Post-Infection A77-1726 Blocks Pathophysiologic Sequelae of Respiratory Syncytial Virus Infection

Ian C. Davis[1]*, Eduardo R. Lazarowski[2], Fu-Ping Chen[3], Judy M. Hickman-Davis[1‡], Wayne M. Sullender[3,4], and Sadis Matalon[1,4,5]

Departments of [1]Anesthesiology, [3]Pediatrics, [4]Microbiology, and [5]Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama; and [2]Department of Medicine, University of North Carolina, Chapel Hill, North Carolina

Despite respiratory syncytial virus (RSV) bronchiolitis remaining the most common cause of lower respiratory tract disease in infants worldwide, treatment has progressed little in the past 30 years. The aim of our study was to determine whether post-infection administration of *de novo* pyrimidine synthesis inhibitors could prevent the reduction in alveolar fluid clearance (AFC) and hypoxemia that occurs at Day 2 after intranasal infection of BALB/c mice with RSV. BALB/c mice were infected intranasally with RSV strain A2. AFC was measured in anesthetized, ventilated mice after instillation of 5% bovine serum albumin into the dependent lung. Post-infection systemic treatment with leflunomide has no effect on AFC. However, when added to the AFC instillate, leflunomide's active metabolite, A77-1726, blocks RSV-mediated inhibition of AFC at Day 2. This block is reversed by uridine (which allows pyrimidine synthesis via the scavenger pathway) and not recapitulated by genistein (which mimics the tyrosine kinase inhibitor effects of A77-1726), indicating that the effect is specific for the *de novo* pyrimidine synthesis pathway. More importantly, when administered intranasally at Day 1, A77-1726, but not its vehicle dimethyl sulfoxide, maintains its beneficial effect on AFC and lung water content until Day 2. Intranasal instillation of A77-1726 at Day 1 also reduces bronchoalveolar lavage nucleotide levels, lung inflammation, and hypoxemia at Day 2 without impairing viral replication at Day 2 or viral clearance at Day 8. Post-infection intranasal or aerosolized treatment with pyrimidine synthesis inhibitors may provide symptomatic relief from the pathophysiologic sequelae of impaired AFC in children with RSV bronchiolitis.

Keywords: paramyxovirus; leflunomide; dihydroorotate dehydrogenase; pulmonary edema

Respiratory syncytial virus (RSV) remains the most common cause of lower respiratory tract infection in infants and children worldwide (1), and recent studies indicate that it has a disease impact comparable to that of nonpandemic influenza A in the elderly (2). However, while infant hospitalization rates for RSV increased 2.4-fold from 1980 to 1996 (3), treatment for infants with moderate to severe RSV bronchiolitis has progressed little in the past 30 years: supplemental oxygen and periodic suctioning to remove excess nasopharyngeal secretions provide clear benefit, but more specific pharmacologic therapies are moderately successful at best (4). The need for new approaches to RSV treatment is therefore paramount.

CLINICAL RELEVANCE

Herein we show that prophylactic intranasal administration of A77-1726, an agent that decreases UTP levels, prevents the respiratory syncytial virus–induced decrease of alveolar fluid clearance and onset of arterial hypoxemia in mice.

The interface between the respiratory epithelium and the air is normally bathed by a thin layer of fluid, the airspace lining fluid (ALF). To permit efficient gas exchange in the bronchoalveolar compartment and effective mucociliary clearance in the airways, the depth of this layer must be tightly regulated. Active transport of sodium (Na^+) ions from the ALF to the interstitial space by bronchoalveolar epithelial cells is critical to the regulation of ALF thickness (5). Inhibition of active Na^+ transport can result in formation of an excessive volume of ALF, impairment of gas exchange (6), narrowing of airway lumens (7), and dilution of the surface-active materials that stabilize small airways (8). The resultant small airway obstruction, which would be exacerbated by any intercurrent inflammatory process, such as that occurring during RSV bronchiolitis, would be predicted to be most severe in infancy and early childhood when airway diameter is lowest.

Moderately severe RSV bronchiolitis is commonly associated with signs of respiratory distress, and admission decisions are often based upon clinical evidence of hypoxemia (4). The underlying causes of hypoxemia in RSV bronchiolitis have not been determined, but its presence must perforce indicate either hypoventilation of the bronchoalveolar compartment (as a result perhaps of airway obstruction by fluid secretions, mucus, inflammatory infiltrates, or necrotic cell debris), an abnormal alveolar ventilation–perfusion ratio, and/or diminished respiratory membrane diffusion. Our previous findings suggest that bronchoalveolar edema, occurring as a consequence of reduced active Na^+ transport by the respiratory epithelium, may be an unrecognized component of RSV disease that plays a role in development of hypoxemia, either by impairing alveolar gas exchange or by contributing to obstruction of small airways (9). We have found that infection of BALB/c mice with RSV significantly impairs alveolar fluid clearance (AFC), an *in vivo* measure of active Na^+ transport in the whole lung, at early time points after infection (by 43% from mock-infected values at Day 2) (10). This decrease in AFC is mediated by *de novo* synthesized UTP acting on P2Y purinergic receptors, and is rapidly reversed by addition to the AFC instillate of agents that degrade UTP, but not ATP (9, 10). Our studies also demonstrated that RSV-mediated nucleotide release, AFC inhibition, and physiologic sequelae thereof can be prevented by pretreatment of mice with the *de novo* pyrimidine synthesis inhibitor leflunomide (9). These findings suggested that inhibitors of *de novo* pyrimidine synthesis, while not being antiviral, might

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*Present affiliation: Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio

‡Present affiliation: Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, Ohio

Correspondence and requests for reprints should be addressed to Sadis Matalon, Ph.D., Department of Anesthesiology, University of Alabama at Birmingham, BMR II, Rm. 224, 901 19th Street South, Birmingham, AL 35205-3703. E-mail: sadis@uab.edu

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be useful in alleviating symptoms of RSV bronchiolitis. However, as a consequence of its poor pharmacodynamic profile (11), leflunomide is only effective in abrogating effects of RSV on AFC when given as a systemic pretreatment regimen. This renders it less valuable as a potential therapy for RSV bronchiolitis. The aim of the current study was to determine whether post-infection treatment with the active metabolite of leflunomide, A77-1726, could produce similar beneficial effects on AFC, lung edema, and hypoxemia in a murine model of RSV infection. Some of the results of these studies have previously been reported in the form of abstracts (12–14).

MATERIALS AND METHODS

Preparation of Viral Inocula and Infection of Mice

Preparation of viral stocks and intranasal infection of 8- to 12-week-old pathogen-free BALB/c mice of either sex with RSV strain A2 (10^6 plaque-forming units in 100 μl) were performed as previously described (10). All mouse procedures were approved by the UAB and OSU Institutional Animal Care and Use Committees.

Reagents

Leflunomide, A77-1726, genistein, and amiloride were reconstituted in dimethyl sulfoxide (DMSO), aliquoted, and stored frozen. Uridine was reconstituted in sterile normal saline. All reagents were from Sigma-Aldrich (St. Louis, MO).

AFC Measurements

AFC was measured as previously described (10). Briefly, mice were anesthetized with diazepam (1.75 mg/100 g, intraperitoneally; Abbott Laboratories, Abbott Park, IL) followed 6 minutes later by ketamine (45 mg/100 g, intraperitoneally) and were placed on a heating pad (Braintree Scientific, Cambridge, MA). The trachea was exposed and cannulated with a trimmed 18-gauge intravenous catheter, which was then connected to a mouse respirator (model 687; Harvard Apparatus, Holliston, MA). Mice were paralyzed with pancuronium bromide (0.04 mg, intraperitoneally; Gensia Pharmaceuticals, Irvine, CA) and ventilated with 100% O_2 with a 200-μl tidal volume (8–10 ml/kg body wt) at 160 breaths/minute. Once stable anesthesia was obtained, mice were positioned in the left decubitus position, and 300 μl of isosmolar NaCl containing 5% fatty acid–free bovine serum albumin was instilled via the tracheal cannula, followed by 100 μl of room air to clear dead space. After instillation, mice were ventilated for a 30-minute period, then the alveolar fluid was aspirated. AFC was calculated from the ratio between the protein concentration of the instillate before instillation and of the alveolar sample at 30 minutes.

All reagents were added to the AFC instillate from stock solutions directly before instillation, in a minimal volume of solvent (1–10 μl/ml). Previous studies have demonstrated that mock infection has no effect on AFC or other measured lung parameters, and that measured declines in AFC are not a consequence of instillate dilution by intrapulmonary edema fluid, but require replication-competent virus (10). No sex difference in AFC rate has been found in BALB/c mice.

Topical (Intranasal) Inhibition of *De Novo* Pyrimidine Synthesis

Mice were lightly anesthetized. One hundred microliters of saline, containing 0.5 μl A77-1726 in DMSO, was administered dropwise, via both nares, at Day 1 after RSV infection. Control animals received an equal volume of saline + DMSO. Mice were placed in lateral recumbency, allowed to recover, and returned to their cage.

Peripheral Blood Arterial Oxygen Saturation

Sp_{O_2} was measured in conscious mice using the MouseOx system (Starr Life Sciences Corp., Allison Park, PA), in accordance with manufacturer's instructions. To ensure accurate sensor placement, Sp_{O_2} data points were excluded from analysis if one of the four measured parameters (Sp_{O_2}, pulse rate, pulse distension, and respiratory rate) received an error code during measurement. Data were collected for a minimum of 10 s (150 data points) per sample. Arterial P_{O_2} was estimated from Sp_{O_2} using the Ventworld interactive oxyhemoglobin dissociation curve tool (http://www.ventworld.com/resources/oxydisso/oxydisso.html), assuming a normal blood pH and arterial P_{CO_2}.

Measurement of Airway Resistance

Mice were anesthetized using the same regimen as for AFC studies. Total lung resistance was measured in mice undergoing mechanical ventilation at 150 breaths per minute on a computer-controlled piston ventilator (flexiVent; Scireq Scientific Respiratory Equipment Inc., Montreal, PQ, Canada), with 3 cm H_2O PEEP, as previously described (15). R was recorded after performance of two TLC maneuvers to standardize volume history and administration of normal saline by Aeroneb nebulizer. Data were analyzed using the single compartment model. Female mice only were used in these studies, since male mice exhibit exaggerated AHR responses to methacholine (MCH) (16).

Other Procedures

All other procedures were performed as previously described (10, 17, 18).

Statistical Analyses

Descriptive statistics were calculated using Instat software (GraphPad, San Diego, CA). Differences between group means were analyzed by ANOVA, with Tukey-Kramer multiple comparison post-tests. All data values are presented as mean ± SEM.

RESULTS

Effect of Post-Infection Systemic Leflunomide Treatment on RSV-Mediated Inhibition of AFC at Day 2

Previously, we had shown that, in the BALB/c mouse model, pretreatment with the dihydro-orotate dehydrogenase inhibitor leflunomide by oral gavage for 7 d blocked RSV-mediated inhibition of AFC at Day 2 after infection (when AFC is most impaired) (9). However, we have found in subsequent studies that post-infection gavage with leflunomide at Day 1 had no beneficial effect on AFC at Day 2 (mean AFC rate 20.5 ± 2.3% in leflunomide-treated mice, $n = 15$, versus 21.4 ± 1.2% in untreated animals, $n = 19$, 34.2 ± 1.1% in uninfected animals, $n = 10$). AFC rates in uninfected mice and at Day 2 in the current study are comparable to those that we have reported previously using this model (9, 10).

Effect of Topical Nucleotide Synthesis Inhibition on RSV-Mediated Inhibition of AFC at Day 2

We hypothesized that the lack of effect of post-infection leflunomide treatment at Day 1 on AFC at Day 2 was a result of the need for its metabolism to an active form, and of poor bioavailability in the lungs after a single dose (since 90% of the drug remains bound to plasma proteins [11]). We therefore decided to investigate the effects of intrapulmonary administration of the active metabolite of leflunomide, A77-1726, on AFC after RSV infection. In agreement with our hypothesis, addition of 25 μM A77-1726 to the AFC instillate resulted in complete blockade of RSV-induced suppression of AFC at Day 2 and returned AFC to control levels (Table 1). A77-1726 also restored normal amiloride sensitivity to AFC at Day 2: amiloride reduced AFC by 56% in the presence of A77-1726 at Day 2, while our previous studies (10) have shown that amiloride reduces AFC by 61% in uninfected mice and has no effect on AFC at Day 2 in untreated, RSV-infected mice.

Dihydro-orotate dehydrogenase inhibitors such as A77-1726 also have nonspecific tyrosine kinase inhibitory activity (19). However, the beneficial effect of A77-1726 on AFC at Day 2 was not replicated by the broad-spectrum tyrosine kinase inhibitor genistein, even at a concentration of 25 μM (Table 1),

TABLE 1. EFFECT OF ADDITION OF INHIBITORS OF *DE NOVO* PYRIMIDINE SYNTHESIS TO THE ALVEOLAR FLUID CLEARANCE INSTILLATE ON ALVEOLAR FLUID CLEARANCE AT DAY 2 AFTER RESPIRATORY SYNCYTIAL VIRUS INFECTION

Treatment	Concentration (μM)	*n**	% AFC_{30}†
None	—	19	21.4 ± 1.2
A77-1726	25	18	34.1 ± 1.1‡
A77-1726 + Amiloride	25 + 1500	6	15 ± 3.2
Genistein	25	7	20.4 ± 0.7
A77-1726 + Uridine	25 + 10	13	24 ± 1.8
Uridine	10	7	21.8 ± 1.9

Definition of abbreviation: AFC, alveolar fluid clearance.
* Number of mice in which AFC was evaluated.
† Mean % AFC over 30 min ± SEM.
‡ % AFC_{30} in uninfected mice is 34.2 ± 1.1 (n = 10) ($P < 0.0005$ versus untreated AFC at Day 2).

which is far greater than that which has been shown previously to be effective at tyrosine kinase blockade in a rat AFC model (1 μM) (20). Furthermore, blockade of RSV-mediated AFC inhibition by A77-1726 was reversed by concomitant addition of 10 μM uridine to the instillate, which allows pyrimidine synthesis via a salvage pathway, indicating the A77-1726 block is specific to the *de novo* pyrimidine synthesis pathway. Uridine alone had no effect on AFC in RSV-infected mice.

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on RSV-Mediated Inhibition of AFC at Day 2

Since topical, post-infection treatment with A77-1726 appeared effective in blocking RSV-mediated inhibition of AFC at Day 2 after infection, we wished to determine whether A77-1726 might provide prolonged therapeutic benefit. When A77-1726 (10–50 μM, but not 1 μM, in DMSO) was administered intranasally at Day 1 after infection, its blocking effect on RSV-induced inhibition of AFC persisted for at least 24 hours: AFC remained at control levels at Day2, even in the absence of further addition of A77-1726 to the AFC instillate (Figure 1A). In contrast, administration of an equivalent volume of DMSO in saline (100 μl of 5 μl/ml solution) alone intranasally at Day 1 had no beneficial effect on AFC at Day 2. However, in spite of this ability to restore normal AFC to RSV-infected mice, intranasal treatment with 10 or 50 μM A77-1726 did decrease AFC in uninfected mice 24 hours later, by 19% and 34%, respectively (Figure 1B). This effect is comparable to that which we observed previously after treatment of uninfected mice with leflunomide (9).

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on Lung Water Content at Day 2

Treatment of mice with 50 μM A77-1726 by intranasal instillation at Day 1 after infection restored normal lung wet:dry weight ratios at Day 2 (Figure 2), but had no significant effect on lung water content in mock-infected mice (mean wet:dry weight ratio 4.54 ± 0.05 in A77-1726–treated mice, versus 4.67 ± 0.03 in untreated animals, n = 8 for both groups). In contrast, intranasal treatment with an equivalent volume of DMSO in saline (100 μl of 5 μl/ml solution) at Day 1 had no beneficial effect on wet:dry weight ratios at Day 2.

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on Bronchoalveolar Lavage Nucleotide Levels at Day 2

Previously, we demonstrated that RSV infection resulted in a doubling of bronchoalveolar lavage (BAL) UTP and ATP

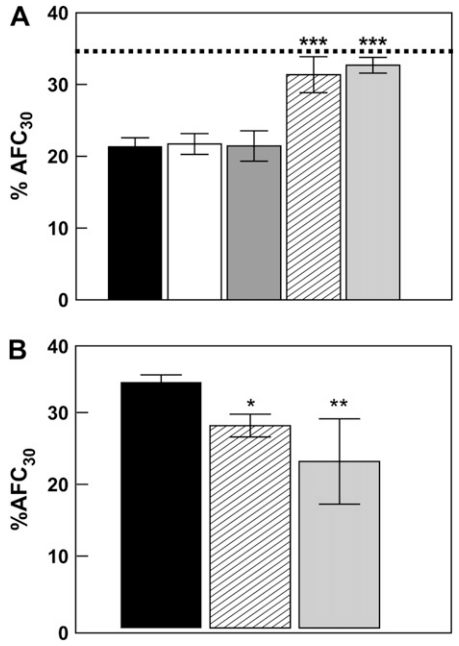

Figure 1. Effect of intranasal A77-1726 treatment at Day 1 after RSV infection on AFC at Day 2. (*A*) Effect of intranasal instillation of 100 μl saline containing DMSO vehicle (*open bar*), or 1 (*darkly shaded bar*), 10 (*hatched bar*), and 50 (*lightly shaded bar*) μM A77–1726 at d1 after RSV infection on % AFC over 30 minutes at Day 2 (n = 19 for untreated mice [*solid bar*]; n = 7 for DMSO; n = 9 for 1 μM A77–1726; n = 7 for 10 μM A77–1726; n = 14 for 50 μM A77-1726). *Dotted line* indicates mean AFC rate in untreated, uninfected mice (n = 10). (*B*) Effect of intranasal instillation of 100 μl saline containing 10 (*hatched bar*) or 50 (*shaded bar*) μM A77-1726 on % AFC over 30 minutes in uninfected mice, 24 hours later (n = 10 for untreated mice [*solid bar*]; n = 9 for 10 μM A77-1726–treated mice; n = 10 for 50 μM A77-1726–treated mice). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, compared with untreated mice.

levels at Day 2 (9). Intranasal treatment with 50 μM A77–1726 at Day 1 after infection returned BAL UTP and ATP content at Day 2 to levels comparable to those in untreated, uninfected mice (Figures 3A and 3B). These findings are consistent with the observation that *de novo* purine and pyrimidine synthesis pathways are usually concordantly regulated (reviewed in Ref. 21). Interestingly, despite causing mild suppression of AFC in uninfected mice 24 hours later, intranasal treatment of uninfected mice with A77-1726 had no stimulatory effect on BAL nucleotide content. This differs from our previous finding after treatment of uninfected mice with leflunomide,

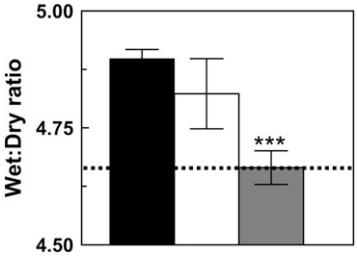

Figure 2. Effect of intranasal A77-1726 treatment at Day 1 after RSV infection on lung water content at Day 2. Effect of intranasal instillation of 100 μl saline containing 0.5 μl DMSO (*open bar*) or 50 μM A77-1726 (*shaded bar*) at Day 1 on lung wet:dry weight ratio at Day 2 after RSV infection (n = 7–8 per group). *Solid bar*, Day 2. *Dotted line* indicates mean wet:dry weight ratio in untreated, mock-infected animals (n = 8). ***$P < 0.0005$, compared with untreated mice.

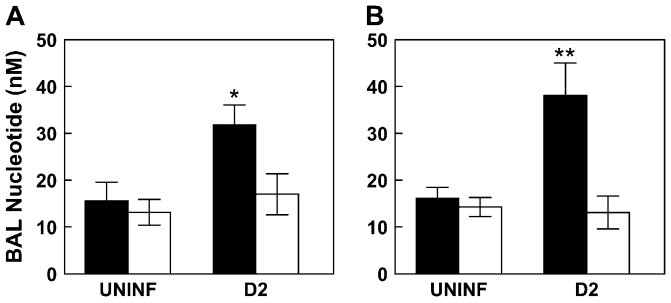

Figure 3. Effect of intranasal A77-1726 treatment at Day 1 after RSV infection on BAL nucleotide levels at Day 2. (*A*) Bal UTP levels ($n = 10$ for uninfected, untreated mice; $n = 7$ for uninfected, A77-1726–treated mice; $n = 14$ for RSV-infected, untreated mice; $n = 11$ for RSV-infected, A77-1726–treated mice). (*B*) BAL ATP levels ($n = 11$ for uninfected, untreated mice; $n = 7$ for uninfected, A77-1726–treated mice; $n = 14$ for RSV-infected, untreated mice; $n = 12$ for RSV-infected, A77-1726–treated mice). BAL nucleotide levels for untreated groups were previously published (9), although samples for all groups were collected contemporaneously. $^*P < 0.05$, $^{***}P < 0.005$, compared with uninfected, untreated mice. *Solid bars*, untreated mice; *open bars*, mice treated with A77-1726.

when suppression of AFC was associated with elevated BAL nucleotide levels (9).

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on Peripheral Blood Oxygen Saturation at Day 2

RSV had a very significant overall effect on peripheral blood oxygen saturation (Sp_{O_2}) in conscious mice over the 8-d infection period ($P < 0.0005$ by ANOVA), and impairment of AFC at Day 2 was temporally associated with a statistically significant ($P < 0.05$, by Tukey-Kramer post-test) reduction (1.95%) in Sp_{O_2} (Figure 4). This corresponds to a decline in arterial P_{O_2} from 82 to 71 mm Hg. No change in either respiratory rate or heart rate was detected at any time after infection (data not shown). Treatment of mice with 50 μM A77-1726 by intranasal instillation at Day 1 prevented the decline in Sp_{O_2} readings seen in untreated, RSV-infected, conscious mice at Day 2, but had no significant effect on Sp_{O_2} at later time points after infection. Mean Sp_{O_2} values in A77-1726–treated RSV-infected mice at Day 2 were significantly higher than in untreated animals. In contrast, intranasal treatment with an equivalent volume of DMSO in saline at Day 1 had no beneficial effect on Sp_{O_2} at d2. Finally, intranasal treatment of uninfected mice with 10 μM A77–1726 did not result in hypoxemia 24 hours later (mean Sp_{O_2} 96.25 ± 0.3%, $n = 10$), despite causing a 19% decrease in AFC.

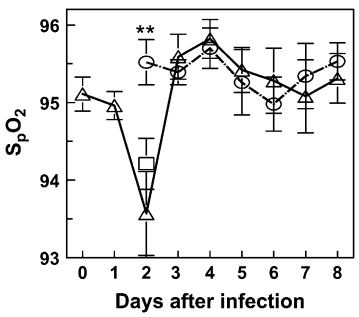

Figure 4. Effect of RSV infection and intranasal A77-1726 treatment at Day 1 after RSV infection on peripheral oxygen saturation. Time course of effect of RSV infection and intranasal instillation of 100 μl saline containing 0.5 μl of DMSO (*squares*) or 50 μM A77-1726 (*circles*) at Day 1 on Sp_{O_2} in conscious BALB/c mice (n = 10–20 per day). $^{**}P < 0.005$, compared with untreated mice (*triangles*).

Effect of RSV Infection and Intranasal A77-1726 Treatment at Day 1 on Baseline Airway Resistance

Infection with RSV resulted in a significant increase in baseline airway resistance at Day 2, but not at other time points after infection (Figure 5). Increased airway resistance at Day 2 was reversed by intranasal administration of 50 μM A77-1726 at Day 1.

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on BAL Cytokines and Chemokines

Post-infection intranasal treatment with 50 μM A77-1726 had a very limited effect on BAL cytokine and chemokine responses after RSV infection (Table 2). Interestingly, treatment with the A77-1726 vehicle DMSO at Day 1 had some proinflammatory effects (induction of BAL IL-1β and TNF-α at Day 2, and of TNF-α at Day 4). These proinflammatory effects of the vehicle at Day 2 were abrogated by A77-1726 itself, resulting in normalization of IL-1β levels and complete suppression of TNF-α. However, the anti-inflammatory effect of A77-1726 on TNF-α did not persist until Day 4. Intranasal A77-1726 treatment at Day 1 also significantly reduced BAL keratinocyte cytokine (KC) (murine homolog of CXCL8) chemokine levels at Day 2 after RSV infection, but not later time points. However, DMSO treatment had a similar effect. Some limited suppression of CCL3 (MIP-1α) was seen after A77-1726 treatment, but only at Day 8. Neither DMSO nor A77-1726 treatment had any effect on BAL IFN-γ or CCL5 (RANTES) levels at any time point after infection. A77-1726 treatment had no significant effect on the minimal levels of proinflammatory cytokines and chemokines detectable in BAL fluid from mock-infected mice.

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on BAL Cell Counts

Post-infection intranasal treatment of mice with 50 μM A77-1726 also resulted in a significant reduction in BAL total cell counts at Day 2 (Figure 6A), although numbers remained elevated above those in uninfected mice. The decline in BAL cellularity was primarily due to a drop in BAL alveolar macrophages—no significant changes in BAL neutrophil or lymphocyte counts were observed after intranasal A77-1726 treatment (Figure 6B).

Effect of Intranasal A77-1726 Treatment at Day 1 after RSV Infection on RSV Replication in Mouse Lungs

Treatment of mice with DMSO or 50 μM A77-1726 by intranasal instillation at Day 1 had no effect on viral growth at any time point after infection (Figure 7). Moreover, unlike with leflunomide treatment (10), viral replication was not prolonged to Day 8—replication of RSV was undetectable at Day 8 in untreated, intranasal DMSO-treated, and intranasal A77-1726–treated animals.

DISCUSSION

Bronchiolitis results in estimated hospitalization rates of 30 per 1,000 children younger than 1 year old in the United States and Europe (22), and RSV remains its most common underlying cause (23). There is much controversy regarding the best approach to treatment of RSV bronchiolitis (24). β-adrenergic agonists are frequently prescribed, primarily as bronchodilators, but there is little conclusive evidence that they are effective (25), and recent studies suggest that RSV may induce insensitivity to their actions (26). Likewise, systemic corticosteroids (27) and ribavirin (28) appear to provide no clinical benefit in

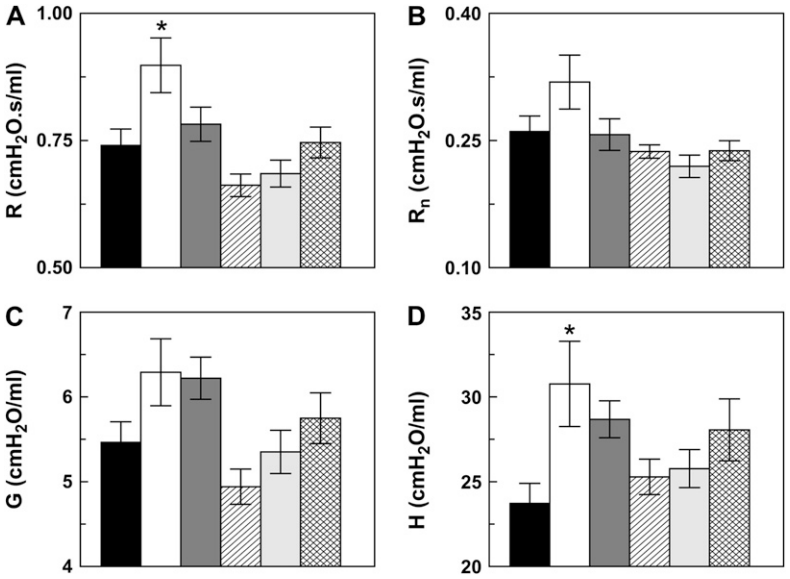

Figure 5. Effect of RSV infection and intranasal A77-1726 treatment at Day 1 after RSV infection on baseline airway resistance. Time course of effect of RSV infection on total lung resistance (R) in BALB/c mice undergoing mechanical ventilation at 150 breaths per minute on a computer-controlled flexiVent piston ventilator with 3 cm H_2O PEEP (n = 10–20 per day). Also shown is effect of intranasal instillation of 100 μl saline containing 50 μM A77-1726 at Day 1 on R at Day 2. *$P < 0.05$, compared with uninfected mice (*solid bars*). *Open bars*, Day 2; *darkly shaded bars*, Day 2 + A77; *hatched bars*, Day 4; *lightly shaded bars*, Day 6; *cross-hatched bars*, Day 8.

children with bronchiolitis. Finally, trials of surfactant, immunoglobulins, heliox, vitamin A, interferon, and erythropoietin in the ICU setting have been inconclusive (29). Only supportive care (supplemental oxygen, suctioning, and parenteral fluid replacement) appears beneficial (4). In these circumstances, new and effective approaches to therapy are urgently needed.

Previously, we had shown that we could prevent RSV-mediated nucleotide release, AFC inhibition, and the physiologic sequelae thereof, including hypoxemia, by systemic pretreatment of BALB/c mice with leflunomide (an inhibitor of *de novo* pyrimidine synthesis) for 7 days (9). However, we found that, as a consequence of its poor pharmacodynamic profile (11), leflunomide is completely ineffective in abrogating effects of RSV on AFC when given after infection. In contrast, its active metabolite, A77-1726, was effective as both a topical treatment (added to the AFC instillate) and as an intranasal pretreatment (administered at Day 1 after infection) in blocking RSV-mediated inhibition of AFC at Day 2.

Results of our previous studies with leflunomide had suggested that UTP release from the respiratory epithelium is limited by UTP availability in cells: the pool of UTP that is normally available for release is small, and primarily derived from *de novo* pyrimidine synthesis (9). The effects of A77-1726 on AFC, when this drug is added directly to the AFC instillate at Day 2, tend to support this contention. For RSV-mediated inhibition of AFC to be blocked so rapidly under these conditions, the extracellular UTP that is activating P2Y receptors must be derived from continuous *de novo* synthesis, since the uridine salvage pathway is not sensitive to inhibition by A77-1726. Furthermore, this result suggests that the half-life of extracellular UTP is very short and that maintenance of the elevated extracellular steady-state UTP concentrations required to inhibit AFC necessitates a constant efflux of newly synthesized intracellular UTP: if the extracellular UTP that mediates AFC inhibition has a prolonged half-life in the bronchoalveolar space, or were derived from an intracellular pool independent

TABLE 2. EFFECT OF INTRANASAL TREATMENT WITH DIMETHYL SULFOXIDE OR 50 μM A77-1726 AT DAY 1 AFTER RESPIRATORY SYNCYTIAL VIRUS INFECTION ON BRONCHOALVEOLAR LAVAGE CYTOKINE AND CHEMOKINE LEVELS

	*n**	IL-1β†	TNF-α†	IFN-γ†	KC†	CCL5†	CCL3†
Uninfected	14	29 ± 10	0	0	63 ± 14	36 ± 19	25 ± 1
Mock-infected	6	47 ± 14	0	0	55 ± 16	26 ± 6	31 ± 3
Mock, DMSO-Tx	6	40 ± 13	0	0	122 ± 33	11 ± 11	28 ± 7
Mock, A77-1726-Tx	6	27 ± 5	25 ± 15	0	116 ± 15	20 ± 3	30 ± 2
Day 2	14	106 ± 19	140 ± 25	0	777 ± 50	508 ± 23	71 ± 8
Day 2, DMSO-Tx	6	266 ± 20‡	366 ± 48‡	0	554 ± 62‡	531 ± 12	92 ± 9
Day 2, A77-1726-Tx	12	95 ± 20	0‡	0	588 ± 42‡	571 ± 25	81 ± 7
Day 4	6	35 ± 13	0	598 ± 99	181 ± 25	208 ± 30	81 ± 13
Day 4, DMSO-Tx	6	18 ± 7	110 ± 9‡	699 ± 82	162 ± 15	203 ± 21	85 ± 10
Day 4, A77-1726-Tx	6	79 ± 18	105 ± 30‡	582 ± 83	164 ± 20	212 ± 17	106 ± 10
Day 6	6	61 ± 13	0	398 ± 83	78 ± 12	117 ± 6	121 ± 24
Day 6, DMSO-Tx	6	101 ± 24	0	300 ± 72	95 ± 8	76 ± 18	125 ± 16
Day 6, A77-1726-Tx	6	169 ± 24	0	368 ± 131	164 ± 42	55 ± 27	133 ± 20
Day 8	6	33 ± 5	0	270 ± 16	153 ± 19	114 ± 19	177 ± 33
Day 8, DMSO-Tx	6	32 ± 9	0	330 ± 42	146 ± 20	108 ± 9	147 ± 46
Day 8, A77-1726-Tx	6	81 ± 18	0	196 ± 37	158 ± 20	88 ± 11	26 ± 11‡

Definition of abbreviations: BAL, bronchoalveolar lavage; KC, keratinocyte cytokine.

* Number of mice in which BAL cytokine/chemokine levels were measured.

† Mean concentration in BAL fluid ± SEM (pg/ml)

‡ $P < 0.0005$ versus untreated mice at the same time point.

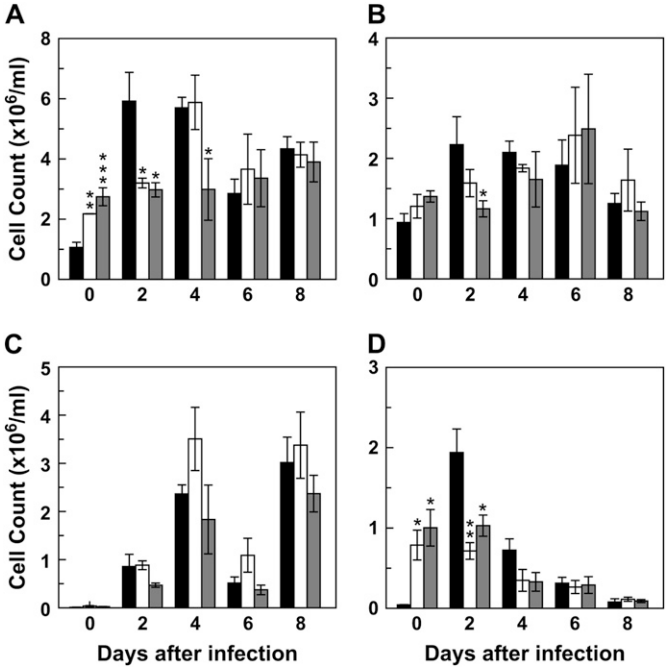

Figure 6. Effect of intranasal A77-1726 treatment at Day 1 after RSV infection on BAL cell counts. (*A*) Total counts. (*B*) Alveolar macrophage counts. (*C*) Lymphocyte counts. (*D*) Neutrophil counts (n = 6–8 per group). $^{**}P < 0.005$, $^{***}P < 0.0005$, compared with uninfected mice. $^{*}P < 0.05$, compared with untreated, RSV-infected mice at Day 2. *Solid bars*, untreated; *open bars*, DMSO; *shaded bars*, A77.

of active *de novo* pyrimidine synthesis (either a storage pool, or one resulting from salvage synthesis), then there would be sufficient UTP to mediate AFC inhibition for at least the duration of the AFC measurement (30 min) and topical A77-1726 would have no effect. Indeed, previous *in vitro* studies have demonstrated that UTP and ATP are rapidly degraded by ectonucleotidases at the respiratory mucosal surface (30), which supports our contention that continuous *de novo* nucleotide synthesis is necessary to maintain AFC inhibition.

We have previously shown that infection of mice with RSV resulted in mild hypoxemia in conscious mice at Day 2, which was reversible following pretreatment with leflunomide (9). However, because of the very rapid pulse rate of the mouse, we were only able to measure mean O_2 saturation values from arterial and venous blood (Sm_{O_2}), which therefore appear low (85%) relative to true Sp_{O_2} values (95%) even in uninfected mice. In the current study, we used the newly available MouseOx system, which can accurately measure true Sp_{O_2}, in addition to other physiologic parameters, in conscious mice at a 15-Hz sampling rate. Moreover, the associated software allows accurate sensor placement, since error codes are displayed when arterial pulse signals do not conform to the software algorithm, so that erroneous data may be excluded. To our knowledge, our study is the first to be published using this system. We found that infection with RSV is associated with a significant reduction in Sp_{O_2} at Day 2 only, which is when AFC is most impaired (by 43% from mock-infected values). The lack of decline in Sp_{O_2} at Day 4 suggests that the degree of AFC impairment at this time point (21%) is insufficient to result in detectable hypoxemia. Indeed, we were similarly unable to detect hypoxemia in uninfected mice 24 hours after intranasal treatment with 10 μM A77-1726, which causes a comparable decline in AFC (19%).

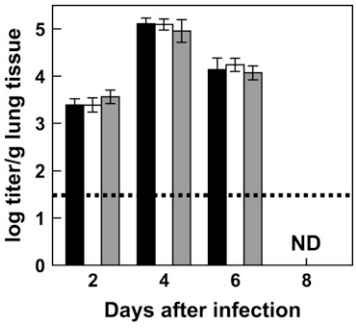

Figure 7. Effect of intranasal A77-1726 treatment at Day 1 after RSV infection on RSV replication in mouse lungs at Day 2. Effect of intranasal instillation of 100 μl saline containing 0.5 μl DMSO (*open bars*) or 50 μM A77-1726 (*shaded bars*) in 0.5 μl DMSO at Day 1 on lung homogenate viral titers at Days 2 to 8 after RSV infection (n = 6–12 per group). ND: none detected. *Solid bars*, untreated.

The time course of effect of RSV on Sp_{O_2} described herein was almost identical to that which we reported previously for Sm_{O_2} (9), and results of the two techniques were very strongly correlated ($P < 0.0005$), confirming the validity of our earlier findings. Interestingly, van Schaik and coworkers (31) previously reported an early peak in respiratory rate at Day 1 to Day 2 after RSV infection, measured by whole-body plethysmography, although they also found a second period of tachypnea at Day 6, which they associated with the onset of the specific immune response to infection. We found no hypoxemia at Day 6 and no tachypnea at any time point after infection, although this latter finding may be a consequence of the greater animal handling required for Sp_{O_2} measurement versus plethysmography, which may have artificially altered respiratory rates. Nevertheless, our findings are supported by the observation of Welliver and colleagues (32) that in human patients, severity of hypoxemia during RSV infection is poorly correlated to T cell cytokine levels.

Although the observed decrease in mean Sp_{O_2} at Day 2 in RSV-infected mice appears small (2%), this nevertheless corresponds to a decline in arterial P_{O_2} of 10 mm Hg. Moreover, an Sp_{O_2} of 94%, which is greater than the mean value observed in our study at Day 2 (93.5%), has also been suggested as a lower acceptable limit for bronchiolitis outpatient therapy (33), and has been defined by some authors as indicative of hypoxemia (34). In one recent study, an Sp_{O_2} below 95% was found to be highly predictive of admission to the pediatric ICU in full-term infants with bronchiolitis that lack other underlying illnesses (35). Most importantly, the limited hypoxemia observed in conscious mice at Day 2 could be prevented by intranasal A77-1726 treatment at Day 1, as we had previously shown for leflunomide pretreatment (9). This finding further supports our hypothesis that hypoxemia during RSV bronchiolitis may result from nucleotide-mediated impairment of AFC. Furthermore, intranasal A77-1726 treatment had no detrimental effect on Sp_{O_2} at later time points after infection.

Unlike systemic leflunomide treatment (9), intranasal A77-1726 treatment at Day 1 had only a limited suppressive effect on the intrapulmonary proinflammatory cytokine/chemokine response at Day 2, since it induced significant reductions only in BAL KC and TNF-α levels. However, a comparable suppressive effect on BAL KC levels was also noted after intranasal DMSO treatment at Day 1, suggesting that the reduction in BAL KC levels at Day 2 after intranasal A77 treatment at Day 1 is an effect of the vehicle rather than the drug itself. DMSO has previously been shown to suppress IL-8 production by RSV-infected A549 cells, as a consequence of its antioxidant properties (36). While the suppressive effect of A77 treatment on BAL TNF-α levels was not replicated by DMSO alone, our previous studies demonstrated that leflunomide's effect on RSV-induced AFC inhibition was reversed by uridine, but its effect on BAL TNF-α levels was not (9). Taken together, these findings suggest that the beneficial effect of A77-1726 treatment on AFC in RSV

infection is not a consequence of its immunosuppressive effects, but instead results from its inhibitory effect on *de novo* pyrimidine synthesis.

Intranasal A77-1726 treatment at Day 1 also blocks the increase in BAL alveolar macrophage counts in response to infection at Day 2, although it has no effect on BAL lymphocyte or neutrophil responses to RSV. Previously, we had found that continuation of leflunomide treatment to Day 8 after infection resulted in impaired leukocyte recruitment to the airspaces and histopathologic evidence of trapping of leukocytes around major blood vessels (9). These data suggest that either nucleotides themselves, or the cytokines and chemokines that they induce, may be important for recruitment of alveolar macrophages to the lung in the early phase of RSV infection. Interestingly, nucleotides have been shown to promote monocyte chemotaxis (37), and our findings may reflect this role. Taken together, the alteration in pulmonary humoral and cellular responses to RSV infection observed after inhibition of *de novo* pyrimidine synthesis suggest that nucleotides may play a significant role in the initiation of the immune response to RSV infection (although this alteration may be partially attributed to the tyrosine kinase inhibitory activity of these drugs [19]). Nevertheless, irrespective of the underlying mechanism, the immunosuppressive activity of A77-1726 may be an additional beneficial property of this drug, since an elevated nasal lavage or lung level of CXCL8, which intranasal A77-1726 reduces, may be an indicator of increased disease severity in children with RSV (38, 39).

Our previous studies had demonstrated that the inhibitory effect of RSV on AFC requires active viral replication (10), and the beneficial effect of A77-1726 on AFC at Day 2 might therefore simply result from an antiviral activity. However, neither intranasal A77-1726 treatment nor intranasal DMSO treatment at Day 1 had any effect on viral replication kinetics in mouse lungs. The beneficial effect of A77-1726 on basal and amiloride-sensitive AFC at Day 2 is not therefore simply a consequence of inhibition of viral replication, but is rather a specific modulatory effect on epithelial cell function. Moreover, unlike leflunomide therapy, intranasal A77-1726 treatment at Day 1 does not prolong viral replication to Day 8. This finding suggests that, despite any immunosuppressive effects, this treatment does not impede normal antiviral clearance mechanisms.

In conclusion, our studies indicate that post-infection intranasal therapy with an inhibitor of *de novo* pyrimidine synthesis can have a prolonged beneficial effect on the pathophysiologic consequences of RSV infection in the BALB/c mouse model—intranasal A77–1726 treatment at Day 1 after RSV infection is able to restore normal AFC and alleviate lung edema 24 hours later, thereby helping to counter the development of hypoxemia, without impairing clearance of RSV from the lung in the long term. Post-infection intranasal or aerosolized therapy with pyrimidine synthesis inhibitors may therefore have the potential to provide symptomatic relief from the pathophysiologic sequelae of impaired AFC in children with severe RSV disease. To our knowledge, this is the first report of an effective post-infection therapy for hypoxemia in RSV bronchiolitis in the murine model, other than supplemental oxygen.

Conflict of Interest Statement: I.C.D., W.M.S., and S.M. have been granted US Provisional Patent Application #60/573558: "Methods for using pyrimidine synthesis inhibitors to increase airway epithelial cell fluid uptake." (Filed May 21, 2004; Inventors: Dr. Ian C Davis, Dr. Wayne Sullender and Dr. Sadis Matalon) which converted to International PCT application (#PCT/US2005/017939);May 2005. I.C.D. has received $500 in consultancy fees from Inspire Pharamaceuticals for advising on licensing issues related to this patent. S.M. received $1000 as an honorarium from Inspire Pharmaceuticals for a seminar delivered at Inspire. S.M. was the principal investigator for a grant from Inspire Pharmaceuticals, entitled: "Assessment of a novel P2Y receptor antagonist in preventing RSV induced injury to the Alveolar epithelium *in vivo*" (07/15/2006–07/15/2007; $130,000, direct costs). W.M.S. and I.C.D. acted as a co-investigator and consultant, respectively. None of the experiments and results presented in this paper were funded from this grant. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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