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The Impact of Viral Genotype on Pathogenesis and Disease Severity: Respiratory Syncytial Virus and Human Rhinoviruses

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRI) and viral death in infants. RSV disease in infants is characterized by epithelial desquamation, neutrophilic bronchiolitis and pneumonia, and obstructive pulmonary mucus. Human rhinoviruses (HRV) are by far the most common cause of symptomatic upper respiratory tract infection (URI) in people and are more recently appreciated as a significant cause of LRI. RSV and HRV are also implicated in asthma pathogenesis. Within both RSV and HRV, viral genetic differences play a role in disease severity and/or prevalence in patient populations, and viral genetic differences affect pathogenesis. Here, we review data on how viral genetic differences impact disease using RSV and HRV as examples, including effects on the host immune response. Virus genotype-phenotype relationships can be exploited in the laboratory to gain insight into mechanisms by which respiratory viruses modulate host immune responses and cause disease.

Introduction: viral genetic variation impacts pathogenesis

Virus-induced disease is determined by a combination of host and viral genetics. There are many examples across virus families of how relatively minor changes in viral genome sequence have a large impact on pathogenesis. For example, virulence of 1918 and avian influenza virus strains in animals can hinge on a few amino acids [1–4]. Mutations in the HIV-1 Vpu and Nef proteins are associated with HIV-1 M (pandemic) and O (non-pandemic) strains and play a role in species-specific host restriction [5,6]. Phenotypically distinct clones of the prototypical arenavirus lymphocytic choriomeningitis virus (LCMV), a

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rodent-borne virus, have been used to elucidate immune regulation of T cell responses. LCMV Clone 13, which causes persistent infection of mice, was isolated from the spleens of mice infected with LCMV Armstrong strain, which causes acute infection. LCMV Clone 13 and Armstrong differ by three amino acids, and LCMV reverse genetics was used to map the persistent infection phenotype to a single amino acid in the viral spike protein that confers infection of dendritic cells [7•]. The virulence factors of influenza A viruses, such as the pandemic swine-origin 2009 H1N1 are well-studied and reviewed elsewhere [8]. Additional examples include papillomavirus types (e.g. HPV-16) associated with cervical cancer and hepatitis C virus genotypes associated with acute or persistent infection. In general, the specific virus strain or genotype as an important determinant of disease is the rule, not the exception, and here we will review the literature on this topic for two important respiratory viruses, human respiratory syncytial virus (RSV) and human rhinoviruses (HRV).

RSV disease in children

RSV is a member of the *Paramyxoviridae* family of non-segmented, negative-sense single-stranded RNA viruses [9]. RSV is the most important pathogen for lower respiratory tract infection (LRI) in infants [10]. RSV, an epidemic winter virus in temperate regions, causes LRI in 20–30% of infants and hospitalizes approximately 1% of the winter infant cohort in the USA, resulting in 100K infant hospitalizations per year. Globally, RSV is a leading cause of infant viral death [11]. Annually, it is estimated to infect 64 million people worldwide and result in 160,000 deaths [12]. Overall, bronchiolitis hospital admissions of children less than two years old in the US cost more than \$500 million annually [13]. Despite the burden of RSV disease, there is no vaccine or effective treatment.

In addition to acute infant bronchiolitis, RSV is implicated in asthma pathogenesis. Many infants with lower respiratory tract infection (LRI)-associated wheeze in the first year of life are transient wheezers [14]. However, severe RSV LRI in infancy is consistently associated with recurrent wheezing to age 6, and RSV LRI may be a risk factor for allergic sensitization [14,15]. It is not clear if LRI-induced recurrent wheezing contributes to progression of atopic asthma in adults. Nevertheless, asthma in children is a significant cause of morbidity. A cohort study of >95K children showed that infants who are ~4 months old at winter virus peak have increased risk of bronchiolitis and childhood asthma compared to children who were 1 year old at winter virus peak [16•]. This suggests a causal role of RSV bronchiolitis in childhood asthma.

RSV targets the bronchiolar epithelium resulting in necrosis and destruction of ciliated epithelial cells. Airway mucus secretion increases during RSV infection, forming thick plugs mixed with cell debris, fibrin, and lymphocytes [17]. Mucus plugging is a pathology causing airway obstruction and airway hyperresponsiveness (AHR) via reduced airway caliber and increased airway resistance [18]. In lung tissue from archived fatal RSV cases, acute bronchiolitis was prominent in mostly medium and small bronchioles. Airways were plugged with debris composed of sloughed epithelial cells, macrophages, fibrin, and mucin [17]. RSV antigen was found in both bronchiolar and bronchial epithelial cells as well as in alveolar spaces and exudates [17]. In another autopsy-based study that compared fatal influenza virus and RSV infections in children, macrophages and neutrophils were abundant

in the lungs of fatal RSV bronchiolitis cases [19]. Viral antigen was detected in the airway epithelium in subjects infected with both influenza and RSV but more prominently in RSV-infected infants [19]. CD8 T cells were not found in autopsy tissues of infants with fatal RSV bronchiolitis in this study [19]. Taken together, the two studies suggest that severe bronchiolitis is not characterized by abundant CD8 T cells but by inefficient viral clearance, cytopathology, and mucus plugging. There is scant evidence for a “cytokine storm” or significant immunopathology in severe RSV infant bronchiolitis, where epithelial destruction, viral antigen in the airways, and mucus expression in the airways are hallmarks of pathogenesis.

RSV strain-specific pathogenesis

RSV has one serotype, within which there are two antigenic subgroups, A and B, defined by reactivity to monoclonal Abs [20]. Within antigenic subgroups, RSV strains can be further classified into clades based on nucleotide (nt) sequence of a hypervariable region of the G gene [21]. Annual RSV epidemics consist of a small number of clades with one or two dominant clades accounting for > 50% of isolates, and a variable number of less prevalent clades. Dominant RSV clades in a given location are frequently replaced every one or two RSV seasons [21]. Subgroup A strains can be divided into at least seven clades (GA1-GA7) and subgroup B strains can be divided into at least four clades (GB1-GB4) [22]. A current hypothesis for waxing and waning RSV clade prevalence is that short-term clade-specific herd immunity drives RSV clade distribution. Residues in defined immunogenic regions of RSV G have a high dN/dS ratio, suggesting immune selection [21].

Antigenic subgroup A RSV is associated with greater clinical severity than subgroup B RSV [23,24]. Within RSV subgroup A, disease severity correlated with RSV clades in a small cohort. Clade GA3 was associated with more severe disease than clade GA2 [25]. GA3 isolates are less frequently isolated than GA2 clade in recent years, and GA3 RSV has not been specifically studied in the laboratory to our knowledge.

The airway epithelium is the primary target of RSV. Primary, polarized human airway epithelial (HAE) cells in air-liquid interface (ALI) cultures have been used to model RSV infection [26,27••]. RSV infects ciliated cells via the apical surface and is shed from the apical surface [26]. Paradoxically, despite clear in vivo data that RSV causes destruction or desquamation of the airway epithelium in children, the virus causes minimal cytopathology in HAE cells ex vivo [26,27••]. In agreement with the comparative autopsy study discussed above where influenza virus infection was associated with greater inflammation [19], comparison of influenza virus and RSV infection of HAE ALI cultures revealed that RSV induces relatively low interferon, cytokine, and chemokine expression [28]. In the system established by Villanave and colleagues, well-differentiated pediatric bronchial epithelial cells (WD-PBECs) are derived from bronchial brushings of children undergoing elective surgery. The WD-PBECs are pseudostratified ciliated, goblet, and basal cells with intact tight junctions. In these cultures, RSV infects mainly ciliated and occasionally non-ciliated epithelial cells but not goblet cells, and the virus induces apical cell sloughing, goblet cell hyperplasia, and occasional syncytia in WD-PBEC, reaching a peak titer 48 hours post-infection. RSV-induced goblet cells hyperplasia/metaplasia similar to infant RSV

bronchiolitis was also seen in the WD-PBEC system [27••]. Two strains of RSV were studied in WD-PBEC, the commonly used laboratory-adapted A2 strain (GA1 clade) and a clinical isolate, BT2a (GA5 clade) [29]. RSV A2 and BT2a grow to equivalent titers in WD-PBEC, but the BT2a isolate causes significantly greater epithelial sloughing and goblet cell hyperplasia than the A2 strain [27••]. Thus, RSV BT2a is more mucogenic than RSV A2 in an ex vivo HAE system. At this time, RSV BT2a has not been sequenced (U. Power, personal communication), and the molecular determinant(s) of its relatively more cytopathic and mucogenic phenotypes are unknown.

The RSV A2 and Long strains are antigenic subgroup A (GA1 clade) reference strains. The A2 strain has been used extensively for all aspects of RSV biology, including studying immune responses in mice. Hunein “John” Maassab, known for isolating and cold-adapting the Ann Arbor influenza master strain that became FluMist vaccine, isolated the line 19 strain of RSV in 1967, the year he published adaptation of influenza at 25° C [30,31]. Infection of BALB/c mice with the laboratory RSV strains A2 or Long results in a predominant T_H1-type antiviral response [32–34]. In contrast to A2 and Long strains, the parental (unattenuated) line 19 strain of RSV induces IL-13-dependent airway hyperreactivity (AHR) and pulmonary mucus in BALB/c mice [35–37].

RSV strain line 19 has been used to probe immune mechanisms in RSV-induced T_H2 responses, mucus expression, and AHR. RSV line 19 infection results in lung IL-17 expression, and IL-17 plays a role in promoting airway mucus in response to line 19 [38•]. A major difference between A2 and line 19 pathogenesis in mice may be that IL-13 protects against A2-induced illness (weight loss) whereas IL-13 plays a central role in mediating line 19-induced mucus and AHR [35,36,39]. Recently, it was shown that repeated RSV line 19 infection of infant mice that were tolerized to ovalbumin via mother’s milk results in breaking of the immune tolerance to ovalbumin, suggesting a possible role for early RSV infection in the inception of allergic airway inflammation [40••]. Thymic stromal lymphopoietin (TSLP), an important cytokine in allergic responses, plays a role in T_H2 and mucus responses to RSV line 19 in mice [41]. However, HAE ALI cultures produce equivalent levels of TSLP in response to RSV A2 and RSV line 19, so TSLP induction may be RSV strain-specific in this system [41].

Our group sought to identify the RSV line 19 strain-specific factor in T_H2 and mucus induction in BALB/c mice. Using a recombinant virus strategy, we showed that the fusion (F) protein of the line 19 RSV strain has a unique sequence and, when expressed as the F gene in the non-mucogenic genetic background of the A2 strain (chimeric A2-line19F), can induce pulmonary IL-13 and mucin expression in mice [37]. Although viral load is likely a key driver of RSV pathogenesis, comparing strains in these models indicates that quantitative peak viral load in mice does not correlate with mucus expression because peak viral load is A2-line19F > A2 > line 19, whereas mucus expression is A2-line19F = line 19 > A2 [36,37].

RSV strain-specific pathogenesis in mice was investigated using six subgroup A RSV clinical isolates maintained at low passage [42]. Three of six RSV clinical isolates induced lung IL-13 expression in BALB/c mice, and two distinct GA2 clade isolates, 2–20 and 3–12

were studied further [42]. RSV 2–20 induces high levels of lung IL-13 and airway mucin expression in BALB/c mice, as well as increases breathing effort, whereas RSV 3–12 does not. Similar to the data on RSV strain line 19, RSV 2-20-induced airway mucus is IL-13-dependent and does not correlate with peak viral load when compared to non-mucogenic strains [42]. Mucogenic strains 2-20 and line 19 induced lower total IFN-gamma-expressing CD8 T cells in the lung 8 days post-infection than non-mucogenic strains A2 and 3-12 [42].

RSV F protein in strain-specific pathogenesis

Similar to the RSV line 19 strain F protein, the RSV 2-20 strain F protein was implicated as a mucus-inducing viral protein, as a chimeric A2-2-20F recombinant strain is mucus inducing like the parental 2-20 strain [43•]. The mucogenic phenotype of 2-20 and A2-2-20F does not correlate with peak viral load but correlates with early histopathologic lesions in the airway epithelium and infiltration of neutrophils to the lung [43•].

Paramyxovirus F proteins are type I fusion proteins that irreversibly undergo a dramatic change from a higher energy pre-fusion conformation to a lower energy post-fusion conformation in the process of fusing the viral membrane with the target cell membrane. F proteins of model paramyxoviruses, such as measles virus, are triggered from pre- to post-fusion forms by interaction of the viral attachment protein with its receptor. However, the RSV attachment G protein is not required for infection or for RSV F triggering. Structures of the pre- and post-fusion forms of RSV F have been determined, major achievements [44•,45]. The molecular mechanisms regulating RSV fusion protein triggering are not yet defined. Paramyxovirus F protein activity can be measured *in vitro* using F-mediated cell-cell fusion as a surrogate of virus-cell fusion. The RSV 2-20 F protein, when expressed *in vitro*, exhibits equivalent fusion activity to the A2 F protein. However, when the RSV F proteins are co-expressed with RSV G, RSV 2-20 F exhibits higher fusion activity than A2 F, suggesting a functional interaction between RSV G and F leads to higher fusion activity, and possibly pathogenicity, for the 2-20 strain [43•]. One hypothesis for the F-specific mechanism of pathogenesis is that variation in F sequence leads to a range of direct fusion activity, and higher fusion activity leads to greater cytopathology in the airway epithelium. The hypothesis is based in part on work with Sendai virus, a murine paramyxovirus which produces lethal infection in mice. In Sendai, an F protein mutant with decreased fusion activity has attenuated pathogenicity, and a hyperfusogenic Sendai F mutant exhibits elevated lethality [46•].

Neutrophils in RSV strain-specific pathogenesis

Neutrophils likely contribute to goblet cell hyperplasia and mucus secretion. Neutrophils are a major source of TNF- α . Intratracheal administration of TNF- α in BALB/c mice induces gob-5, Muc5ac, and mucus expression in the airways [47]. Intratracheal IL-13 instillation in rats results in neutrophil TNF- α expression [48]. Neutrophil elastase can increase Muc5ac expression *in vitro* [49]. Neutrophils are prominent in RSV pathogenesis. They are the predominant airway leukocytes observed in RSV bronchiolitis. Neutrophils are overwhelmingly present in BAL samples from both lower and upper respiratory airways of infants infected with RSV in numerous studies. The addition of neutrophils to RSV-infected

A549 cells increased cell damage and detachment, suggesting that neutrophils may induce epithelial damage and contribute to RSV pathogenesis [50]. Neutrophils could provide an explanation as to why RSV-infected primary HAE cells show little cytopathology compared to RSV-infected airways in vivo, but this remains to be demonstrated.

The role of neutrophils was examined in RSV strain-specific pathogenesis in BALB/c mice. Infection with the 2-20 strain and the chimeric A2-2-20F strain resulted in more neutrophils in the lung one day post-infection than infection with the A2 strain, suggesting that the F protein of the 2-20 strain is a mucus factor in the setting of infection [43•]. Depletion of neutrophils from the lungs of RSV 2-20-infected mice resulted in lower TNF- α levels in the lung one day post-infection, fewer IL-13-expressing CD4 T cells in the lung six days post-infection, and lower airway mucin expression in the airways eight days post-infection [43•]. Thus, strain variation in the RSV F protein sequence can lead to a range of fusion activity and initial cytopathology (discussed above), which may lead to variable neutrophil responses, exacerbation of epithelial damage, and ensuring T_H2 IL-13 and mucus expression (Figure 1).

HRV species and types

Human rhinoviruses (HRVs) are members of the genus *Enterovirus* within the *Picornaviridae* family. HRVs have long been recognized as the predominant cause of acute URI in children and adults, a major public health and socioeconomic problem. HRV respiratory tract infection actually causes a wide range of clinical phenotypes, including asymptomatic infections, frequently common colds (URI), and severe LRI.

HRV was first isolated in 1957. By the late 1960s, over 50 HRV serotypes had been defined, and vaccination of volunteers did not result in heterologous neutralizing Abs. By the mid-1970s, the outlook for a HRV vaccine was dour, due the large number of serotypes, technical challenges associated with producing high valency vaccines, and poor performance of a decavalent, formalin-inactivated HRV vaccine, although the vaccine study was limited by low input titers of several HRV strains [51,52]. There is some cross-reactivity between HRV serotypes, and experimental infection of volunteers can result in protective immunity lasting at least one year [53,54]. HRVs are well characterized at the molecular level. The icosahedral atomic structure of HRV14 was determined, as well as neutralizing epitopes [55,56]. Rescue of infectious poliovirus (also *Picornaviridae*) from cloned cDNA paved the way for infectious clones of HRV, and many aspects of fundamental molecular biology of poliovirus translate to HRV. HRV biology was recently reviewed along with HRV antivirals, an area of active research and development [57].

In contrast to RSV, HRVs are highly genetically and antigenically diverse. There are three species groups, A, B, and C (C being more recently discovered). Classically, 75 A serotypes and 25 B serotypes were defined by cross-neutralization assays [58]. The “serotype” designation is increasingly replaced with “type,” as cDNA sequencing technologies have led to expansion of known HRV types and clarification of phylogenetic relationships between them [59••]. A recently proposed classification protocol was based on sequence of the HRV capsid genes revealed approximately 78 A types, 30 B types, and 51 C types [58].

Neutralization is not a practical method to type HRV C species viruses because cultivation of HRV C is currently limited to organ culture, itself a remarkable and recent advancement [60•]. HRVs are also classified by cellular receptor utilization. About 90% of A and B HRV types belong to the “major group” which binds to intercellular adhesion molecule I (ICAM-I), and “minor group” types bind to low-density lipoprotein receptor (LDLR). Antibodies that block major and minor group HRV failed to block HRV C, suggesting HRV C viruses utilize an alternative, yet to be discovered receptor [60•]. Recently, an anti-ICAM-I mAb specific for domain 1 of ICAM-I was shown to block major group HRV entry without blocking ICAM-I interaction with its ligand, LFA-I, suggesting that Abs blocking HRV can be developed that do not interfere ICAM-I/LFA-I functions [61].

HRV species-specific disease

The development of sensitive and specific molecular diagnostic detection techniques in the 1990s led to greatly improved detection of respiratory tract viruses and what followed was a clear demonstration of the important link between viral respiratory tract infections, in particular HRV, and asthma exacerbations [62]. Evidence supports that HRV causes greater morbidity in persons with asthma than in healthy populations, with differences in the antiviral host response demonstrated among asthmatic subjects [63–65]. New detection methods and sequencing methods ultimately led to identification of HRV species C. HRV C was associated with a high proportion of HRV hospitalizations, especially for asthma exacerbation [66,67]. More recently, both HRV A and C species have been shown to cause moderate to severe illness in young children than HRV B, and host and seasonal factors appear to play a role in phenotypic disease expression [68••]. In this study, A and C types were overall seven times more likely to be associated with moderate to severe disease than B types. Interestingly, this study ranked the observed virulence of individual types and found that some C types may be more virulent than other C types, suggesting that HRV genotype-specific disease extends beyond the species level to the type level [68••]. Overall, the viral molecular determinants of HRV species- and potentially type-specific pathogenesis are largely undefined, although recent cultivation and mutagenesis of HRV C, as well as advancement in HRV mouse models, will enable progress in this area [60,69,70].

HRV infection in early life has been also demonstrated to be an independent risk factor for subsequent wheezing illnesses and early childhood asthma [71,72]. Early wheezing episodes associated with human rhinovirus in the first three years of life are the most significant predictor of asthma at six years of age [72]. Whether HRV is causal in the development of asthma, however, has not been yet been worked out. The hypothesis that early life wheezing with HRV may be one of the first manifestations of asthma, rather than causal, is supported by data demonstrating that allergic sensitization precedes wheezing illnesses with HRV, and that most infants who wheeze with HRV have a maternal history of asthma [73,74]. Association of a susceptibility locus on chromosome 17q21, rhinovirus infection in early childhood and wheezing indicates the existence of a complex interaction between a genetic predisposition and environmental factors [75]. However, it remains an unresolved question as to whether HRV LRI in early life alters immunologic or normal lung development in a way that predisposes to subsequent wheezing, or whether wheezing and HRV LRI is the first manifestation of asthma in the genetically susceptible host. Of course, these aren't mutually

exclusive options, and it could be that HRV is both causal and one of the first manifestations of asthma.

Summary

RSV and HRV are important respiratory viruses for which there are no vaccines or proven effective therapies available. Both viruses cause acute URI and LRI, and both are associated with asthma pathogenesis. As with many other viruses, the specific strain or genotype of RSV and HRV has an impact on pathogenesis. Virus strain-specific phenotypes (e.g. virulence, cytopathology, or TH2 responses) provide an additional tool or approach to elucidating the role of infection in lung disease.

RSV has relatively limited genetic diversity (single serotype). RSV antigenic subgroup A is associated with greater disease severity in infants than antigenic subgroup B, and there are reports of clade-specific virulence. Within antigenic subgroup A, RSV strain-specific phenotypes have been reported in primary cells *ex vivo* and in experimentally infected mice. RSV reverse genetics derived chimeric RSV strains from mucus-inducing and non-mucogenic RSV strains has mapped the mucus-inducing RSV phenotype to variation in the RSV fusion protein, in agreement with fusion mutants of Sendai virus shown to exhibit virulence variation in mice.

HRVs have tremendous genetic diversity (>100 serotypes). Discovery of the C species led to great interest in genotype-specific disease in patients. HRV species A and C are associated with greater acute illness severity than species B, and C species is associated with LRI and asthma. Recently, HRV C reverse genetics and propagation (albeit in organ culture) was described, which should enable dissection of HRV C virulence factors.

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Highlights

- RSV and HRV cause LRI, are linked to asthma, and show strain-specific virulence.
- RSV strain-specific mucus induction has been mapped to the viral fusion protein.
- HRV species C is associated with asthma, and A and C cause more disease than B.
- Viral strain-specific phenotypes can be used to dissect pathogenesis mechanisms.
- Distinct RSV fusion proteins induce more or less neutrophils, which regulate IL-13.

Figure 1.

Working model of RSV strain-specific induction of airway mucin expression. (A) Electron micrograph of RSV exhibiting glycoprotein spikes. The sample was cell-free supernatant from RSV A2-infected primary human airway epithelial cells processed for negative staining. The RSV fusion (F) protein is a major spike protein necessary for virus fusion to target cells and for cell-cell fusion in the formation of syncytia. Recent work showed the “mucogenic” strain 2-20 F protein is capable of enhanced fusion compared to the laboratory A2 strain F protein [•43]. (B) Enhanced fusion correlates with the ability to infect the mouse airway epithelium. RSV strain 2-20 infects the BALB/c mouse airway epithelium, resulting in early necrotic cell debris in the airways as well as lung neutrophils [42, •43]. (C) Six days post-infection with mucogenic RSV 2-20, IL-13-expressing CD4⁺ T cells are found in the lung [•43]. IL-13 plays a critical role in airway mucin induction by both RSV strains line 19 and 2-20 [35, 42]. (D) Robust PAS⁺ airways day 8 post-infection in lungs of a mouse infected with RSV 2-20. Digitized H&E-stained section as described [42]. Depletion of neutrophils results in significantly fewer IL-13-expressing CD4⁺ T cells in the lung and less airway mucin expression, although the mechanism by which neutrophils promote T_H2 cytokine expression and mucus are unknown.