Asthma and respiratory syncytial virus. New opportunities for therapeutic intervention

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INTRODUCTION

Asthma is the most frequent chronic illness in childhood. Approximately 5 million children have asthma in the United States, and more than 155 million individuals are affected worldwide[1]. Asthma is a complex and heterogeneous disease; genetic and environmental factors influence its clinical expression and progression, which is characterized by bronchial obstruction, airway inflammation and recurrent airway hyperresponsiveness (AHR).

Respiratory syncytial virus (RSV) is an RNA virus belonging to the *Pneumovirus* genus of the *Paramyxoviridae* family. It is an enveloped, single-stranded, negative-sense RNA virus that encodes 11 proteins with different molecular weights[2]. Only two are non-structural proteins (NS1, NS2) and are therefore present in infected cells only and not in the virions. Among the structural proteins, the surface glycoproteins, G and F, are the most interesting from the immunological point of view. These glycoproteins play a role in adhesion and fusion, respectively, and are responsible for maintaining viral cell-to-cell replication. They represent the major antigenic determinants of the virus, inducing the production of neutralizing antibodies. Antigenic differences in the G protein, as well as in the F, N and P proteins, give rise to two types of RSV: A and B[3,4].

EPIDEMIOLOGY

RSV was discovered in 1956 when a group of chimpanzees was noted to have cold-like symptoms[5]. The virus was isolated for the first time in infants with bronchiolitis in 1957, and in 1975 it was named "respiratory syncytial virus" because of its ability to form syncytia in *in vitro* cell cultures

RSV is currently the leading viral pathogen associated with bronchiolitis and lower respiratory tract disease requiring hospitalization in infants and young children worldwide[6]. Moreover, RSV is a major cause of severe respiratory illness not only in the elderly or immunocompromised individuals[7,8] but also in previously healthy adults[9,10]. In the United States, RSV is responsible for more than 150,000 hospitalizations per year and the incidence is increasing[11,12].

In addition to the acute morbidity caused by RSV, numerous studies over the past 40 years have described a strong association between RSV infection in infancy and the subsequent development of recurrent wheezing and AHR later in life[13-17]. Rooney et al[16] followed-up 62 infants with a diagnosis of RSV bronchiolitis until they were 7 years old. Fifty-seven percent of the patients subsequently developed recurrent wheezing and 71% of these infants had a familial history of asthma. Thirty-eight percent of the patients experienced no further wheezing episodes and only 18% had a family history of asthma. These initial findings have been confirmed over the years and recent evidence demonstrates a strong association between RSV and AHR not only in infants with severe bronchiolitis requiring hospitalization[14], but also in children with RSV lower respiratory tract infection (LRTI) who did

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Received: June 2004.
Accepted for publication: June 2004.

not require hospitalization and who were followed-up as outpatients[18]. Most studies, regardless of their design (inpatients *vs* outpatients, prospective *vs* retrospective), reach a similar conclusion: RSV LRTI in infancy is significantly associated with an increased risk of recurrent wheezing in up to 71% of patients and is accompanied by long-term alterations in pulmonary function tests. Both forced expiratory volume in 1 second (FEV_1) and forced expiratory flow at 25-75% of forced vital capacity ($FEF_{25-75\%}$/FVC) are lower in school-aged children who had symptomatic RSV LRTI during infancy than in those in control groups[13,14,18-21].

RSV is able to trigger acute exacerbations in adults with chronic bronchitis and recurrent episodes of wheezing in infancy[14,18,22]. Hall et al[10] prospectively studied 10 previously healthy adults with a mean age of 30 years who developed an acute respiratory illness while working in an infant's ward during a community outbreak of RSV infection. All the subjects, in addition to developing upper respiratory tract symptoms (fever, pronounced cough, nasal congestion, rhinorrhea and/or hoarseness) in the acute phase of the disease, also developed altered airway reactivity characterized by exaggerated responses of pulmonary resistance to carbachol challenge that persisted through the first 8 weeks of evaluation, when signs or symptoms of the acute disease were no longer present.

CAUSATION OR ASSOCIATION?

Because most studies describing the association between RSV and AHR are observational, it is still unclear whether RSV infection alone causes the development of pulmonary sequelae or whether the combination of predisposing factors coupled with RSV infection early in life contributes to respiratory sequelae and AHR in later childhood[23,24]. Does severe RSV infection during infancy cause the differences in pulmonary function observed later in life? Or do inherent abnormalities of the host, such as different polymorphisms of the interleukin (IL)-8 promoter gene, or in the surfactant protein A gene, combined with RSV infection at certain developmental stages of life, alter the susceptibility of the host to asthma as evidenced by changes produced in lung physiology after this infection[25,26]? Well known risk factors for developing RSV-associated AHR include maternal smoking, infants with smaller-than-normal airways, or infants with a familial history of asthma or atopy[27,28]. However, most children with RSV bronchiolitis who develop recurrent wheezing and AHR do not have clear predisposing risk factors.

RSV infection ⟶ Abnormal pulmonary function
or
Airway hyperreactivity
Atopy + RSV ⟶ Abnormal pulmonary function
Small airways

PATHOGENESIS

The pathogenesis of RSV-induced pulmonary inflammation is complex. The clinical manifestations of RSV bronchiolitis result not only from the direct cytopathic effect of the virus on the respiratory epithelial cells but also from the host inflammatory response, innate and adaptive immunity, and the distinct neurogenic mechanisms involved such as substance P and neurokinin. A number of mechanisms have been proposed to explain the association of RSV infection early in life with subsequent AHR but the specific mechanism and cell types involved in the long-term sequelae of RSV infection remain to be elucidated. These mechanisms include:

1. Neuropeptides and increased expression of specific receptors (substance P and neurokinin 1 [NK1]), which not only provoke AHR but also act as pro-inflammatory molecules, attracting leukocytes into the airways and activating them[29,30].

2. Prostaglandins, leukotrienes and other cellular metabolites.

3. Activation of mast cells, eosinophils and IgE, reflecting a persistent shift toward a Th2 cellular response[31]. Most children produce RSV-specific IgE responses during their first episode of RSV bronchiolitis. However, it is the amount, the persistence and duration of this response that is critical in determining which patients will develop recurrent wheezing and/or asthma[31].

4. Release of immunomodulatory and pro-inflammatory cytokines such as IL-4, IL-6, RANTES (*regulated on activation normal T-cell expressed and secreted*), macrophage inflammatory protein 1α (MIP-1α), IL-8 and tumor necrosis factor-α (TNF-α) that recruit different cells to the respiratory tract[32].

The immune response to RSV: an unfavorable balance

The immune response to primary RSV infection is generally inefficient and consequently subsequent reinfections are common throughout life. During reinfections a significant booster effect of mainly IgG and IgA with sustained immunologic reactivity is observed in serum and respiratory mucosa. The virus does not normally replicate outside the respiratory tract and the infection is restricted to the respiratory mucosa. However, the development of viremia and extrapulmonary disease has been observed in certain T and B cell immunodeficiency states[33-35].

Under normal circumstances the immune system is composed of two interrelated and extremely well balanced parts, the innate and adaptive immune responses. The innate immune response provides the first line of defense against infection and is capable of immediately recognizing and responding to microbial invasion via nonspecific mediators. The adaptive, or specific response, in addition to recognition and elimination of the patho-

gen, is characterized by the property of antigenic memory, which induces the generation of specific memory cells. In RSV infection, innate and adaptive immunity are out of balance. The innate immune response generates pro-inflammatory molecules and recruits neutrophils, eosinophils and monocytes to the site of infection. The adaptive immune response is characterized by an exaggerated CD8+ cellular immune response and CD4+ lymphocyte production of Th1/Th2 cytokines. Together all these factors lead to lung inflammation, bronchiolitis and the development of recurrent wheezing.

Innate Immunity (cytokines and chemokines)

As a rule, primary infections are symptomatic. Between 30 and 70 % of children with primary RVS infection develop bronchiolitis or pneumonia. A leading hypothesis is that cell-mediated immune responses with imbalanced production of Th1 vs Th2 cytokines and chemokines is correlated with disease severity, although data published in the literature are contradictory, probably because of factors related to differences in study design. Th1 cytokines, such as interferon-γ (IFN-γ), IL-12 and IL-18, promote a cell-mediated immune response and are required for effective responses to intracellular pathogens including viruses. Th2 lymphocytes release IL-4, IL-5, IL-13 and IL-9, cytokines that mediate allergic inflammation.

Initial reports suggested that the IL-4/IFN-γ ratio produced by peripheral blood mononuclear cells (PBMC) from children hospitalized for RSV bronchiolitis was higher than that of children with less severe forms of the disease[36]. Subsequently, infants with severe bronchiolitis were observed to have a more balanced Th1-Th2 response, similar to that observed in infants with upper respiratory tract infection (URTI) alone, while infants with mild bronchiolitis seemed to have a predominantly Th1 response[37]. Recently, in a cohort of children with at least one familial antecedent of atopy who were prospectively followed-up from birth, the immune response to natural infection with RSV was studied *in vivo*. In this selected cohort, samples of nasal washings were obtained in the first 2 days after identifying RSV infection, as well as at days 5 and 7. The patients were subdivided in two groups according to severity: bronchiolitis vs. URTI. The IL-4/IFN-γ ratio was significantly higher in the nasopharyngeal samples from infants with RSV bronchiolitis ($p < 0.01$) compared with that in the group with URTI. Concentrations of IFN-γ and IL-18 in PBMC were significantly lower in the group of patients with bronchiolitis, regardless of age[38].

The chemokines RANTES, monocyte chemotactic protein-1 (MCP-1), eotaxin and macrophage inflammatory protein-1α (MIP-1α) have been related to the pathogenesis of RSV infection. Recently MIP-1α concentrations have been directly correlated with the degree of hypoxia in infants hospitalized with severe RSV bronchiolitis. MIP-1α stimulates B-cell production of IgE, recruiting basophiles and eosinophils into the airways. Additional support for a pathogenic role of MIP-1α in RSV infection is provided by studies using a murine model. Mice with a targeted gene deletion for MIP-1α have decreased inflammatory response in the lung after RSV infection[39,40]. Other chemokines, such as MCP-1 and RANTES, have also been related to RSV infection and are found in greater concentrations in severe bronchiolitis.

The mechanisms through which infants with RSV bronchiolitis develop recurrent wheezing later in life remain to be elucidated. There is mounting evidence in experimental models that RSV may persist "latently" in immunologically privileged sites within the lung, avoiding immune detection and elimination. These findings have led some investigators to propose that the long-term effects observed after RSV infection may be partly explained by the persistence of the RSV in the respiratory tract. Hence, RSV would maintain a constant stimulation of the immune system, which could be responsible for chronic lung inflammation and the different pattern of IFN, cytokine and chemokine responses observed in children with RSV-induced AHR. This latent virus could also be an important source of new community outbreaks[41].

Cubie et al[42] detected RSV by *in situ* hybridization in archival lung tissue from infants who died from sudden infant death syndrome. RSV was found not only when death had occurred during the winter, when the infection is more prevalent, but also in some infants who died in the summer months, suggesting the persistence of RSV in the lungs of these infants[42]. Another study was recently conducted in the UK in patients with chronic obstructive pulmonary disease (COPD) to determine the effects of respiratory virus infections on the time course of COPD exacerbations. Surprisingly, this study found that RSV RNA detected by polymerase chain reaction (PCR) in nasopharyngeal aspirates was more frequently isolated in patients with COPD and stable disease than in those with exacerbations (24 % vs 14.2 %) in whom, moreover, higher titers of the virus were not detected, probably reflecting a low level of replication. What is even more interesting is that the group of COPD patients with positive viral detection when stable were more likely to have greater concentrations of plasma fibrinogen and IL-6 and an increased frequency of exacerbations than those in whom the virus was not detected[43]. Recently, the use of PCR in the murine model has demonstrated the persistence of the virus in the lungs of RSV infected mice for up to 100 days after intranasal inoculation. No RSV RNA was isolated in other sites (lymphoid tissue, brain, spleen or bone marrow)[41]. The intracellular location of the virus may partly explain the mechanisms through which RSV avoids the humoral immune response, since intracellular virus is not accessible to circulating immunoglobulin and would not be neutralized[41].

The mechanisms through which RSV avoids the cellular immune response are not completely understood. Could RSV persist latently in immunologically privileged sites such as neurons or special antigen presenting cells, where restricted expression of viral genes and low expression of major histocompatibility complex class I molecules prevents effective presentation of viral particles? Experimental data has shown that *in vitro* RSV infection of human cord blood-derived macrophages and dendritic cells induces the production of IL-10. These findings raise the possibility that RSV may be able to redirect the quality of antigen presentation, possibly resulting in anergy or apoptosis of RSV-specific cytotoxic T cells and even in some degree of RSV tolerance.

New therapeutic interventions

Several studies have suggested that RSV viral load in the respiratory tract correlates with disease severity[44-46]. However, as the disease progresses, it is mainly the immune response, rather than viral replication, that is responsible for the clinical manifestations and disease severity. Numerous studies have investigated whether the initial treatment of RSV bronchiolitis could modify or have an impact on the long-term consequences of RSV infection in the respiratory tract. In some studies, but not all, ribavirin administered early in the course of the illness decreased episodes of wheezing after RSV infection[47-48]. Data on the use of systemic or inhaled steroids in RSV infection are contradictory. In children intubated for severe RSV bronchiolitis, we previously demonstrated that the administration of systemic steroids did not modify the acute course of the disease and had no impact on the inflammatory parameters measured (leukocyte counts in nasal or tracheal aspirates and concentrations of cytokines and chemokines) or on clinical outcomes defined as a total days of mechanical ventilation, days of intensive care unit admission and total hospitalization days. Moreover, at 48 hours of therapy there was a significantly faster decline of RSV load in the placebo-treated group than in the dexamethasone-treated group ($p < 0.05$)[44].

A recent pilot study evaluated the long-term effects of the prophylactic administration of intravenous RSV immunoglobulin (RSV-IVIG). Thirteen children with bronchopulmonary dysplasia who received monthly prophylactic RSV-IVIG during the winter and 26 controls matched for gestational age and risk factors who did not receive RSV-IGIV were included. The frequency of lower respiratory tract RSV infection was lower in the group that had been treated prophylactically (6/13) than in the controls (21/26) ($p < 0.001$). Pulmonary function test (FEV_1/FVC) ($p < 0.02$) as well as the incidence of atopy ($p < 0.04$), missed school days ($p = 0.006$) and asthma attacks ($p = 0.03$) were significantly lower in children who had received RSV-IVIG than in controls. These results suggest that prophylaxis of RSV infections in infancy may have long-term effects on the respiratory and immunologic parameters related to the development of asthma[49]. No data are available yet on the long-term effects of palivizumab on respiratory parameters and asthma/AHR prevalence in high-risk infants who received palivizumab monthly as prophylaxis, although one study is underway (protocol W00-353)[50].

During RSV bronchiolitis, cysteinyl-leukotrienes are released. These potent pro-inflammatory molecules are known to increase the permeability of the respiratory mucosa, favoring the production of edema and bronchoconstriction, as well as the development of AHR. Several studies with leukotriene-antagonists have been performed both in human and animal models, with contrasting results[51,52]. The only study conducted in humans was carried out in infants hospitalized with RSV bronchiolitis with a mean age of 9 months at diagnosis. Sixty-one patients vs 55 controls received 5 mg daily of montelukast, a cysteinyl-leukotriene receptor antagonist for 28 days, starting from days 3 to 7 of hospitalization. The effects of montelukast began to be evident after 2 weeks of therapy. A greater number of infants treated with montelukast were free of symptoms after the acute episode of bronchiolitis compared with infants receiving placebo (6/28 vs 1/28, $p = 0.015$). Daytime cough was significantly reduced while on treatment and exacerbations were significantly delayed by montelukast (23 days) compared with placebo (8 days). During follow-up starting 4 weeks after finishing the intervention there were no significant differences between treatment groups in any of the outcomes[51].

Lessons from the animal model

To examine the immunopathogenesis of RSV infection in both the acute and chronic phases of the disease, we established a mouse model in our laboratory, which allowed us to study the dynamics of RSV replication in a more controlled setting. This model enabled us to quantitate the viral load and inflammatory mediators in the lower respiratory tract and to evaluate the impact of these inflammatory changes on pulmonary function. These analyses were based on:

1. A standardized scoring system (histopathologic score [HPS]) to evaluate the lung inflammatory changes in the acute and chronic phases of the disease and to assess mucus the overproduction in the airway.

2. Whole-body plethysmography to measure both basal airway obstruction (bronchiolitis phase) and airway obstruction after methacholine challenge (asthma phase) to determine AHR as defined by a calculated, dimensionless value, called enhanced pause (Penh), which reflects the degree of airway resistance.

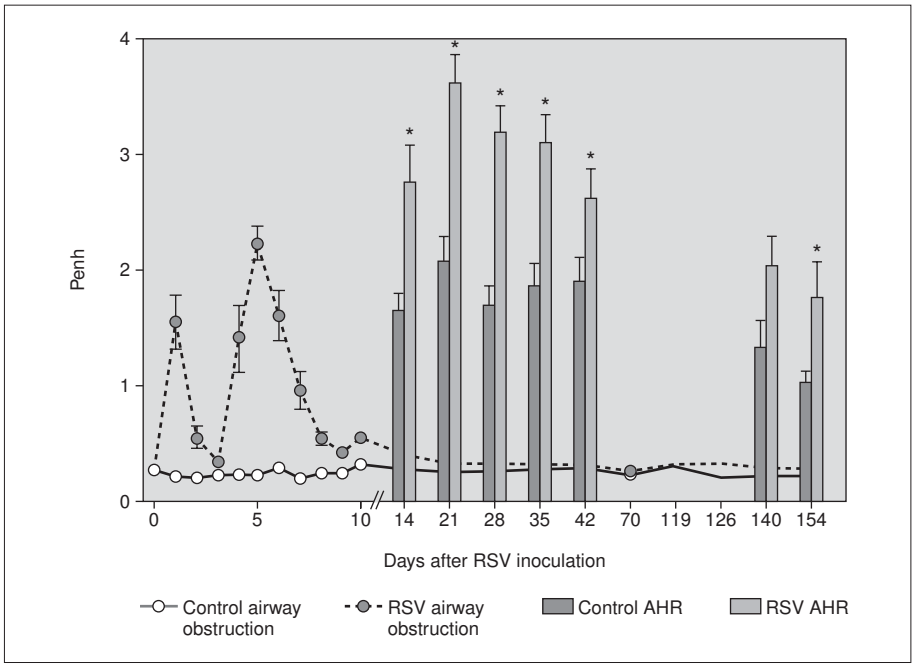

Figure 1. *Airway hyperresponsiveness (AHR) (chronic phase). After the initial phase of acute bronchial obstruction, which resolves in 14 days, AHR was evaluated after inhalation of methacholine, a nonspecific airway irritant. The changes produced after methacholine inhalation, shown as an increase in Penh (bars), began to be evident after the bronchiolitis phase, 14 days after infection, and persisted until day 154 after inoculation. Although minimal increases in Penh can also be seen in noninfected animals after methacholine inoculation, the magnitude of the response was markedly higher in animals infected with respiratory syncytial virus (RSV). *$p \leq 0.05$.*

Using this model, we demonstrated that RSV induces a significant inflammatory response in the lungs, which continues to worsen and progresses into a chronic phase characterized by AHR and persistent airway inflammation[53] when the virus is no longer detectable by cell culture[40]. Compared with infection-free control mice, the lungs from mice inoculated with live RSV continued to demonstrate persistent airway inflammation for up to 154 days after inoculation (fig. 1). Perhaps even more importantly, these histological changes were accompanied by functional changes in pulmonary function parameters.

To determine whether the possible persistence of RSV in the lower respiratory tract contributes to the development of the long-term histopathologic and functional changes observed after RSV infection, we measured RSV loads by real time RT-PCR in lung specimens from mice inoculated with RSV during both the acute (days 1 to 5) and chronic phases of the disease (days 12 to 42). In contrast to viral cultures, which were consistently negative after the first week post-inoculation, RSV RT-PCR remained positive in infected mice for up to 6 weeks after inoculation. Moreover, the RSV RNA copy number was significantly correlated with AHR ($r = 0.83$, $p < 0.001$)[54].

During the *acute phase* (bronchiolitis/pneumonia phase), the histopathologic changes included a dense perivascular and peribronchial/peribronchiolar inflammatory infiltrate composed of mononuclear cells and scattered neutrophils. At its peak (days 4 to 7), this inflammatory infiltrate extended into surrounding alveolar septa in a stellate manner with patchy involvement of the parenchyma and abundant macrophages, occasional lymphocytes and neutrophils in alveolar spaces. No intraluminal exudates were identified in the airways. In the *chronic phase* (asthma phase), no involvement of alveolar septa or air spaces was observed. Occasional hemosiderin-laden macrophages were also seen in this phase, but no neutrophils or eosinophils. With time (days 21 to 154), the inflammatory infiltrates changed from circumferential to partial involvement around vessels or airways, and there was a tendency for involvement of the larger central vessels and airways, rather than the smaller peripheral broncho-vascular bundles. Control mice evaluated at different time-points demonstrated only rare small lymphocytic infiltrates involving only a small portion of the circumference of central vessels or airways. Concerning mucus production, in the acute phase (day 5) infected mice had increased PAS-positive cells in central and peripheral airways, compared with controls, and these cells showed a greater degree of hypertrophy. On approximately day 14, there continued to be more mucus producing cells in the central airways of infected mice than in controls. No PAS-positive cells were seen in peripheral airways on day 14. These results clearly demonstrate the presence of abnormal chronic inflammatory changes and mucus overproduction, which probably contributes significantly to the long-term airway disease induced by RSV infection in both the acute and chronic phases of the disease[40].

To determine the effect of decreasing RSV replication on disease severity, distinct groups of mice were treated with palivizumab, a neutralizing anti-RSV monoclonal antibody against the F protein[55], recently approved in the US and Europe for the prevention of severe RSV in-

fection in high risk patients[56,57]. Palivizumab was administered once either a) 24 hours before infection as prophylaxis or b) 48 hours after inoculation with RSV. Figure 2 demonstrates that RSV-infected mice treated with palivizumab at either –24 h or + 48 hours showed complete suppression of RSV loads compared with untreated controls that received saline intraperitoneally as a placebo. We also measured the concentrations of different cytokines in the bronchiolar lavage (BAL) of RSV infected mice after palivizumab administration. Only the mice that received palivizumab as prophylaxis had lower BAL concentrations of RANTES, eotaxin, MIP-1α, IL-10 and IFN-γ ($p < 0.01$) in the acute phase of the disease (bronchiolitis phase) compared with the other regimes evaluated. HPS was assessed during the acute (day 5) and chronic phases (day 70). We demonstrated that although all RSV-infected mice had histopathologic abnormalities, administration of palivizumab 24 h before infection was associated with a significant reduction of HPS during the acute phase of the disease (day 5, $p < 0.007$) (fig. 3). Although the mice received a single dose of the antibody, the reduction of HPS compared with infected untreated controls continued to demonstrate a marked trend even at 10 weeks after infection. These studies demonstrate that decreasing RSV replication in the lower respiratory tract with palivizumab significantly reduced the cytopathic effect of the virus in the respiratory epithelial cells and in the immune response elicited by RSV.

Subsequently, we analyzed the effect of the anti-RSV monoclonal antibody on pulmonary function. Figure 4A shows that mice treated with the anti-RSV antibody at 24 h before inoculation showed a significant reduction in airway obstruction ($p < 0.001$) that was not observed in the other treatment group. Indeed, mice treated with palivizumab 24 h before infection looked clinically similar to uninfected controls that were inoculated with sterile medium. Aerosolized methacholine challenge 4 weeks after the infection elicited a significantly larger increase in Penh (AHR) in untreated RSV infected mice than in mice treated with palivizumab either before or after inoculation with RSV. On day 70, 10 weeks alter RSV inoculation, however, only mice that received the antibody at –24 h had a significant reduction in AHR compared with untreated controls (fig. 4B).

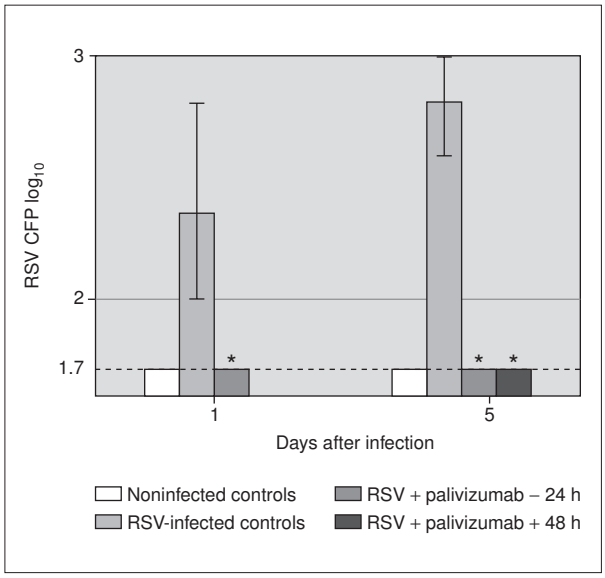

Figure 2. *Palivizumab administration 24 hours before (prophylaxis) or 48 hours after (treatment) inoculation reduced the number of respiratory syncytial virus (RSV) colony-forming plaques (CFP) by more than one logarithm compared with the untreated group. *p < 0.01 compared with RSV infected controls (untreated).*

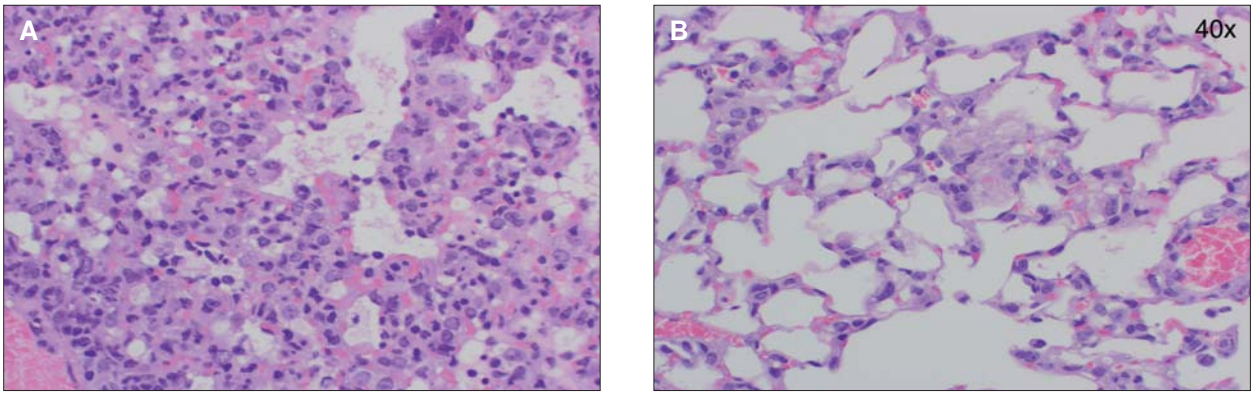

Figure 3. ***A)*** *In the pneumonia/bronchiolitis phase (day 5 after inoculation), lung sections from untreated mice infected with respiratory syncytial virus included dense perivascular mononuclear inflammatory infiltrates at the expense of mononuclear cells in the peribronchial area. The air spaces contained numerous monocytes and neutrophils as well as proteinaceous fluid.* ***B)*** *Mice treated with palivizumab 24 h before inoculation showed significantly decreased inflammation in the air spaces, which consisted mainly of intra-alveolar macrophages. No significant intra-alveolar edema was seen in this group of mice.*

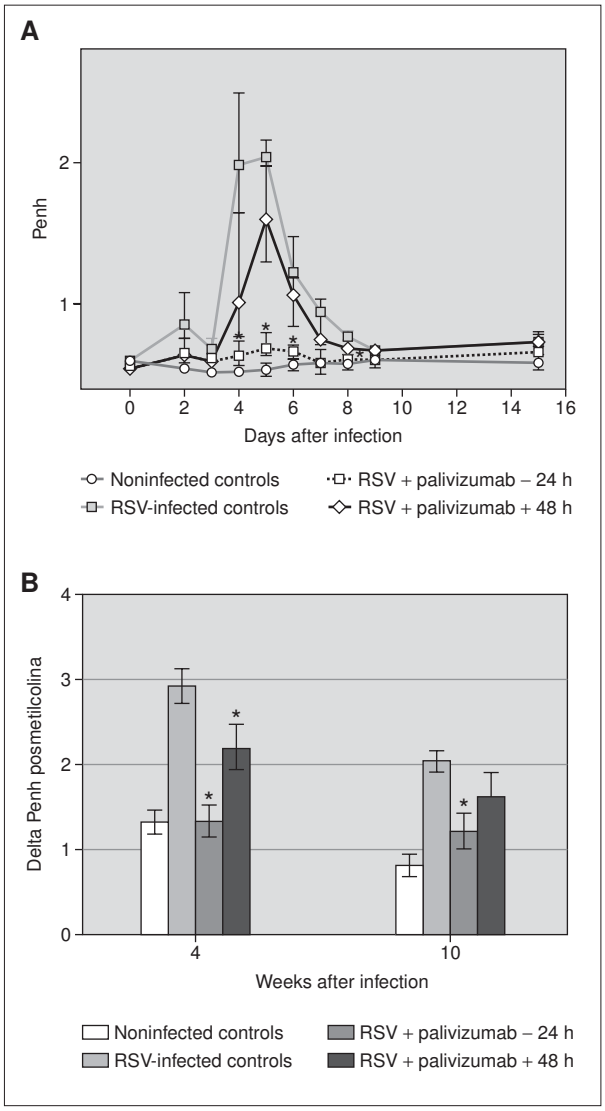

Figure 4. *Basal airway obstruction was evaluated daily in the first 2 weeks of infection* ***(A)****. Then, airway hyperreactivity (AHR) was evaluated after the mice (n = 8) underwent methacholine challenge weekly. Bars show delta Penh values reflecting the difference between Penh values before and after methacholine challenge (n = 12)* ***(B)****. *p < 0.001 compared with respiratory syncytial virus (RSV)-infected controls (untreated).*

Taken together, these results demonstrate that reducing RSV replication is significantly associated with parallel reductions in lung inflammatory changes, concentrations of inflammatory cytokines and airway obstruction during the acute phase of the disease, as well as with a remarkable attenuation of the long-term airway disease induced by RSV infection.

Future clinical studies to determine the role of palivizumab in preventing the long-term morbidity associated with RSV in the pediatric population should be performed.

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