Pathogenesis of Respiratory Syncytial Virus Infection in the Murine Model

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There is a wide spectrum of illness caused by respiratory syncytial virus (RSV) infection that is caused in large part by host-related factors, such as age of the patient and degree of host immunocompetency. Although the vast majority of persons infected with RSV experience symptoms of mild upper respiratory tract infection, in some people these infections cause significant morbidity and are sometimes fatal. Although a great deal of investigation in both humans and animals has explained the timing and tropism of RSV infection and the general principles by which the immune system responds to this infection, at present we only partially understand the disparity in illness severity that can occur. This article briefly reviews the clinical sequelae of RSV infection and then focuses on the mechanisms of viral pathogenesis.

Keywords: airway hyperreactivity; interleukin-17; mucus; pathology; T cells

Respiratory syncytial virus (RSV) is a leading viral cause of wheezing-related hospital admissions in children younger than 2 years, despite the fact that the majority of admissions caused by this virus typically only occur in a narrow time window between November and March each year in the United States (1). Risk factors for RSV hospitalization in infancy include the following: day care attendance, birth between November through January, pre-school-age siblings, birth weight less than the tenth percentile, being male, having two or more smokers in the household, and having households of five or more persons (2, 3). RSV infection can also cause asthma exacerbations in older children (4). Patients who have undergone bone marrow and stem cell transplants are at risk for severe RSV pneumonia, and RSV infection in the preengraftment period after autologous hematologic transplantation has been reported to delay engraftment (5). RSV, as well as other respiratory viruses, such as parainfluenza, influenza, and adenovirus, can cause bronchiolitis obliterans syndrome and death in lung transplant recipients (6). RSV is less severe in the setting of other solid organ transplantation and does not appear to cause enhanced disease in patients with AIDS. In the elderly, RSV is an important cause of morbidity and mortality. In a retrospective cohort study, RSV was responsible for an annual average of 15 hospitalizations and 17 deaths per 1,000 nursing home residents, whereas influenza accounted for an average of 28 hospitalizations and 15 deaths in the same setting (7) (Table 1). In addition to the consequence of an acute infection on lung health, RSV infection has been proposed to have important immunomodulatory effects in predisposing individuals to the development of allergic disease and asthma. For instance, RSV

bronchiolitis in infancy so severe as to warrant hospitalization has been reported to result in increased sensitivity to common aeroallergens by both skin testing and allergen-specific serum IgE levels, as well as more reactive airways, up to age 13 (8). However, in another prospective longitudinal study, RSV lower respiratory infection in early life was associated with wheezing up to age 11, but not to age 13, and was not associated with an increased incidence of allergic sensitization (9). Therefore, although RSV infection is almost universal by age 3 and reinfection occurs throughout life (10), certain populations are at greater risk for more severe acute disease, and in turn, may have longlasting RSV-induced physiologic changes. Defining the complete cellular and immunologic consequences of RSV infection will be important in understanding the mechanisms by which these high-risk populations have greater disease severity and allow us to create more effective and targeted treatment strategies (Table 2).

INITIAL EVENTS IN RSV INFECTION

RSV has three virion surface proteins, which include the heavily glycosylated G (attachment) protein (11), the F (fusion) protein, which modulates membrane fusion virus penetration at the cell surface (12), and the SH protein, which has no known function (13). An experimental human primary airway epithelial cell culture technique has helped elucidate how RSV infects these cells, which are the primary point of entry of the host (14). RSV preferentially infects the apical, but not basolateral, surface of the airway epithelial cells. RSV seems to specifically target luminal columnar cells after they have developed cilia. RSV is also shed from the apical surface, and the beating of the cilia may facilitate spreading of the infection to neighboring ciliated cells. In this system, RSV is not cytopathic, implying that the immune response to RSV infection in epithelial cells results in the epithelial damage and cellular desquamation that is a hallmark of human infection (14).

One of the earliest host responses to infection is generation of the initial innate cytokine response (10). These cytokines include the type I IFNs, of which IFN- α and IFN- β are key members, as well as IFN- λ 1, IFN- λ 2, and IFN- λ 3 (15). These cytokines can be induced by viral infection or double-stranded DNA, activate the identical JAK/STAT signal transduction pathway, mediate the expression of genes that lead to cellular resistance to infection, and perhaps regulate the subsequent adaptive immune response (15). RSV expresses two nonstructural (NS) proteins, NS1 and NS2, which reduce the induction of the type I IFNs, IFN- λ 1, IFN- λ 2, and IFN- λ 3 (15). When the NS1 and/or NS2 genes are deleted from the RSV genome, the replication of the virus is inhibited in cultured cells that produce the type I IFNs (15). These results suggest there is a link between the NS1 and NS2 inhibition of the host cytokine response and the ability to control viral replication. Interleukin-12 (IL-12) and IL-18 are also part of the initial innate cytokine response and have been detected in the respiratory secretions of human infants with RSV infection (16).

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TABLE 1. DISTINCT DISEASE SYNDROMES ASSOCIATED WITH RESPIRATORY SYNCYTIAL VIRUS INFECTION

Bronchiolitis in infants
Postinfection wheezing and childhood asthma
Severe disease in the institutionalized elderly leading to excess mortality and exacerbation of underlying disease conditions
Giant cell pneumonia in persons with deficient T-cell immunity
Vaccine-enhanced disease
Apnea and sudden infant death syndrome

In response to RSV infection, the airway epithelium also produces chemokines that modulate the influx of inflammatory cells into the infected tissues. Polarized, differentiated respiratory epithelial cells produce IL-8 and CCL5 (RANTES [regulated on activation, normal T-cell expressed and secreted]) after RSV infection (17). Chemokines identified in the respiratory tract secretions of children who have naturally occurring RSV infection include the β -chemokines CCL3 (macrophage inflammatory protein-1 [MIP-1 α]), CCL2 (monocyte chemoattractant protein-1 [MCP-1]), CCL11 (eotaxin), and CCL5 (18). Levels of these β -chemokines in the respiratory tract secretions have been reported to correlate with severity of RSV-induced illness, presumably by initiating an augmented inflammatory response to RSV (18), although it may also reflect the magnitude of the overall RSV antigen stimulus and therefore be simply a byproduct of virus load.

Autopsy studies of children who die of RSV bronchiolitis reveal that the inflammation generated by the immune response may be an important pathogenic factor, and that airway obstruction is an important component of the disease. Not only does this infection result in loss of cilia and sloughing of epithelial cells into the airway but the pathologic features of bronchiolitis include collections of desquamated airway epithelial cells, polymorphonuclear leukocytes, fibrin, lymphocytes, and some mucus within the airway, and cellular infiltration and edema around the airway. In addition, there is an accumulation of macrophages within the alveoli and a mononuclear interstitial infiltrate. Desquamation of airway epithelial cells and inflammation are more extensive in bronchiolitis than in asthma. In acute bronchiolitis, sloughed epithelial cells, neutrophils, lymphocytes, fibrin, and mucus appear to be major contributors to airway obstruction.

Intervention studies often elucidate mechanisms of disease. Corticosteroids are not effective as might have been suggested by the histopathologic description of the autopsy studies of RSV bronchiolitis. In a randomized, double-blind, and placebo-controlled study of 147 infants younger than 2 years hospitalized with RSV infection, 5 days of therapy with systemic prednisolone had no effect on adjunctive medication usage, length of hospital stay, or duration of illness (19). In another randomized trial, administration of intravenous dexamethasone every 12 hours for 4 days had no effect on duration of mechanical ventilation, intensive care unit stay, or hospital stay (20). A meta-analysis review of the use of bronchodilators for bronchiolitis showed some short-term improvement in clinical scores, but no improvement in measures of oxygenation or in the rate of hospitalization (21). In addition, a recent randomized trial revealed that nebulized epinephrine did not improve the time until an infant with bronchiolitis was ready for discharge (22). However, among infants who required supplemental oxygen and intravenous fluids, the time until the infant was ready for discharge was significantly longer in the epinephrine group than in the placebo group, suggesting that epinephrine treatment may be harmful in infants with more severe bronchiolitis (22).

Our knowledge of the pathogenesis of RSV in humans is limited because of the dearth of experimental studies using this

virus because of safety concerns, because persons at risk for more severe RSV infections are not always identifiable before infection. As a result, animal models have been developed to better understand the mechanisms by which RSV causes disease. These models include infection of mice, rats, guinea pigs, sheep, cows, and monkeys. Although there are advantages and disadvantages for each of these systems, most of the experimental work focused on immune mechanisms of disease has been in the murine model of RSV infection. We will therefore primarily focus on what murine models have taught us about pathogenic mechanisms of RSV-induced disease, and give a perspective on how that data should be interpreted. The murine model offers easy accessibility to the primary tissue infected by RSV (the lung), relatively low cost compared with other animal models, and a plethora of reagents, inbred strains, and genetically modified strains that can be used to define the mechanisms of RSV disease pathogenesis. However, there are significant shortcomings in using the murine system, including lack of infection in bronchiolar epithelium, failure to propagate infection from the upper to lower airway, and relatively high inoculum required for robust infection and induction of pathology and illness. Nevertheless, the mouse is no less permissive to infection than other small animal models, and many elements of the mouse and human response to RSV infection are similar, particularly in the cytokines and chemokines produced in response to infection as well as the cellular patterns of lung inflammation.

MURINE MODEL OF RSV INFECTION AND IMMUNOPATHOGENESIS

The murine model of RSV infection has been used effectively to study the following: (1) pathogenesis of acute self-limited respiratory virus infection, (2) CD8⁺ T-cell biology, (3) CD4⁺

TABLE 2. MAJOR MECHANISMS OF RESPIRATORY SYNCYTIAL VIRUS-INDUCED DISEASE

Virus-induced cytopathology
Severe combined immunodeficiency
Bone marrow and lung transplantation
Airway obstruction
Small airways
Exudate with inflammatory cells, epithelium, fibrin, and mucus
Hyperreactivity and remodeling
Immune-mediated
Proinflammatory cytokine production
Polymorphonuclear leukocytes
Excessive CD8 ⁺ T-cell response
Immune evasion or deviation
Inhibition of type I IFN activity
CD4 ⁺ T cells producing IL-4, IL-5, or IL-13
Immune complex deposition
Inhibition of CD4 ⁺ T-cell proliferation
Altered CD8 ⁺ T-cell function

Definition of abbreviation: IL = interleukin.

Figure 1. Murine model of respiratory syncytial virus infection. Anesthetized mice can be inoculated intranasally with high-titer respiratory syncytial virus (RSV) stocks to establish infection in the upper and lower respiratory tract. Virus titer peaks in nasal and lung tissue between Days 3 and 6 after inoculation. Illness begins on Day 5, peaks between Days 8 and 10, and resolves by Day 12. Illness is temporally associated with the level of mononuclear cell infiltration into lung. A number of clinical, physiologic, cellular, and molecular endpoints can be measured in the system, providing the opportunity to define mechanisms and correlates of disease. These assays are particularly informative when designed to measure *in vivo* processes at a moment in time, and avoid *in vitro* manipulations. BAL = bronchoalveolar lavage; CTL = cytotoxic T lymphocyte; FACS = fluorescence-activated cell sorting; PFU = plaque-forming unit; RPA = RNase protection assay.

T-cell biology, and (4) virus-induced innate immune responses. However, there are murine models for other viruses that can be used for similar purposes. The model has also been successfully used for preclinical testing of vaccines, passive antibody, immunomodulators, or antiviral therapy, even though other small animal models like the cotton rat can serve this purpose. Most important, the murine model of RSV provides a singular tool for studying basic aspects of the interaction between allergic inflammation and virus-induced immune responses (Figure 1). Using the unique antigenic stimulus of RSV after immunization, coadministered with allergen, or in selected genetically modified mice, it is possible to investigate the regulation and consequence of a virus-induced inflammatory process that may be relevant to the pathogenesis of asthma and airway hyperreactivity.

The primary target of RSV infection in the mouse model is the type 1 alveolar pneumocyte, and illness is a consequence of infection at this level of the lung. RSV has been reported to inhibit alveolar fluid clearance, a crucial function of bronchoalveolar epithelium (23). In these experiments, RSV-infected mice had a significant decrease in alveolar fluid clearance both 2 and 4 days after infection that returned to baseline at later time points. These investigators confirmed that the decrease in alveolar fluid clearance was dependent on the virus being alive by showing that ultraviolet light-inactivated virus had no effect on alveolar fluid clearance. This RSV-induced reduction in alveolar fluid clearance was associated with increased water content that was not a consequence of increased epithelial permeability or cell death. RSV infection also led to an increase in concentration of uridine triphosphate in bronchoalveolar lavage fluid from an unidentified source (23). Uridine triphosphate has been shown by other groups to inhibit respiratory epithelial sodium resorption *in vitro*, therefore inhibiting alveolar fluid clearance. Thus, RSV may increase lung water content leading to airway wall edema and obstruction. In addition to RSV's effect on fluid clearance, it also may change the epithelium's interaction with other environmental factors. In an epithelial monolayer system, RSV augmented expression of toll-like receptor 4, or TLR4, which resulted in an increased response to LPS with increased mitogen-activated protein activity, IL-8 production, and tumor necrosis factor- α production (24).

As in humans, RSV infection in the mouse leads to the production of a number of chemokines. Murine airway epithelial cells infected with RSV produced CCL2, CCL5, CXCL10 (IFN-inducible protein 10), and keratinocyte cytokine, which is the murine homolog of IL-8 and a potent neutrophil chemoattractant (25). RSV infection of a transformed murine alveolar macrophage

cell line, MHS, expressed CCL5 and CXCL2 (MIP-2) (25). *In vivo* infection of mice leads to production of CCL2, CCL3, CCL5, CXCL10, and keratinocyte chemoattractant (25). Several intervention studies have shown that a number of these chemokines have pathogenic effects in RSV infection. For instance, treatment with anti-CCL5 antibodies reduced RSV-induced airway hyperresponsiveness (AHR) (26). In a combined RSV/allergen challenge model, anti-CCL5 antibody decreased recruitment of inflammatory cells to bronchoalveolar and peribronchial regions of the lungs, and these reductions were associated with a reduction in both T-cell recruitment into the bronchoalveolar space, leukotriene release, and chemokine generation (27). Mice deficient in CCL3 had a significant reduction in lung inflammation after RSV infection, compared with control littermates, whereas there was no effect on viral replication (28). Blocking chemokine receptors also prevents RSV-induced airway disease. RSV infection of mice treated with anti CXCL2 antibody or mice deficient in CXCL2 results in reduced AHR and airway mucus while having no impact on viral replication (29). Thus, chemokines have an important role in regulating RSV-induced illness and inflammation.

Natural killer (NK) cells are one of the earliest cell types to enter into the lung after RSV infection. The number of NK cells producing IFN- γ peaks between Days 3 and 5 after infection and decreases by Day 7 (30). Antibody depletion of NK cells results in an increased viral load 5 days after infection (31). Just as RSV NS1 and NS2 proteins modulate type 1 IFN production, infection with an RSV deletion mutant lacking the G and SH genes resulted in an increased number of NK cells in bronchoalveolar lavage fluid compared with mice infected with wild-type RSV, suggesting that RSV has multiple mechanisms to modify the innate immune response (32). NK T cells are a subset of lymphocytes that also have an immunomodulatory role in RSV infection. In BALB/c mice, NK T cells contribute to the expansion of RSV-specific CD8 T lymphocytes and to disease severity (33).

Nowhere is it more apparent that the immune response to RSV is responsible for a substantial portion of RSV-induced illness than in studies in which CD4 and CD8 cells have been depleted in the *in vivo* murine model of RSV infection. Mice treated with either anti-CD4 or anti-CD8 antibodies have decreased weight loss and illness and delayed viral clearance compared with mice administered an isotype control antibody (34). However, mice depleted of both CD4 and CD8 cells have no weight loss and no discernable illness, despite persistent viral replication for several weeks (34). Therefore, T-cell-mediated illness is the price paid for viral clearance.

AIRWAYS DYSFUNCTION AND IMMUNE DEVIATION IN THE MOUSE MODEL

The mouse model of RSV was critically important in understanding the mechanisms of enhanced illness to a formalin-inactivated, alum-precipitated RSV vaccine (FI-RSV) that was tested in clinical trials in the 1960s. This vaccine caused respiratory disease in a high percentage of vaccinees who were subsequently infected with wild-type RSV (35). This enhanced illness was characterized by typical features of severe wild-type RSV infection, including bronchiolitis, hypoxemia, and radiographic evidence of pneumonia; in addition, eosinophil infiltration, not usually associated with RSV-induced disease, was also present (35). Many of the features of vaccine-enhanced illness in children were found in mice that were similarly vaccinated and subsequently infected with RSV (36). In these studies, mice immunized with the FI-RSV had a dominant Th2-like lymphocyte response in the lungs with high levels of IL-4 expression, whereas RSV infection alone led to a dominant Th1 phenotype with IFN- γ production (36). Neutralization of IL-4 at the time of FI-RSV immunization resulted in reduced clinical illness after live virus challenge with decreased weight loss, illness score, and virus replication, and augmented CD8⁺ cytotoxic T-lymphocyte activity (37).

Other immunization studies have provided important clues about the determinants of RSV-induced lung disease. For instance, immunization with recombinant vaccinia viruses that express the RSV G glycoprotein promote differentiation of Th2 CD4⁺ T lymphocytes and induce an eosinophilic response in lungs of RSV-infected mice (38). Further studies revealed that the form of G protein available for initial antigen processing and presentation is an important factor in promoting Th2-like immune responses, including the induction of lung eosinophilia. Immunization with a vaccinia construct expressing the secreted G protein (vvGs) causes greater airway eosinophilia than constructs expressing either wild-type G or membrane-bound G (38). Further studies revealed that, in contrast to FI-RSV, which causes airway eosinophilia that is dependent on IL-4 at the time of immunization, the secreted form of RSV G can directly induce IL-5 and IL-13, producing pulmonary eosinophilia and enhanced illness in RSV-challenged mice by an IL-4-independent mechanism (39). However, depleting both IL-4 and IL-13 by treatment of IL-4-deficient mice with the IL-13 antagonist (IL-13Ra), reduced IL-5, IL-13, eotaxin, and pulmonary eosinophilia after RSV challenge (40). The G-induced response is mediated largely through a single immunodominant epitope recognized by a V β (14+) subset of CD4⁺ T cells that produce both IFN- γ , IL-5, and IL-13 after *in vitro* stimulation (41). Immunization with the secreted form of the RSV G glycoprotein also results in mucus induction with significant increases in Muc-5ac gene expression (42). The regulation of mucus in this system is complex. For instance, the mucus produced by RSV G immunization followed by RSV infection was IL-13-dependent because treatment with the IL-13R abrogated RSV-induced increases in mucin gene expression (42). The mucus production in this system also appeared to depend on IL-10 production because comparable levels of induction of mucin gene expression were not seen after virus challenge of vvGs-immunized IL-10-deficient animals, and the administration of neutralizing antibodies against IL-10 during the viral challenge phase of this protocol abrogated Muc-5ac mRNA induction (42).

Examination of the effect of preexisting allergic lung disease on RSV-induced airway dysfunction has also provided important information on viral pathogenesis and the role of mucus induction in airway obstruction. Whether airway responsiveness is measured in endotracheally intubated and mechanically ventilated animals (43) or by whole-body plethysmography (44), mice

infected with RSV after ovalbumin-induced allergic pulmonary inflammation (OVA/RSV) have increased airway reactivity compared with those infected without allergic sensitization (RSV) or with noninfected (OVA) control animals. This airway responsiveness (AHR) occurs despite the fact that RSV infection diminishes allergen-induced cytokines in the OVA/RSV mice, such as IL-5 and IL-13 (45). However, RSV infection in the setting of allergic lung inflammation has an important impact on airway mucus production. For instance, OVA/RSV mice had more abundant airway epithelial mucus production compared with OVA mice 14 days after infection, whereas there was virtually no mucus in mice that were RSV infected but not allergically sensitized (46). In OVA/RSV mice, there was significantly increased staining for gob-5 and Muc5ac in the airways compared with either OVA mice or allergically sensitized mice that were challenged with inactivated RSV, and virtually no detectable staining in the RSV group (46). These findings were confirmed by Western blot analysis. The increased mucus expression in the OVA/RSV group was associated with increased lung levels of IL-17, a factor known to stimulate airway mucin gene expression. The impact of virus infection combined with allergic inflammation on mucus production may partially explain the more severe disease and AHR associated with RSV in the setting of atopy. IL-17 is also induced in mice deficient in signal transducers and activators of transcription 1 (STAT1) and is associated with AHR and mucus production in the airways of these animals after RSV infection (47). Thus, IL-17 as well as IL-10 and IL-13 may be important independent factors in virus-induced airway mucus production. However, AHR in the mouse model of RSV infection is not solely dependent on airway mucus production. For instance, administration of a selective Rho kinase inhibitor that inhibits smooth muscle contraction, Y-27632, prevented AHR in OVA/RSV mice in which either methacholine or serotonin were used as airway constrictors while having no significant effect on airway inflammation (48).

It is clear that the immune interaction between RSV infection and allergen sensitization critically depends on the timing of virus challenge relative to the allergic inflammation. For instance, RSV infection before allergic sensitization decreased allergen-induced airway responsiveness, production of IL-13 in lung tissue, and lung eosinophilia (49). In contrast, allergic sensitization before RSV infection increased AHR and decreased RSV-related weight loss and lung levels of IFN- γ , but did not alter viral clearance (49). These data provide evidence that RSV-associated AHR occurs in hosts with allergic responses and that allergic inflammation is diminished when preceded by RSV infection.

CONCLUSIONS

RSV pathogenesis is a complex process dependent on the interaction of viral and host determinants. Although RSV has the capacity for direct cytopathology of the respiratory epithelium, in the immunocompetent host, the immune response is a more important factor in disease pathogenesis. RSV-induced cytokines and chemokines have direct effector functions that impact disease, and are important for the recruitment and differentiation of the T-lymphocyte response. In typical primary RSV infection, the host response is dominated by IFN- γ produced by NK, CD4⁺, and CD8⁺ T cells. However, immunization with either FI-RSV or the RSV G glycoprotein followed by RSV infection results in a response dominated by type 2 cytokines and is associated with lung eosinophilia and airway mucus production. RSV infection in the setting of allergic inflammation or the absence of STAT1-mediated signaling induces airway epithelial mucus that is associated with the expression of IL-17, a cytokine recently described to regulate mucus production. Further exploration of

the factors that regulate RSV pathogenesis, including investigation beyond the classical Th1/Th2 paradigm, will be critical for developing effective therapies and safe vaccines against this important pathogen.

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