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Virology and Molecular Epidemiology of Respiratory Syncytial Virus (RSV)

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1. Introduction

Human respiratory syncytial virus (RSV) is a ubiquitous virus of worldwide distribution and is the leading cause of infant morbidity from respiratory infections. By the age of two years nearly all children have been infected and can cause severe bronchiolitis and pneumonia in this age group (Hall et al., 2009). Nearly 100% of children in the USA are infected with the virus by 2 to 3 years of age, several hundred infants may die directly from the infection, while the deaths of an additional several thousand may be attributed to RSV-related complications (Nair et al, 2010). The World Health Organization estimates that (RSV) is responsible for 64 million infections worldwide and 160,000 deaths per annum (Openshaw, 2002).

Although mostly young infants are affected, it is increasingly recognized as a significant cause of disease in the elderly population and can often be fatal for patients with impaired immune systems (Collins and Malero., 2011). The incubation period of RSV respiratory disease is estimated to be three to five days (Black, 2003). The virus can remain viable on hard surfaces (e.g., countertops) for up to 6hr, on rubber gloves for 90min, and on skin for 20min. (Hall et al., 1980). This prolonged survival highlights the need (and effectiveness) for hand washing and contact precautions in limiting the spread of RSV infection (Koetz et al, 2006). Viral shedding is significantly prolonged in immunocompromised individuals, and can continue for several months (Hall et al., 1981; Simoes, 2003).

2. Spectrum of respiratory disease

The spectrum of clinical manifestations ranges from mild upper tract illness, infection in middle ear which progresses to acute otitis media, croup, to apnoea in premature infants,

pneumonia and bronchiolitis (Hall et al., 1975). At the beginning of the illness, the virus replicates in the nasopharynx. Common symptoms of an upper respiratory tract infection include a productive cough and mild to moderate nasal congestion with clear rhinorrhea. A low-grade fever presents in the early stages of the infection and symptoms may persist for one to three weeks before complete recovery (Welliver, 2003).

Symptoms of lower respiratory tract disease include tachypnea (ie rapid breathing greater than 60 to 70 breaths/min), wheezing, and/or rales, that usually appear up to three days following onset of rhinorrhea. These symptoms are indicative of the virus spreading into the bronchi and bronchioles. A chest radiograph exhibits hyperinflation with flattened diaphragms. If the RSV further spreads to the alveoli, an interstitial pneumonia may develop, with middle and upper lobes affected. In such patients, tachypnea becomes severe respiratory distress, with deep retractions and grunting respirations. The risk for cardiovascular failure secondary to hypoxemia, acidosis, and dehydration increases significantly (Greenough, 2002). Immunocompromised and young infants, who have very narrow bronchioles, are at particularly high risk for complete bronchiolar obstruction. The risk for vomiting also increases, which is often related to respiratory distress and can increase the likelihood of gastroesophageal reflux. This may result in decreased oral intake with dehydration. Premature babies born at 30–35 weeks of gestation, HIV-infected patients, infants with cyanotic congenital heart disease, and other immunosuppressive therapy such as bone marrow transplant are at increased risk for morbidity and mortality during RSV infection (Resch, 2011).

3. The structure of respiratory syncytial virus

RSV is a member of the subfamily *Pneumovirinae* in the family *Paramyxoviridae*, order *Mononegavirales*. RSV is closely related to several other RNA viruses, including measles, mumps, and parainfluenza types 1, 2, and 3. Respiratory syncytial virus is a medium-sized (120-200nm) enveloped virus that contains a lipoprotein coat and a linear minus-sense RNA genome. The entire genome of RSV is composed of approximately 15,000 nucleotides long (Dickens et al., 1984).

The genome contains 10 mRNAs, each coding for an individual protein (McIntosh, 1997). The structural proteins are divided into three functional groups, the nucleocapsid (N) protein, phosphoprotein (P) and viral polymerase (L). These proteins together, have been demonstrated to function as the RSV replicase. The outer envelope is lined internally with matrix (M) protein and is spiked externally with fusion (F) and attachment (G) glycoprotein projections, these are responsible for the initiation and propagation of an RSV infection (Empey et al., 2010). Another protein (M2) is also present in the viral envelope. The viral capsid is made up of a nucleoprotein, a phosphoprotein, and a polymerase protein (C). The F protein is a type 1 transmembrane glycoprotein with a Cleaved N-terminal signal sequence and a transmembrane anchor near the C terminus. It is cleaved into two subunits, F1 and F2 that are linked by disulfide bonds, after synthesis and

modification by the addition of N-linked sugars. The G protein is of particular interest because variability in this protein is greater than that in the other proteins both between and within the major antigenic groups of RSV (Sullender, 2000).

4. Mechanism of infection

RSV does not normally replicate outside of the bronchopulmonary tree and is restricted to the respiratory mucosa (Othumganpat et al, 2009). The G protein initiates attachment of the virus to the epithelial cell. The virus fuses with the epithelial cell membrane and enters the cytoplasm after the F protein is cleaved by proteolytic enzymes of the infected cell. If the precursor is not cleaved it has no fusion activity, virion penetration will not occur and the virus particle is unable to initiate infection. Fusion by F1 occurs at the neutral PH of the extra cellular environment, allowing release of the viral nucleocapsid directly into the cell. This enables the virus to bypass internalization through endosomes. After replication the virus matures by budding from the cell surface. The new progeny nucleocapsids form in the cytoplasm and move to the cell surface. The M protein is essential for particle formation, probably serving to link the viral envelope to the nucleocapsid. During budding, most host proteins are excluded from the membrane. If appropriate host cell proteases are present, precursor proteins in the plasma membrane will be activated by cleavage. Activated fusion protein will then cause fusion of adjacent cell membranes, resulting in formation of large syncytia (Jawetz et al., 2004). The viral RNA can spread without forming complete viral particles. The infection results in the destruction of the epithelial cells of the upper respiratory tract. Exposure to RSV triggers humoral immune responses. Primary RSV infection results in only a weak antibody response with IgM, IgG, and IgA produced. This response is not sufficient to completely destroy the virus or to prevent upper respiratory tract replication of the virus, thus an upper respiratory tract illness develops (Smith et al., 2009).

Although, RSV mostly infects the nasal epithelial cells. However, development of extrapulmonary disease has been observed in certain T and B cell immunodeficiency states. The association of RSV with asthma and reversible reactive airway disease in early childhood has attracted significant attention (Mohapatra et al., 2008). Recurrent wheezing for up to 5 to 7 years of age and established airway disease has been observed in a significant number of children with a strong family history of allergy, after primary infection or reinfection with RSV (LeManske, 2004). Immune response to primary infection is relatively small but on reinfection, a significant booster effect with sustained immunologic reactivity is observed in serum and respiratory mucosa. Both CD4- and CD8-specific as well as Th1- and Th2-cell specific immune responses have been observed during human infection. In addition, proinflammatory as well as immunoregulatory cytokines and chemokines are induced in the respiratory tract after natural and induced (in vitro) infection. In vulnerable patients, the RSV infection will spread into the lower respiratory tract (Becker et al., 2006). In premature infants and immunocompromised hosts, the infection quickly progresses to the LRT. As the virus is destroyed within the lung by T cells, immunopathological responses cause further lung injury. Infected cells release proinflammatory cytokines and chemokines, including in-

terlukins (IL-1, IL-6, and IL-8) and tumor necrosis factor-alpha (TNF- α). These actions result in activation of inflammatory cells, including macrophages, eosinophils, neutrophils, and T lymphocytes, into the airway lining and surrounding tissues.

5. Molecular epidemiology of RSV

Variability between RSV strains contributes to the ability of the virus to infect people repeatedly and cause annual outbreaks. Consistent shifts in RSV group dominance have been reported worldwide in which RSV group A viruses are more frequently detected. A new BA genotype was identified in Buenos Aires in 1999 that is characterized by a 60-nucleotide duplication starting after residue 791 of the G protein. Subsequently, strains with this duplication have been found in clinical specimens from distantly related places in the world, including Kenya in East

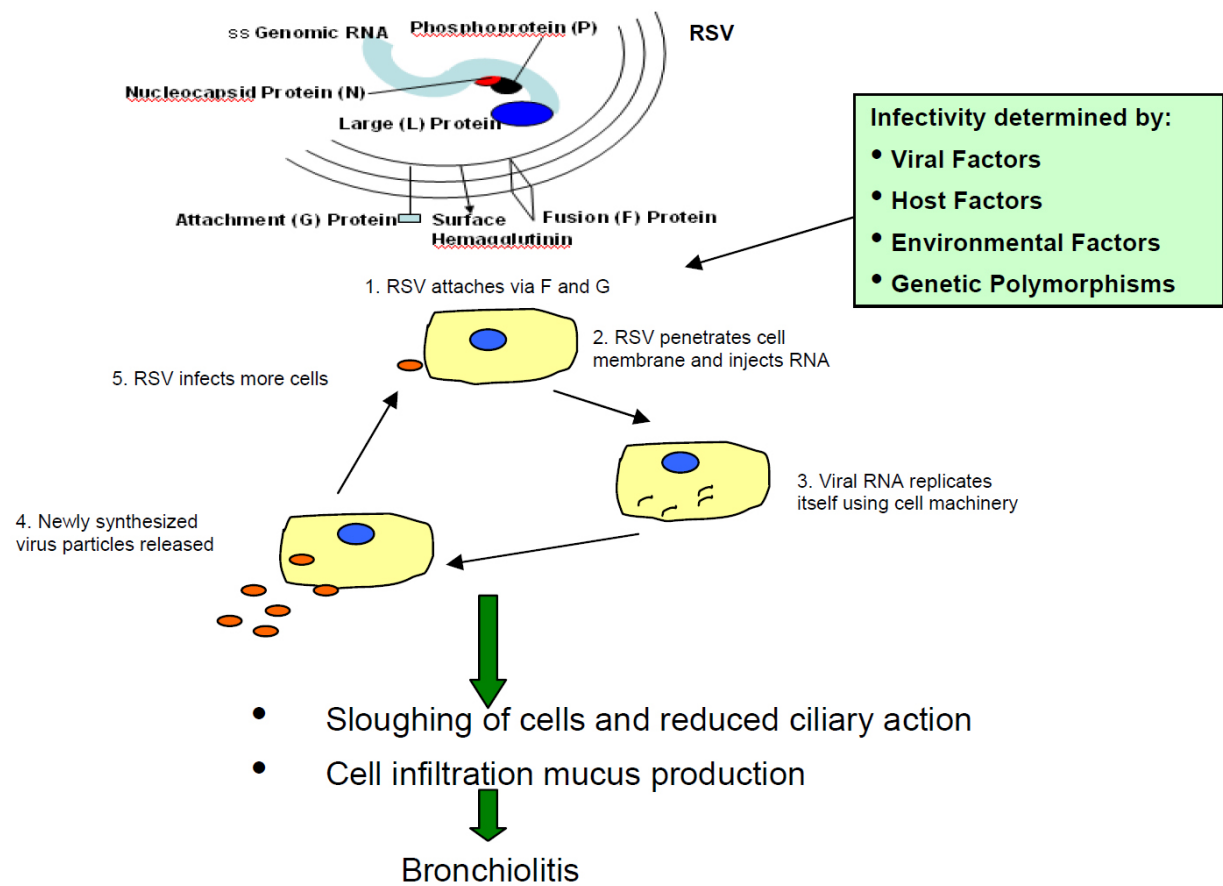


Figure 1. Mechanism of RSV Virus Entry and Replication in Respiratory Virus Infection

Africa. This BA genotype was first discovered in South Africa during the investigation of a nosocomial outbreak in Pretoria in 2006. (Niekerk and Venter, 2011). In Germany, RSV group A was dominant in seven out of nine epidemic seasons predominating between

1999 to 2007 seasons. During the same periods, mainly RSV group A dominated in several other countries.

The subgroup prevalence and genotype distribution patterns of RSV strains were investigated in a community in Belgium during 10 successive epidemic seasons (1996 to 2006). A regular 3-year cyclic pattern of subgroup dominance was observed, consisting of two predominant RSV-A seasons, followed by a single RSV-B-dominant year. RSV infections with both subgroups were more prevalent among children younger than 6 months and had a peak incidence in December. The most frequently detected genotypes were GA5 and GB13, the latter including strains with the 60-nucleotide duplication in the G gene (Zlateva et al, 2007). A study in India reported on RSV group A dominance for three consecutive epidemic seasons. RSV group B predominated for a single season in the countries investigated and at the same or similar time as observed in Germany. Thus, it can be concluded that RSV group A predominated within similar seasons, implicating that most RSV infections were caused by RSV group A worldwide at the same time.

The results of the first study to investigate the circulation and genetic diversity of RSV in Cambodia among different age ranges of population over 5 consecutive years (Arnott, et al., 2011). Circulation of RSV was seasonal, coinciding with the rainy season between July and November. The majority of RSV group B strains belonged to the BA genotype, with the exception of 10 strains classified as belonging to a novel RSV group B genotype,

In an Iranian study during the season 2009, samples were obtained from several provinces: Tehran, Hamadan, Isfahan, Kordestan, Zanjan, Lorestan and West Azarbayjan, and were tested for G protein gene of RSV by RT-PCR (Faghihloo et al., 2011). Of the respiratory samples tested, 22% were positive for RSV, of which 67% belonged to subgroup A and 33% to subgroup B. Phylogenetic analysis revealed that subgroup A strains fell in two genotypes GA1 and GA2, whereas subgroup B strains clustered in genotype BA. This study revealed that multiple genotypes of RSV cocirculate in Iran. Subgroup A strains are more prevalent than subgroup B strains, with genotype GA1 predominant.

There has been a distinct lack of data from the Middle East in terms of molecular typing of RSV. For example, In Saudi Arabia, only few reports have described the prevalence of RSV infection in sporadic districts of the kingdom including Riyadh, Al-Quassim and Abha (Jamjoom et al., 1993; Bakir et al., 1998; Al-Shehri et al., 2006; Meqdam and Subiah, 2006, Akhter and Johani., 2011). These reports covered short periods of time extending from 1991-1996, 2003-2004 and 2004-2010. Both virus subtypes in RSV infection of Saudi Arabia children are found to occur with a greater dominance of type A viruses in a three year cyclical pattern. However, a greater interest in RSV is needed for proper virus characterization. Molecular typing studies are required to elucidate the nature of RSV spread in these populations.

Similar viruses were first isolated around the same time in Europe, USA, The Gambia, Malaysia, Uruguay and Australia (Cane and Pringle, 1995). Further studies in North America (Peret et al., 2000), Europe (Lukic-Grlic et al., 1999), and Africa (Cane et al., 1999, Venter et al., 2002) have shown that similar strains of RSV appear simultaneously in indistinct geographic locations in these areas. It is clear that infections with very similar viruses may be

occurring world-wide during the same season. Overall there appears to be very little geographic clustering of RSV strains and where this has been reported it may often be due to inadequate sampling or delayed reporting of strain variability, particularly from less developed parts of the world. As the virus is constantly accumulating genetic and antigenic change, this implies that when a new strain arises it is able to spread very rapidly around the world.

6. Vaccines

There have been exhaustive attempts to identify pharmacological therapies to improve the clinical course and outcomes of this disease. However, presently, there is no licensed vaccine against RSV. High-risk infants can be substantially protected by monthly intramuscular injections of a commercially available RSV-neutralizing antibody (palivizumab) administered during the RSV epidemic season (American Academy of Pediatrics, 2003). RSV is the focus of antiviral- and vaccine-development programmes because of the morbidity and mortality associated with bronchiolitis early in life. However, these goals are now being aided by an understanding of the virus genome architecture and the mechanisms by which it is expressed and replicated (Meisner and Long, 2003).

The reasons why an RSV vaccine is not yet available emanate from numerous problems with its development. First, is the possibility that vaccination will potentiate naturally occurring RSV disease, as observed with the formalin-inactivated vaccine (Domachowske and Rosenberg, 1996). Second, young infants exhibit relative immunologic immaturity or because of suppression of their immune response due to circulating maternally derived anti-RSV antibodies (Falsey and Walsh, 1996). Another important consideration is the need to provide protection against multiple antigenic strains of RSV in the two major groups, A and B. A number of strategies have been implemented recently to generate safe and effective subunit, inactivated, and live attenuated virus vaccines (Falsey and Walsh, 1997).

7. Inactivated and subunit respiratory syncytial virus vaccines

The first attempt at an RSV vaccine in the 1960s employed formalin-inactivated RSV particles (FIRSV). This vaccine failed to induce a protective immune response and led to enhanced disease upon natural infection with wild type (*wt*) RSV. As a result, 80% of the children vaccinated needed to be hospitalized following *wt* RSV infection, and two children died (Kim et al., 1969). Consequently, there was great apprehension to study any nonreplicating RSV vaccine in seronegative infants and children. Enhanced disease has never been observed with live attenuated vaccines and it is not seen in seropositive subjects, neither with inactivated nor with subunit RSV vaccines (Collins et al., 2001).

8. Sub unit vaccines

A large number of subunit vaccines have been studied in preclinical trials, and some have progressed into clinical trials. Most of these candidate vaccines consist of either or both of the RSV surface glycoproteins that mediate membrane fusion (F) and virus attachment (G). These vaccines were administered in various ways, such as using alum phosphate or alum hydroxide. Others were evaluated conjugated to bacterial toxins, as ISCOMs (immunostimulating complex), or with adjuvants such as CpGs (CpG-DNA), monophosphoryl lipid A, saponines, or oil-in-water emulsions (Piedra, 2003; Kneber and Kimpen, 2004). The use of inactivated and subunit vaccines seems safe in RSV seropositive individuals, however, two exceptions have been noted in seronegative infants: FI-RSV and subunit vaccines induced high RSV-binding antibody titers with low neutralizing activity and the enhanced disease seen with FI-RSV can be replicated in rodent models using subunit vaccines. (Openshaw et al., 2001; Johnson et al., 2004)

One of the more recent trials evaluated PFP-3 (purified F protein with aluminum phosphate) in seropositive children 1 to 12 years of age with cystic fibrosis. In this trial, PFP-3 was found to be safe and immunogenic but not protective. Similarly, in a trial of PFP-2 in healthy pregnant women and their offspring, PFP-2 was found to be safe but there was no significant increase in RSV neutralizing IgG titers following vaccination in the third trimester so that a protective effect in the offspring could not be expected (Munoz et al., 2003). A different subunit vaccine was tested consisting of copurified F, G, and M proteins from RSV subgroup A (sanofi-aventis, Bridgewater, NJ) in a phase 1 trial in healthy adult volunteers. Of those vaccinated, 80% developed a greater than fourfold increase in neutralizing antibody titers to RSV A and RSV B. However, these titers did not persist so that annual vaccination would be necessary to maintain potentially protective antibody titers with this candidate vaccine (Wright et al., 2000). BBG2Na (Pierre Fabre, Castres, France), a promising vaccine candidate that was developed by fusing the conserved central domain of the RSV G protein to the albumin-binding region of streptococcal protein G, was found to be safe and immunogenic in phase 1 and phase 2 studies. But in the phase 3 trial purpura / type III hypersensitivity resulted as an unexpected side-effect in a small number of vaccine recipients and so halted further development (Kneiber and Kimpen., 2003).

9. Live attenuated respiratory syncytial virus vaccines

Several live attenuated RSV vaccines have been identified by passage of RSV under different conditions such as lower temperatures, temperature-sensitive (*ts*), chemical mutagenesis and non-*ts* attenuating mutations. Several candidate vaccines went into clinical trials, but it was not possible to find an appropriate balance between immunogenicity and attenuation, particularly in young infants.

The development of a method to generate infectious virus from a cDNA copy of the negative-sense single-stranded virus genome initiated the era of rational RSV vaccine design. It

allowed the use of site-directed mutagenesis to introduce desired mutations into the RSV genome and to evaluate the contribution of each mutation to the attenuation phenotype by introducing individual mutations or sets of mutations into the RSV genome. Reverse genetics has also been used to generate attenuated deletion mutants that are useful as live attenuated RSV vaccines. Deletion of the genes for the interferon antagonists NS1 or xlink, the small hydrophobic gene SH, or the M2-2 regulator of transcription and replication produced viable viruses that displayed restricted replication in nonhuman primates. Candidate vaccines bearing these deletions with or without additional attenuating point mutations were identified as suitable for clinical development (Collins and Murphy, 2005).

A promising candidate vaccine is an RSV subgroup A mutant designated *cp248/404/1030ΔSH*. This mutated virus has 11 mutations identified in the biologically derived *cp248/404RSV*, a further mutation in the large polymerase protein L (the 1030 mutation), and a deletion of the SH protein. It was evaluated in a phase 1 trial in 1- to 2-month-old infants, replication was highly restricted in seronegative infants and the vaccine was well tolerated (Karron et al., 2005). Although neither systemic nor mucosal antibody responses were repeatedly observed in the youngest vaccinated infants, but complete restriction of a second dose of vaccine given 4 weeks after the first dose indicated that protective immunity could be induced.

10. Future strategies

One of the most promising current strategies for protection against respiratory tract infection is intranasal treatment with vectors capable of generating RNAs that block viral replication. RNA interference (RNAi) is a natural defense of the innate immune system against viruses (Dallas and Vlassov, 2006). Following viral replication, double-stranded viral RNA is recognized by the host RNAi system which cuts it into short oligoribonucleotides, 20–30 bases long. These short sequences activate the cell's RNA cleavage machinery (the RNA interference silencing complex, or RISC) to destroy the viral RNA. By introducing siRNAs complementary to specific viral mRNAs, double-stranded activating RNA can be generated that activates the RISC cleavage system and destroys the viral message. This approach using antiviral siRNA has been reviewed by Manjunath et al, 2009. The use of siRNA as an antiviral agent involves a relatively straightforward attack on one or more key viral genes and should be effective against many human pathogens. The use of non-integrating plasmid vectors removes the risk of mutagenesis caused by some viral vectors.

11. Summary

The burden of acute respiratory infections (ARIs) caused by viral pathogens is impressive and leaves no doubt that effective and affordable vaccines are urgently needed. The impact of respiratory viral infection is greatest in the very young, the elderly, and people with an

impaired immune system or other chronic conditions. LRIs are a common cause of hospital admission and excess mortality. RSV infection is the most frequent etiology of a child's first LRI. Presently, passive protection against RSV is achieved successfully through monthly intramuscular injection of the humanized monoclonal anti-RSV antibody palivizumab. However, immunoglobulin products are expensive to administer.

Attempts to develop a vaccine against RSV have been unsuccessful to date. A formalin-inactivated RSV vaccine was developed in the 1960s. Although initial serological responses to this vaccine appeared promising, children who received this vaccine developed more severe disease, with a number of deaths, when exposed to natural RSV infection. The development of a successful RSV vaccine must address this issue and achieve protection of very young children if it is to have an impact on severe RSV disease. Recent progress in this area has included development of stable, live-attenuated RSV vaccines that can be administered as nasal spray. Despite progress in this area, a vaccine that is ready for use in clinical practice is still many years away.

Another approach is the development of an RSV vaccine that involves use of cloned RSV surface proteins as potential subunit vaccines. RSV fusion (F) and glycoprotein (G) can induce neutralizing and protective antibodies and are the components in development. Phase 1 trials of Fusion (F) protein nanoparticle RSV vaccine candidates have shown that they are generally well-tolerated, highly immunogenic and produce functional antibodies that neutralize RSV. These are being evaluated for potential immunization of young children and also for administration to pregnant women during the last trimester to boost anti-RSV antibody levels transferred to the infant (Schmidt, 2007).

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