use in anti-viral therapy. In addition, motavizumab was engineered further to increase its serum half-life by increasing its affinity for a receptor that recycles antibodies taken up intracellularly for degradation (Dall'Acqua et al., 2006). This antibody (MEDI-557) has been evaluated for tolerability and pharmacokinetics in health adults (clinicaltrials.govNCT00578682). However, given the issues with its motavizumab parent, it seems unlikely that this derivative will be developed further for prophylaxis.

As noted, fully human G-specific MAbs have been developed from human B cell clones and were shown to compare favorably with palivizumab in neutralizing activity *in vitro* and in rodents (Collarini et al., 2009). In another development, llama-derived heavy chain antibody fragments (nanobodies) directed against the F protein were reported (Hultberg et al., 2011). Multimeric nanobody constructs (either multiparatopic or multivalent) showed improved neutralizing potencies that compare favorably with palivizumab.

8. Pediatric vaccines

Given the early incidence of RSV hospitalization, vaccination should be initiated during the first weeks of life. The reduced immune responses characteristic of infancy probably will necessitate multiple vaccine doses, which might be given as part of the routine vaccination schedule at 2, 4, and 6 months of age.

Development of a pediatric RSV vaccine has been complicated by the experience with a formalin-inactivated RSV vaccine (FI-RSV) evaluated in the 1960s in infants and young children. This vaccine consisted of FI-RSV that was concentrated with alum adjuvant and administered intramuscularly. The vaccine was well tolerated and appeared at the time to be moderately immunogenic, but it proved to be poorly protective against natural RSV infection. Furthermore, vaccinees that were subsequently infected by natural exposure experienced immune-mediated enhancement of disease: nearly 80% of infected vaccinees required hospitalization. Subsequent analysis indicated that the immune response to FI-RSV in the vaccinees was markedly different from that to natural RSV infection, including poor induction of virus-neutralizing serum antibodies (Murphy and Walsh, 1988) and an exaggerated CD4+ T lymphocyte response (Kim et al., 1976). These observations were confirmed and extended in animal models. The poor neutralizing response likely involved denaturation of neutralization antigens as well as deficient antibody affinity maturation (Delgado et al., 2009). These antibodies were unable to efficiently restrict viral replication, but they bound to antigen and created antibody-antigen complexes that activated complement and contributed to disease (Melendi et al., 2007). The exaggerated CD4+ T cell response was found to involve the Th2 subset (Connors et al., 1994; de Swart et al., 2007; Waris et al., 1996), which was directly confirmed to be involved in disease enhancement in the mouse model (Connors et al., 1992). The Th2 biased response appeared to result from poor stimulation of NK cells and CD8+ CTL, which otherwise down-regulate Th2 and inflammatory responses to RSV antigens, as already noted. In addition, the formalin fixation creates carbonyl groups that enhance the Th2 response (Moghaddam et al., 2006). In summary, the aberrant responses to FI-RSV included induction of antibodies that bound viral antigen but did not neutralize infectivity, resulted in poor stimulation of cells and intracellular signaling pathways important in immunoregulation, and increased stimulation of Th2 cells.

This phenomenon of vaccine-primed disease enhancement also has been observed following RSV infection of experimental animals that were immunized with purified RSV F and G glycoprotein preparations as experimental vaccines (Murphy et al., 1990). In contrast, it has not been observed with natural RSV infection and re-infection, or in most cases with viral or DNA vectors expressing RSV antigens. These different outcomes for killed-virus/subunit RSV vaccines versus live/vectored RSV vaccines likely reflect a greater efficiency of the latter in inducing a broad, regulated immune response that down-regulates Th2 lymphocytes and generates antibodies that efficiently neutralize infectivity. Thus, inactivated RSV vaccines are generally considered unsuitable for pediatric use. It may be that the use of improved adjuvants (Boukhvalova et al., 2006) or improved antigen constructs (Swanson et al., 2011) will yield inactivated vaccines that are safe in RSV-naïve infants. However, it may be hard to establish safety for any new inactivated vaccine, since disease enhancement sometimes only becomes evident with time (Murphy et al., 1990), and because it is unclear how accurately experimental animals model the human response.

Efforts to develop a pediatric RSV vaccine have focused on live-attenuated vaccines, which were free of disease enhancement in experimental animals and in clinical trials (Wright et al., 2007). Intranasal administration of a live vaccine stimulates local as well as systemic immunity. The topical route of administration also was found to partly reduce the

immunosuppressive effects of passive serum antibodies (Crowe et al., 1995). Beginning in the 1960's a number of live attenuated RSV strains were developed by serial passage at suboptimal temperatures (cold-passage, cp) (Friedewald et al., 1968) and growth in the presence of mutagens followed by identification of temperature sensitive (ts) mutants (Crowe et al., 1994). Among the biologically-derived viruses that were evaluated clinically, the most promising one (called *cpts*248/404) was well-tolerated and immunogenic in seronegative infants and children older than 6 months of age, but caused mild congestion in younger infants and thus was insufficiently attenuated (Wright et al., 2000).

All subsequent live attenuated viruses have been developed by reverse genetics, which provides the means to introduce attenuating mutations into RSV in desired combinations to make "designer" vaccines (Collins and Murphy, 2005). Attenuating mutations were identified by analysis of existing attenuating viruses (Whitehead et al., 1998a) or were designed using new strategies such as deleting accessory proteins, or introducing host-range restriction elements (Bermingham and Collins, 1999; Buchholz et al., 2000; Teng et al., 2000; Whitehead et al., 1999). Novel mutations also may have desirable properties such as improved immunogenicity and increased genetic stability, as noted below. Reverse genetics also provides the possibility to increase the genetic stability of amino acid substitutions (McAuliffe et al., 2004) and to produce virus with short, well-defined passage histories, important for safety (Surman et al., 2007).

Several of these new attenuated RSV strains have been evaluated in clinical trials (Karron et al., 2005; Wright et al., 2006). The most promising candidate, called rA2cp248/404/1030ΔSH or MEDI-559, contains a number of introduced mutations, including two amino acid substitutions in the L polymerase, a nucleotide substitution in the M2 gene-start signal, and deletion of the SH gene. The MEDI-559 virus is strongly temperature-sensitive, which would restrict replication *in vivo*, especially in the warmer lower respiratory tract. In 1- to 2-month old, RSV-naive infants, the virus was well tolerated, which represents an important advance. Furthermore, a single dose provided substantial restriction of a second vaccine dose administered two months later, indicative of protective immunity. Despite this observed protection, the majority of vaccinees did not have detectable rises in serum antibodies. This reflects the general difficulty in detecting correlates of immunity to RSV in young infants, due to weak responses and limitations on sampling. Importantly, it remains to be determined whether this lead vaccine candidate will provide protection against natural infection. MEDI-559 presently is in expanded studies in young infants to further monitor tolerability and immunogenicity (clinicalstudies.govNCT00767416), and subsequently will be evaluated in expanded studies for the ability to induce protection against medically attended RSV disease from natural exposure in the community. Of note, this vaccine candidate exhibited genetic instability involving the loss of one of the two amino acid substitutions in the L protein (Karron et al., 2005). These partial revertants remained highly attenuated and were not associated with disease, but it would be preferable to have a more stable vaccine candidate, and efforts to stabilize this virus are underway (Luongo et al., 2009).

Other candidates also are entering clinical trials. One has the deletion of most of the M2-2 coding sequence (Teng et al., 2000). As noted, the lack of M2-2 results in increased transcription and antigen synthesis (Bermingham and Collins, 1999) and thus may be more immunogenic, and a gene deletion would be expected to be stable. Another potential vaccine candidate contains deletion of the major IFN antagonist NS1 gene (Teng et al., 2000). This would result in increased IFN production and signaling during infection and might thereby increase immunogenicity, which was observed with BRSV lacking the major NS IFN antagonist protein (Valarcher et al., 2003). Thus, attenuation can involve various mechanisms: point mutations in the L polymerase or gene-start signal likely reduce viral

RNA synthesis; deletion of M2-2 increases transcription at the expense of RNA replication, and deletion of NS1 reduces the ability of the virus to inhibit the host IFN system. It will be interesting to characterize the properties of these vaccines in humans.

Another live attenuated RSV vaccine strategy uses an attenuated PIV as a vector to express the RSV F and/or G protein from one or two added genes. This provides a pediatric vaccine against both the PIV vector and RSV, and has the advantage of the improved *in vitro* growth (important for vaccine manufacture) and stability of PIV compared to RSV. In one example, bovine PIV3 (which is attenuated in humans due to the host range difference) was modified so that its F and HN major protective antigen genes were replaced with those of HPIV3 in order to provide homologous protection against HPIV3. This attenuated B/HPIV3 virus was further modified by the insertion of RSV F and/or G, creating a bivalent vaccine against HPIV3 and RSV (Schmidt et al., 2002; Tang et al., 2008). One such construct, in which B/HPIV3 expresses RSV F from a gene added between the N and P genes (MEDI-534), is presently in clinical trials in children >6 mo of age who are seronegative to both RSV and HPIV3 (clinicaltrials.govNCT00686075). Another related approach uses Sendai virus (murine PIV1), which has substantial antigenic cross-reactivity with HPIV1 and may be attenuated in humans, as the vector to express RSV protective antigens (Jones et al., 2009). This provides a potential bivalent vaccine against HPIV1 and RSV. Other vectored approaches, such as using replication-defective adenoviruses (Shao et al., 2009) or alphaviruses (Elliott et al., 2007b), also are being developed for possible pediatric use.

There also is a need for an RSV vaccine in older children and adults who are at risk for severe RSV infection due to underlying disease or old age. Life attenuated RSV vaccines appear to be too restricted in replication in these RSV-experienced individuals to be immunogenic (Gonzalez et al., 2000). Vectored vaccines might be feasible, but would need to be based on a vector that is not a common human pathogen in order to avoid immune restriction. Also, the development of vector-specific immunity would interfere with subsequent doses. Another alternative would be protein vaccines, for which suppression by existing immunity can be partly overcome by increased dose (Murphy et al., 1991). Observations from the original FI-RSV vaccine trials and studies in experimental animals (Waris et al., 1997) indicated that disease enhancement is observed only in RSV-naïve recipients. This indicated that RSV protein vaccines do not prime for disease enhancement in RSV-experienced individuals, which was confirmed in clinical trials outlined below.

Subunit vaccines based on the RSV F protein isolated from infected cell culture have been evaluated in healthy adults, in children over 12 months of age who were healthy or who had chronic lung disease from prematurity or cystic fibrosis, in the elderly, and in pregnant women in whom the goal was to increase the titer of maternal antibodies in the newborn (Belshe et al., 1993; Falsey and Walsh, 1997; Groothuis et al., 1998; Munoz et al., 2003; Paradiso et al., 1994; Piedra et al., 1996; Tristram et al., 1993). In these studies, the vaccines were well tolerated but were not very immunogenic. More recently, a vaccine consisting of the RSV F, G, and M proteins from RSV-infected cells was evaluated in elderly individuals in conjunction with the inactivated seasonal influenza virus vaccine, and was well tolerated and moderately immunogenic (Falsey et al., 2008). However, development has been suspended. Another protein based vaccine, called BBG2Na, consisted of a fragment of the G protein containing the central conserved domain that was fused to the albumin-binding domain of the streptococcal G protein and was expressed in bacteria. However, this vaccine may have induced an increased Th2 response in infant macaques (de Waal et al., 2004) and in clinical trials in adults it was not very immunogenic (Power et al., 2001) and was associated with hypersensitivity reactions in some recipients. More recently, as already noted, a post-fusion form of the F protein was produced with deletion of the major hydrophobic regions (McLellan et al., 2011; Swanson et al., 2011). Importantly, this

expressed protein forms stable trimers that were recognized by a number of neutralizing MAbs. In rodents, this antigen induced high titers of neutralizing serum antibodies and protection against RSV challenge. This may represent an improved RSV subunit vaccine.

Virus-like particle (VLP) vaccines represent another approach (Jennings and Bachmann, 2008). VLP vaccines for hepatitis B virus and papillomavirus are in wide use. In addition, a VLP vaccine against influenza virus has been in use in a number of countries since 1997 and compares favorably with the standard inactivated influenza vaccine (Herzog et al., 2009; Kanra et al., 2004). VLPs may mimic some aspects of the superior immunogenicity of virus compared to purified protein vaccines (Jennings and Bachmann, 2008). In the case of RSV, a single formulation is presently reported to be in early clinical trials (clinicaltrials.govNCT01290419). This vaccine is called an “RSV-F Particle Vaccine” but is not otherwise described. In other work, the production of RSV VLPs was enhanced by co-expression of the NDV N and P proteins, which stimulated particle formation (McGinnes et al., 2011). Whether a VLP-based RSV vaccine would be free from disease enhancement in RSV-naïve infants is unknown.

Numerous additional approaches to an HRSV vaccine continue to be evaluated in pre-clinical studies, including synthetic peptides, engineered multi-epitope vaccines, antigen expressed by recombinant baculoviruses or produced in plants, antigen fused to carrier proteins such as cholera toxin B subunit, immune-stimulating complexes containing RSV antigens, the use of adjuvants such as CpG oligonucleotides or biopolymer nanoparticles, and vectors including vesicular stomatitis virus, vaccinia virus (Modified Vaccinia Ankara), rhinovirus, Newcastle disease virus, *Mycobacterium bovis* BCG, and *Salmonella typhimurium*. Whether any of these offers advantages remains to be seen.

Can successful RSV vaccines be developed? In the case of older children and adults, it seems likely that improved methods of producing RSV antigen, or alternatively the use of a VLP, will result in an effective subunit vaccine that could be given periodically, possibly in conjunction with the yearly influenza virus vaccine. In the case of RSV-naïve infants and children, it remains to be seen whether vaccine candidates will be sufficiently immunogenic in infancy to provide effective protection against RSV disease. It seems unlikely that complete protection against infection will be achieved, but a substantial reduction in RSV replication should be effective in controlling severe disease. The recent successful development of live-attenuated pediatric vaccines against rotavirus, which faced many of the same obstacles, gives hope for success.

9. Future prospects and thanks

Since the first isolation of RSV in the 1950’s, research has come a long way in understanding this important pathogen, but major challenges remain. Beginning in the 1980’s, cloning and reverse genetics technologies and improved immunological methods have augmented classical virological approaches, and have uncovered a number of intricacies of RSV replication, biology, and the host response that have been summarized here. More recently, this has been further augmented by the beginning of descriptions of viral structures at the atomic level. We anticipate that this will lead to a fuller understanding of virus replication and viral antigens, and will help develop improved antiviral drugs and vaccines for control of RSV infections.

For basic science, major challenges include (i) understanding the basis of the pathology associated with RSV infections, and (ii) understanding the effects that RSV has on the host immune response. As with many human pathogens, these goals are impeded by the lack of a convenient, available animal model that faithfully reproduces RSV infection and disease. Another complication is that the first exposure to RSV typically occurs in young infants

with immature immune systems and lungs, and in the presence of immunosuppressive maternal antibodies, and thus multiple factors are in play. A number of additional approaches are helping to fill the gaps, including genomic and proteomic technologies, increased study of innate immune responses, genetic susceptibility studies, challenge studies in adults, more detailed analysis of hospitalized infants, improved *in vitro* models such as airway epithelium and immune cells, and other approaches. These have begun to yield data on isolated aspects, but an integrated picture is still missing.

Despite this incomplete understanding, research led to the previous development of palivizumab for prophylaxis of high-risk infants, and at the present time a number of vaccine candidates and antiviral drugs seem at the doorstep of clinical application. It seems likely that, in the upcoming years, new medicines will be on the market for the prevention and/or therapy of RSV disease. But, it should not be forgotten that much of the burden of RSV disease is in developing countries, which must be able to benefit from any progress made to control RSV.

Finally, we would like to thank Brian Mahy for inviting us to write this review. Brian has been a mentor – directly or indirectly - for many of us over the years in many areas of virology. His authoritative and wide-ranging editorial work has contributed to fundamental books and journals, like this one. But for us, we are particularly grateful for Brian’s (and Penny’s) major contributions to the organization of the Negative Strand RNA Virus Meetings that since the 1970’s have been the most enjoyable and rewarding event for virologists in this field, for the excellent quality of science, for the enjoyable social events that have been a very important part of those meetings, and for the spirit of collegiality that Brian helped infuse into this research community.

Abbreviations

6HB	six-helix bundle
CCR	chemokine receptor
DC	dendritic cell
eIF-2a	eukaryotic translation initiation factor 2a
FI-RSV	formalin-inactivated RSV
GAG	glycosaminoglycan
HAE	human airway epithelium
HIV	human immunodeficiency virus
HMPV	human metapneumovirus
HPIV	human parainfluenza virus
HR	heptad repeat
IFN	interferon
IKKε	inhibitor of NFκB kinase ε
IL	interleukin
IRF3	interferon regulatory factor 3
JAK	Janus kinase
LRI	lower respiratory tract illness

MAb	monoclonal antibody
MALRI	medically-attended LRI
MAVS	mitochondrial antiviral-signaling protein
MDA-5	melanoma differentiation-associated gene 5
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PKR	dsRNA-regulated protein kinase
RIG-I	retinoic acid inducible gene I
RNP	ribonucleoprotein
RSV	respiratory syncytial virus
RSV-IVIG	intravenous immunoglobulin selected for increased RSV-neutralizing activity
STAT	signal transducer and activator of transcription
Th	T helper cell
TNF	tumor necrosis factor
TLR	toll-like receptor
TRAF3	TNF receptor-associated factor 3
URI	upper respiratory tract illness
VLP	virus-like particle

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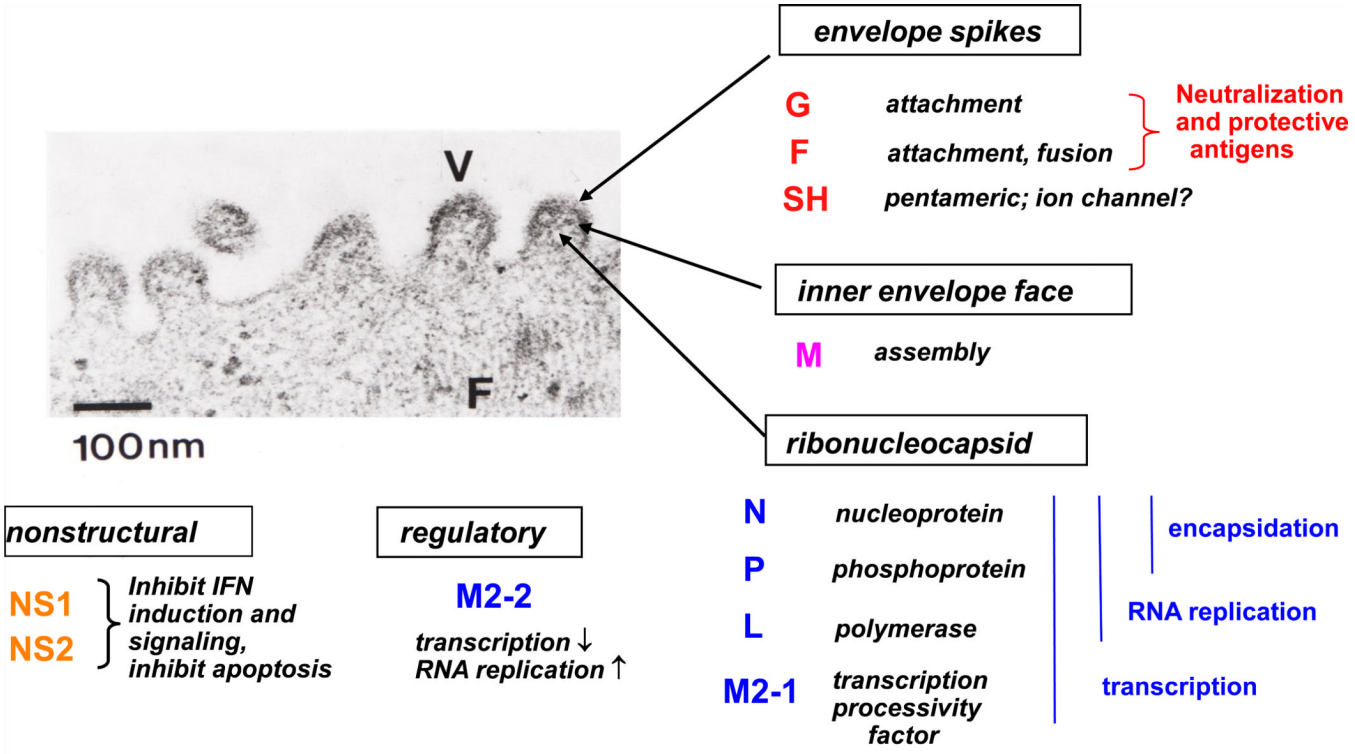
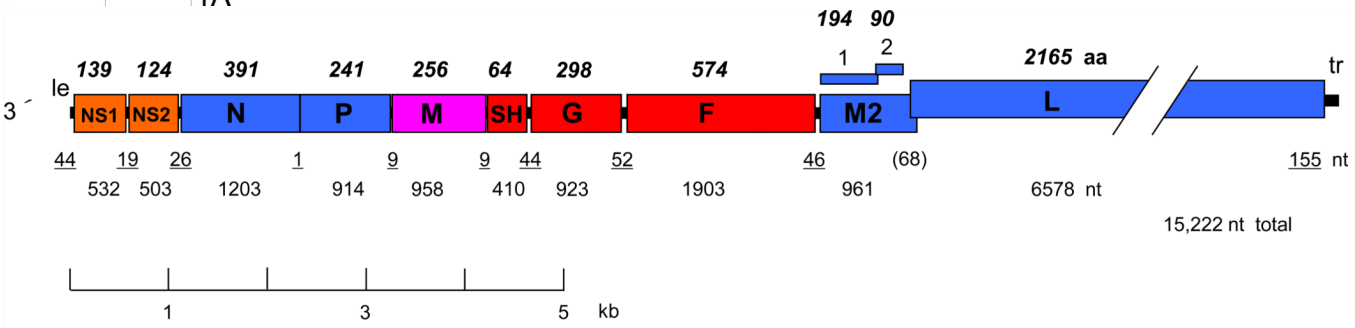


1A



1B

Figure 1.

RSV proteins (A) and gene map (B). Panel A shows a negatively-stained electron micrograph of budding RSV virions: V indicates a budding virion and F indicates filamentous cytoplasmic structures thought to be nucleocapsids (courtesy of Dr. Robert M. Chanock) (Kalica et al., 1973). The locations of viral proteins in the virion, and their functions when known, are indicated. Panel B shows a map of the negative sense genome (RSV strain A2), approximately to scale. The overlapping M2-1 and M2-2 ORFs are shown over the gene. Numbers below the map indicate nucleotide (nt) lengths: those of the 3´ leader (le) and 5´ trailer (tr) and intergenic regions are underlined, and the length of the gene overlap in parentheses. Italicized, bold numbers over the map indicate the amino acid (aa) lengths of the unmodified proteins. The viral proteins are as follows: G, attachment glycoprotein; F, fusion glycoprotein; SH, small hydrophobic glycoprotein; M, matrix protein; N, nucleoprotein; P, phosphoprotein; L, large polymerase protein; M2-1, product of the first ORF in the M2 mRNA; M2-2; product of the second ORF in the M2 RNA.

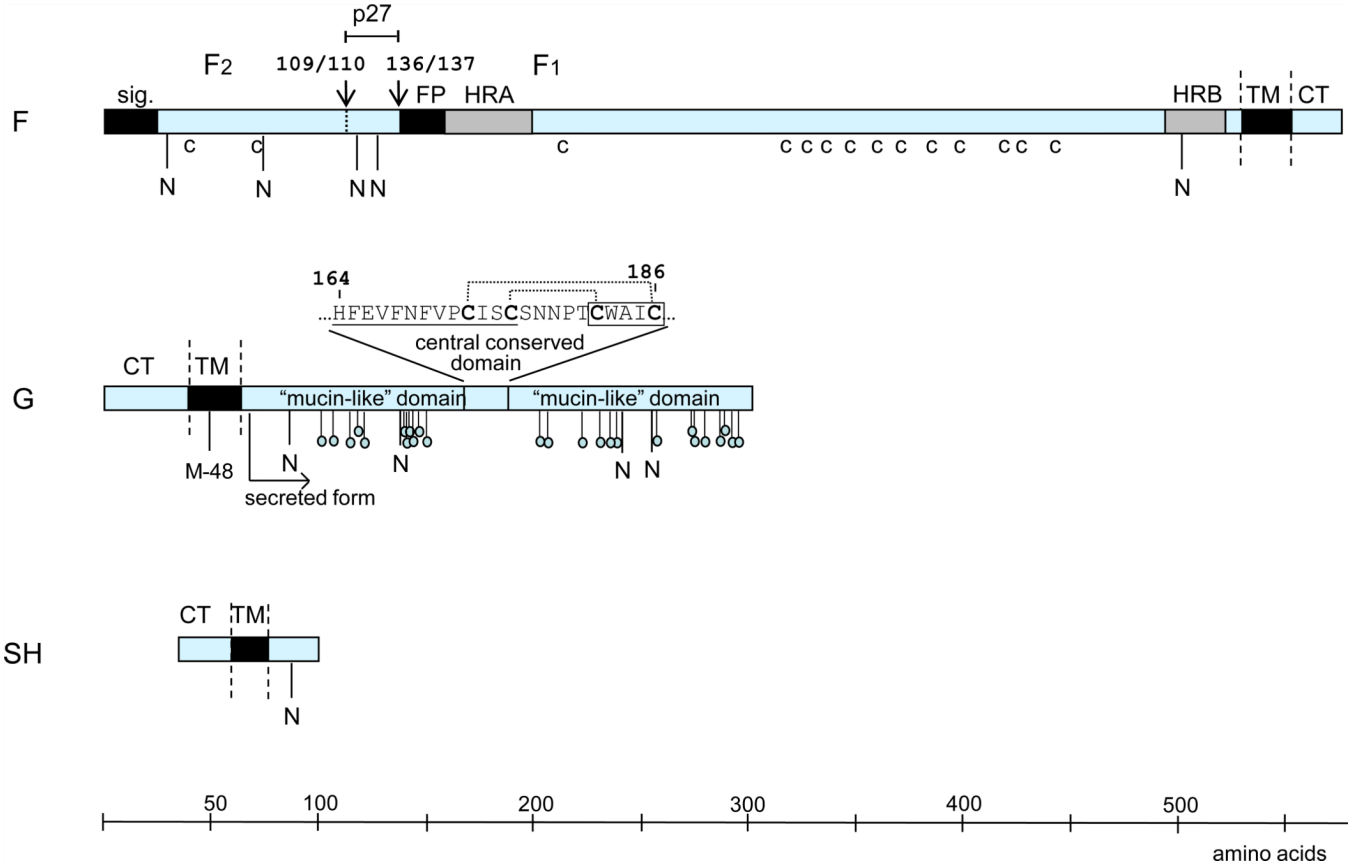


Figure 2.

The surface glycoproteins of RSV (strain A2), drawn approximately to scale. Hydrophobic domains are shown as black boxes (sig., signal peptide; FP, fusion peptide; TM, transmembrane anchor). Heptad repeats (HRA and HRB) in F are in grey. CT: cytoplasmic tail. The cleavage sites in F are indicated with downward facing arrows and identified by amino acid position. Cysteine residues in F that are conserved among human pneumoviruses are indicated (c). Potential acceptor sites for N-linked carbohydrate are indicated as downward facing stalks with N. The 25 potential acceptor sites for O-linked sugars in G that are predicted by NetOGlyc 2.0 to be the most likely to be utilized are indicated as downward facing stalks with small circles. The expanded sequence above G protein shows the conserved 13-amino acid segment (underlined) and cystine noose; cysteine residues are bold; the disulfide bonding pattern is indicated by dotted lines (Gorman et al., 1997); and the fractalkine CX3C motif is boxed. M-48 in the HRSV G protein is the translational start site for the secreted form, and the mature secreted form is indicated (Roberts et al., 1994).

Table 1

Notable features of RSV genetics and biology

- Replication and budding *in vitro* are inefficient, infectivity is unstable, particles are mostly large filaments
- RSV is the most complex member of *Paramyxoviridae*, encoding additional proteins that are either unique (NS1, NS2) or found only in a subset of viruses within this family (SH, M2-1, M2-2)
- Two genes, NS1 and NS2, are dedicated to expressing proteins that interfere with the host type I interferon system, among other functions
- Overlapping ORFs in the M2 gene encode novel factors that confer transcription processivity (M2-1) or shift RNA synthesis from transcription to RNA replication (M2-2)
- The M2 and L genes overlap and are expressed by a scanning mechanism
- The F protein precursor is activated by cleavage at two furin recognition sites
- Viral attachment appears to involve both the F and G proteins; nucleolin is a newly identified binding partner for F
- The G protein is heavily glycosylated, non-globular, and highly variable
- G is a poor antigen; G-specific monoclonal antibodies usually are poorly neutralizing
- The G protein bears a CX3C fractalkine-like motif that may reduce the cellular immune response
- The G protein is expressed in membrane-bound and secreted forms; the latter interferes with antibody-mediated neutralization
- The F protein induces signaling from TLR4, whereas the G protein inhibits signaling from TLR2, 4, and 9

Table 2

Notable features of RSV pathology and epidemiology

- RSV efficiently infects very early in life, with the peak of severe disease before 6 months of age
- RSV readily re-infects throughout life – especially during the first years of life - without need for antigenic change
- Severe RSV infection is frequently followed by abnormalities in pulmonary function that may persist for 10 years or more; possible link to asthma
- The relative contributions of direct viral cytopathology versus the host immune response to viral pathogenesis remain controversial
- RSV infection in an *in vitro* model of airway epithelium is non-destructive and non-invasive
- Immunity to RSV is commonly thought to be incomplete and transient
- A formalin-inactivated RSV vaccine evaluated in the 1960's caused enhanced disease upon subsequent infection
- Clinical immunoprophylaxis of high-risk infants with neutralizing antibody confers substantial protection from severe RSV disease