ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2004, p. 1811–1822 Vol. 48, No. 5
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.5.1811–1822.2004

Anti-Respiratory Syncytial Virus (RSV) Neutralizing Antibody Decreases Lung Inflammation, Airway Obstruction, and Airway Hyperresponsiveness in a Murine RSV Model

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Received 7 June 2003/Returned for modification 17 August 2003/Accepted 19 January 2004

Numerous studies have described a strong association between respiratory syncytial virus (RSV) infection in infancy and the development of recurrent wheezing and airway hyperresponsiveness. We evaluated the effect of an anti-RSV neutralizing monoclonal antibody (palivizumab) on different aspects of RSV disease by using a murine model. BALB/c mice were intranasally inoculated with RSV A2. Palivizumab or an isotype-matched control antibody was administered once at 24 h before inoculation, 1 h after inoculation, or 48 h after inoculation. Regardless of the timing of administration, all mice treated with the neutralizing antibody showed significantly decreased RSV loads in bronchoalveolar lavage (BAL) and lung specimens compared with those of infected controls. Pulmonary histopathologic scores, airway obstruction measured by plethysmography, and airway hyperresponsiveness after methacholine challenge were significantly reduced in mice treated with the anti-RSV antibody 24 h before inoculation compared with those for untreated controls. Concentrations of interferon-gamma, interleukin-10, macrophage inflammatory protein 1α, regulated on activation normal T-cell expressed and secreted (RANTES), and eotaxin in BAL fluids were also significantly reduced in mice treated with palivizumab 24 h before inoculation. This study demonstrates that reduced RSV replication was associated with significant modulation of inflammatory and clinical markers of acute disease severity and significant improvement of the long-term pulmonary abnormalities. Studies to determine whether strategies aimed at preventing or reducing RSV replication could decrease the long-term morbidity associated with RSV infection in children should be considered.

Respiratory syncytial virus (RSV) is the leading viral pathogen associated with lower respiratory tract disease in infants and young children worldwide. In addition to acute morbidity, numerous studies have described a strong association between RSV infection in infancy and the development of recurrent wheezing and airway hyperresponsiveness (AHR) (34, 45, 50). More recently, RSV has also been demonstrated to be an important cause of severe respiratory illness among the elderly and immunocompromised individuals (11, 12). Accordingly, efforts have been focused on both prevention and treatment of this common infection.

Despite almost half a century of active clinical research on humans, as well as with animal and in vitro models, to define the immunopathogenesis of the disease, no effective vaccine is available yet, and the relationship between RSV and AHR remains to be completely characterized. In the United States, RSV is responsible for more than 150,000 hospitalizations per year in the pediatric population (49), resulting in an estimated annual cost of $300 million to $500 million for children below the age of 5 years (51, 57). Moreover, the long-term morbidity associated with severe RSV infection results in health care burdens and costs disproportionately greater than the estimated hospitalization costs associated with treatment of the acute infection.

Licensed in the United States in 1998, palivizumab (Synagis; MedImmune, Gaithersburg, Md.), a humanized monoclonal antibody (MAb) (immunoglobulin G1 [IgG1]) against a neutralizing epitope on the RSV F glycoprotein (28), is the first MAb approved for prevention of an infectious disease (1, 47). When administered to high-risk infants once a month during the RSV season, palivizumab resulted in a 55% reduction in hospitalizations for RSV illness compared with placebo (26).

To elucidate the specific responses that are associated with RSV-induced AHR, our laboratory has adapted the murine model of RSV infection to analyze lung inflammatory abnormalities during both the acute and chronic phases of the disease (27). The present study was designed to test the hypothesis that reducing RSV replication by use of an anti-RSV neutralizing antibody will result in modulation of the immune response in the respiratory tract, with a subsequent decrease in acute disease severity and possibly in the long-term sequelae associated with RSV infection in the murine model.

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MATERIALS AND METHODS

Animals. Specific-pathogen-free BALB/c mice (7 to 8 weeks old; female) were purchased from Charles River Laboratories (Wilmington, Mass.) and housed in

the animal care facility of our institution in separate filter-top cages in a temperature-controlled room (22°C). Water and chow were provided ad libitum. Mice were allowed to acclimate to the new environment for 1 week and were housed in groups according to the experimental setup. Their virus-free status was confirmed by use of sentinel mice that were regularly tested for various pathogens, including mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reo-3 virus, mouse encephalitis virus (GD-7), mouse rotavirus (EDIM), minute virus of mice, and *Mycoplasma hominis*, as well as pinworms and mites.

The guidelines of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center at Dallas and the appropriate federal guidelines were followed.

RSV growth conditions. Human RSV stock A2 (RSV A2) was grown and quantified on Hep-2 cells and was prepared and stored as described previously (14). Hep-2 cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with glutamine, amphotericin, gentamicin, penicillin G, and 10% fetal bovine serum (10% EMEM). Virus titers were determined by a methylcellulose plaque assay, with a lower level of detection of 1.7 $\log_{10}$ PFU. Because RSV titers can decrease over time, the RSV aliquot used for each individual experiment was tested in duplicate at the time of inoculation. UV-inactivated RSV was generated by exposing RSV A2 to UV radiation (UV Cross-linker; Fisher Biotech) for 40 min. Plaque assays confirmed the absence of viable virus.

RSV infection and inoculation. Methoxyflurane, an inhaled anesthetic, was used for sedation during inoculation. BALB/c mice were intranasally inoculated once (on day 0) with $10^{7.5}$ to $10^{8.2}$ PFU of human RSV A2/ml in 100 μl of 10% EMEM. A similar inoculum of UV-inactivated RSV was used for appropriate control experiments. Control mice were sham inoculated with 100 μl of sterile tissue culture medium supernatant processed in the same way as uninfected cells in the preparation of stock virus (14). Under these conditions, liquid materials inoculated intranasally are delivered predominantly into the lungs by direct aspiration.

Experimental design and sample collection. In each separate time course experiment, four mice per treatment group were evaluated and euthanized at different time points: on day 1, day 5, and day 70 after inoculation. These time points were selected on the basis of previous studies that defined the peak concentrations of the various inflammatory markers evaluated (27). Mice were anesthetized by intraperitoneal injection of 75 mg of ketamine/kg of body weight and 5 mg of acepromazine/kg before cardiac puncture. These animals weighed, on average, 25 g at the time of therapy. Bronchoalveolar lavage (BAL) specimens were obtained by instilling 500 μl of 10% EMEM once through a 25-gauge needle into the lungs via the trachea. Approximately 70 to 80% of the instilled volume was consistently retrieved. Previous work from many laboratories has demonstrated the value of this technique for assessing RSV loads and cytokine concentrations in respiratory secretions (10, 25, 41, 53). Whole-lung specimens, including the trachea and both lungs, were collected and fixed with 10% buffered formalin solution for further histological evaluation. To determine the effect of suppressing RSV replication on BAL cytokine concentrations, the severity of pneumonia as defined by a standard histopathologic score (HPS) (see below), and respiratory parameters measured both spontaneously and after methacholine exposure, mice were treated with a high dose of a humanized neutralizing MAb (IgG1) against the RSV F protein (palivizumab) at different time points. After reconstitution from lyophilized powder in 100 μl of sterile water, a dose of 50 mg/kg (1.25 mg per mouse) was administered once intraperitoneally, either at 24 h before intranasal inoculation, as an immunoprophylaxis (anti-RSV MAb −24 h); at 1 h after inoculation, as an early treatment (anti-RSV MAb +1 h), or 48 h after inoculation with RSV, as a late treatment (anti-RSV MAb +48 h). We selected a high dose (50 mg/kg) of the anti-RSV MAb because the purpose of the study was to assess the consequences of a significant reduction in viral replication on inflammatory markers and disease severity and not to characterize the pharmacodynamic properties of this antibody in the mouse model.

Control groups included medium-inoculated (uninfected) mice, RSV-infected mice treated with phosphate-buffered saline (untreated), and RSV-infected mice treated with an IgG1 isotype-matched control antibody against human CD2 (MEDI-507), also administered 24 h before, and 1 and 48 h after, the inoculum. Both antibodies were provided by MedImmune, Inc. Control experiments were performed to determine whether the circulating antibody present in the pulmonary vessels at the times of sampling (days 1 and 5) could interfere with quantification of the RSV load measured in BAL specimens. To this end, in one set of animals, the blood was rinsed from the lungs by injection of 2 ml of normal saline into the right cardiac ventricle before the BAL specimens were obtained. Experiments showed similar results in both groups of animals, regardless of whether the blood was rinsed or not. Another set of experiments was performed to compare the effects of the neutralizing antibody on RSV loads measured in BAL samples versus whole-lung specimens from the same animals. For this purpose, after BAL samples were obtained, the lungs were embedded in 1,000 μl of 10% EMEM, homogenized, and then centrifuged at 1,000 × *g* for 10 min. The clarified supernatant was used to determine RSV loads by the quantitative plaque assay.

RSV quantitative culture. Two-day-old Hep-2 cells, 80% confluent in Costar (Cambridge, Mass.) 12-well plates, were used for the plaque assay. Twenty microliters of undiluted BAL fluid and serial 10-fold dilutions in 10% EMEM (50 μl of undiluted BAL fluid was used for the initial dilution) were immediately cultured on Hep-2 cell plates, while the remainder of the BAL specimens were stored at −80°C for further analysis. After incubation for 5 days at 37°C, monolayers were fixed with 10% formalin phosphate and stained with hematoxylin-eosin (15). Quantification was performed by counting the syncytia on plate specimens under a dissecting microscope; counts were expressed as log PFU per milliliter. If plated dilutions were negative for growth, the specimen was assigned a value of 1.7 $\log_{10}$ PFU/ml, the lower limit of detection of the assay (4, 32, 33).

Histopathology. Lung tissue was fixed in buffered formalin, and transverse sections (thickness, 5 μm) were stained with hematoxylin and eosin. The HPS was based on grading of five different parameters: (i) peribronchiolar and bronchial infiltrates, (ii) bronchiolar and bronchial luminal exudates, (iii) perivascular infiltrates, (iv) amount of monocytes, and (v) parenchymal pneumonia. This HPS system assigned values from 0 to 21; the higher the score, the greater the inflammatory changes in the lung (8, 19). The HPS was determined by a pathologist who was unaware of the infection statuses of the animals from which specimens were taken.

Cytokine concentrations in BAL samples. Concentrations of cytokines, including gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1α (MIP-1α), and eotaxin, were measured in BAL sample supernatants by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn.). All assays were carried out according to the manufacturer's instructions, and samples from each mouse were assayed in duplicate. The lower limits of detection for these assays were 50 pg/ml for IFN-γ, 60 pg/ml for TNF-α, 20 pg/ml for IL-10 and eotaxin, 25 pg/ml for MIP-1α, and 80 pg/ml for RANTES. For statistical analysis, samples with optical density readings below the limit of the standard curve of the assay were assigned a value half that of the detection level.

Pulmonary function tests: measurement of airway obstruction and long-term hyperresponsiveness. Whole-body unrestrained plethysmography (Buxco, Troy, N.Y) was utilized to monitor the respiratory dynamics of mice in a quantitative manner before and after methacholine exposure. During the first 2 weeks after infection, all groups of mice were monitored daily to assess the differences in airway obstruction. In this acute phase, enhanced pause (Penh) values were recorded without methacholine challenge. After the acute phase, mice were evaluated before and after challenge with methacholine (50 mg/ml) once a week for as long as 10 weeks after inoculation to assess differences in AHR. Data are expressed as delta Penh, representing the difference between the maximum value registered post-methacholine challenge and the baseline Penh.

The plethysmograph used has been specifically designed for the low tidal-air volumes in mice (5, 6, 29). Prior to methacholine challenge, previously inoculated mice were first allowed to acclimate to the chamber; then plethysmograph readings were recorded to establish baseline values. Next, mice were exposed once to aerosolized methacholine previously dissolved in phosphate-buffered saline (acetyl-β-methylcholine chloride; Sigma, St. Louis, Mo.), and plethysmograph readings were recorded again. Mean Penh values were calculated from recordings. Penh is a value without dimension that represents a ratio of peak expiratory flow to peak inspiratory flow and is a function of the timing of expiration. Penh correlates with pulmonary airflow resistance or obstruction. Penh as measured by plethysmography has been validated in animal models of AHR previously (13, 18, 20, 30, 48, 52).

Noninvasive plethysmography allowed us to assess the illness in an objective manner and to monitor the changes in pulmonary dynamics in the same group of animals for as long as 10 weeks after inoculation in order to characterize the chronic phase of the disease.

Statistical analysis. For all statistical analyses, Sigma Stat 2000 software (SPSS Science, San Rafael, Calif.) was used. Since most of the data did not follow a normal distribution, they are presented as medians and 25th to 75th percentiles. Differences between different groups of animals were tested by using the Kruskal-Wallis test as a nonparametric one-way analysis of variance (ANOVA). When this test demonstrated a significant difference between groups ($P < 0.05$), two different methods of correcting for multiple comparison were used to evaluate statistical significance between the different regimens evaluated (Dunn's test and Tukey's test).

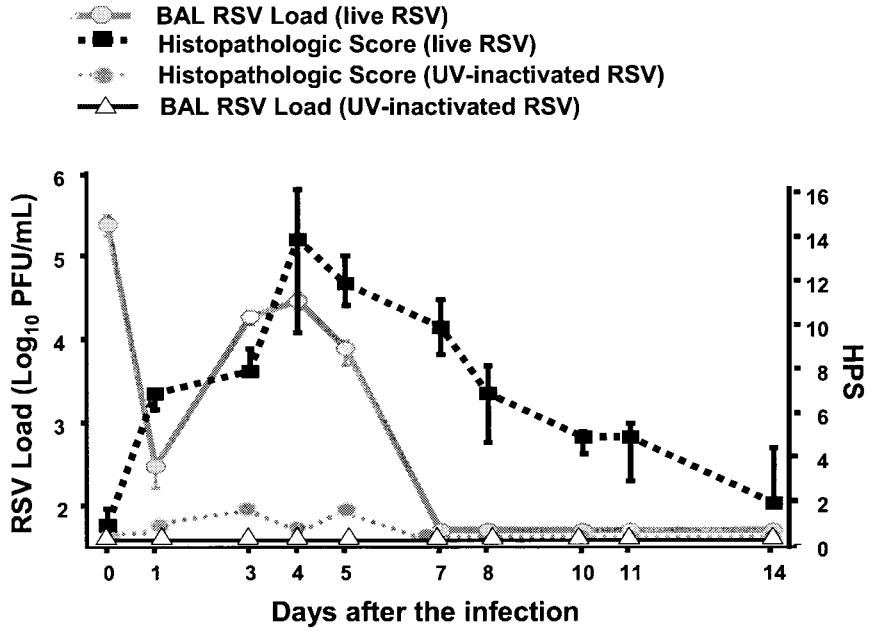

FIG. 1. Dynamics of RSV replication and histopathologic changes in RSV-infected, untreated mice. Mice were intranasally inoculated with live or UV-inactivated RSV. Two hours after live-RSV inoculation, RSV loads in BAL fluids were significantly elevated; they declined within 24 h and rose gradually thereafter, peaking at days 3 to 5. By day 7, no virus was detected by plaque assay. Lungs from RSV-infected mice revealed a progressive increase in the HPS. By the time that disease severity peaked (days 5 to 7), the quantity of replicating virus was already declining. RSV-infected mice showed significantly greater HPS than uninfected controls or mice inoculated with UV-inactivated RSV. Light shaded circles and unbroken shaded line, viral loads in mice infected with live RSV; solid squares and dotted solid line, HPS in mice infected with live RSV; medium shaded circles and dotted shaded line, HPS in mice infected with UV-inactivated RSV; open triangles and unbroken solid line, viral loads in mice infected with UV-inactivated RSV.

RESULTS

Dynamics of RSV replication and histopathologic changes in RSV-infected, untreated mice. RSV loads in BAL samples obtained from mice 2 h after live RSV inoculation were significantly elevated, representing the inoculum. However, viral loads declined significantly within 24 h and then showed a progressive increase, suggesting active replication (Fig. 1); they peaked at days 3 to 5 and had fallen below the limit of detection by day 7. BAL viral loads remained undetectable by plaque assay (limit of detection, 1.7 $\log_{10}$ PFU/ml) from day 7 after inoculation up to day 70 (data not shown). These results were consistent regardless of the size of RSV inoculum used.

RSV infection induced acute and chronic pulmonary inflammatory infiltrates. Compared with the lungs of control mice, lungs obtained from mice inoculated with live RSV showed a persistent progression of severity of pneumonia, as determined by an increasing HPS, which peaked between days 5 and 7 (Fig. 1). RSV-induced histopathologic abnormalities gradually decreased in the first week after infection. However, RSV-infected mice continued to demonstrate significantly greater HPS than the sham-inoculated controls up to 154 days postinfection (data not shown). Mice inoculated with UV-inactivated RSV showed responses similar to those of control mice inoculated with sterile medium in terms of viral load, HPS (Fig. 1), and cytokine profiles, suggesting that the changes observed in mice inoculated with live RSV were induced by actively replicating virus (27).

Effect of the anti-RSV neutralizing MAb (palivizumab) on RSV replication. Administration of the anti-RSV neutralizing antibody at a dose of 50 mg/kg resulted in significant reductions in RSV replication in BAL samples at all time points evaluated in all treatment groups. Treatment of mice before inoculation (anti-RSV MAb −24 h) and after inoculation but before the onset of disease (anti-RSV MAb +1 h and +48 h) was effective in diminishing RSV loads in BAL samples. On days 1 and 5, the numbers of plaques were reduced more than 1 log unit in treated mice (to $\leq$1.7 $\log_{10}$ PFU/ml on days 1 and 5, i.e., at or below the limit of detection of the assay) compared with those in RSV-infected, untreated mice (medians [25 to 75th percentiles], 2.33 [2.0 to 2.85] and 2.79 [2.55 to 3.05] $\log_{10}$ PFU/ml on days 1 and 5, respectively) (Fig. 2A). By day 70 postinoculation, no virus was detected by plaque assay.

RSV loads were also significantly reduced in lung specimens of mice treated with the anti-RSV MAb compared with those in infected, untreated mice ($P < 0.001$). Lung RSV loads were reduced by >1 log unit in treated mice regardless of the time of MAb administration. This effect was most evident in the anti-RSV MAb −24-h group on day 5 (the day of maximal replication). However, some virus was still detected in the lung specimens of mice that received the anti-RSV antibody after inoculation (mean lung RSV loads [in $\log_{10}$ PFU per milliliter], 3.87 in infected, untreated mice versus 2.05 for anti-RSV MAb −24 h, 2.35 for anti-RSV MAb +1 h, and 2.41 for anti-RSV MAb +48 h (Fig. 2B). RSV loads in mice treated with the isotype-matched control antibody were similar to those in RSV-infected, untreated mice (Table 1).

Chemokine and cytokine concentrations in BAL samples of RSV-infected mice following treatment with the anti-RSV neu-

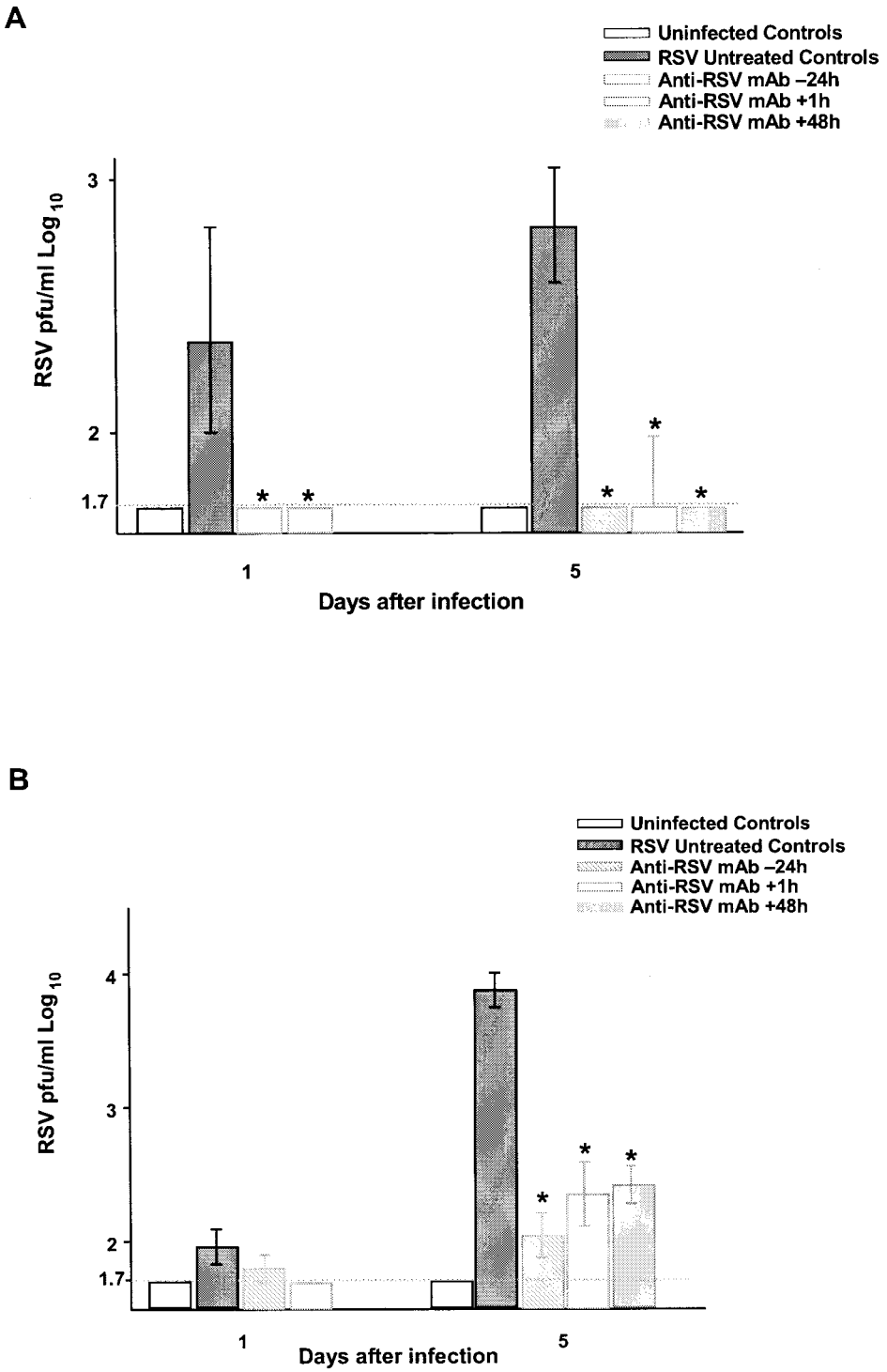

FIG. 2. Effect of the anti-RSV neutralizing MAb (palivizumab) on RSV replication. Mice were intranasally inoculated with RSV ($10^{7.5}$ to $10^{8.2}$ PFU/ml) or Hep-2 cell supernatants (uninfected controls) and treated with a single dose of the anti-RSV neutralizing MAb administered at different time points. On days 1 and 5 after inoculation, mice were sacrificed, and BAL samples and lung specimens from the same mice were obtained. RSV loads in BAL samples (A) and lung specimens (B) were determined by plaque assay. Treatment groups consisted of uninfected controls (open bars), RSV-infected, untreated mice (dark shaded bars), and RSV-infected mice receiving the anti-RSV MAb at −24 h (hatched bars), +1 h (open bars with shaded outlines), or +48 h (light shaded bars). Values are median $\log_{10}$ PFU of RSV per milliliter of BAL fluid; error bars, 25th to 75th percentiles. *, $P < 0.001$ by Kruskal-Wallis ANOVA on ranks for comparison with RSV-infected, untreated controls.

TABLE 1. RSV loads in BAL samples of mice treated with the anti-RSV MAb or an isotype matched control MAb

Group	Day 1		Day 5	
	RSV load (range)[a]	P[b]	RSV load (range)	P[b]
Uninfected controls	<1.70 (1.7–1.7)	<0.05	<1.70 (1.7–1.7)	<0.05
RSV-infected mice				
Untreated controls	2.66 (2.39–2.89)		2.70 (2.45–2.84)	
Anti-RSV MAb −24 h	<1.70 (1.7–1.7)	<0.05	<1.70 (1.7–1.7)	<0.05
Anti-RSV MAb +1 h	<1.70 (1.7–1.7)	<0.05	<1.70 (1.7–1.7)	<0.05
Anti-RSV MAb +48 h	ND	ND	<1.70 (1.7–1.7)	<0.05
Isotype MAb −24 h	2.56 (2.38–2.70)	NS	2.88 (2.80–3.18)	NS
Isotype MAb +1 h	2.36 (2.24–2.62)	NS	2.63 (2.44–2.97)	NS
Isotype MAb +48 h	ND	ND	2.54 (2.32–3.12)	NS

[a] Values are median $\log_{10}$ PFU of RSV per milliliter of BAL fluid (25th to 75th percentiles) on days 1 and 5 postinoculation. ND, not done.
[b] For comparison with RSV-infected, untreated mice. NS, not significant.

tralizing antibody. On days 1, 5, and 70, concentrations of IFN-γ, MIP-1α, eotaxin, RANTES, IL-10, and TNF-α in BAL samples were measured by ELISA. On day 1 after inoculation, RANTES and IL-10 concentrations in uninfected control mice and RSV-infected mice treated with the anti-RSV MAb at −24 h were significantly lower ($P = 0.006$ for RANTES; $P < 0.001$ for IL-10) than those in RSV-infected, untreated mice and in mice treated at +1 or +48 h. TNF-α concentrations peaked at day 1. Higher concentrations of this cytokine were detected in mice treated with the anti-RSV MAb than in untreated controls, but the difference did not reach statistical significance (Fig. 3). On day 5, concentrations of IFN-γ ($P = 0.018$), MIP-1α ($P < 0.001$), RANTES ($P = 0.016$), eotaxin ($P = 0.018$), and IL-10 ($P < 0.001$) in the anti-RSV MAb −24 h group and in uninfected controls were significantly lower than in RSV-infected, untreated mice and in mice treated with the anti-RSV MAb at +1 or +48 h. The concentrations of all cytokines and chemokines, except for IL-10, in mice treated with the isotype-matched control antibody were similar to those in RSV-infected, untreated mice (Table 2).

By day 70, the concentrations of all cytokines measured were below the limit of detection of the assay, except for eotaxin, levels of which were slightly elevated in uninfected mice and in mice that received the anti-RSV MAb at +1 h (medians [25th to 75th percentiles], 20.57 [8.0 to 29.94] and 13.66 [8.0 to 23.23] pg/ml, respectively).

Effect of the anti-RSV neutralizing MAb on HPS of RSV-infected mice. RSV replication resulted in marked lung inflammation. In the acute phase (days 1 to 5), changes included perivascular edema and margination of neutrophils and monocytes on day 1, progressing to dense perivascular, peribronchial, and peribronchiolar inflammatory infiltrates composed of lymphocytes, macrophages, and dispersed neutrophils. These infiltrates, which peaked around day 5, extended into alveolar septa with patchy involvement of the parenchyma. No intraluminal exudates were identified in the airways. These findings are consistent with pneumonia (Fig. 4).

HPS at days 5 and 70 demonstrated that although all RSV-infected mice had histologic abnormalities, administration of the anti-RSV MAb at −24 h was associated with a significant reduction in the HPS during the acute phase of the disease (day 5; $P < 0.001$). Although mice received a single dose of the antibody, the reduction in the HPS relative to that of infected, untreated controls continued to demonstrate a marked trend even at 10 weeks after infection (Fig. 5). When the antibody was administered as an early treatment (anti-RSV MAb +1 h), it had no significant effect on the HPS at day 5, the time of peak pulmonary inflammation. On day 70, the HPS was modestly elevated in this treatment group compared with that of infected, untreated mice. Administration of the anti-RSV MAb as a late treatment (anti-RSV MAb +48 h), although not significant, was associated with a trend toward less severe inflammatory changes at day 5 after infection, but at day 70 the scores were similar to those of infected, untreated mice. Likewise, the HPSs of animals treated with the isotype-matched MAb-were similar to those of RSV-infected, untreated mice (Table 3).

Effects of the anti-RSV neutralizing antibody on pulmonary function. (i) Airway obstruction. RSV-infected, untreated mice developed significant airway obstruction, objectively defined by measuring Penh, compared with uninfected controls and RSV-infected mice treated with the anti-RSV MAb at −24 h. The latter treatment-group showed significant reductions in airway obstruction at all time points evaluated ($P < 0.001$) and remained asymptomatic throughout the disease course. Although mice to which the anti-RSV MAb was given after RSV inoculation but prior to the establishment of disease also showed very significant reductions in viral loads, the severity of clinical illness, assessed objectively by plethysmography, was not modified (Fig. 6A).

(ii) AHR. Aerosolized methacholine challenge elicited an increase in Penh in RSV-infected, untreated mice compared with values for mice that received the anti-RSV MAb either before or after inoculation with RSV (Fig. 6B). This effect was clearly evident at day 14, reached statistical significance 4 weeks after inoculation, and persisted until day 70, 9 weeks after RSV infection had cleared. Data are shown as delta Penh, representing the difference between the maximum value registered post-methacholine challenge and the baseline Penh. Although modest increases in Penh after methacholine challenge were also observed in control uninfected animals, the magnitude of the response was markedly more elevated in RSV-infected, untreated animals.

Our results also indicate that the timing of administration of the anti-RSV MAb in relation to inoculation had a significant effect on delta Penh. Four weeks after infection, animals in all the anti-RSV MAb groups evaluated had significantly lower delta Penh values than infected, untreated mice, but only the

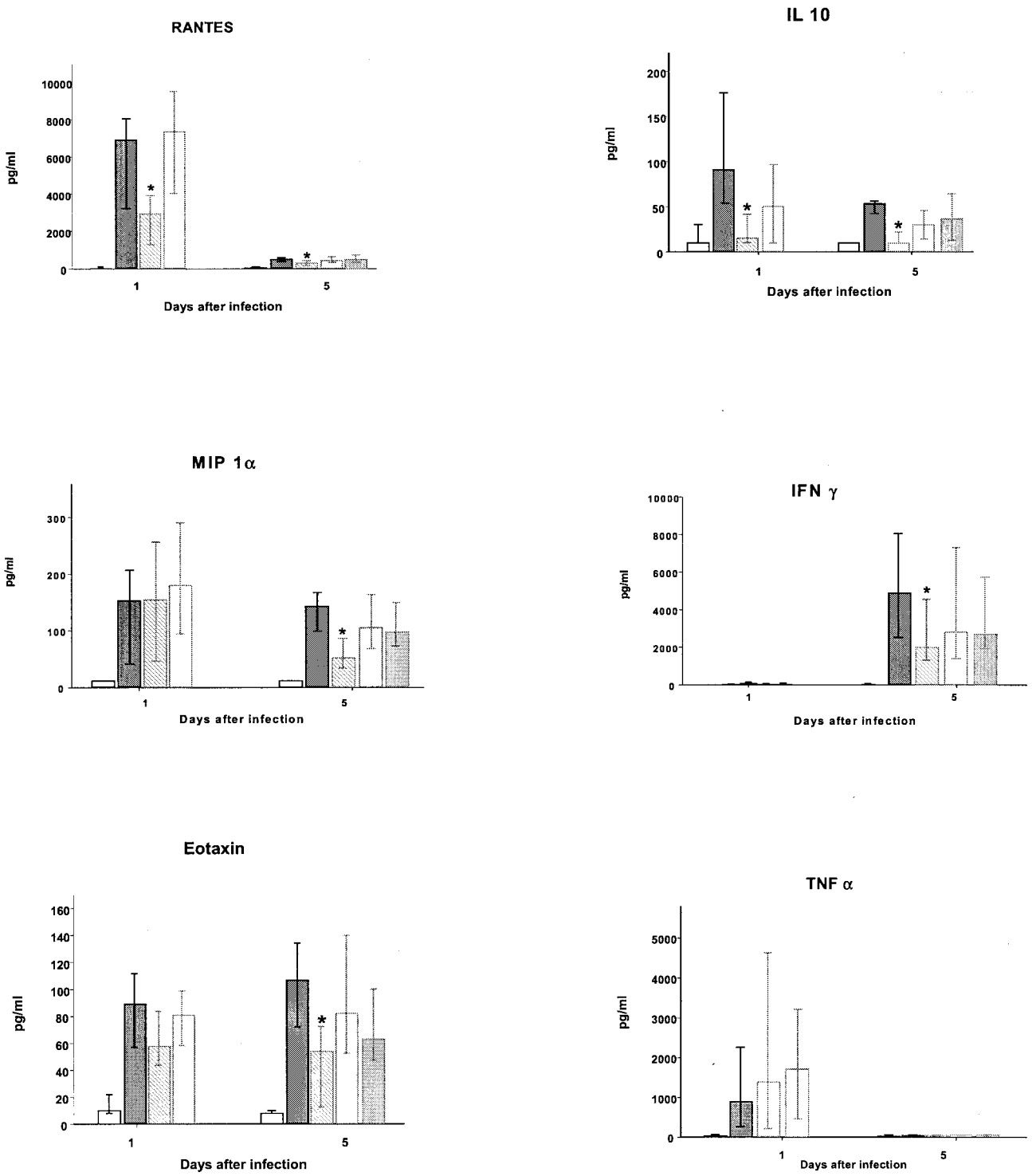

FIG. 3. Chemokine and cytokine concentrations in BAL samples of RSV-infected mice following treatment with the anti-RSV neutralizing antibody. Mice were inoculated intranasally and treated as described in Materials and Methods. On days 1 and 5 after inoculation, mice were sacrificed and BAL samples were obtained. Concentrations of TNF-α, MIP-α, IFN-γ, IL-10, eotaxin, and RANTES in BAL fluids were measured by ELISA. Bars represent results from five independent experiments, with 16 to 20 mice per time point in each treatment group. Treatment groups consisted of uninfected controls (open bars), RSV-infected, untreated mice (dark shaded bars), and RSV-infected mice receiving the anti-RSV MAb at −24 h (hatched bars), +1 h (open bars with shaded outlines), or +48 h (light shaded bars). Values are medians; error bars, 25th to 75th percentiles. *, $P < 0.05$ by Kruskal-Wallis ANOVA on ranks for comparison with RSV-infected, untreated controls.

TABLE 2. BAL cytokine concentrations in RSV-infected mice treated with the isotype-matched control MAb compared with untreated controls

Day postinoculation and group	BAL cytokine concn (pg/ml)[a]					
	IFN-γ	MIP-1α	Eotaxin	RANTES	IL-10	TNF-α
Day 1[b]						
Untreated controls	131.90 (74.05–155.35)	184.1 (156.8–229.1)	7.41 ± 32.34	8,271.00 ± 2,208.13	90.96 (52.97–176.00)	1,362.16 ± 1,388.45
Isotype MAb −24 h	50.00 (25.00–185.00)	275.40 (200.17–346.45)	83.96 ± 22.05	7,046.12 ± 1,603.85	10.00 (10.00–10.00)	1,899.50 ± 1,103.64
Isotype MAb +1 h	25.00 (25.00–106.83)	233.8 (207.25–267.07)	111.44 ± 15.14	8,215.00 ± 2,420.02	10.00 (10.00–10.00)	931.52 ± 534.89
Day 5[c]						
Untreated controls	2,794.50 (2,203.00–5,633.00)	99.02 (91.84–135.15)	79.12 ± 41.49	488.65 (384.85–570.75)	55.59 (43.99–60.65)	48.23 (22.76–60.00)
Isotype MAb −24 h	1,587.00 (1,100.00–2,229.00)	91.86 (32.96–139.15)	52.53 ± 37.40	465.80 (100.00–713.10)	64.91 (42.65–75.90)	60.00 (60.00–60.00)
Isotype MAb +1 h	2,131.00 (1,733.00–3,242.50)	62.13 (58.27–292.6)	81.95 ± 37.24	435.60 (343.35–1,353.10)	53.45 (47.50–172.03)	60.00 (60.00–98.15)
Isotype MAb +48 h	1,810.50 (1,374.50–2,320.00)	87.58 (65.17–137.85)	70.36 ± 40.83	668.20 (461.22–698.65)	26.39 (13.66–40.39)	60.00 (60.00–60.00)

[a] Expressed as means ± standard deviations or as medians (25th to 75th percentiles) according to whether data were normally distributed.
[b] For day-1 comparisons between untreated infected mice and mice treated with the isotype-matched MAb, *P* values were 0.091 for IFN-γ, 0.16 for MIP- 1α, 0.014 for eotaxin, 0.41 for RANTES, <0.001 for IL-10, and 0.56 for TNF-α.
[c] For day-5 comparisons between untreated infected mice and mice treated with the isotype-matched MAb, *P* values were 0.058 for INF-γ, 0.68 for MIP- 1α, 0.42 for eotaxin, 0.68 for RANTES, 0.02 for IL-10, and 0.13 for TNF-α.

anti-RSV MAb −24-h group appeared like the uninfected control mice. At this time, the delta Penh values for these two groups of mice were exactly the same (mean delta Penh, 1.27). This trend persisted up to 10 weeks after inoculation, at which point only uninfected mice and those treated with the anti-RSV MAb at −24 h had statistically significantly lower delta Penh values than RSV-infected, untreated mice (Fig. 6B).

Administration of the isotype-matched control MAb had no effect on the pulmonary-function test results of RSV-infected mice, either at baseline (airway obstruction) or after methylcholine challenge (AHR) (data not shown). RSV-infected mice with persistent AHR also demonstrated consistent inflammatory changes, predominantly a lymphocytic perivascular infiltrate, up to 70 days after inoculation. These findings provide a histologic correlation with the abnormal pulmonary responses presented in Fig. 6.

DISCUSSION

The immune response to RSV infection is primarily directed against the two major surface viral glycoproteins: the G (attachment) and F (fusion) glycoproteins. The F glycoprotein appears to be more important for induction of protective immunity and is associated with production of a high serum neutralizing antibody response (9) and activation of CD14-Toll-like receptor (17, 21). Several MAbs against the F glycoprotein provide passive protection against RSV infection (28); therefore, this glycoprotein has been a major focus for therapeutic intervention in RSV disease. Previous studies with both animals and humans have demonstrated a role for a passively administered antibody in the prevention of RSV disease (14). Both polyclonal-antibody (RSV-IVIG, also called RespiGam; MedImmune, Inc.) and MAb (palivizumab, also called Synagis; MedImmune, Inc.) preparations have been approved by the Food and Drug Administration for prevention of severe RSV infection in high-risk children (1, 2, 16, 26, 46).

The pathogenesis of RSV lower respiratory tract infection is not completely understood, but it is likely to be mediated both by the direct effects of the virus and by the host's inflammatory response. Despite more than 3 decades of effort, current options for treatment of acute RSV disease are limited. Treatment with anti-RSV human immune globulin or anti-F glycoprotein neutralizing antibodies is effective at decreasing the viral load, but it does not appear to ameliorate the disease process, suggesting that as the disease progresses, it is predominantly the immune response rather than virus replication that determines the clinical manifestations and disease severity (4, 7, 32, 33, 54, 56). We have demonstrated that a passively administered anti-RSV neutralizing MAb effectively diminished the replication of RSV in mice when used as preexposure prophylaxis or early therapy. When the anti-RSV MAb was administered at −24, +1, or + 48 h in relation to virus inoculation, RSV loads as measured by plaque assay in BAL samples were all below the limit of detection of the assay. Cultures of homogenized whole lungs, however, demonstrated the presence of residual RSV, possibly cell associated, indicating that virus neutralization was not complete. In addition, there was a trend suggesting that the reduction in RSV loads was more prominent in mice treated at −24 h.

By limiting RSV replication, direct virus-induced cytopa-

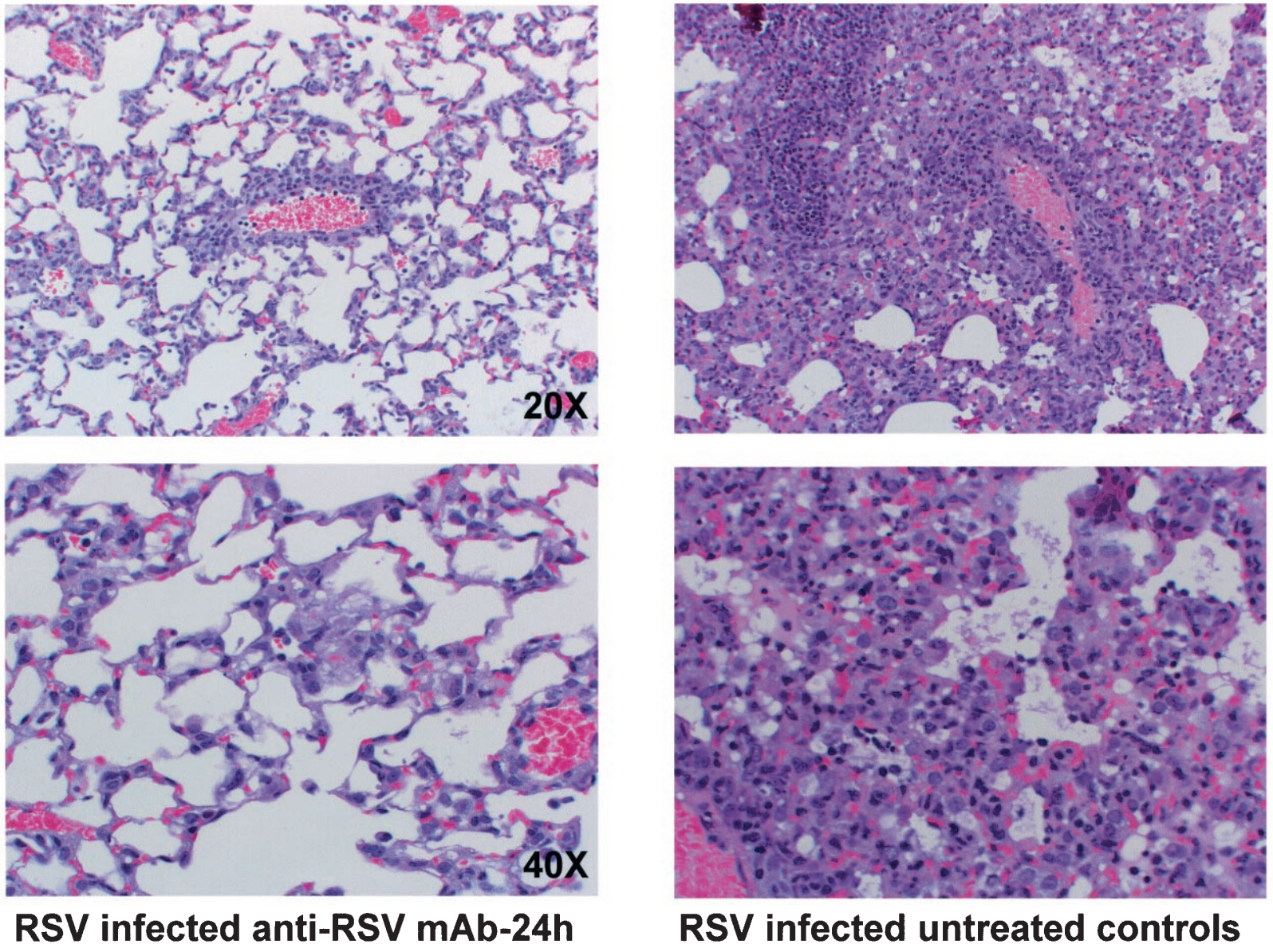

FIG. 4. Effect of the anti-RSV neutralizing MAb on the lung histopathology of RSV-infected mice. At day 5 after inoculation, lung sections from RSV-infected, untreated mice demonstrated dense perivascular mononuclear inflammatory infiltrates. Scattered areas of peribronchial mononuclear inflammation were also seen. In many areas, the air spaces contained numerous monocytes and neutrophils as well as proteinaceous fluid. Sections of lungs from RSV-infected mice treated with the anti-RSV MAb at −24 h also showed areas containing dense perivascular infiltrates consisting of mononuclear cells, as well as scattered foci of mononuclear peribronchial infiltrates. Inflammation of the air spaces, however, was much less dense and consisted mostly of intra-alveolar macrophages. No significant intra-alveolar edema was seen in mice treated with the anti-RSV MAb at −24 h.

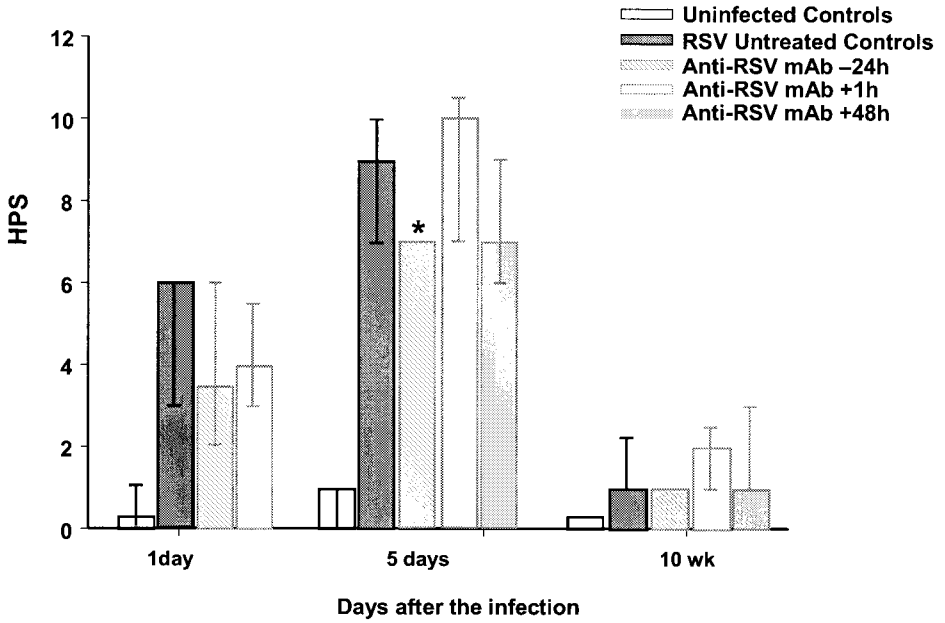

FIG. 5. Impact of treatment with the anti-RSV neutralizing MAb on lung HPS of mice infected with RSV. Mice were inoculated and treated as described in Materials and Methods. On days 1, 5, and 70 after inoculation, mice were sacrificed. Lung specimens were obtained, fixed, stained, and evaluated by a pathologist in a blinded fashion as described in Materials and Methods. Bars represent results of five independent experiments, with 16 to 20 mice per time point in each treatment group. Treatment groups consisted of uninfected controls (open bars), RSV-infected, untreated mice (dark shaded bars), and RSV-infected mice receiving the anti-RSV MAb at −24 h (hatched bars), +1 h (open bars with shaded outlines), or +48 h (light shaded bars). Values are medians; error bars, 25th to 75th percentiles. *, $P < 0.05$ by Kruskal-Wallis ANOVA on ranks for comparison with RSV-infected, untreated controls.

thology was reduced, and by decreasing the viral antigen load, the magnitude of the immune inflammatory response, which is also a factor in the severity of the illness, was diminished (7). Obviously, important aspects are still unclear. Mice that received the anti-RSV MAb at +1 or +48 h had a disease course similar to that of infected, untreated mice, and the concentrations of cytokines and chemokines measured, as well as the severity of pneumonia evaluated by HPS, were not very different in these two groups of mice compared with the infected, untreated mice. Since there were no differences in RSV loads measured in BAL samples among the different groups treated with the anti-RSV MAb, it can be argued that the minor differences in RSV loads found in lung cultures between the −24-h group and the other two groups, together with the time of administration, could have contributed to the significant differences observed in inflammatory response and disease severity. Taken together, these results also suggest that direct viral cytopathology played an initial role in the pathogenesis of RSV-induced disease in the murine model, and it appears that much of the lung injury caused by RSV infection is a result of the host inflammatory response.

TABLE 3. HPS of RSV-infected mice treated with the isotype-matched control MAb compared with those of RSV-infected, untreated controls

Group	HPS[a]		
	Day 1	Day 5	Day 70
Untreated controls	6.0 (3.0–6.0)	9.0 (7.0–10.0)	1.5 (1.0–2.5)
Isotype MAb −24 h	3.0 (2.0–5.50)	9.0 (7.0–11.0)	1.0 (1.0–2.0)
Isotype MAb +1 h	6.0 (2.0–6.0)	11.0 (8.75–11.0)	1.0 (1.0–3.0)
Isotype MAb +48 h	ND	7.0 (6.0–9.0)	1.0 (1.0–1.0)

[a] Data are medians (25th to 75th percentiles). ND, not done.

Infants with RSV-induced wheezing mount an immune response involving a cascade of cytokines, chemokines, and cell mediators that regulate local immunity. The relationships among these factors are quite complex. They operate in a coordinated manner, activating and recruiting mononuclear cells and neutrophils to the respiratory tract. A wide range of cytokines and chemokines induced by RSV have been found in the respiratory tracts of children with RSV infection, including proinflammatory TNF-α and IL-6, the TH1 cytokine IFN-γ, the beta-chemokines MIP-1α, eotaxin, and RANTES (23, 24, 33, 36, 55), and the regulatory or anti-inflammatory cytokine IL-10, recently described as a TH3 cytokine (3). TNF-α has a critical role in inflammation, but the function of this cytokine in RSV disease is not completely understood. Some authors have suggested that TNF-α has a protective role in RSV infection (35), while others have related the lung disease caused by RSV to the overproduction of TNF-α (25). In our experiments, the mice that received the neutralizing MAb at −24 h had the highest TNF-α concentrations in BAL samples but remained asymptomatic during the course of the infection. The production of TNF-α could explain, at least in part, why this group of mice, although clinically asymptomatic, had a moderate degree of lung inflammation.

Because of the similarities between asthma and RSV-induced bronchiolitis in terms of airway inflammation and wheezing, experimental models of allergen sensitization and RSV infection have been developed to elucidate the links

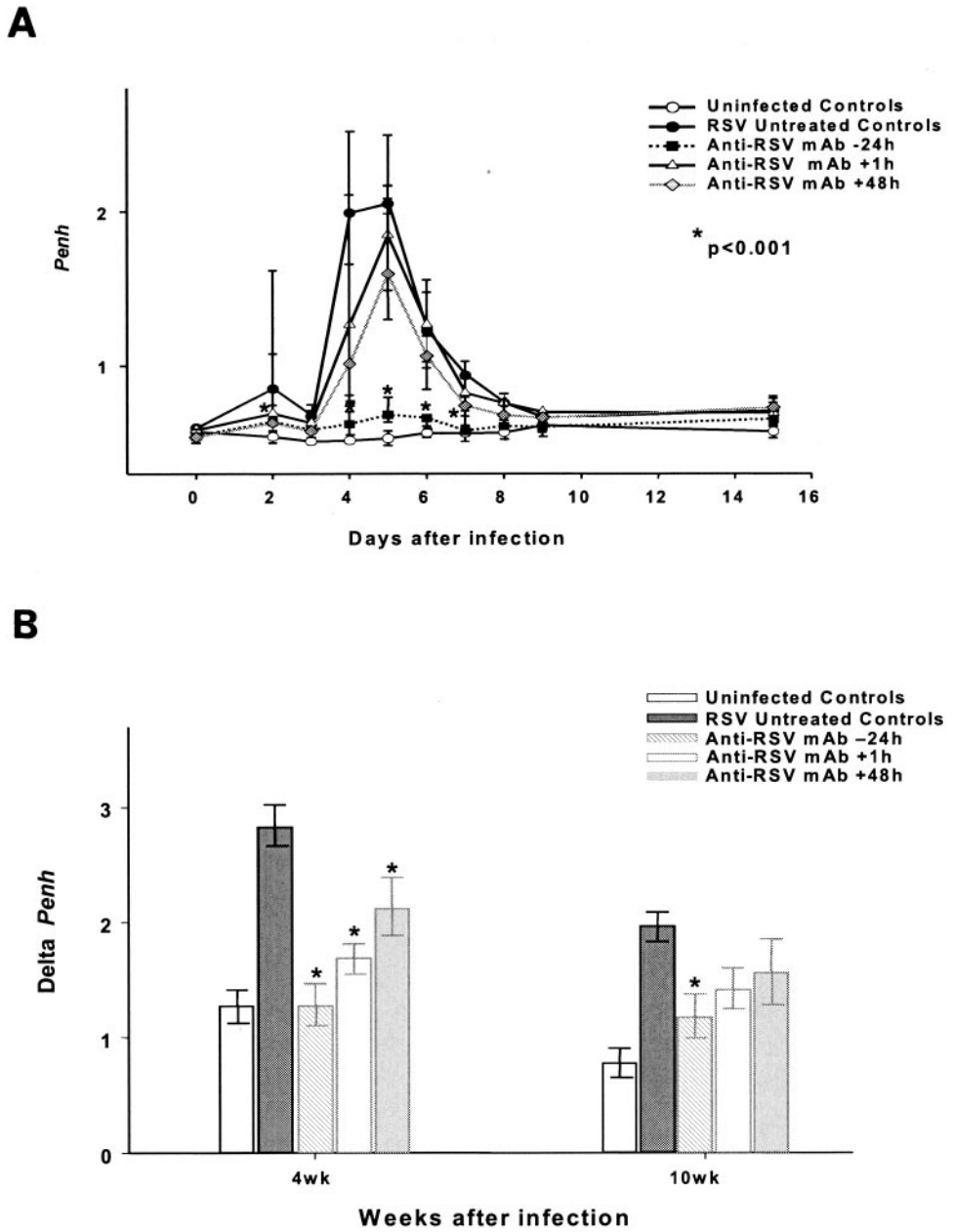

FIG. 6. Effects of the anti-RSV neutralizing MAb (palivizumab) on pulmonary dynamics. Mice were inoculated with RSV or Hep-2 cell supernatants and treated with anti-RSV as described in Materials and Methods. (A) Airway obstruction was assessed by whole-body plethysmography by measuring Penh without methylcholine challenge daily during the first 2 weeks as described in Materials and Methods. Values are medians; error bars, 25th to 75th percentiles. Each group consisted of eight mice. Results of two experiments representative of five independent experiments are shown. *, $P < 0.001$ by Kruskal-Wallis ANOVA on ranks for comparison between RSV-infected mice treated with the anti-RSV MAb at −24 h and the other groups of RSV-infected mice. (B) To assess AHR, mice were challenged weekly with aerosolized methalcholine, and delta Penh was recorded up to 10 weeks postinfection. Bars represent means for 12 to 16 mice per group; error bars, standard errors of the means. *, $P < 0.001$ by one-way ANOVA for comparison with RSV-infected, untreated controls.

between severe RSV bronchiolitis and allergic wheezing (39). Studies have demonstrated that RSV infection induced IL-13 production, which correlated with pulmonary eosinophilia and RSV-induced AHR (30). IL-10, an anti inflammatory cytokine, has been shown to be an important component in the enhanced AHR that occurs after RSV infection (31). In contrast to those animal experiments, which suggest that RSV infection elicits a type 2 immune response, there is also evidence that the IFN-γ response plays a significant role in RSV-induced AHR (37, 40, 53). Our experiments showed that RSV infection, without allergic sensitization, elicited a type 1 response as well as the production of IL-10 and beta-chemokines. This immune response was significantly reduced in the group of mice that received the anti-RSV MAb before inoculation. These cytokines may act in a coordinated manner, and the chemokines released may even be responsible for the pattern of cytokines

present. Whether any of these cytokines plays a key role in RSV pathogenesis is still unclear. The reduced concentrations of IL-10 in BAL fluids from mice treated with the isotype-matched control MAb was an isolated finding of unclear significance at this point. The rest of the variables evaluated in this group of mice, such as RSV loads, HPS, Penh (airway obstruction), and delta Penh (AHR), were similar to values measured in RSV-infected, untreated mice.

The mechanisms by which RSV causes airway inflammation and hyperreactivity have not been fully elucidated. Previous studies have demonstrated that RSV lower respiratory tract infection causes a strong potentiation of neurogenically mediated inflammation, which has been proposed to play an important role in the pathogenesis of asthma (42). It has been demonstrated that the combination of an anti-RSV MAb with an antibody directed against substance P, a neuropeptide produced by afferent neurons and a variety of immune cells that affects inflammation by mediating vasodilation, given either as treatment or as prophylaxis, significantly reduced pulmonary inflammation (22). However, the long-term effect of this therapeutic combination on pulmonary function has not been evaluated. Other investigators have demonstrated a protective role of palivizumab on RSV-induced neurogenic inflammation, as determined by capsaicin sensory nerve-mediated extravasation in F-344 rats (43).

Recent studies in the cotton rat model have shown that palivizumab, when given after infection, reduced viral replication without altering the severity of inflammation. In contrast, when the anti-RSV MAb was administered in combination with systemic steroids, both viral replication and inflammatory changes were greatly reduced (38, 44). In our study, the anti-RSV MAb administered prior to inoculation had effects on both viral replication and pulmonary inflammation. These results would predict that the anti-RSV neutralizing MAb administered prophylactically would offer the greatest protection against RSV lower respiratory tract illness, suggesting that prophylaxis of RSV infection may have long-term effects on respiratory and immunologic parameters relevant to the development of RSV-induced AHR. Nevertheless, even when mice treated with the anti-RSV MAb on day 2 after infection did not recover from the acute disease more rapidly than untreated mice, the long-term consequences of RSV in the lower respiratory tract were diminished, as evidenced by a significant reduction in AHR documented at 4 weeks after infection.

Even though administration of the neutralizing antibody as a prophylaxis had a clear impact on pulmonary function and viral replication, it did not completely suppress the effect of RSV in the lower respiratory tract, as demonstrated by the presence of virus in lung specimens and the observation that although histologic abnormalities and cytokine concentrations were significantly modulated, they were still elevated compared with those for uninfected control mice. Thus, the antibody did not prevent establishment of the infection, but it modulated its effects. It will be critical in future studies to analyze the sequence of events occurring in the upper and lower portions of the respiratory tract in the first few hours after inoculation in order to ascertain which of these early events determine whether the infection progresses into the lower respiratory tract and causes more severe disease manifestations, and by what mechanisms they do so.

In conclusion, the present study in the mouse model demonstrated that decreasing RSV replication in the lower respiratory tract resulted in significantly reduced manifestations of acute disease and a remarkable attenuation of the long-term airway disease induced by RSV infection. Future studies to determine whether strategies aimed at preventing or reducing RSV replication could decrease the long-term morbidity associated with RSV infection in children should be considered.

ACKNOWLEDGMENTS

This work was supported in part by grants by MedImmune Inc. and the American Lung Association to O.R. A.M. was supported in part by the Ausonia & Arbora grant from the Asociación Española de Pediatría.

We thank Lonnie Roy and Naveed Ahmad for excellent statistical advice.

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