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## Influence of Respiratory Syncytial Virus Strain Differences on Pathogenesis and Immunity

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### Abstract

Molecular epidemiology studies have provided convincing evidence of antigenic and sequence variability among respiratory syncytial virus (RSV) isolates. Circulating viruses have been classified into two antigenic groups (A and B) that correlate with well-delineated genetic groups. Most sequence and antigenic differences (both inter- and intra-groups) accumulate in two hypervariable segments of the G protein gene. Sequences of the G gene have been used for phylogenetic analyses. These studies have shown a worldwide distribution of RSV strains with both local and global replacement of dominant viruses with time. Although data are still limited, there is evidence that strain variation may contribute to differences in pathogenicity. In addition, there is some but limited evidence that RSV variation may be, at least partially, immune (antibody) driven. However, there is the paradox in RSV that, in contrast to other viruses (e.g., influenza viruses) the epitopes recognized by the most effective RSV-neutralizing antibodies are highly conserved. In contrast, antibodies that recognize strain-specific epitopes are poorly neutralizing. It is likely that this apparent contradiction is due to the lack of a comprehensive knowledge of the duration and specificities of the human antibody response against RSV antigens. Since there are some data supporting a group- (or clade-) specific antibody response after a primary infection in humans, it may be wise to consider the incorporation of strains representative of groups A and B (or their antigens) in future RSV vaccine development.

### 1. RSV antigenic groups and clades

An early study of the seroepidemiology of RSV in Sendai, Japan found that patient sera did not differ in neutralization of a small number of homologous and heterologous RSV strains, as measured by reduction in tissue culture infectious dose (TCID<sub>50</sub>) in HEp-2 cells (Suto et al. 1965). Using a methylcellulose overlay plaque assay developed in 1966, sera from infected ferrets detected limited strain antigenic variability, reflected in slightly different plaque reduction neutralization (PRN) titers for homologous (Long) versus heterologous (CH18537) strains (Coates et al. 1966). However, it was also found in those early days that children could be naturally infected in consecutive years with RSV strains indistinguishable

by cross-PRN, and adults were naturally re-infected despite pre-existing neutralizing antibodies (Abs) (Beem 1967).

Despite the previous comments, antigenic groups of RSV strains were definitively identified by enzyme-linked immunosorbent assay (ELISA) using a panel of ten monoclonal Abs (mAbs) obtained from mice immunized with different RSV strains, such as A2, Long, and CH18537 (Anderson et al. 1985). In a separate study from the same year, RSV isolates from West Virginia were probed with a panel of mAbs generated against RSV Long (Mufson et al. 1985). RSV proteins recognized by the mAbs were identified by radioimmunoprecipitation assay (RIPA) and SDS-PAGE of <sup>35</sup>S-labelled infected cell extracts. When these mAbs were tested against RSV field isolates by RIPA, it was revealed that RSV separated into two antigenic groups, A and B, based on eight epitope differences in the attachment glycoprotein (G), one epitope difference in the fusion glycoprotein (F), and one epitope difference in the nucleoprotein (N). The antigenic groups correlated with genetic differences identified by sequencing cDNA clones of the G genes of RSV A2 (A group), Long (A group), and CH18537 (B group) strains. Thus, while the deduced G protein sequences of A2 and Long strains shared 94% amino acid identity, those of CH18537 and A2 strains shared only 53% amino acid identity, with the majority of the diversity residing in the predicted extracellular domain (Johnson et al. 1987b). The classification of RSV isolates into A and B antigenic groups is now more often done via sequencing of variable region(s) of the G extracellular domain, rather than by mAb reactivity. The RSV A and B group designation is also referred to as antigenic “subgroups” in the literature, group A being more prevalent than group B (Hall et al. 1990; Matheson et al. 2006).

Sequence-based molecular epidemiology of RSV led to the identification of genetically distinct, co-circulating genotypic lineages. Evidence of RSV lineages within group A was revealed in isolates from Birmingham, U.K. (1989) using partial sequences of the small hydrophobic (SH) gene and restriction patterns of RSV nucleoprotein (N) gene PCR amplicons (Cane and Pringle 1991). RSV G gene sequences from 27 group A isolates from Montevideo, Uruguay and Madrid, Spain (1987 to 1993) were aligned with those of A2, Long, and six isolates from Birmingham, UK to analyze the phylogenetic relatedness of group A strains, and distinct lineages were evident (Garcia et al. 1994). Similarly, lineages were observed by analyzing sequences of the two variable domains of the G gene from 48 group A RSV isolates collected from 1956 to 1993 in the US, Australia, UK, Norway, Sweden, and Finland (Cane and Pringle 1995). Both studies also found local co-circulation of group A lineages and a high ratio of nonsynonymous to synonymous (dN/dS) mutations in the C-terminal variable region of G, suggesting positive selection. Furthermore, both studies probed isolates with panels of mAbs to the G protein and found that the strength of reactivity roughly paralleled the position on the phylogenetic dendrogram, consistent with contribution of immune selection to RSV G variability (Cane and Pringle 1995; Garcia et al. 1994).

A more detailed picture of RSV genetic lineages emerged with additional sequences. It was determined that the C-terminal 270 nt of the G gene can serve as a proxy for full length G gene variability (Peret et al. 1998). Phylogenetic analysis of G sequences from 204 RSV isolates collected in Rochester, New York from winter 1990/1991 to winter 1994/1995 revealed a number of genetically distinct clusters of genotypes (clades) within A and B

groups of RSV (Peret et al. 1998). These clades were designated GA1 to GA5 for group A and GB1 to GB4 for group B. This work provided a clade nomenclature and framework that was consistent with earlier observations of distinct RSV lineages and aided ongoing investigations of RSV molecular epidemiology. Subsequent RSV studies with large sequence data sets of RSV isolates over time from around the globe confirmed these co-circulating clades and identified additional clades (Gaunt et al. 2011; Matheson et al. 2006; Reiche and Schweiger 2009; Shobugawa et al. 2009; Venter et al. 2001; Zlateva et al. 2007; Botosso et al. 2009). Genetic relatedness of RSV group A strains is depicted in Figure 1 by a phylogenetic tree composited from multiple studies, showing clades and representative isolates. Since the late 1990s, the GA2 and GA5 clades have dominated among group A RSV clades, with season-to-season fluctuation in relative rate of isolation. Group B RSV clades will be discussed in more detail below. In general, there is greater diversity between group A clades than there is between group B clades.

## 2. Temporal and geographical distribution of RSV strains

Initial analysis of multi-sequence alignments of RSV G gene sequences showed clustering of strains temporally and not geographically. Phylogenetically related RSV strains could be found at similar times in different continents, whereas isolates collected in the same place from the same epidemic may show greater diversity (Garcia et al. 1994). Additional evidence for temporal and not geographic clustering came from a study comparing 106 group A and 38 group B RSV isolates from New Zealand (collected 1967–2004) with published isolates from around the world. The New Zealand isolates did not cluster with each other, and RSV isolates clustered by clade, not country (Matheson et al. 2006). However, despite data on wide geographic dissemination of RSV strains, community-based transmission likely plays a role in RSV epidemiology. RSV isolates were collected from the 1994–1995 RSV season in the following cities, states/province: Birmingham, Alabama, Rochester, New York, Houston, Texas, St. Louis, Missouri, and Winnipeg, Manitoba (Peret et al. 2000). The GA1 clade was most prevalent in Birmingham, Rochester, and St. Louis, whereas the GA5 clade was dominant in Houston, and the GA5 and GA7 clades co-dominated that season in Winnipeg. RSV isolates from Japan generally clustered with known clades, but some were genotypically unique, suggesting a role for geographic clustering and community-based spread (Kuroiwa et al. 2005).

One way to gauge community-specific RSV distribution is to compare RSV studies reporting RSV isolates from different locations spanning the same time frame. Table 1 compares the prevalence of RSV group A and group B as well as the prevalence of the dominant group A clades (GA2, GA5 and GA7) over time in different regions. Dominant group B clades were omitted in Table 1 because their classification is less consistent. Table I shows some patterns of widespread RSV distribution. For example, the relative A to B and relative GA2 to GA5 rates are roughly similar in Belgium, Germany, and Buenos Aires over three RSV seasons, 1998–99, 1999–00, 2000–01 (Table 1). Yet, site-specific group and clade restrictions are also evident. For example, Belgium and Germany differ in whether A or B is more prevalent in 3 of the 8 overlapping seasons (Table 1). Also, GA5 was the dominant group A clade in Belgium, Germany, and Japan in the 2003–04 season. However, GA2 was dominant the next season in Belgium and Japan, whereas GA5 remained dominant in

Germany (Table 1). In tropical and subtropical regions, the relative prevalence of A and B differed between Buenos Aires and Kenya in two out of three overlapping seasons (Table 1). RSV seasonality in tropical regions is distinct, and epidemics occur during the rainy season from July to November, with a biennial pattern of low and high incidence seasons, as observed in Cambodia (Arnott et al. 2011). In Cambodia and China, the dominance of group A or group B in a particular season was strong (Table 1). In summary, factors determining RSV group and clade compositions of epidemics are complex. Region-specific factors play a role, and, as discussed in more detail below, specific genotypes can also spread globally.

Although RSV groups and clades co-circulate and can appear in successive years, year-to-year changes in the predominance of group A and group B and year-to-year changes in the predominance of clades were observed in a given location (Peret et al. 1998). Subsequent studies confirmed that clades co-circulate locally and alternate in predominance over time, potentially due to immune selection but without evidence of progressive evolution as defined by new strain emergence (Botosso et al. 2009; Gaunt et al. 2011; Matheson et al. 2006; Reiche and Schweiger 2009; Venter et al. 2001). Two key questions about the temporal distribution of RSV strains are: i) what drives season-to-season changes in clade predominance? and ii) do RSV clades impact natural infection and re-infection by, for example, providing some degree of immune evasion? A recurring hypothesis has been that alternating clade prevalence is a result of short-lived strain- or clade-specific herd immunity that favors circulation of a heterologous clade. Although it has yet to be shown definitively, there are published data consistent with the hypothesis of selection by herd immunity. Thus, several of the amino acid residues in the C-terminal hypervariable region of the G protein identified as having a high dN/dS ratio map within known Ab epitopes (Botosso et al. 2009; Garcia et al. 1994). Furthermore, a number of positively selected amino acid sites in the C-terminus of G show a reversion (“flip-flop”) pattern of evolution, consistent with rising and waning strain-specific immunity (Botosso et al. 2009). The infant serum Ab response to primary RSV infection contains clade-specific Abs, as measured by ELISA using plates coated with polypeptides corresponding to the C-terminus of G (Scott et al. 2007). More recently, the RSV group and clade of infecting and reinfecting strains was identified in a birth cohort in Kenya. Excluding 7 reinfections that occurred during the same epidemic, there were 46 reinfections documented (Agoti et al. 2012). Twenty eight (61%) of those reinfections were group heterologous (A then B, or B then A). Among the 46 reinfections, there were only six instances where group A infection was followed by group A in a subsequent season. Of those six, four were heterologous for GA2/GA5 clade. Although the numbers are small, the authors state that the majority of reinfections are heterologous at the group or clade level (Agoti et al. 2012). It would be more compelling if higher rates of heterologous clade reinfection could be documented when the re-infecting clade is subdominant.

A better understanding of the potential for global spread of RSV strains has been facilitated by the discovery, in 1999, of a novel group B RSV genotype. It was isolated for the first time in Buenos Aires and contained a 60 nt duplicated region in the C-terminal one-third of G, and the clade was named BA (Trento et al. 2003). These initial BA isolates had an exact 60 nt duplication, resulting in two tandem sequences of TERDTSTSQTSTVLDTTTSKH from amino acids 240 to 280 in G (Trento et al. 2003). This insertion was then used as a “tag” to

analyze the global distribution and evolution of a new RSV genotype since its initial emergence (Trento et al. 2006; Trento et al. 2010). The BA clade rapidly disseminated worldwide and became dominant, and these findings have been confirmed by a number of RSV molecular epidemiology studies with relatively large data sets (Baek et al. 2012; Gaunt et al. 2011; Zhang et al. 2007; Botosso et al. 2009). The BA clade continued to evolve, the two exact 60 nt repeats diverged slightly, and a new BA-IV lineage essentially replaced all other group B RSV G strains. The biological significance of the duplicated region in BA G is not known.

Besides evolutionary studies, analysis of sequence variation in the G gene may have other practical applications as, for instance tracing the origin of infecting viruses during outbreaks in hospitalized patients (Mazzulli et al. 1999; Taylor et al. 2001).

### 3. Clinical differences between RSV strains

Early work showed that group A RSV is associated with slightly greater clinical severity than group B RSV (Hall et al. 1990). RSV disease severity was correlated with RSV clades in small cohorts, but these studies are not definitive because clade GA2 was found to be less pathogenic in one study and more pathogenic in others (Gilca et al. 2006; Martinello et al. 2002). Thus, the role of RSV strain differences in disease remains to be elucidated.

### 4. Phenotypic Differences between RSV strains in vitro and in animal models

Small changes in viral gene sequences may have a large impact on pathogenesis. For instance, the elevated virulence of 1918 and avian influenza strains hinges on few amino acids differences (Conenello et al. 2007; Tumpey et al. 2005). Passage of viruses in animal hosts can lead to adaptation. The RSV Long strain was adapted to mice (line 19) by serial intracranial inoculation, resulting in higher virus replication presumably due to mutations that have not been characterized (Cavallaro and Maassab 1966). Infection of BALB/c mice with the laboratory RSV strains A2 or Long results in a predominant  $T_H1$ -type antiviral response (Moore and Peebles, Jr. 2006). In contrast, the line 19 strain of RSV induces a  $T_H2$  type and IL-13-dependent airway hyperreactivity (AHR) and pulmonary mucus in BALB/c mice (Lukacs et al. 2006). The F protein of the line 19 RSV strain has a unique sequence and is a factor that can induce pulmonary IL-13 and mucin expression in RSV infection (Moore et al. 2009). In addition, six RSV isolates have been screened for lung IL-13 levels and airway mucin expression in BALB/c mice. Three of these isolates induced lung IL-13 and gob-5 (a marker of mucin) expression, and were found to be differentially mucogenic in BALB/c mice (Stokes et al. 2011). RSV clinical isolates also infect the airway epithelium of mice to a greater extent than the laboratory adapted A2 strain (Stokes et al. 2011). In differentiated primary pediatric airway epithelial cells cultured at air-liquid interface, a RSV clinical isolate exhibited enhanced infectivity (but not virus yield) and induced greater mucus production than the laboratory A2 strain (Villenave et al. 2012). The molecular bases for these strain-specific phenotypes are largely unknown.

## 5. Protective immunity

Animal and human studies have provided a wealth of evidence indicating that protection against RSV infection is afforded mainly by neutralizing antibodies. In early experiments (Prince et al. 1983), it was found that cotton rats infected with RSV developed complete resistance to pulmonary reinfection which lasted at least 18 months. Adaptive transfer studies with the convalescence blood showed that serum antibody, but not circulating lymphocytes, conferred resistance. Immune pregnant cotton rat females transmitted protective antibodies to their young mainly through colostrum and milk (Prince et al. 1983). Similar findings were observed in pregnant ferrets (Prince and Porter 1975) and guinea pigs (Buraphacheep and Sullender 1997). Human convalescent serum and human immunoglobulin (Ig) preparations were also found to confer pulmonary protection against RSV in the cotton rats. Serum neutralizing antibody titres of 1:380 or higher were required for complete protection in the lungs whereas about 10 fold higher titres were required for protection in the nose (Prince et al. 1985). Therapeutic administration of neutralizing Abs reduced significantly the level of RSV replication in the lungs of cotton rats and owl monkeys but showed only a slight trend of beneficial effects in a limited number of RSV infected children (Hemming and Prince 1990). In addition to polyclonal antibodies, administration of certain monoclonal antibodies (mAbs) directed against either G or F glycoproteins protected the lungs of mice (Taylor et al. 1984) and cotton rats (Walsh et al. 1984) against a RSV challenge. In one study, it was found that passive protection of mice afforded by a non-neutralizing mAb directed against the G glycoprotein was dependent on both the Fc fragment of the antibody and the host complement (Corbeil et al. 1996), explaining the lack of an strict correlation between *in vitro* neutralization in the absence of complement and *in vivo* protection (Taylor et al. 1984; Walsh et al. 1984).

In humans, protection of adult volunteers to an RSV challenge was correlated with high titres of preexisting serum neutralizing antibodies (Hall et al. 1991). Additionally, an inverse correlation was observed between high titres of RSV-neutralizing serum antibodies and risk of infection in children (Glezen et al. 1986). However, whereas in animal models the quantitative aspects of passive protection to RSV infection by neutralizing antibodies is well established, the situation in human is less certain. For instance, contradicting results were reported by the same group, about correlation (Falsey and Walsh 1998) or lack of correlation (Falsey and Walsh 1992) between neutralizing antibody titers and risk of RSV infection in the elderly. Nevertheless, the consensus inferred from the majority of data is that neutralizing antibodies protect against RSV infection and particularly against RSV-associated pathology. Perhaps the best evidence for this assertion was provided by the clinical studies carried out with an Ig preparation (RS-IVIG, Respigam<sup>TM</sup>), selected for high titers of RSV-neutralizing antibodies which was administered prophylactically to high risk infants (Groothuis et al. 1993). The beneficial effect of RS-IVIG was noticed in the reduction of hospitalizations (55%) and days of intensive care (97%) rather than frequency of RSV infections. These studies led to licensing of Respigam in 1996 for prophylaxis of RSV infections in high-risk infants. Respigam was replaced in 1998 by a humanized neutralizing mAb (MEDI-493, palivizumab) directed against the RSV F glycoprotein

(Beeler and Van Wyke 1989) that showed similar efficacy but it was easier to administer than Respigam.

Despite the beneficial effects of antibodies in protection against RSV, some caution is needed because:

1. Passive serum Abs have been shown to inhibit the antibody responses to F and G glycoproteins expressed by recombinant vaccinia viruses (Murphy et al. 1988) or administered as purified antigens adjuvanted with alum (Murphy et al. 1991); however, they did not suppress the T-cell response nor the priming for a strong secondary antibody response (Fisher et al. 1999; Crowe, Jr. et al. 2001). Therefore, a well-balanced dose of prophylactic neutralizing antibodies may be required to avoid interference with the host immune response to either RSV infection or RSV vaccination.
2. Weakly neutralizing Abs may have detrimental effects that contribute to the enhanced respiratory disease observed in seronegative children that were vaccinated in the 1960's with a formalin inactivated RSV preparation (Kim et al. 1969). It has been shown that antibodies may lead to formation of immune complexes that correlate with enhanced pathology in mice after an RSV challenge. Immune complex activation of complement was also observed in post-mortem lung sections from children with enhanced RSV disease (Polack et al. 2002). Non-replicating RSV vaccines that fail to promote antibody affinity maturation may prime for immune complex formation upon RSV infection (Delgado et al. 2009).

Although antibodies are important for resistance to infection, T cells are imperative for virus clearance. Thus, individuals with compromised T cell immunity can shed virus for months (Hall et al. 1986). Prolonged virus shedding is also observed in nude or irradiated mice (Cannon et al. 1987) and in mice depleted of both CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes (Graham et al. 1991). Furthermore, CD8<sup>+</sup> cytotoxic lymphocytes may provide some protection in mice against infection, but this effect is short-lived (Connors et al. 1991; Connors et al. 1992). In infants with severe RSV infection, the peak of activated CD8<sup>+</sup> T cell numbers in bronchoalveolar lavage (BAL) samples and in blood correlated with convalescence, consistent with a role for CD8<sup>+</sup> T cells in recovery (Heidema et al. 2007).

## 6. Protective antigens

Identification of antigens able to induce a neutralizing and protective immune response was achieved initially by immunization of mice or cotton rats with recombinant vaccinia viruses encoding individual RSV gene products (Stott et al. 1987; Stott et al. 1986; Wertz et al. 1987; Olmsted et al. 1986). It was promptly found that only the external F and G glycoproteins were able to confer long-lasting protection against RSV infection and that this protection correlated with induction of neutralizing antibodies. The nucleoprotein (N) and the M2-1 (or 22k) proteins were also able to induce partial and short-lived protection which, at least in the case of the M2-1 was mediated by cytotoxic T lymphocytes (Connors et al. 1992).

It was also noticed that: i) recombinant vaccinia viruses expressing the F protein conferred higher level of protection against RSV than those expressing the G protein (Olmsted et al. 1986) and ii) the neutralizing immune response against the F protein was cross-protective against viruses of a different antigenic group whereas the neutralizing and protective response against the G protein was restricted to viruses of the same antigenic group (Johnson et al. 1987a;Stott et al. 1987). These differences reflect the dissimilar structural and antigenic characteristics of the F and G glycoproteins and their differences at the level of antigenic and genetic relatedness between RSV isolates.

In the case of the F glycoprotein (the viral glycoprotein that mediates fusion of the virus and cell membranes), there is 89% amino acid sequence identity between the proteins of groups A and B of human RSV (Johnson and Collins 1988). This is reflected in the high level of antigenic relatedness observed with murine mAbs (Garcia-Barreno et al. 1989). Epitopes have been mapped in the F protein primary structure primarily by isolation and sequencing of mutants that grow in the presence of individual mAbs. These escape mutants normally contained single amino acid substitutions that obliterated the epitopes recognized by the antibodies used in their selection. Figure 2 summarizes the amino acid changes in escape mutants reported up to date by different groups (Arbiza et al. 1992;Lopez et al. 1998;Crowe et al. 1998;Zhao et al. 2004b;Zhao et al. 2004a). In most cases, selection was done with murine mAbs, except in the case of the mutant I266M that was selected with a recombinant human Fab fragment (Crowe et al. 1998). It is worth stressing that frequently the same mutation could be repeatedly selected with different antibodies (e.g., K272N isolated with mAbs 151, 1200, 47F and palivizumab). Occasionally, more than one amino acid change was selected at the same position (e.g., N262S or N262Y) and one of the changes (N268I) was also selected after passing the virus in the presence of a polyclonal rabbit antiserum raised against purified F protein (Tome et al. 2012). These findings probably reflect the dominance of certain epitopes and the propensity of the F protein to incorporate certain mutations but not others. The amino acid changes depicted in Figure 2 and therefore the corresponding antibody epitopes are clustered in three regions (antigenic sites) of the F protein primary structure, named sites II, I and IV and corresponding to sites A, B and C as designated by Beeler et al. (Beeler and Van Wyke 1989). The epitopes of antigenic sites II and IV (Anderson et al. 1985;Beeler and Van Wyke 1989;Garcia-Barreno et al. 1989) are very conserved among RSV strains while those of antigenic site I are group-specific; i.e., they are conserved only in RSV strains of the same antigenic group (Garcia-Barreno et al. 1989).

In contrast to the antigenic and genetic conservation of the F protein, the G glycoproteins of RSV group A and B strains share less than 50% amino acid sequence identity (Johnson et al. 1987b). This genetic variation is reflected in the high level of antigenic differences detected with anti-G mAbs (Garcia-Barreno et al. 1989). As with the F protein, the epitopes recognized by anti-G MAb have been mapped in the protein primary structure mainly by selection and sequencing of escape mutants (Figure 3). Three types of epitopes were identified: i) epitopes *conserved* among all RSV strains that mapped in the central unglycosylated segment of the G protein ectomain, ii) *group-specific* epitopes that overlapped partially with the conserved epitopes and that were retained only in RSV strains



of the same antigenic group and iii) a majority of *strain-specific* epitopes that were located in the C-terminal third of the G protein ectodomain and that were present only in some RSV strains of the same antigenic group (Martinez et al. 1997). Additionally, and in clear distinction with the escape mutants selected with anti-F MAbs, those selected with anti-G mAbs frequently contained drastic genetic alterations, other than single amino acid changes and which included: i) frame-shift mutations that altered the C-terminal third of the G molecule (Garcia-Barreno et al. 1990), ii) premature stop codons that shortened the G polypeptide between 1 and 42 amino acids (Rueda et al. 1991;Rueda et al. 1995;Martinez et al. 1997), iii) multiple A-G transitions (A-G hypermutations) that change several amino acids, including some cysteines in the G protein ectodomain (Rueda et al. 1994;Martinez et al. 1997;Walsh et al. 1998) and iv) amino acid changes that prevent insertion of G in the viral membrane (Walsh et al. 1998). All these findings emphasize the extreme plasticity of the G molecule to adopt sequence changes, something that might be related to the fact that G is not required for RSV replication in Vero cells, although viruses lacking G are attenuated in HEP-2 cells and in vivo (Karron et al. 1997).

Another major difference between mAbs specific for the F and G glycoproteins is their potency and mechanism of neutralization. Whereas certain mAbs reacting with epitopes in sites II or IV of the F glycoprotein are potent neutralizers of RSV infectivity *in vitro* (Lopez et al. 1998) most mAbs specific of the G glycoprotein neutralize RSV very poorly, even if tested against the homologous strain used in their isolation. However mixtures of anti-G mAbs show a synergistic effect in neutralization that is not observed with anti-F MAbs (Martinez and Melero 1998;Anderson et al. 1988). It has then been postulated that neutralizing antibodies directed against the F glycoprotein inhibit RSV infectivity by blocking the conformational changes that follow activation of RSV F to initiate the process of virus-cell membrane fusion (Magro et al. 2010). In contrast, results obtained with anti-G mAbs suggest that neutralization is afforded in this case by steric hindrance of G protein interactions with cell surface components (likely proteoglycans). This steric inhibition requires simultaneous binding of several antibodies to the same G molecule, explaining the synergistic effect found with combinations of these antibodies (Martinez and Melero 1998). Despite these findings, it has been reported that a minority of human antibodies with high affinity for the central conserved region of RSV G are potent neutralizers of virus infectivity (Collarini et al. 2009) and may neutralize RSV infectivity by mechanisms others than steric hindrance.

In summary, most data indicate that F is more potent than G in inducing neutralizing and protective antibodies. The potent neutralizing antibody response afforded by anti-F antibodies is widely cross-reactive, while the poorer neutralizing anti-G response is rather strain or group-specific. These differences are probably deep-rooted in the level of genetic variability of F and G between RSV isolates and in the mechanism of virus neutralization afforded by different antibodies, particularly *in vivo*.

## 7. Human antibody response

As noted in previous sections, most studies carried out so far with neutralizing antibodies have been done with murine mAbs. Therefore, two relevant questions are: i) what are the specificities of human neutralizing Abs? and ii) are they the same as those of murine Abs?

Not much is known about the specificities of human anti-RSV neutralizing Abs. This gap between human and animal studies is due at least in part to the inherent difficulty of experimenting with humans. The use of technologies to clone and express human antibodies produced by individual B cells should facilitate in the future dissecting the repertoire of specificities represented in the human neutralizing antibody response against RSV.

Nevertheless, it has been reported already that human Ig preparations contain antibodies that compete with most murine anti-F antibodies (Sastre 2004); however, human neutralizing Abs have been identified that recognize F protein fragments laying outside of the antigenic sites demarcated by murine mAbs (Sastre et al. 2004). Additionally, neutralizing Abs specific for the untriggered form of RSV F, not represented in the collections of murine mAbs so far described were recently unveiled in human Ig preparations (Magro et al. 2012). Therefore, although the specificities of murine anti-F Abs are present in human sera, human Abs seem to embrace a broader range of specificities than murine Abs.

Similarly certain specificities of anti-G mAbs also seem to be relevant in the human antibody response. Thus, human Igs were able to out compete binding of certain anti-G mAbs that recognized epitopes of the unglycosylated central segment of the G protein ectodomain (Sastre 2004). Furthermore, neutralizing antibodies could be purified from human Ig preparations by affinity chromatography with a fragment of the G glycoprotein corresponding to the conserved ectodomain segment (Sastre et al. 2004). In agreement with these results, group-specific serum antibodies directed against the central non-glycosylated G protein segment have been found after primary infections of children (Murata et al. 2010b; Murata et al. 2010a). These antibodies are likely responsible for the partially group-specific neutralizing antibody response reported in previous studies after primary RSV infections (Hendry et al. 1988; Muelenaer et al. 1991). However, it remains uncertain whether or not human sera contain Abs reacting with strain-specific epitopes of the C-terminal third of the RSV G glycoprotein. Detection of this type of antibodies in convalescent sera is likely dependent on the use of an appropriate G protein (or fragment thereof) that matches the antigenic properties of the infecting virus. Since detailed information about the infecting virus is not available for most sera, detection of strain-specific antibodies has been reported only in a few cases (Palomo et al. 2000; Cane et al. 1996; Jones et al. 2002; Scott et al. 2007)

In summary, it is apparent that the antibody specificities of murine mAbs are also represented in human convalescent sera, but human antibodies seem to recognize a broader range of F and G protein epitopes than murine Abs. It will be important in the future to elucidate the actual relevance of the different antibody specificities for protection against infection and pathology. This might be particularly relevant in the case of anti-G Abs; for instance, it has been reported that certain murine Abs that block the interaction of the

fractalkine-like motif of RSV\_G with the CX3CR1 receptor reduced pulmonary inflammation and virus replication in mice (Zhang et al. 2010b). Likewise, other Abs may block additional, but still unrecognized activities of the F or G glycoproteins involved in virus replication and/or pathology.

## 8. Antigenic variation, immune selection and RSV evolution

It has been observed that generally viral antigens accumulate amino acid changes at the sites that are recognized by neutralizing Abs as exemplified by the influenza virus haemagglutinin (HA) (Knossow and Skehel 2006). In this case, most of the sequence changes that are retained in viruses of the same subtype over the years involve residues on the surface of the HA head that are part of the epitopes recognized by neutralizing Abs. In other words, to escape neutralization by pre-existing antibodies new influenza strains are positively selected that can infect again the same human population.

The situation described for influenza virus is in apparent contradiction with that found in RSV and outlined in previous sections. Thus, in the case of human RSV, the most effective neutralizing antibodies are those directed against antigenic sites II and IV of the F glycoprotein (Figure 2), which are highly conserved among RSV isolates. In contrast, the antibodies that recognize epitopes in the C-terminal third of the G glycoprotein are only weakly neutralizing (Figure 3), although this segment of the G molecule represents an extreme example of sequence variation, compared with antigens of related viruses (e.g. influenza virus). In other words, the paradoxical situation in RSV is that the epitopes recognized by the most potent neutralizing antibodies are highly conserved whereas those recognized by poorly neutralizing antibodies are extremely variable.

As mentioned before, two types of evidence support the notion that the sequence variation found in the G protein is the result of positive selection: i) whereas synonymous nucleotide changes have a uniform distribution along the G protein gene, non-synonymous changes accumulate in the two variable mucin-like regions of G (Melero et al. 1997), in analogy with the accumulation of non-synonymous changes at the antigenic sites of influenza HA due to positive Darwinian evolution (Fitch et al. 1991) and ii) phylogenetic methods have identified several positively selected sites in group A and B of RSV isolates (Woelk and Holmes 2001; Botosso et al. 2009). The fact that some of the positively selected sites coincide with epitopes recognized by anti-G mAbs and that the antigenic changes detected with a panel of anti-G mAbs correlated with the position of RSV isolates in a phylogenetic tree (Garcia et al. 1994) support the notion that positive selection of changes in the G glycoprotein might be immune driven.

Data from human studies offer conflicting interpretations about the relevance of antigenic variation and immune selection in protection against infection and in RSV evolution, respectively. On the one hand, as noted before the epitopes recognized by the most efficient neutralizing anti-F mAbs are highly conserved among RSV isolates and antibodies with the same specificities are present in human Ig preparations (Sastre et al. 2004). These results would argue against immune selection playing any role in the generation of RSV variability and support the idea that RSV vaccines may require only one virus strain or its antigens.

On the other hand, studies conducted with convalescent sera have provided evidence that upon primary RSV infections there was some dominance of group-specific neutralizing antibodies (Muelenaer et al. 1991; Cane et al. 1996; Scott et al. 2007). This group-specific response was also observed by ELISA with sera from convalescent primary infections using segments of the central core of the G glycoprotein as antigens (Murata et al. 2010a; Scott et al. 2007; Cane et al. 1996). However, the group-specificity of the neutralizing and antigen-binding antibodies was blurred upon secondary infections (Murata et al. 2010a). Consequently, there is a critical need for detailed assessment of the antibody specificities (including strain-specific) induced upon RSV infection and re-infection and their actual contribution to the human neutralizing immune response.

It is possible that the immune selective pressure on RSV is not as strong as that operating in influenza virus; for instance, if the antibody response is short-lived in RSV in comparison with influenza virus, replacement of pre-existing RSV strains by new variants may be a slower process than in influenza. Of note, the new variants of RSV group B with a 60-nucleotide duplication in the G-protein gene replaced the pre-existing strains of the same antigenic group worldwide but after a 6–7 year period (Trento et al. 2010), which is clearly longer than the observed replacement of influenza A viruses by new variants (usually 2–3 years). A combination of immune selection and high plasticity of the G protein may be at the basis of the extreme sequence variation observed for this gene among RSV isolates. In consequence, until new information concerning the specificities of human Ab responses is available, it would be wise to include viruses (or antigens) representing the two RSV antigenic groups in vaccine development.

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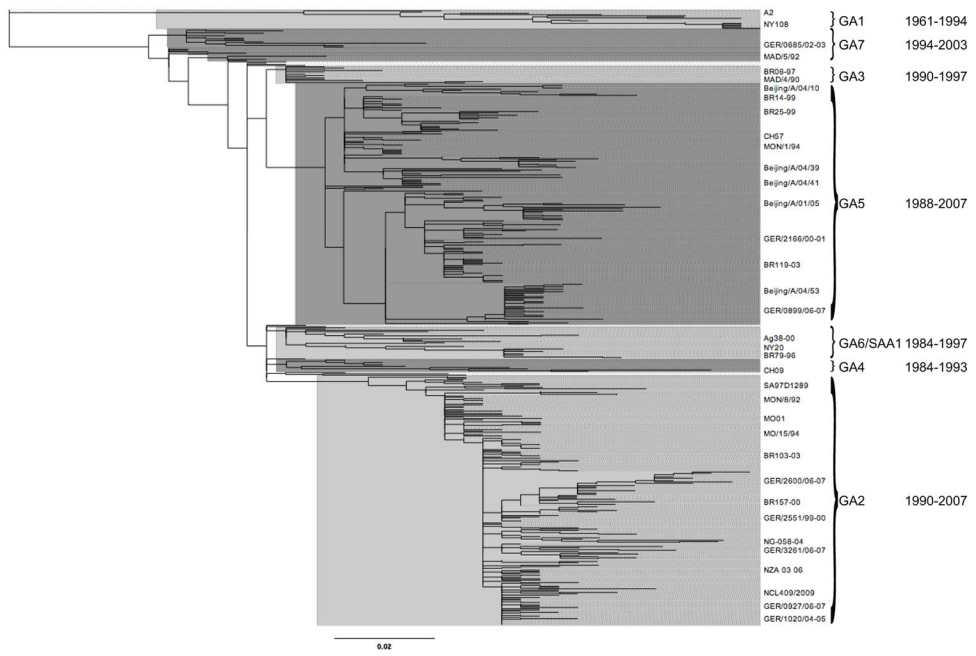
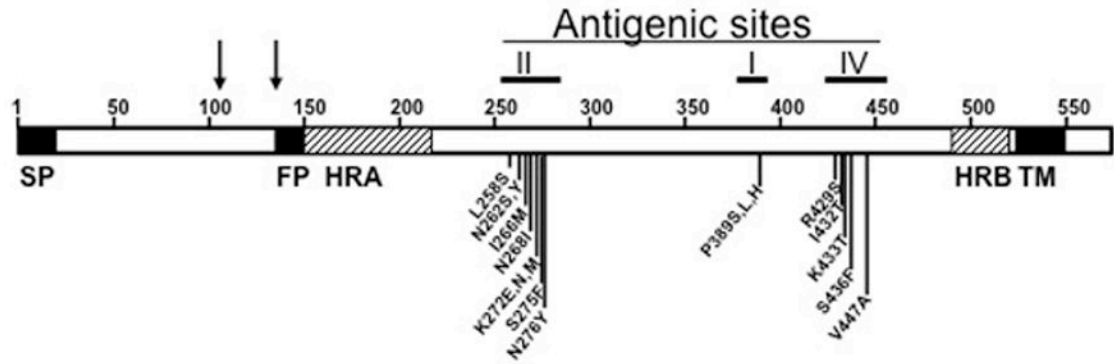
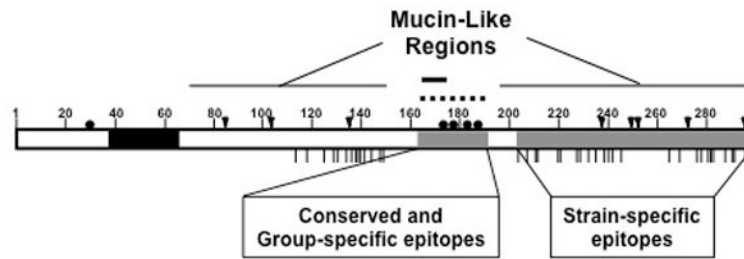


Figure 1.



**Figure 2. Scheme of the F protein primary structure**

The sequence length is indicated above the main rectangle. Black boxes denote the hydrophobic signal peptide (SP), fusion peptide (FP) and transmembrane region (TM). Shaded boxes symbolize heptad repeat sequences, HRA and HRB and the two arrows indicate the location of the proteolytic cleavage sites. Vertical lines denote the location of the indicated amino acid changes selected in escape mutants described in the following articles: (Arbiza et al. 1992; Lopez et al. 1998; Crowe et al. 1998; Zhao et al. 2004a; Zhao et al. 2004b). These amino acid changes have been grouped in antigenic sites I, II and IV as shown above the main rectangle.



**Figure 3. Scheme of the G protein primary structure**

The sequence length is indicated above the main rectangle. Two mucin-like variable regions of the G protein ectodomain are indicated. Black dots denote Cys residues, black arrowheads denote N-glycosylation sites and short vertical lines (below the main rectangle) O-glycosylation sites. The black box delineates the transmembrane region. The grey boxes delineate the location of conserved and group-specific epitopes in the central segment and the strain-specific epitopes in the C-terminal mucin-like region, respectively (Garcia-Barreno et al. 1990; Rueda et al. 1991; Rueda et al. 1994; Rueda et al. 1995; Martinez et al. 1997; Walsh et al. 1998). The continuous horizontal line denotes the segment of identical sequence (amino acids 164–176) in all RSV isolates and the broken horizontal line the segment of identical sequence (amino acids 163–189) in all RSV group A isolates.

RSV group prevalence and group A clade prevalence by location, study, and epidemic season

Table 1

	Temperate Region Winter Epidemic										
	1997-1998	1998-1999	1999-2000	2000-2001	2001-2002	2002-2003	2003-2004	2004-2005	2005-2006	2006-2007	2007-2008
Leuven, Belgium <sup>a</sup>											
A/B <sup>b</sup>	64/34	25/75	75/24	82/18	15/85	55/45	61/37	22/76	84/15		
GA2/GA5 <sup>c</sup>	70/30	42/58	26/74	41/59	83/17	20/80	0/100	75/25	22/78		
Germany <sup>d</sup>											
A/B	20/80	93/7	58/42	67/33	34/66	75/25	80/20	64/36	53/47		
GA2/GA5/GA7	50/50/-	37/58/5	38/62/-	50/50/-	4/48/48	28/72/-	13/87/-	26/74/-	57/43/-		
Niigata, Japan <sup>e</sup>											
A/B					94/6	22/78	89/11	80/20	70/30	70/30	
GA2 <sup>f</sup> /GA5/GA7					-97/3	-69/31	-100/-	81/19/-	91/1/-	100/-/-	
Lanzhou and Chongqing, China <sup>g</sup>											
A/B									100/-	97/3	
GA2/GA5									100/-	100/-	
	Subtropical or Tropical Epidemic										
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	
Buenos Aires, Argentina <sup>h</sup>											
A/B	38/62	100/0	83/17	29/71	74/26	82/18					
GA2/GA5	64/36	30/70	43/57	-100	78/22	50/50					
Kilifi District, Kenya <sup>i</sup>											
A/B			100/0	98/2	39/61						
GA2/GA5			5/95	27/73	-100						

	Temperate Region Winter Epidemic										
	1997-1998	1998-1999	1999-2000	2000-2001	2001-2002	2002-2003	2003-2004	2004-2005	2005-2006	2006-2007	2007-2008
Cambodia/ <sup>a</sup>											
A/B							0/100		100/-	92/2	8/92
GA2 <sup>k</sup> /GA5							-		100/-	98/2	100/-

<sup>a</sup>Data from (Zlateva et al. 2007).

<sup>b</sup>% of isolates that were RSV group A/% of isolates that were RSV group B

<sup>c</sup>% of group A isolates that were clade GA2/% of group A isolates that were clade GA5

<sup>d</sup>Data from (Reiche and Schweiger 2009).

<sup>e</sup>Data from (Shobugawa et al. 2009).

<sup>f</sup>Niigata isolates assigned to NA1 and NA2 clades are represented as GA2 because these three clades are more closely related to each other than to GA5.

<sup>g</sup>Data combined from (Zhang et al. 2010a; Zhang et al. 2010c).

<sup>h</sup>Data from (Viegas and Mischenko 2005).

<sup>i</sup>Data from Scott et al. 20(Scott et al. 2004).

<sup>j</sup>Data from (Arnott et al. 2011).

<sup>k</sup>Cambodia isolates assigned to NA1 clade are represented as GA2.