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Immune Responses and Disease Enhancement during Respiratory Syncytial Virus Infection

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INTRODUCTION

It is increasingly appreciated that symptoms and signs of many viral diseases are caused less by viral cytopathic effects than by the host's response to infection. The peak of viral infection often precedes the period of maximal illness, which coincides with cellular infiltration of infected tissues and the release of inflammatory mediators. In this review, we discuss the role of overexuberant immune responses in disease caused by respiratory syncytial virus (RSV).

RSV is the most important cause of viral respiratory tract infection in infants. Previous reviews have described the clinical impact of RSV disease (63), its pathogenesis (102), and the molecular biology of paramyxoviruses (30) and have compared RSV to other paramyxoviruses (40). The aim of this review is to provide an up-to-date summary of the host-RSV interaction and how this can cause disease. We will discuss the burden of disease caused by RSV infection, factors which affect disease severity, and what is known of the mechanisms of viral bronchiolitis.

It is useful to consider inflammation in RSV disease in three distinct scenarios: (i) the response to first infections in previously nonexposed hosts, (ii) the pathogenesis of enhanced disease in RSV-infected recipients of formalin-inactivated RSV (FI-RSV) vaccines, and (iii) specific animal models of disease augmentation. By comparing and contrasting the immunopathogeneses of primary bronchiolitis and enhanced disease, we attempt to identify common mechanisms that are shared or distinct in these conditions.

THE BURDEN OF RSV DISEASE

Epidemiology and Clinical Presentation

RSV is a negative-strand, nonsegmented RNA pneumovirus of the family *Paramyxoviridae*. It is the single most important cause of acute respiratory tract viral infections in infants (62). In 2002, the World Health Organization estimated that 18.3

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million people died of infectious diseases; of these, 3.96 million died of respiratory infections, over 95% of which were lower respiratory tract infections (LRTI) (188). Viral LRTI are particularly serious during infancy, during which time the lungs are adapting to extrauterine life. Viral bronchiolitis is the most common single cause of infantile hospitalization in the developed world, and about 70% of bronchiolitis hospitalizations are associated with RSV infection (70). RSV has been estimated to cause 91,000 hospital admissions per year in the United States, with associated costs of $300,000,000 per year. In Europe, RSV accounts for 42 to 45% of hospital admissions with LRTI in children younger than 2 years of age, with inpatient populations tending to be younger and experiencing greater disease severity (153). The potential burden of RSV reinfection of adults on the health care system has been underappreciated; RSV also causes significant disease in healthy adults (especially those with contact with children) and generally passes undiagnosed (63). RSV causes high morbidity and mortality in patients with underlying cardiopulmonary illnesses (178), the elderly (44), and the immunosuppressed, particularly bone marrow transplant patients (68, 135).

Primary infantile RSV infection typically presents as a winter upper respiratory tract infection, which is followed by mild lower respiratory tract symptoms in about 40% of cases. Otitis media is common. The mechanism of progression may involve aspiration of virus-containing upper respiratory tract secretions (by, for example, inhalation of postnasal drip) or short-range intercellular spread via the extracellular fluid or sol phase of the surface mucus (136). Lower respiratory tract signs include tachnypea, hyperinflation, recession, crackles, and expiratory wheezing (leading to a clinical diagnosis of bronchiolitis). RSV is extremely common in children; for example, in the Houston Family Study, the infection rate was 68.8/100 in children less than 12 months of age and 82.6/100 during the second year of life; virtually all children had been infected at least once by 24 months of age, and about half had experienced two infections (53).

RSV infections usually pass in less than a week and tend to be more severe in children aged 8 to 30 weeks. About 1 to 2% of all infants require hospitalization for bronchiolitis; among these, mechanical ventilation is needed in 2 to 5% (92). In affluent countries, mortality from RSV infection has been estimated as 0.005% to 0.02%. However, very few previously healthy children suffer life-threatening infections, and deaths are practically confined to those who are immunocompromised or who have preexisting cardiorespiratory disease (70, 138). Risk factors for severe RSV disease include premature birth, male sex, concurrent heart or lung disease, the presence of multiple siblings in the household (especially those sharing a bedroom), day care attendance, having parents who smoke, lower family income, and lack of breast feeding (152).

In recent years there have been some excellent studies of RSV in developing countries such as Indonesia (39), South Africa (97), and the Gambia (184). In these regions RSV infections are usually seasonal and are not necessarily most frequent at the coldest time of year (164). Risk factors differ from those in developed countries and include, for example, the presence or absence of a flushing toilet and exposure to cooking fires; however, high sibling number remains an important risk factor in all settings (183).

Acute and Delayed Pathology of RSV Infection

RSV bronchiolitis is pathologically similar to bronchiolitis caused by other respiratory viruses such as influenza virus, parainfluenza virus type 3, and adenovirus. RSV has a direct cytopathic effect on cells in the lung epithelium, leading to loss of specialized functions such as cillial motility and sometimes to epithelial destruction (4). In addition, a peribronchiolar mononuclear cell infiltrate forms and is accompanied by submucosal edema and mucus secretion. This inflammation leads to bronchiolar obstruction with patchy atelectasis and areas of compensatory emphysema (47). Syncytium formation is not often seen in vivo and varies considerably from one RSV strain to another in vitro (146). In explanted human epithelial cultures, RSV infects the apical surface of ciliated columnar cells, is shed exclusively from the luminal surface, and spreads to neighboring cells by cillial motion (191).

It is important to recognize that only a small minority of RSV-infected children develop severe disease and that the disease in ventilated children (i.e., those from whom it is possible to obtain samples from the lung) may be very different from that in hospitalized nonventilated children; we know virtually nothing about the pathogenesis of disease in the great majority of children, who develop mild respiratory symptoms and are neither seen by doctors nor sent to hospital. Since only a few RSV-infected children get very ill, the causative factor or factors do not need to be universal or even common.

Severe RSV infection in the first 6 months of life is often followed by recurrent childhood wheezing, an association which is lost by 11 to 13 years (100, 150, 163). In a study by Sigurs (148), it was shown that children with severe RSV bronchiolitis in infancy had a significantly higher rate of asthma than age- and sex-matched controls (11% versus 0% at age 1, 23% versus 1% at age 3, and 23% versus 2% at age 7.5). In this same cohort studied at 13 years of age, asthma had been diagnosed in 37% of the RSV bronchiolitics and 5.4% of the control group. Allergic rhinoconjuntivitis was present in 39% and 15%, respectively, and skin prick tests were also more often positive in ex-bronchiolitics (50% versus 28%; $P = 0.022$) (149). There are also variable reports of an association with atopic disease (124), with some studies reporting a positive association (112, 116), which was not found by others (154).

Accepting that RSV bronchiolitis and recurrent childhood wheeze are associated, the fundamental question remains: is the association causal, or does bronchiolitis act as a marker for an increased risk of allergy and wheezing illness due to genetic predisposition or impaired respiratory reserve (11, 118)? Direct interventional studies demonstrating a causal relationship have not been published. However, administration of anti-RSV immune globulin to children at high risk of RSV disease seems to improve asthma scores and reduce atopy (185). It is possible that an anti-RSV neutralizing monoclonal antibody (e.g., palivizumab) also has long-term beneficial effects, but studies are still to be published.

The mechanisms that could account for delayed effects of RSV infection are not clear but could include immune "imprinting" (see "Effects of Age" below) and viral persistence. A sustained increase in interleukin-2 (IL-2) receptor levels is seen after RSV infection, suggesting that inflammation may continue after the acute symptoms and signs have resolved

(155), but direct histological confirmation of persistent inflammation has not been possible. RSV has been shown to be persistent in vitro in a macrophage-like cell line (57, 58, 173) and in cattle (172), mice (143), and guinea pigs (23). If persistence occurs in humans, it could explain the apparent delayed effects and serve as a reservoir for future RSV outbreaks in infants.

Although severe RSV disease in infancy may cause recurrent wheezing, this is not the case with most viral infections. Uncomplicated common colds (without wheeze), type I herpetic stomatitis, chicken pox, and exanthema subitum seem to protect against wheeze in children up to 7 years of age. The risk of asthma diagnosis by this age is reduced by about 50% in children with two or more reported common colds by the age of 1 year (79). Viral infections typically induce T-helper type 1 responses, characterized by high levels of gamma interferon (IFN-γ) production; by contrast, asthma and atopy are typically characterized by T-helper type 2 cells producing IL-4 and IL-5 (Th2 cells). In contrast, analysis of nasal lavage and peripheral blood samples from RSV-infected children shows elevated IL-4/IFN-γ ratios in infants during the first week of acute bronchiolitis compared with infants with upper respiratory tract signs alone. These data are consistent with excessive type 2 and/or deficient type 1 immune responses in RSV bronchiolitis (93).

Viral infection could act to permit inhaled antigen to penetrate the mucosal barrier of the respiratory tract and meet relevant antigen-presenting cells and specific T cells, thereby leading to systemic sensitization. Prior allergic sensitization potentiates the physiologic and structural changes induced by acute RSV bronchiolitis, suggesting that an allergic diathesis may increase the severity of RSV infections in children (137). Animal models favor a role for RSV bronchiolitis in triggering asthma and in promoting a Th2 bias in immune responses to other antigens (117). Using palivizumab to prevent infection reduces airway obstruction and airway hyperreactivity to methacholine challenge in mice (104).

Whatever the role of RSV in the inception of asthma, it (and other viruses) can certainly lead to asthma exacerbations in older children and adults (63). Rhinoviruses are commonly found during acute exacerbations of chronic obstructive pulmonary disease, but there is intriguing preliminary data suggesting that RSV may also be present in some patients during remission (144).

HOST FACTORS AFFECTING PATHOGENESIS

Host Genetics

Even in a single outbreak of RSV disease, the severity is highly variable. Viral strain variations seem to play only a minor role in causing this variation, implying that host factors are important in determining disease severity, even when taken in the context of age-specific effects, current or recent infection, atmospheric pollution, and concurrent allergen exposure. For example, bronchiolitis risk is linked to polymorphisms in the wide-spectrum chemokine receptor CCR5 (74) and the IL-8 locus (54, 75). Further studies on IL-8 gene haplotype variants suggest that variation in susceptibility to RSV-induced bronchiolitis occurs via an increase in IL-8 transcription, possibly mediated by functional polymorphisms (59). Genetic associations have also been found with the IL-4 gene (28, 73); with promoter variants of IL-9, IL-10, and tumor necrosis factor (TNF) alpha (72); and with the protein surfactant D (91). An association has also been found between soluble CD14 and wheezing following RSV bronchiolitis (156). Toll-like receptor 4 (TLR4) and CD14 are part of a receptor complex involved in the innate immune response to RSV, and TLR4 mutations have been associated with severe disease (165). In animal models, the host genotype has a large effect on the severity of disease (77, 77, 162).

Disease in Immunosuppressed Individuals

RSV infections tend to be prolonged in patients with defects in immunity (102), and bone marrow transplant recipients are at particularly high risk of severe RSV disease. In one study 8.2% of adult hematological inpatients were diagnosed with RSV infection, of whom 50% developed LRTI. Two of the 16 patients (12.5%) died of respiratory failure due to RSV pneumonia, despite intensive care unit admission and supportive ventilation (1). Immunosuppression caused by human immunodeficiency virus also affects RSV pathogenesis, and patients with AIDS have an increased duration of viral shedding (26).

It is an apparent paradox that RSV causes severe problems in immunodeficient individuals, given that the disease is in large part due to excessive immune responses. Clearly, if viral replication is unchecked, RSV causes progressive cytopathic damage to the lung, leading to viral pneumonia and respiratory failure, as seen in immunodeficient (athymic nu/nu or irradiated) mice (25). On the other hand, partial immune reconstitution (e.g., during engraftment of bone marrow transplantation) is associated with an exuberant immunopathogenic response, representing an unbalanced reaction that is poorly antiviral.

Effects of Age

RSV has its greatest effects at the extremes of age. First infections in neonates may be severe, particularly in premature infants (139); reinfections are generally milder in older children and in adults but again can have serious consequence in the elderly (44, 113, 168). In neonates, the onset of air breathing is associated with a relatively high dead space, inelastic lungs, and flexible ribs arrayed horizontally (123); in older persons, the lungs normally decline in elasticity and trap air, which limits expiration (35). The immune system has distinctive features which may account for increased disease susceptibility in the young and the old (10, 105). The effects are most evident in high-risk premature infants, particularly those born before 28 weeks of gestation, before the transfer of maternal antibody occurs (36). Immune immaturity in neonates and immune senescence in the elderly may be associated with imbalanced RSV-specific immune responses that favor disease enhancement.

In the mouse model, subjecting animals to primary infection at up to 1 week of age leads to increased disease severity during adult reinfection with RSV. Mice that were infected with RSV neonatally are sicker and have greater cell recruitment to the lung, increased IL-4 production, and a mixture of lung eosin-

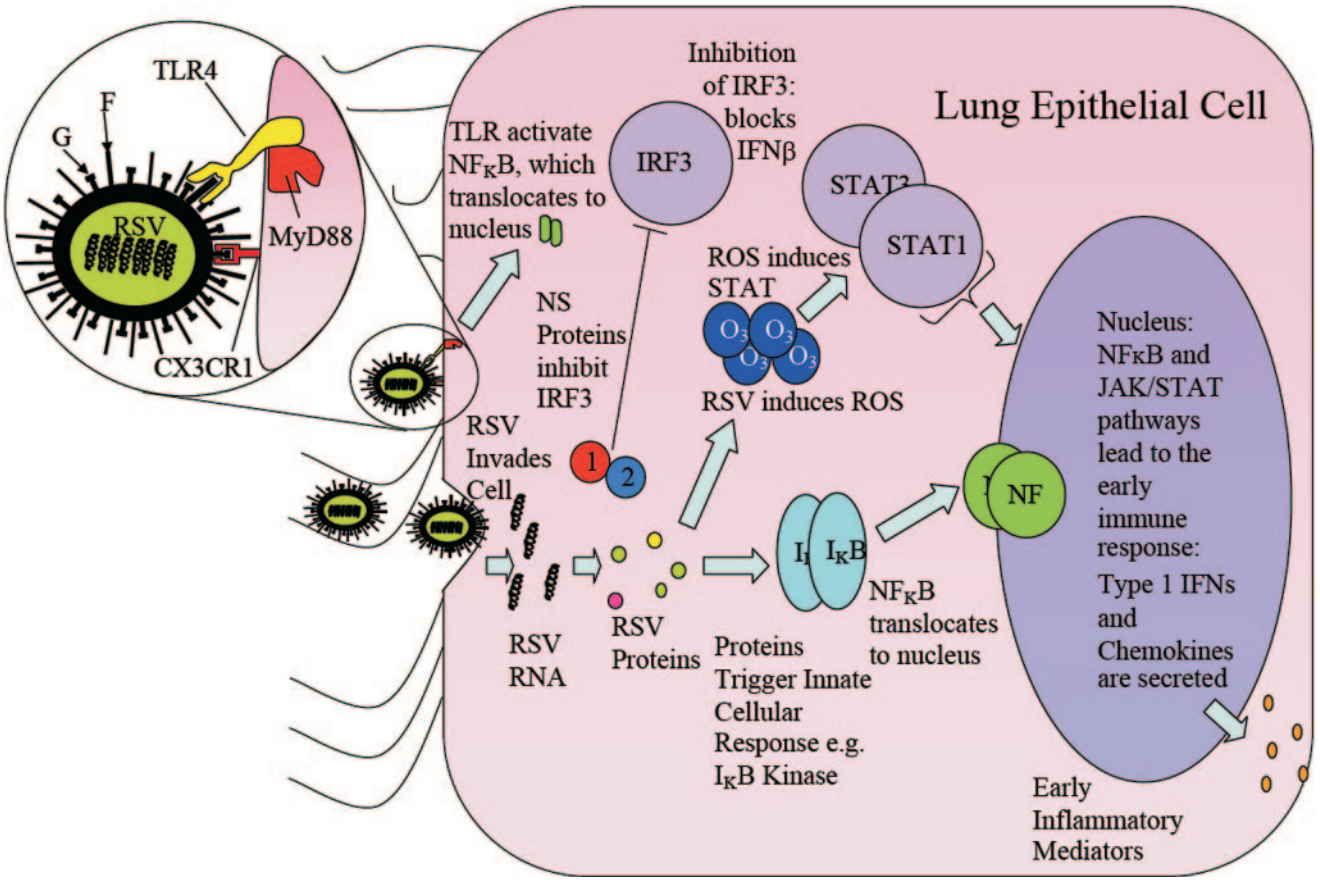

FIG. 1. RSV binding and triggering of cellular responses. RSV is bound by surface glycosaminoglycans, and the F protein binds to TLR4, while G glycoprotein (virus associated or secreted) binds fractalkine receptor CX3CR1. The interaction with TLR4 leads to upregulation of NF-κB via MyD88. RSV upregulates NF-κB via IκB and STAT1 and -3 via reactive oxygen species (ROS), and RSV RNA activates protein kinase R. Viral NS proteins inhibit the interferon response factor (IRF3) pathway.

ophilia and neutrophilia during adult RSV challenge (34). Therefore, in the mouse model at least, the timing of neonatal infection establishes and determines the subsequent "imprinted" pattern of T-cell responses and, consequently, the nature and severity of disease during of reinfection in adulthood. The practical implication of these studies is that delaying RSV infection beyond early infancy could have long-term benefits.

VIRAL INTERACTIONS AFFECTING DISEASE PATHOGENESIS: THE INNATE IMMUNE RESPONSE AND VIRAL EFFECTS ON THE HOST

RSV surface proteins bind glycosaminoglycans (e.g., heparin or chondroitin sulfate), removal of which reduces the infectibility of HEp2 cells in vitro (65). RSV can also interact with annexin II and L-selectin (99). The RSV glycoprotein G has been shown to have structural homologies with the CX3C chemokine fractalkine. G binds the human CX3CR1 receptor and mediates chemotaxis of cells that respond to CX3CL (169). It is possible that this interaction facilitates binding to CX3CR1-bearing cells, including mast cells and neuronal cells. The fusion (F) protein binds TLR4 (89), upregulating its surface expression and sensitizing airway epithelial cells to endotoxin (109). The frequency of $TLR4^+$ monocytes is increased in the peripheral blood of some infants with RSV bronchiolitis (46), but the role of TLR4 in vivo is unclear (41).

Events during the first minutes and hours after viral entry are of key importance, not only in determining the balance between viral multiplication and elimination but also in setting the pattern that will be followed by acquired immune responses. Early viral proteins therefore frequently interfere with innate immune mechanisms (Fig. 1).

Once within cells, RSV upregulates the STAT pathway via reactive oxygen species (95). Nitric oxide production is associated with the upregulation of IL-8 (polymorphisms in the promoter of which have been shown to be associated with bronchiolitis severity [54]), leading to pulmonary neutrophilia (159). RSV infection also upregulates proapoptotic factors in the cell (88) and activates the nuclear factor κB (NF-κB) pathway (19), which stimulates the transcription of genes directly involved in the antiviral response via IκB kinase (60). NF-κB is an upstream mediator of many of the innate responses, especially alpha/beta interferon and chemokine production, which leads to the recruitment and activation of cells and the production of further inflammatory mediators. RSV's nonstructural proteins, NS1 and NS2, cause species-specific resistance to alpha/beta interferons (141, 157) via interferon

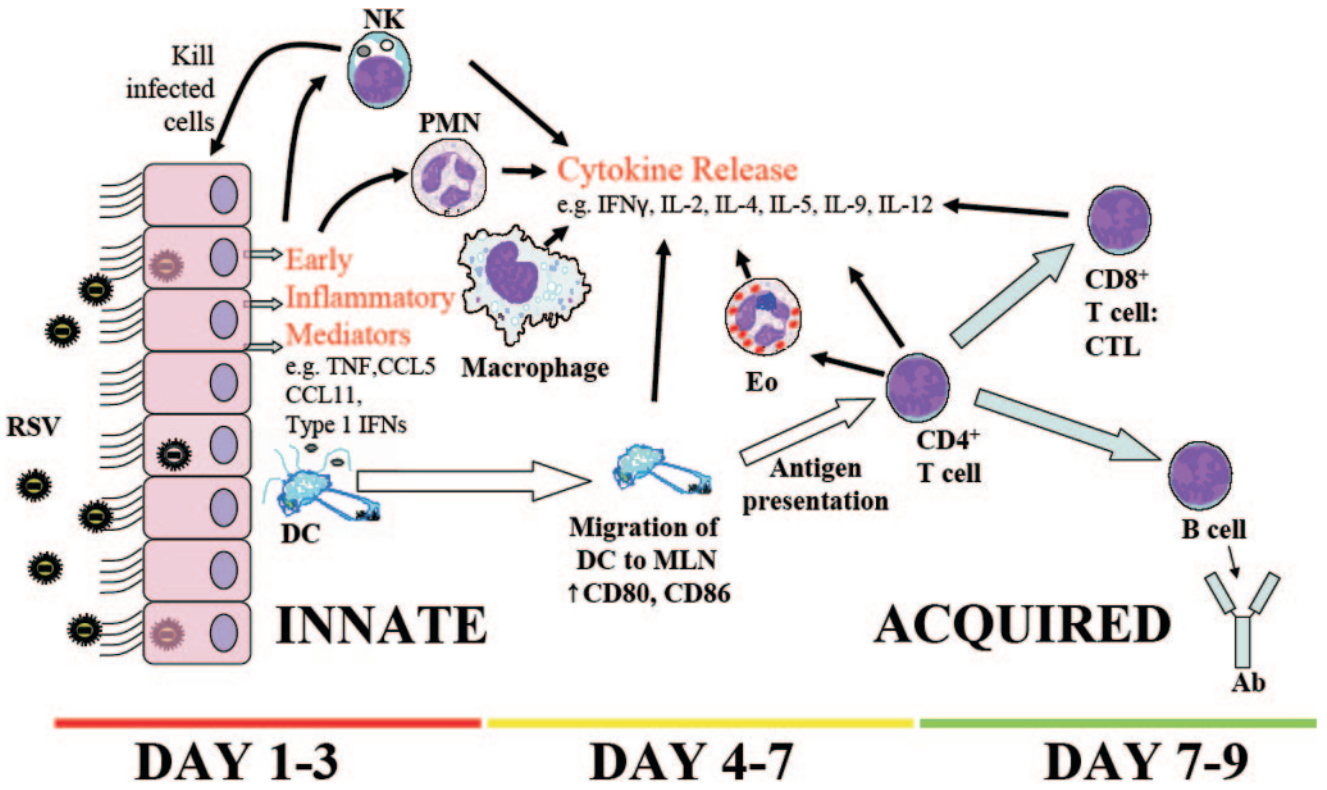

FIG. 2. Cells involved in the immune response to RSV. Cellular infection triggers the release of early inflammatory mediators, e.g., TNF and IFN-α/β. NK cells and PMN are recruited in the first 3 days of infection, at which time DC carry viral antigen to local lymph nodes and present it to $CD4^+$ T cells. Once primed, these cells migrate back to the infected epithelium, release further mediators, and recruit additional inflammatory cells, including mononuclear cells (including $CD8^+$ T cells and B cells) and granulocytes (e.g., neutrophils [PMN] and eosinophils [Eo]). Ab, antibody.

regulatory factor 3 (IRF3) (21, 22). Similar effects have been demonstrated with other paramyxoviruses, simian virus 5, and Sendai virus (38).

Chemokines are crucial in directing the recruitment of different cell subsets and make attractive targets for intervention. Double-stranded RNA selectively induces the secretion of chemokines such as CCL5 and IL-8, a factor that promotes neutrophils (49). Chemokines are produced in abundance during RSV infection in humans (90, 115, 145), and RSV infection of BALB/c mice induces expression of CXC, CC, and C chemokines in the lung (61, 106). Cytokine depletion, receptor blockade, or genetic deletion of chemokines or their receptors generally reduces disease severity and pathology during RSV infection. For example, antibody depletion of CCL5 (167) or CCL11 (101) reduces eosinophilia and disease severity in immune-augmented RSV disease, and MIP1α knockout mice have less severe disease during primary RSV infection (61).

CCL5 (RANTES) seems of particular interest. It is produced in response to stimuli such as IFN-γ, IL-1α, IL-1β, and TNF by many cells, including fibroblasts, smooth muscle cells, and epithelial cells; in later stages of infection it is made by infiltrating cells, including γδ T cells. It selectively recruits monocytes and memory T cells and eosinophils and (at high concentrations) activates T cells. Treatment of HEp-2 cells with recombinant human CCL5 inhibits infection with RSV in vitro, an effect not seen with other chemokines. This action may result from blocking RSV fusion with host cells (42). CCL5 increases after RSV infection of mice and correlates with the severity of disease. Anti-CCL5 antibody administration decreases airway hyperreactivity and increases IL-12 production. Moreover, CCL5 production appears to be regulated by IL-13 which is also important in RSV-induced airway hyperreactivity (167). CCL5 may also be important in humans, since genetic studies show that polymorphisms of CCR5 affect disease severity (74). Moreover, CCL5 levels in nasal secretions during acute RSV bronchiolitis, although not correlated with disease severity, may be predictive of the later development of recurrent wheeze (29).

Cytokine production has also been extensively studied in RSV bronchiolitis. For example, IL-9 is a cytokine associated with Th2 responses and with asthma (114). IL-9 mRNA and protein production is elevated in the lungs of infants with RSV bronchiolitis. Intriguingly, polymorphonuclear cells (PMN) seem to be a major source of IL-9 in this situation (103).

RSV infection thus triggers innate immune responses that influence the developing acquired immune response (71). NK cells are an abundant source of IFN-γ, which has potent effects on developing αβ T cells and thus on the immunopathology of RSV infection (81). IL-12 from antigen-presenting cells has potent effects on NK cells, enhancing IFN-γ production (78). This cascade of innate and acquired events during RSV infection is outlined in Fig. 2.

AUGMENTATION OF DISEASE BY VACCINATION

Disease Enhancement by Vaccination with FI-RSV

The best-studied model of RSV disease enhancement by the immune system is the formalin-inactivated vaccine FI-RSV. Following the success of other chemically inactivated viral vaccines (e.g., polio vaccine), studies using FI-RSV were conducted in 1966 and 1967. Vaccines were administered to infants and children aged 2 months to 9 years in two or three intramuscular doses separated by 1 to 3 months (27). After subsequent RSV exposure, the rate of virus infection in children receiving FI-RSV was no less (and was perhaps even greater) than that in a control group immunized with a control parainfluenza vaccine. Most remarkably, 80% of RSV vaccinees needed hospitalization, whereas only 5% of RSV-infected children given the control parainfluenza vaccine required admission. Illnesses among RSV-infected FI-RSV-vaccinated children included pneumonia, bronchiolitis, rhinitis, or bronchitis; two of the vaccinated children died (87). Importantly, these trials were conducted in the absence of prior animal testing.

Postmortem examinations showed bronchopneumonia with emphysema and pneumothorax. Microscopically, there was an intense inflammatory infiltrate, including mono- and polymorphonuclear cells and eosinophilia. These changes suggested an immunopathological cause of enhanced disease. Analysis of sera from children immunized with FI-RSV shows that antibodies to the F and G proteins were generated but were poorly neutralizing (110). The severity of illness was remarkably dependent on the age of the vaccinees, with the younger children suffering more severe symptoms. The reasons for this are not clear, but animal models suggest that there is an age-dependent factor as a major determinant of the pathogenic immune response (34, 125).

Immune Enhancement in Dengue and Measles Virus Infection

Like FI-RSV, formalin-inactivated measles virus (FI-MV) can cause severe and generalized disease during subsequent natural infection (45). This 'atypical' measles is seen after a delay of about 7 years, whereas enhanced RSV disease occurs within 2 years of FI-RSV administration (128). Therefore, a window of protection may be followed by a phase of disease enhancement (111), followed by a final period during which immune memory is still measurable but neither protection nor enhancement is seen.

The pathogenesis of dengue hemorrhagic fever is imperfectly understood, but epidemiological data suggest that it occurs when a dengue virus-immune person becomes infected with a second viral serotype (140). In that prior infection leads to a more severe disease on reinfection, this resembles RSV disease following FI-RSV immunization. Antibody-dependent enhancement has been suggested, whereby preexisting nonneutralizing antibodies may opsonize dengue virus and enhance its uptake and replication in macrophages. Higher viral loads have been demonstrated in preimmune primates (66). However, T-cell activation may also contribute to disease in that virus-specific CD8 T cells disappear from the peripheral blood during acute infection. It has been suggested that "original antigenic sin" in the T-cell responses may suppress or delay viral elimination, leading to higher viral loads and increased immunopathology (108).

TABLE 1. Advantages and disadvantages of animal models of RSV disease

Characteristic of model
Ideal animal model:
Determines which antigens to use in vaccines
Allows optimization of route, dose, frequency, etc.
Predicts effects of key genetic and environmental variables
Anticipates vaccine failures and adverse effects
Animal models can:
Allow study of complex biological systems
Allow controlled interventional experiments
Test genetic influences
Illustrate principles
Generate hypotheses
Animal models cannot:
Give quantitative information about human responses
Determine exactly which protective or pathogenic mechanisms operate in humans
Accurately determine the effects of genetic variations
Usually anticipate adverse effects in humans

FI-RSV IN ANIMAL MODELS

Given the tragic results of the FI-RSV trials in human infants, it is essential to determine the nature of pathogenesis in animal models. Animal models have been used extensively to elucidate possible mechanisms linking RSV disease with subsequent wheezing (Table 1). These include the cotton rat, calf, monkey, mouse, and guinea pig models. Each has advantages and drawbacks.

Mice

Factors favoring the mouse model include the easy availability of immunological reagents (exceeding that for any other species); the many inbred and congenic strains, gene knockouts, and transgenics; and a complete genome sequence. The mouse and human genomes are very similar; each has about 30,000 genes, of which only 1% are species specific. Equivalent mouse genes have been found for all genes known to cause human disease, and 99% of mouse genes have a human homologue. Although the human and mouse immune systems have diverged during the 75 million years since separation, the same immunological niche is sometimes occupied by nonhomologous proteins (e.g., KIR and Ly49) because of convergent functional development (182).

The role of T cells in augmented lung pathology has been highlighted in the mouse model of FI-RSV. Connors et al. showed that $CD4^+$ T cells are crucial to the immunopathogenesis of FI-RSV disease and that RSV-specific antibodies (in the absence of $CD4^+$ and $CD8^+$ T cells) are not sufficient to cause disease enhancement (31). Further studies revealed a marked increase in the expression of Th2-type cytokines (IL-5, IL-13, and IL-10) and reduced expression of IL-12 in FI-RSV-immunized mice, indicating a Th2 bias in the increased inflammation

(181). The same authors found a positive correlation between the signal for IL-5 mRNA and the eosinophil infiltration. Moreover, skewing to a T-helper 1 (Th1) pattern of cytokine production by priming with live RSV prevented subsequent enhanced disease (180).

Cotton Rats

FI-RSV-vaccinated cotton rats show disease augmentation during intranasal challenge with RSV. The lungs of immunized animals are infiltrated with a mixture of cells, including neutrophils, macrophages, and lymphocytes. Serum contained only low levels of neutralizing antibodies (134). It has been suggested that epitopes against which neutralizing antibody is directed could have been modified by formalin treatment, leaving nonprotective epitopes undamaged and therefore inducing high titers of antibodies binding in enzyme-linked immunosorbent assay that form immune complexes. However, FI-RSV does reduce RSV titers in the lung by 90% while increasing peribronchiolitis and alveolitis (132), demonstrating a dissociation between effects on viral load and immunopathology. While the cotton rat model demonstrates clear disease enhancement and immunopathology, it has hitherto been difficult to dissect the precise mechanisms because of the paucity of immune reagents. A good range of reagents are now being developed, enhancing the utility of the cotton rat model (20).

Calves

Bovine RSV (bRSV) is a natural infection of cattle that is of considerable economic importance. Primary bRSV infection can cause severe lower respiratory tract disease in calves, although asymptomatic infections also occur. The effects of bRSV are similar in some ways to those of human RSV in humans, but bRSV tends to cause an acute interstitial pneumonia with alveolitis, emphysema, and bronchiolitis, especially in calves and yearlings (174).

Immunization with FI-bRSV generally results in strong immunoglobulin G antibody responses against F and G, without an adequate neutralizing antibody response (50). In one study, 6-month-old calves were vaccinated with FI-bRSV, live bRSV, or control material. One month after the second vaccination, vaccinees were infected with a field isolate of bRSV. The FI-bRSV recipients developed pyrexia and dyspnea more rapidly than controls but showed inconsistent changes in pulmonary pathology (186). Increased disease after vaccination was also shown during a bRSV outbreak in 60 calves less than 8 months old that were housed in barns. During this outbreak, FI-bRSV-vaccinated calves showed more severe disease than unvaccinated animals, with 30% of the immunized calves dying of respiratory distress. In the calves that died, an eosinophilic infiltrate was present in the lungs (142). Another study examined the role of antibody subtypes and suggested that FI-bRSV enhances Th2 immune responses (86). Antonis et al. also showed that immunization with FI-bRSV mainly primes a Th2-like inflammatory response, which is associated with an eosinophilic influx into the bronchial alveolar lung fluid and lung tissues and high levels of immunoglobulin E serum antibodies (8).

FI-bRSV therefore causes enhanced disease in bRSV-infected cattle in a pattern highly reminiscent of the disease seen in infants, mice, cotton rats, and monkeys.

Primates

Studies with nonhuman primates generally show patterns of FI-RSV-induced disease augmentation similar to those seen in other species. In rhesus macaques, FI-RSV vaccination leads to RSV-specific T cells, predominantly producing the Th2 cytokines IL-13 and IL-5. Intratracheal challenge with RSV 3 months after the third vaccination elicited a hypersensitivity response associated with lung eosinophilia, and two out of seven FI-RSV-vaccinated animals died 12 days after RSV challenge with pulmonary hyperinflation (37). This result is compatible with those of other studies showing decreased lung viral titer following RSV challenge in association with enhanced pathology (85).

Bonnet monkeys also develop enhanced disease after FI-RSV vaccination, but with increased viral replication in perivascular sites of the lung (130). The site of RSV replication may be mononuclear cells which have taken up RSV because of infection-enhancing antibodies. Enhancement of infection was not observed in animals with primary and tertiary infections or in those immunized with FI-Vero cell culture. Serum antibody from animals immunized with FI-RSV increased RSV infection of U937 cells, and the enhancement index correlated positively with the pathological scores of the FI-RSV-vaccinated monkeys (131). This finding suggests that antibody may play a role in FI-RSV enhancement of disease.

It has been suggested that waning levels of maternal antibody might cause spontaneous disease augmentation in children undergoing natural primary RSV infection between 2 and 6 months of age (43). However, maternal antibody is clearly protective in mice (166) and cotton rats (133). In many hundreds of studies of passive antibody transfer in animals, disease augmentation has not been observed. In a prospective study of 68 infants with RSV infection and 575 controls, neutralizing antibody titers in cord blood correlated with protection against disease, not with infection. Moreover, the level of antibody at birth directly correlated with the age at the time of infection and severity of disease (52). More conclusively, passive immunization with palivizumab induces solid protection in most infants and does not cause enhanced disease. It is possible that the anti-RSV antibody induced by FI-RSV blocks epitopes on the RSV surface proteins that bind receptors (for example, TLR4) on the target cells which normally elicit a downstream immune response. If this binding is blocked, the initial protection afforded by interferons or other early innate immune systems may also be blocked, leading to uncontrolled virus replication.

The FI-RSV models are useful because they provide information pertinent to the design of future vaccines, especially as to what strategies may be unsuccessful; they give a guide towards how RSV interacts with the body; and they have led to the development of animal models of RSV disease, which can be used to better study it.

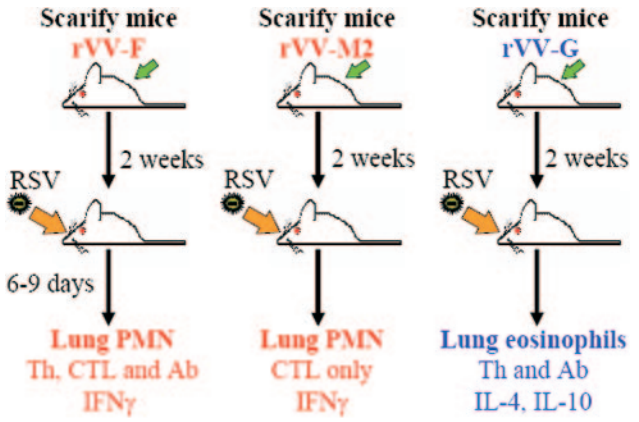

FIG. 3. Immunopathology to RSV surface proteins. In the BALB/c mouse, different RSV proteins expressed by recombinant vaccinia viruses after infection by scarification of the skin cause markedly different effects on subsequent RSV challenge intranasally. The F protein primes CD4 and CD8 T cells and leads to intense inflammation characterized by efflux of PMN. The G attachment glycoprotein primes for CD4 T cells and no CD8 response and is associated with a relatively weak NK cell response; this leads to eosinophilia. The transcription antiterminator (M2) protein primes only CD8 T cells and induces almost no CD4 T-cell response and virtually no antibody. This is often the most illness inducing of the sensitizing protocols, but the effect is not as durable and that induced by G or F. Ab, antibody.

Immune Priming with Individual RSV Antigens

Sensitization of BALB/c mice by dermal scarification with recombinant vaccinia viruses (rVV) expressing individual RSV proteins makes it possible to dissect the contribution of different RSV antigens and T-cell subsets to protection and pathology. The three most studied RSV proteins are the major surface glycoprotein (G), the fusion protein (F), and the transcription antiterminator (formerly called the second matrix protein, M2). These vectors induce contrasting outcomes (Fig. 3). While rVV-G primes Th2 cells and leads to secondary RSV disease characterized by lung eosinophilia (5), rVV-F primes cytotoxic T lymphocytes (CTL) and Th1 responses, resulting in secondary RSV disease with PMN efflux (122, 160), and rVV-M2 primes for a secondary RSV disease characterized by a strong CTL response (120). This situation is reminiscent of that seen in lymphatic filarial infection, where some antigens tend to prime Th1 cells and others prime Th2 cells (190).

Using deletion mutations of regions of the G protein, a site within G which seems to be of key importance to induction of Th2 cells has been identified. Deletion mutants of this region of G no longer prime an eosinophilic response to infection but do prime the immune system to clear the virus more effectively (158). Sensitizing mice with recombinant vaccinia virus expressing the secreted soluble form of the G protein leads to a greater eosinophilic influx into the lungs following RSV challenge than in mice sensitized with vaccinia virus expressing only the membrane-anchored form (15, 82). Engineered recombinant RSV expressing only the membrane-bound form of G is immunogenic but grows poorly in vivo and is unable to generate the eosinophilic response seen with nonrecombinant virus (98). Antibody depletion of cells bearing T1/ST2 (a marker of the Th2 cells [96]) reduces eosinophilia in RSV-infected rVV-G-primed mice (179).

rVV-G does not prime for eosinophilia in all strains of mice. In tests of 15 different inbred mouse strains, eosinophilia developed in all $H\text{-}2^d$ mice but not in $H\text{-}2^k$ mice. Among $H\text{-}2^b$ mice, 129 and BALB/B mice developed eosinophilia, whereas C57BL/6 and C57BL/10 mice did not. Testing of F_1 crosses between sensitive and resistant strains showed that eosinophilia developed in all $H\text{-}2^d \times H\text{-}2^k$ mice but not in $H\text{-}2^d \times H\text{-}2^b$ mice, so that inheritance of the eosinophilic trait could be either recessive or dominant depending on the strain combination (77). Treatment of otherwise eosinophilia-resistant mice with anti-CD8 antibody (or use of mice genetically deficient in functional $CD8^+$ cells) allows the development of eosinophilic inflammation in previously resistant strains (76). These results support the concept that RSV disease arises because of the balance between different subsets of T cells.

If an engineered, secreted form of the F protein is used for vaccination, IL-4 and IL-5 production is increased but pulmonary eosinophilia following RSV challenge is not seen (16). The transcription antiterminator protein (M2) contains an immunodominant K^d-restricted peptide epitope (121) which can be used for mucosal vaccination in conjunction with the adjuvant LTK63 (151). M2 primes strong CTL responses and severe disease enhancement on RSV challenge. Crucially, this type of priming induces virtually no RSV-specific antibody, and transfer of CTL to naive mice results in accelerated viral clearance but greater disease (24).

Immunization with vaccinia virus expressing individual RSV proteins gives valuable information about the possible pathways of RSV disease pathogenesis but does not reproduce the pathology seen after FI-RSV vaccination. Lung eosinophilia is seen in RSV-challenged mice primed with FI-RSV or with rVV-G, but G protein is not necessary for formalin inactivation-enhanced disease (129). In another study, formalin-inactivated mutant RSV strains with truncated or deleted G or deleted SH induced lower protective antibody levels, but immunopathological effects were still seen, with increased illness and eosinophilia (83). This suggests that immunity to G is important for protective immunity but is not necessary for FI-RSV-enhanced disease.

DISSECTING IMMUNOPATHOGENESIS

Many different cell types are involved in the responses to RSV. Some of these responses are clearly protective but can also cause increased damage. The interactions between these cell types occur through cognate interaction and by cytokines and chemokines (Fig. 4). Some of these mediators are produced early during virus infection, but others predominate in the later phases. They may therefore influence both primary and delayed diseases.

Antigen-Presenting Cells

Dendritic cells (DC) are able to initiate potent responses in naive T cells. Influenza A leads to an increase in DC numbers in the lung (189), which may in turn lead to excessive lymphocyte infiltration and immune augmentation. DC are also associated with atopic patients (107) and are seen in allergy models (161). In mice, there is a sustained increase in DC numbers following RSV infection (18). RSV has also been shown to

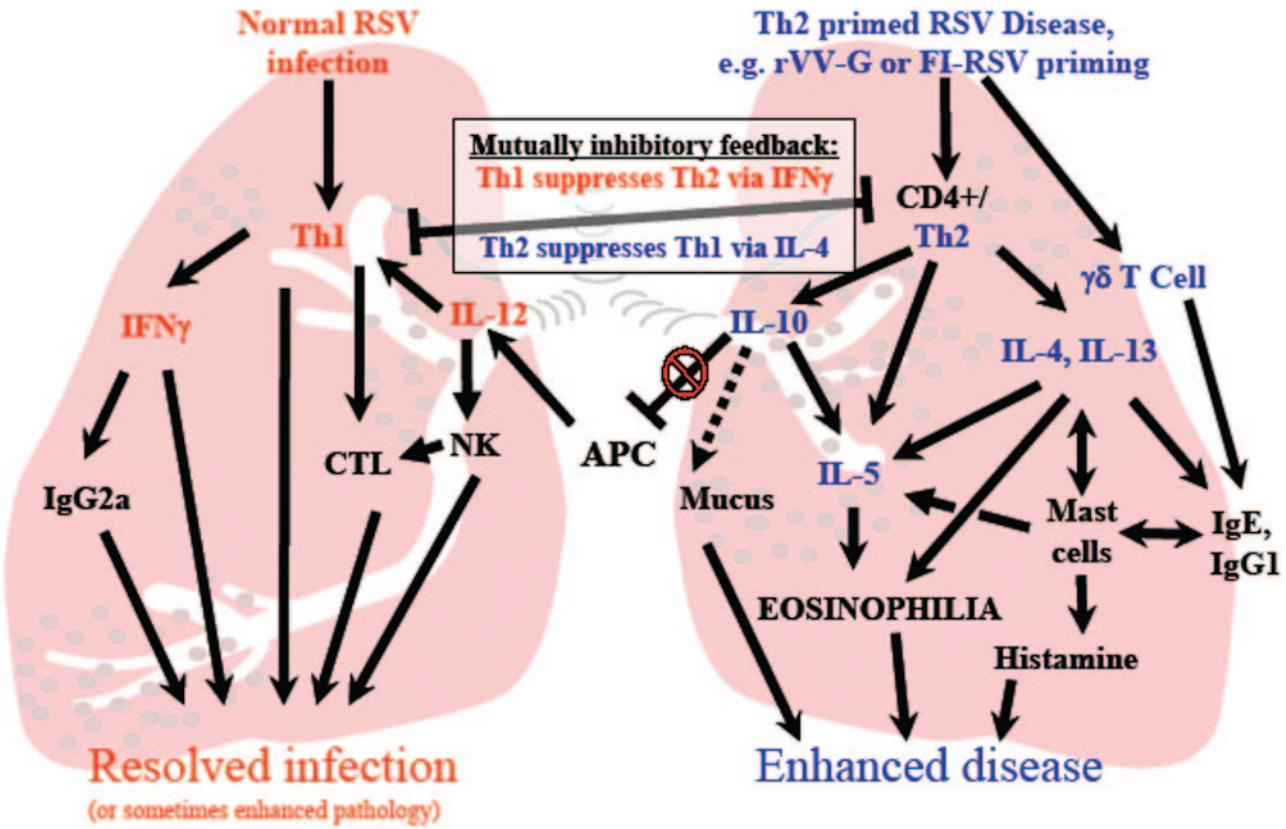

FIG. 4. Interactions of T cells and cytokines. Normal mild or asymptomatic infections are cleared with a predominantly Th1 response, with IFN-γ being generated from NK cells and CD4 and CD8 T cells (left). This inhibits the Th2 cytokine pathways, which are generally inactive. In disease enhanced by prior immunity or in some otherwise predisposed individuals, there is a rapid and strong Th2-type system (right). This Th2-type response may be caused by reduced levels of key cytokines (e.g., IL-12) at an early stage of infection or by vaccination (e.g., with FI-RSV) leading to preferential production of cells that make IL-4 and IL-5. IL-4 suppresses Th1 cytokines and upregulates Th2 cytokines, most importantly IL-5 and IL-13, which leads to eosinophilia and airway narrowing. However, the Th1 response can also be pathogenic if it exceeds that necessary for simple viral clearance, and it may be associated not with eosinophilia but with PMN efflux (see also Fig. 3). IgE, immunoglobulin E.

decrease the production of IFN-γ (14) and to increase the production of prostaglandin E2, IL-10, and IL-11 in cord blood-derived DC, suggesting that RSV might drive the immune system towards a Th2-type environment by effects on DC (13).

Role of CD4 Helper T Cells

It seems some T cells enhance disease, while others control it. In mice, primary signs of disease are reduced by CD4 and/or CD8 T-cell depletion (56). Depletion of $CD4^+$ T cells (32) or transfer of $CD8^+$ T cells modulates the eosinophilia seen in rVV-G-primed RSV-infected mice (6), while eosinophilia can be made to appear in strains that normally do not develop it if CD8 T cells are depleted or impaired in function (76).

This suggests that Th2 cells promote RSV-induced eosinophilia and that CD8 cells generally inhibit it (Fig. 3). As described above, RSV G-induced pathology is caused mainly by the overactive Th2 $CD4^+$ T cells (5). It has been shown that these CD4 T cells are oligoclonal, with approximately half of the cells expressing Vβ14, and that Th2-like pulmonary injury can be abolished by elimination of this $CD4^+$ $V\beta14^+$ subpopulation (177). This intriguing finding suggests that this novel subset of $CD4^+$ T cells is crucial to the development of pathology and that G may have a "superantigen" effect. Investigation of this phenomenon has not been followed through in studies on bronchiolitic infants. Another subset of Th2 CD4 T cells, T1/ST2, has been shown to play a role in RSV-driven eosinophilia (179).

As described above, the effects of RSV are mediated through the cytokines and chemokines that it induces, and these mediators will necessarily affect the outcome of an infection. The immune background of the neonatal lung is different from that of the adult lung (3, 119), with a general bias towards Th2 responses. For example, $CD4^+$ T cells show hypermethylation in the promoter region of the IFN-γ gene, affecting transcription efficiency (187), and IL-12 gene transcription is reduced in neonatal human monocyte-derived dendritic cells (55). It may be that these factors in part account for enhanced disease severity in RSV-infected infants.

Role of CD8 Cytotoxic T Cells

Although murine FI-RSV and rVV-G vaccination models of disease augmentation have focused on the pathogenesis of lung eosinophilia and have emphasized the role of Th2 cells, RSV immunopathology is certainly not solely Th2 related. During primary infection RSV, like most viral infections, tends

to induce inflammation dominated by Th1 cytokine production even in BALB/c mice (which are prone to Th2 responses). Cell transfer studies show that CD8 T cells can cause viral clearance but also can result in remarkable disease enhancement (24). If rVV-M2 is used to prime BALB/c mice, RSV infection results in extreme sickness due to a massive pulmonary influx of $CD8^+$ CTL and pathology reminiscent of acute respiratory distress syndrome. In a few cases, RSV bronchiolitis has also been associated with acute respiratory distress syndrome in humans (67), but the normal pathology of infantile bronchiolitis is very similar to that seen in mice with highly activated CD8 T-cell responses.

CD8 T cells are a major source of IFN-γ. In the mouse model, IFN-γ is produced following primary infection but not sufficiently to control RSV-induced allergy to OVA (12). The role of IFN-γ in RSV disease is unclear; in the mouse model it has been shown to be necessary for protection (126), and airway obstruction is decreased in IFN-γ knockout mice (175). There is a considerable evidence that IFN-γ is produced following RSV infection (see, for example, reference 176) but possibly in an inverse relationship to bronchiolitis severity (48). Clinical data point either towards at least a balanced Th1/Th2 cytokine production (170) or towards a deficiency of Th1 cytokines characterized by infants with RSV producing less IFN-γ (84). Compared to the case for other severe respiratory viral infections leading to LRTI, IFN-γ production by peripheral blood mononuclear cells appears to be decreased in RSV disease (2).

Unconventional T Cells

It seems that numerically small subsets of T cells may play a major role in regulating immune enhancement in RSV disease. NK cells are transiently present in the early stages of RSV infection of mice and are a major source of IFN-γ at day 4, when cells of the acquired immune system are differentiating. Whereas $CD8^+$ T cells can cause enhanced weight loss, IL-12-activated NK cells inhibit lung eosinophilia without causing enhanced illness. However, depletion of both NK and CD8 T cells allows RSV to spread to mediastinal lymph nodes, showing that either subset alone can have antiviral effects (77). Cells of the innate immune system can therefore direct the pattern of subsequent specific immunity.

γδ T cells are defined by the use of the γδ T-cell receptor instead of the more common αβ type associated with classical T lymphocytes. While they are relatively scarce, γδ T cells appear early in thymic ontogeny and are associated with mucosal surfaces (69). In virus-infected infants, γδ T cells produce more IL-4 and less IFN-γ during RSV infection than during reovirus infection (9). It seems possible that these small subsets of cells can be crucial to directing the form of pathology.

CD1d-deficient mice have normal numbers of T lymphocytes and natural killer cells but lack $V\alpha14^+$ natural killer T cells. CD8 T-cell recruitment is reduced in $CD1d^{-/-}$ mice, which show alterations in illness, viral clearance, and IFN-γ production. Activation of NK T cells in normal mice by α-GalCer results in reduced illness and delayed viral clearance (81). Thus, early IFN-γ production and efficient induction of CD8 T-cell responses during primary RSV infection require CD1d-dependent events.

Effects of Virus-Specific Antibody

As described above, it was initially thought that FI-RSV pathology was antibody mediated. This view went out of favor but gained support from studies in vitro and in primates that suggested that antibody may increase viral replication (51, 130, 131). In addition to possible infection-enhancing antibody, antibody may enhance disease by forming immune complexes and activating complement. This has been shown to be important for both FI-RSV (129) and FI-MV (127). This effect appears to preferentially affect lung function, but it possibly also affects Th2 differentiation (7).

SUMMARY OF IMMUNE MECHANISMS OF RSV DISEASE

Studies of immune-augmented secondary disease are sometimes interpreted as being informative about the origins of disease in primary bronchiolitis, but the relationship between the pathogeneses of these conditions is disputed. It is important to resist overinterpreting studies of immune sensitization when attempting to explain the pathogenesis of primary bronchiolitis. However, there are common themes linking the two conditions.

The incubation period of primary RSV is about 5 days; children hospitalized with bronchiolitis have usually already been ill for 3 to 6 days. Virus replicates to higher levels and remains detectable for longer during primary infection than after prior sensitization. It is therefore possible that "acquired" T- and B-cell responses are developing by this time and contribute to disease. This situation is exacerbated by prior sensitization or in those otherwise predisposed to particular specific immune responses.

Important common and distinct factors in different forms of RSV disease are illustrated in Fig. 5. In primary infection of nonvaccinated individuals, the disease is characterized by a relatively high viral load and the delayed appearance of disease, the timing of which coincides with the development of specific acquired T-cell immunity (Fig. 5A). In previously sensitized individuals (or perhaps those in some way predisposed to enhanced disease), viral replication is more limited and viral elimination occurs more rapidly (Fig. 5B). However, the heightened immune response leads to even more disease than is typical during first infections.

Age and host genetics certainly affect the balance of immune responses during primary infection, making first RSV infections sometimes resemble secondary disease. In the neonatal period, Th1 responses are generally poor or short-lived and IL-12 production weak. One possibility is that both Th1 and Th2 responses are formed during the initial infection; however, there is specific IL-4-dependent apoptosis of Th1 cells in the neonatal environment (94). This may explain why at this age, there is a natural tendency towards strong Th2 responses. An autocrine Th2 feedback loop driven by IL-4 might therefore tend to cause a cascade of events causing immune damage or future skewing of response to disease.

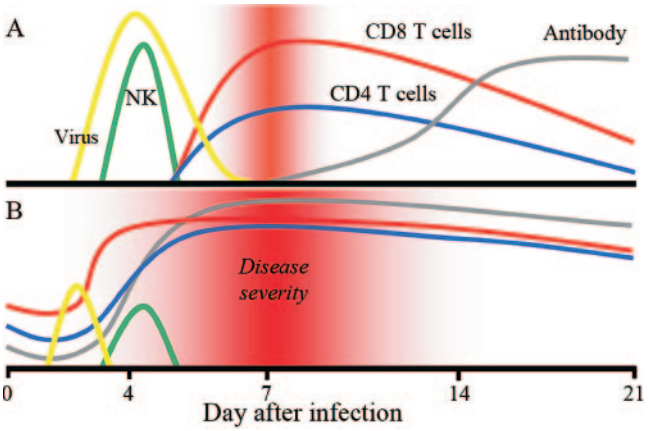

FIG. 5. Overview of the sequence of immune events in viral clearance and disease. In primary infection of nonvaccinated individuals (A), virus peaks on about day 4, associated with recruitment of NK cells, which make IFN-γ. Virus is eliminated between days 5 and 8, during which time activated CD4 and CD8 T cells are recruited and produce local cytokines. The peak of disease coincides with this phase. Anti-RSV serum antibody appears relatively late. In previously vaccinated or sensitized individuals (B), the virus titer is typically 100- to 1,000-fold less than in primary infection and peaks earlier (e.g., day 2). However, the rapid and potent cellular response enhances disease severity, which is usually much greater than in primary infection. Notably, high levels of preexisting specific antibody can prevent infection completely and do not cause disease enhancement.

Vaccine Development and Future Therapies for RSV Disease

Despite continuous efforts to develop safe and effective vaccines, spanning 40 years, none have been successful. The basic difficulties to be overcome include the fact that natural infection gives only transient and partial immunity to reinfection of the upper respiratory tract (64). Many established vaccines are ineffective in the first 6 months of life (147), and it is in this period that most children suffer from RSV bronchiolitis. Vaccines would be useful in the elderly, but many established vaccines are poorly immunogenic in older people. Nonetheless, the potential benefits of an effective vaccine are undoubted. Vaccines under development include cold-passaged live viruses (33), purified proteins (171), and conventional inactivated and DNA vaccines (17).

Given that disease appears to be in some part caused by immune overactivity, it is logical to test specific short-lived immune inhibitors. Steroids appear to be of little or no value, possibly because they lack specificity and reduce the severity of the immune response while potentially increasing viral replication. Anti-TNF, anti-IL-9, and anti-immunoglobulin E therapy all merit consideration, perhaps in combination with antiviral treatments. Such experimental treatments would need to be carefully justified in patients with the most severe forms of bronchiolitis. Effective prophylaxis is available in the form of a biosynthetic humanized monoclonal anti-F antibody, palivizumab. This is administered as a monthly intramuscular injection and is highly effective in preventing infection. However, its cost prohibits its use in resource-poor settings.

CONCLUSION

Severe RSV disease appears to be associated with a misdirected immune response, characterized by enhanced release of mediators and infiltration of a range of monocytes and polymorphonuclear cells. Animal models are essential to understanding disease enhancement and to the development of safe and effective vaccines, but none is ideal in all aspects (Table 1). While it is clear that primary and immune-augmented RSV diseases are not identical, these models shed light on which components of the immune response warrant further study and give rise to important general conclusions about immunopathogenesis: (i) single clinical syndromes (bronchiolitis, asthma, etc.) can result from diverse pathogenic pathways, (ii) the time and place of sampling are critical in acute transient diseases, (iii) samples from remote locations may be misleading, and (iv) the most numerous cells may not be the most influential.

The effects of formalin-inactivated RSV are remarkably similar in all species studied, including cattle, rodents, and primates. Such vaccines enhance disease by multiple pathways, leading to overactive acquired immune responses. In animal models, this disease is relatively hard to block by specific immunomodulation. By contrast, the immunopathogenesis of augmented disease in inbred mice vaccinated with single RSV proteins is highly specific and relatively easy to prevent by treatments that take out single components of the immune response (80, 83).

It seems clear that the immunopathogenesis of RSV disease during primary infection varies considerably from one individual to another and is affected by the postnatal age. Severe RSV bronchiolitis occurs only in a small minority of children and is usually transient and self-limiting. However, studies of disease augmentation by prior sensitization are capable of reproducing many features of the severe primary disease seen in susceptible individuals and may therefore give indications of what type of immunomodulation should be attempted. A great deal has been learned in recent years, and it is to be hoped that clinical application of this knowledge will soon benefit the large numbers of children who suffer from RSV infections each year.

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