Zileuton Reduces Respiratory Illness and Lung Inflammation, during Respiratory Syncytial Virus Infection, in Mice

R. Charles Welliver II, Karen H. Hintz, Maria Glori, and Robert C. Welliver, Sr.
Department of Pediatrics, Division of Infectious Diseases, State University of New York at Buffalo, and Children’s Hospital, Buffalo, New York

To determine the role of leukotrienes in a mouse model of respiratory syncytial virus (RSV) infection, we correlated the quantity of leukotrienes in lung lavage fluids of infected mice with respiratory rates and measures of outflow obstruction and then determined the effects of the leukotriene inhibitor zileuton on clinical features and lung function. Concentrations of leukotrienes were correlated with both increasing respiratory rates and the degree of prolongation of expiratory time. Administration of zileuton 1 day before infection and through day 5 after infection markedly reversed airway constriction, reduced numbers of inflammatory cells in the lung, and prevented the weight loss associated with infection. Leukotrienes appear to contribute substantially to the pathogenesis of RSV-related disease.

Respiratory syncytial virus (RSV) infection is the most important viral respiratory pathogen of infancy and early life, accounting for an estimated 120,000 hospitalizations and several hundred deaths annually [1, 2]. Therapeutic options are limited to ribavirin, an expensive antiviral compound with limited efficacy [3], and drugs such as bronchodilators and corticosteroids, which are ineffective in preventing hospitalization or reducing hospital stays [4, 5].

The results of past studies by our institution demonstrate the presence of cysteinyl leukotrienes in respiratory secretions of human infants with RSV infection [6, 7]. These compounds provoke airway mucus secretion [8], which is an important feature of severe RSV disease [9]. They also promote bronchoconstriction [10] and airway infiltration by inflammatory cells [11, 12]. At present, there are no studies published on the effect of leukotriene inhibitors or antagonists in RSV-induced illness in humans.

In mice, RSV infection results in tachypnea, prolongation of the expiratory phase of respiration (lowering of the ratio of inspiratory time [Ti] to expiratory time [Te]) [13], and evidence of airway obstruction (increases in enhanced pause [P enh]) [14]. In addition to these changes in respiratory pattern, infected mice frequently lose 15%–20% of body weight and exhibit altered behavior, characterized by huddling in groups in the corners of cages and limited activity [15].

The present study was undertaken to determine the relationship, during experimental RSV infection, of the concentration of leukotrienes in the lungs to various clinical features and abnormalities of respiration. In addition, the effects of treatment with zileuton, an inhibitor of leukotriene synthesis [16], on these clinical and respiratory features was determined.

MATERIALS AND METHODS

Study design. In the first phase of this study, we determined, in BALB/c mice after RSV infection, the relationship of the quantity of leukotrienes released in
the airways to the degree of illness. A group of 4 mice (2 males and 2 females) was killed by lethal intraperitoneal injection of barbiturates (Abbott Laboratories) on day 0 (before infection). Other groups of 4–6 mice were then infected with RSV and were killed on either day 1, 3, 6, 11, or 14 after infection. For each group of mice, measurements of respiratory rate and the ratio of $T_i/T_e$ were performed, just before death, by plethysmography, initially with a unit developed by Goran Enhorning [13]. Bronchoalveolar lavage (BAL) was then performed for cell counts and leukotriene determinations. In mock-infected mice (mice receiving virus-free tissue culture medium), respiratory rates and $T_i/T_e$ ratios remained unchanged, and weight increased slightly, over a similar interval.

In the second phase of the study, we determined the effect, on respiratory measures and weight change, after infection, of inhibiting the synthesis of leukotrienes before and during the course of RSV infection. In this phase, we determined respiratory measures, after purchase of the Buxco apparatus (Buxco Electronics), which simplifies all measurements and also affords the use of $P_{eh}$ as a measure of airway resistance [17]. In our other studies, we found that, as a measure of airway obstruction, $P_{eh}$ is preferable to the ratio of $T_i/T_e$ [14]; our studies demonstrated that $P_{eh}$ measurements have considerably less variability than do $T_i/T_e$ measurements.

In this second phase, 20 mice (10 males and 10 females) were each intraperitoneally given a single 35-mg/kg dose of zileuton (gift of R. Shalwitz, Ross Division of Abbott Laboratories) 1 day before infection (day −1), and this daily dose was repeated on days 0–5. Zileuton was dissolved in a 50% solution of DMSO (Sigma) in saline, with a final volume of 100 μL, containing a zileuton concentration of 35 mg/kg. A similar volume of DMSO was given intraperitoneally to 10 infected mice (5 males and 5 females) on day −1 through day 5, as a control. The intraperitoneal, rather than the oral, route of administration was chosen to ensure adequate delivery of the drug. Measurements of respiratory rate and $P_{eh}$ were performed on all animals just before infection on day 0 and were repeated on days 3 and 6. Measurements were performed 1–2 h after the intraperitoneal injection on day 3. All mice were weighed on days −1 and 6. Mice were then killed on day 6, and BAL was performed as described above.

After this part of the experiment was completed, the question arose whether zileuton was preventing or simply postponing disease. Therefore, the second phase was repeated on a second set of 20 mice. However, mice were not killed on day 6 but, instead, were monitored through day 10.

The study was then repeated in a third phase, with the only change being that administration of zileuton began on day 3 (when respiratory illness was already evident) and continued through day 6. Measurements of respiratory rate and $P_{eh}$ were made 1–2 h after the intraperitoneal injection on days 3 and 5.

**Infection of mice.** All mice used in these experiments were 10–12-week-old BALB/c mice (Harlan-Sprague-Dawley). Mice were lightly anesthetized by inhalation of methoxyfluorane (Pitman-Moore) and were infected intranasally by laboratory stocks of the A2 strain of RSV ($5 \times 10^6$ pfu of virus in 100 μL of saline/mouse) grown in Hep-2 cells. Uninfected control mice received an equal volume of clarified, uninfected Hep-2 cell culture lysate. Virus growth in lungs was quantified by a plaque assay of homogenized whole lung tissue, as described elsewhere [14].

**BAL.** Recovery of bronchoalveolar fluid was accomplished as described elsewhere [13, 14]. In brief, a tracheal cannula was inserted postmortem, and 0.5 mL of normal saline supplemented with 2 μg/mL aprotonin (Sigma) was gently instilled into the airway, under a positive pressure of 20 cm of H2O. BAL fluid was recovered from the lung by supplying a negative pressure of 20 cm of H2O. The cycle was repeated 6 times with the same fluid, with ~80% of the instilled volume recovered. BAL samples were centrifuged for 10 min at 12,200 g, and supernatants were stored at −70°C, until assayed.

Cell pellets were resuspended in 100 μL of normal saline, and a 10-μL aliquot was mixed with a solution of 0.1% acetic acid/0.1% crystal violet, for cell counts, by means of a hemocytometer. The remainder was processed by cytospin (Cytospin-3; Shandon) and was stained with Diff-Quik (Dade Diagnostics). Differential determinations were made on 200 cells/slide.

**Leukotriene determinations.** Kits for detection, by ELISA, of leukotrienes were purchased from Cayman Chemical. Samples of BAL fluids were processed, and assays were completed by use of the manufacturer’s instructions. The detector antibody in the kit was raised against leukotriene C4, and cross-reactivity is listed in the package insert as 100% for leukotriene C4, ~45% for leukotriene D4 and leukotriene D5, 28% for N-acetyl leukotriene E4, 7% for leukotriene E5, 2% for leukotriene Eα, and <0.01% for leukotriene B4 and leukotriene B3.

**Statistical analysis.** Changes, over time, in respiratory rate, $T_i/T_e$, and $P_{eh}$ were compared between treatment groups by Student’s $t$ test. When a normal distribution of data was ensured, weight changes and numbers of white blood cells types were also compared between treatment groups by use of Student’s $t$ test. Results are expressed as the mean ± 1SE. Correlations of leukotrienes with other measures were determined by linear regression. Leukotriene concentrations in zileuton recipients were not normally distributed (most values were undetectable), and so the Mann-Whitney test was used to compare median values of leukotrienes between zileuton recipients and control mice. Increases in leukotriene concentrations, between baseline and various study days, were determined first by use of the Kruskal-Wallis test, to detect any differences, and then by use of the Mann-Whitney test, to compare individual days.
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Figure 1. Changes in respiratory rate, ratio of inspiratory time (T_i)/expiratory time (T_e), and leukotriene release, on various days after respiratory syncytial virus (RSV) infection in mice. Mice were inoculated with RSV on day 0; 4–6 mice are represented at each time. Leukotriene values represent micrograms per milliliter (±1SE) of bronchoalveolar lavage fluid.

RESULTS

Changes in respiratory rate, T_i/T_e, and leukotriene content, after infection. The temporal relationship of respiratory changes and leukotriene release was determined in infected but untreated animals, in phase 1 experiments. As illustrated in figure 1A, the respiratory rate of infected mice rose on day 1 and reached a maximum on day 6, before declining on days 11 and 14. Figure 1B depicts the changes in ratio of T_i/T_e after infection. The ratio remained unchanged from baseline through day 3 and then declined on day 6, before returning to baseline values on days 11 and 14. The decline in the ratio on day 6 was due to a prolongation of T_e, as has been established elsewhere [13]. The concentration of leukotrienes present in BAL fluids is shown in figure 1C. Quantities of leukotrienes appeared to be elevated above baseline values on day 3, but, because of the high variability, the difference was not significant (P = .28). However, significantly greater quantities of leukotrienes were noted on days 6 (P = .0074) and 11 (P = .013), compared with day 0. Therefore, the peak of leukotriene release occurred generally at the same time as did the maximum abnormalities in respiratory pattern.

Correlation of leukotriene concentrations with respiratory changes. The correlation of measured quantities of leukotrienes in BAL fluids, on various days after infection, with respiratory rates and T_i/T_e ratios is illustrated in figure 2. All data from days 0–14 are included, with 4–6 samples represented at each data point. In the lower panel, a weak correlation (r = .422; P = .039) between respiratory rate and leukotriene release is described, whereas the upper panel shows a stronger, inverse relationship (r = −.502; P = .009) between the ratio of T_i/T_e and leukotriene release. Therefore, greater degrees of leukotriene release correlated with both increase of tachypnea and prolongation of the expiratory phase.

Correlation of leukotrienes with inflammatory cell number, in BAL fluid. Leukotrienes may create airway obstruction by several mechanisms, including promotion of inflammation [11, 12]. We therefore determined the relationship between leukotriene content of BAL fluid and the number of inflammatory cells present after infection. As shown in figure 3, the quantity of leukotrienes present in BAL fluid was directly correlated with the number of macrophages (r = .756; P = .0002) and lymphocytes (r = .638; P = .002) present, but not with the number of neutrophils (r = .175; P = .45) (data not shown). Neither eosinophils nor basophils were recognized in BAL fluid at any time after infection.

Effect of zileuton prophylaxis on respiratory rate and P_{ENO}, after RSV infection. In phase 2 of this study, mice were given...
a single daily dose of zileuton, beginning 1 day before infection and continuing through day 5 after infection. This schedule would mimic the preventive administration of zileuton to infants at risk for RSV infection. The results are shown in figure 4.

Neither respiratory rates nor $P_{emb}$ differed between groups on day 0. The administration of zileuton resulted in a marked reduction in respiratory rate on day 3 ($P < .0001$) but not at day 6 (figure 4, lower panel). In contrast, pretreatment with zileuton prevented the increase in $P_{emb}$ that was observed in control mice on both day 3 and day 6 ($P < .0001$ in each case) (figure 4, upper panel). Thus, preinfection treatment with zileuton prevented the postinfection increase in airway resistance.

To confirm the validity of using $P_{emb}$ as a substitute for $T_{i}/T_{e}$, we correlated the 2 measures by use of simple regression. We found the expected negative relationship ($r = -0.249; P = .0103$). In addition, a comparison of figures 1 and 4 demonstrates that, on day 6, $P_{emb}$ increases as $T_{i}/T_{e}$ decreases, a finding that indicates that increases in airway resistance (increased $P_{emb}$) and prolongation of expiration (decreased $T_{i}/T_{e}$) occur at the same time.

**Effect of zileuton pretreatment on weight loss, after RSV infection.** Control mice lost a mean ($\pm$ SE) of 15.58% $\pm$ 2.33% of weight between day 0 and day 6, whereas zileuton-treated mice gained a mean ($\pm$ SE) of 0.686% $\pm$ 1.13% ($P < .0001$) (data not shown). Infected, untreated mice remained huddled in the corner of their cages throughout the course of infection and exhibited ruffling of their fur, whereas zileuton-treated mice moved freely around their cages and exhibited no ruffling of their fur.

**Effect of zileuton prophylaxis on viral replication.** After respiratory measures were performed on day 6, mice were killed and lungs were harvested for quantitation of virus in lung tissues. The quantity of virus in the lungs of zileuton-treated mice ($n = 5$) was $4.83 \times 10^{4}$ pfu/g of lung tissue, whereas that of control mice ($n = 5$) was $13.0 \times 10^{4}$ pfu/g of lung tissue, a difference that was not statistically significant ($P = .14$).

**Effect of zileuton prophylaxis on airway inflammation.** After mice were killed on day 6, BAL was performed, as were cell counts and differentials. Total cell counts (mean $\pm$ SE) were $8.98 \pm 0.535 \times 10^{3}$ cells/mL in control mice, versus $5.0 \pm 0.43 \times 10^{3}$ cells/mL in zileuton-treated mice ($P < .0001$). As illustrated in figure 5, zileuton pretreatment reduced the number of macrophages ($P = .0002$), lymphocytes ($P < .0001$), and neutrophils ($P < .0001$), in BAL fluids. Eosinophils were uncommon in zileuton-treated animals ($0.008 \pm 0.003 \times 10^{3}$ cells/mL) and were not observed in control mice ($P = .14$).

**Leukotriene concentrations in BAL fluid of zileuton recipients and control mice.** The median (interquartile range [IQR]) concentration of leukotrienes in BAL fluids of control
Figure 5. Effect of preventive inhibition of leukotriene synthesis on inflammatory cell numbers in bronchoalveolar lavage (BAL) fluid, after respiratory syncytial virus (RSV) infection in mice. Mice were intraperitoneally given either an inhibitor of leukotriene synthesis (zileuton) or a diluent (DMSO), on days −1 through 5, and were infected with RSV on day 0. BAL was performed after death on day 6. Data are mean ± 1SE and were compared by Student's t test.

Figure 6. Effect of preventive inhibition of leukotriene synthesis on delayed respiratory pattern, after respiratory syncytial virus (RSV) infection in mice. Mice were intraperitoneally given either an inhibitor of leukotriene synthesis (zileuton) or a diluent (DMSO), on days −1 through 5, and were infected with RSV on day 0. Ten mice were included in each group at each time. Enhanced pause ($P_{\text{enh}}$) was used as a measure of airway obstruction. In contrast to previous experiments, mice were not killed on day 6 but, instead, were monitored through day 10, to determine whether illness resolved or increased over this interval. The results of this experiment are illustrated in figure 6.

Determination of whether zileuton prevents or postpones illness. The preceding experiments seem to show that zileuton prevented increase in respiratory rate on day 3, but respiratory rates began to increase when zileuton was withdrawn (day 6). Because mice were killed at this time, we could not exclude the possibility that respiratory rates might have increased even further on subsequent days. Thus, it was possible that zileuton was only postponing illness, rather than preventing it. To exclude this possibility, we repeated the experiment, following the exact same protocol (i.e., zileuton was administered from day −1 through day 5), except that mice were not killed on day 6. Instead, mice were monitored through day 10, to determine whether illness resolved or increased over this interval. The results of this experiment are illustrated in figure 6.

As seen in figure 6, during days 5–8, in zileuton recipients, respiratory rates and $P_{\text{enh}}$ remained below those of the control group, and, in each experimental group, resolution of illness was nearly complete by day 10. Thus, illness was not postponed by zileuton therapy. Respiratory rates did increase slightly in the first few days after administration of zileuton was stopped, an increase that is probably a result of the effect of the drug being lost over this interval.

Effect of zileuton in established RSV infection. In the third experimental phase, zileuton treatment was withheld until day 3 and was given again on days 4 and 5. This corresponds to the clinical setting in which zileuton would be started early in the course of RSV infection, just as airway obstruction begins. The results are demonstrated in figure 7.

When the drug was withheld until days 3–5, respiratory rates...
Figure 7. Effect of inhibition of leukotriene synthesis on respiratory pattern, in previously established respiratory syncytial virus (RSV) infection in mice. Mice were infected with RSV on day 0 and were intraperitoneally given either an inhibitor of leukotriene synthesis (zileuton) or a diluent (DMSO), on days 3–5. Five mice were included in each group at each time. Enhanced pause (Penh) was used as a measure of airway obstruction. Data are mean ± SE. Respiratory measurements were made after administration of zileuton on days 3–5 (zileuton was not administered on day 6).

were similar in control mice and zileuton recipients (figure 7, lower panel). However Penh was reduced on days 3 (P = .07), 5 (P = .0014), and 6 (P = .044) in zileuton-treated mice, compared with that in control mice (figure 7, upper panel).

In addition, zileuton-treated mice lost less weight (4.96% ± 1.2% of baseline weight) than did infected, untreated control mice (10.03% ± 2.4%; P = .047). Zileuton-treated mice moved freely around their cages and maintained a normal appearance, whereas control mice again huddled in the corners of their cages and exhibited ruffled fur. Thus, therapeutically administered zileuton reduces airway resistance, weight loss, and changes in physical appearance, after RSV infection.

**DISCUSSION**

The results of this investigation demonstrate that leukotrienes are elevated in a mouse model of RSV infection. The maximum release of leukotrienes occurs simultaneously with both the development of evidence of airway obstruction and the greatest degree of tachypnea. The administration of an inhibitor of leukotriene synthesis (zileuton), before infection with RSV (and continuing through day 5 after infection), markedly inhibits the development of airway obstruction, reduces the degree of tachypnea (at least early in the course of infection), and prevents weight loss associated with infection. These effects remain evident for several days after zileuton has been discontinued.

When treatment is begun on day 3, a time when respiratory abnormalities are already in evidence, a single daily dose of zileuton does not alter respiratory rates but does modestly reduce airway obstruction and weight loss. The effects are observable for 24 h after zileuton has been discontinued. Further experiments with multiple daily doses of zileuton are planned. The dose of zileuton used in these experiments (a single dose of 35 mg/kg/day) was chosen because it had been demonstrated elsewhere to be effective in preventing inflammation, after ovalbumin challenge, in sensitized mice [18].

These findings indicate that leukotrienes contribute to the pathogenesis of RSV-related disease in the mouse model. Leukotrienes are increased in human infants with RSV bronchiolitis [6, 7], although the exact nature of leukotrienes’ contribution to illness has not been identified.

The mechanism by which inhibition of leukotrienes reduces the degree of respiratory illness is not entirely clear. Leukotrienes induce airway mucus secretion [8], which is prominent in human infants with RSV bronchiolitis [9]. However, mucus obstruction of the airway is not an important feature of RSV infection in mice [15], so the improvement in respiratory pattern after zileuton must be related to some other action of leukotrienes. Leukotrienes constrict airway smooth muscle, but there is little or no smooth muscle surrounding the bronchioles. An alternative mechanism is based on the proinflammatory effect of leukotrienes.

In atopic humans, the administration of zileuton, before allergen challenge, reduces the degree of airway infiltration by eosinophils, lymphocytes, and neutrophils [11]. In another study, blockade of the cysteinyl leukotriene type 1 receptor, by zafirlukast, reduced the accumulation of lymphocytes and basophils and reduced macrophage activation, after allergen challenge [12]. In the present study, preventive administration of zileuton reduced the number of macrophages, lymphocytes, and neutrophils induced after RSV infection (eosinophils and basophils were not observed in BAL fluids of untreated, infected mice). Because airway obstruction was related to the number of macrophages and lymphocytes present in the airway, the beneficial effect of zileuton might be related to the reduction in the number of macrophages and lymphocytes. Leukotrienes also induce vascular permeability [19], a potential mechanism that was not evaluated in the present study.

Zileuton inhibits 5-lipoxygenase, an enzyme that is important in the synthesis of leukotriene B4, as well as in the synthesis of the cysteinyl leukotrienes C4, D4, and E4. Further studies with leukotriene B4 antagonists and cysteinyl leukotriene type 1 blockers will be needed to determine whether leukotriene B4 or the group of cysteinyl leukotrienes contributes more sig-
nificantly to the pathogenesis of RSV infection in mice. Also, the source of leukotrienes in mice with RSV infection is not known. One study demonstrated that, in epithelial cells, RSV infection induces the expression of mRNA for 5-lipoxygenase; however, production of leukotrienes is increased only 1.5-fold, and, in addition, for <72 h [20]. Macrophages, which are present in the lungs throughout the course of RSV infection in both mice and humans [9, 13], represent another possible source of leukotrienes [21]. The strong correlation of leukotriene content with the number of macrophages present in BAL fluids, in the present study (r = .756; P = .0002), suggests that this cell type may be the source. Mast cells and basophils in the lung parenchyma may also release leukotrienes, especially in response to activation by the macrophage-derived chemokines macrophage inflammatory protein 1–α and monocyte chemotactic protein–1 [22, 23].

The Buxco apparatus probably measures total airway resistance, including that caused by airway obstruction and that caused by parenchymal disease [17]. Our pathological material (data not shown), and that of others [15], indicates that most of the inflammatory infiltrate is concentrated in the peribronchial area, whereas there is minimal alveolar and interstitial inflammation in the mouse model. Thus, although a contribution of parenchymal disease cannot be excluded, we expect that most of the increase in P_e (not P_a) is caused by increases in airway resistance.

In summary, we find that leukotrienes contribute to respiratory illness, particularly to airway obstruction, after RSV infection in mice. These findings should stimulate investigations of leukotriene inhibitors or antagonists, in human infants with RSV bronchiolitis. These compounds may be more effective when given prophylactically to infants at high risk for the development of severe bronchiolitis but should also be evaluated early in the course of established RSV infection.

References