Oral Administration of L-Arginine Potentiates Allergen-Induced Airway Inflammation and Expression of Interleukin-5 in Mice

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ABSTRACT

The role of nitric oxide in the airway hyperresponsiveness and inflammation of bronchial asthma has not yet been established. However, L-arginine, the substrate for nitric oxide synthases, reportedly alleviates airway hyperresponsiveness caused by parainfluenza virus and reduces granulocytic inflammation induced by ischemia-reperfusion. We investigated the effects of L-arginine on a murine model of allergic asthma that included airway hyperresponsiveness, eosinophilic inflammation and expression of interleukin (IL)-5 in the lung. The mice received drinking water with or without L-arginine for 9 weeks. Histologic evaluation and cellular profiles in bronchoalveolar lavage fluid showed that p.o. administration of L-arginine (72 μmol/kg/day) significantly enhanced eosinophilic airway inflammation and goblet cell proliferation that were associated with intratracheal instillation of ovalbumin. L-Arginine also increased protein levels of IL-5 and IL-2 in supernatants from the lung exposed to ovalbumin. The number of eosinophils in bronchoalveolar lavage fluid correlated significantly with the expression of IL-5. L-Arginine did not reverse ovalbumin-associated airway hyperresponsiveness to inhaled ACh. These results suggest that p.o. administration of L-arginine aggravates allergen-induced eosinophilic airway inflammation via expression of IL-5, and in this model it does not show therapeutic efficacy against airway hyperresponsiveness associated with allergen exposure. Oral administration of L-arginine, the precursor of nitric oxide, may not be an effective intervention in allergic asthma.

Bronchial asthma is characterized by airway hyperresponsiveness and inflammation in which activated eosinophils and lymphocytes are pivotal (Expert Panel Report, 1991). Various lines of evidence have indicated that IL-5 is a central pathophysiologic mediator of bronchial asthma (Robinson et al., 1993; Foster et al., 1996; Takano et al., 1997a). The effects of NO on bronchial asthma are incompletely understood, because NO can exert broad actions on airway hyperresponsiveness and inflammation including cytokine expression, at least in part because three distinct isoforms of NO synthases are present. NO appears to possess bronchodilator properties (Dupuy et al., 1992; Nijkamp et al., 1993; Li and Rand, 1991), and it may afford protection against airway inflammation by inhibiting platelet aggregation and leukocyte adhesion to vascular endothelium (Radomska et al., 1992; Kubés et al., 1991) and by the maintenance of microvascular integrity (Erjefalt et al., 1994). Besides these anti-inflammatory actions, however, NO exerts a variety of pro-inflammatory effects (Bernareggi et al., 1997; Rettori et al., 1992; Salvemini et al., 1995; Lander et al., 1993) that may be detrimental in airway inflammation. NO also can react with superoxide to form the highly reactive anion peroxynitrite, which initiates lipid peroxidation and rapidly oxidizes sulfhydryl groups (Radi et al., 1991).

In clinical studies, inhalation of NO by patients with mild asthma significantly reduces bronchospasm induced by methacholine inhalation (Kacmarek et al., 1996), whereas asthmatics apparently exhale a greater amount of NO than healthy volunteers (Kharitonov et al., 1994). Experimentally, an aerosol containing L-arginine, the substrate for NO synthases, prevented airway hyperresponsiveness to histamine caused by intratracheal inoculation of parainfluenza virus in guinea pigs (Folkerts et al., 1995). In addition, systemic administration of L-arginine reduced granulocytic inflammation induced by ischemia-reperfusion (Weyrich et al., 1992). However, the effects on bronchial asthma of L-arginine, the precursor of NO, have not been elucidated.

The present study was undertaken to examine the pathophysiologic effects of L-arginine on a murine model of allergic asthma that involves airway hyperresponsiveness, eosinophilic airway inflammation and expression of IL-5 in the lung.

ABBREVIATIONS: NO, nitric oxide; IL, interleukin; Rrs, respiratory resistance; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assays; PC_{150}%, provocative concentration of ACh causing a 50% increase in Rrs.
Materials and Methods

Animals and experimental protocol. Male ICR mice 6 to 7 weeks old and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. The animals were fed a commercial diet (Japan Clea Co.) and housed in a facility maintained at 24°C to 26°C with 55% to 75% humidity and a 14 h/10 h light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by our Institutional Review Board.

Mice were divided into four experimental groups: a water-vehicle group, a water-ovalbumin group, an L-arginine-vehicle group, and an L-arginine-ovalbumin group. The mice in the water-vehicle group and the water-ovalbumin group received plain drinking water for a continuous 9-week period, whereas the animals in the L-arginine-vehicle group and the L-arginine-ovalbumin group received drinking water containing L-arginine (Sigma Chemical Co., St. Louis, MO) for the same period. The dose of L-arginine administered was 72 μmol/kg/day. In preliminary studies this dose of L-arginine had no significant effect on water consumption or gain in body weight (data not shown). The normal mouse reportedly drinks about 100 ml of H2O/kg b.wt. every 24 h (Grisham et al., 1994). In the present study, the mice consumed 139 ml/kg/day of plain water and 144 ml/kg/day of water containing L-arginine (50 μmol in 100 ml of water).

The mice in the water-vehicle group and the L-arginine-vehicle group received intratracheal instillations of 0.1 ml of phosphate-buffered saline (pH 7.4; Nissui Pharmaceuticals, Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once weekly for 9 weeks (10 times), beginning 3 days after initiation of p.o. medication. The mice in the water-ovalbumin group and the L-arginine-ovalbumin group intratracheally received 1 μg of ovalbumin suspended in 0.1 ml of vehicle every 3 weeks for 9 weeks instead of the vehicle alone (4 times for ovalbumin and 6 times for vehicle alone). The intratracheal instillations were performed via a polyethylene tube after the animals were anesthetized with 4% halothane (Hoechst Japan, Tokyo, Japan). The animals were studied 24 h after the last intratracheal instillation by evaluation of airway responsiveness, cellular profiles of BAL fluid, lung histology and expression of cytokine proteins in the lung tissue supernatants.

Airway responsiveness. Measurements of pulmonary function were conducted by the method of Sorkness et al. (1994) with a minor modification. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and underwent a tracheostomy with an 18-gauge cannula. Each mouse was mechanically ventilated with a rodent respirator (Model 683; Harvard Apparatus, South Natick, MA) in a plethysmograph box with a pneumotachometer (Model PLYAN-M; Buxco Electronics, Inc., Sharon, CT) at a constant tidal volume (0.2 ml) and at 120 breaths/min. Spontaneous respiration was inhibited by pancuronium bromide (1 mg/kg i.v.). Endotracheal pressure and flow were determined by a differential pressure transducer (Model DP45-28; Buxco) connected to the tracheal cannula. Flow was measured using a pneumotachometer, a differential pressure transducer (Model DP45-14; Buxco) and a preamplifier (Model PREAMP/VAL; Buxco). A continuous measurement of Rrs was computed from the endotracheal pressure and flow using a Pulmonary Mechanics Analyzer (Model-6; Buxco). Endotracheal pressure, flow and Rrs were recorded on a six-channel recorder (Model WR3701-6; Buxco). Four-second averages of Rrs were also recorded on a terminal (Model TI-703; Buxco) via a Datalogger (Model LS-12; Buxco). The ACh challenge was administered by inhalation. ACh solutions (0.01–10 mg/ml in saline) were cumulatively inhaled, each concentration being given for 2 min with an interval of 2 min between administrations, by aerosolizing the solution with an ultrasonic nebulizer (NE-U07; Omron, Kyoto, Japan) designed to generate 5-μm aerosol particles.

PC150 was estimated in each mouse, calculated from the curve determined by the concentration of ACh and the percent increase in Rrs.

BAL. In a separate series of animals, the trachea was cannulated after exsanguination by cardiac puncture. The lungs were lavaged three times with 1.2 ml of sterile saline at 37°C used at each lavage. The average volume of BAL fluid retrieved was 90% of the volume of 3.6 ml that was instilled. This amount was similar with each treatment. The lavage fluid was centrifuged at 300 × g for 10 min. The total number of cells was counted in a fresh specimen of fluid stained with Turk’s solution. Differential counts were assessed on cytologic preparations. Slides were prepared using a Cytospin (Tomy Seiko, Tokyo, Japan) and stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 300 cells in each lavage sample were counted under oil immersion microscopy. After the BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen and stored at −80°C.

Cytokine protein levels in lung tissue supernatants. The frozen lungs were homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA (Sigma), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque), 1 μM pepstatin (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute). The homogenates were then centrifuged at 105,000 × g for 1 h. The supernatants were stored at −80°C. ELISA were conducted for IL-5 and IL-2 in lung tissue supernatants using matching antibody pairs (Endogen, Cambridge, MA). The following antibody pairs were used for detection of IL-5 and IL-2: TRFK5 and TRFK4 for IL-5 and 5B4B6 and 5H 4.1.1. for IL-2. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of recombinant IL-5 and IL-2 with limits of detection of 5 pg/ml and 3 pg/ml, respectively.

Histologic evaluation. In a separate series of animals, the lungs were removed after exsanguination and fixed in 10% neutral phosphate-buffered formalin instilled intratracheally at a pressure of 20 cm H2O for at least 72 h. Slices of each pulmonary lobe 2 to 3 mm thick were embedded in paraffin. Sections 3 μm thick were stained with Diff-Quik for determination of the number of infiltrating eosinophils and neutrophils or with periodic acid-Schiff for quantitation of the goblet cells. Examinations were conducted in blind fashion by two of us. The length of the basement membrane of the airways on each sample slide was measured by videomicroimeter (Olympus, Tokyo, Japan). The number of eosinophils and neutrophils around the airways, and the number of goblet cells in the bronchial epithelium, were counted with a micrometer under oil immersion. Results were expressed as the number of cells per millimeter of basement membrane.

Statistical analysis. Data are reported as mean ± S.E. Differences in the numbers of inflammatory cells and goblet cells, airway responsiveness and cytokine proteins among groups were determined using analysis of variance (Statview version 4.0; Abacus Concepts, Inc., Berkeley, CA). If the differences among groups were statistically significant (P < .05), Fisher’s protected least significant difference test or Scheffe’s F test was used to distinguish between pairs of groups. The correlations between the number of eosinophils or neutrophils and the protein levels of cytokines were evaluated by Pearson’s correlation coefficient (Statview version 4.0; Abacus Concepts, Inc.).

Results

L-Arginine does not affect airway responsiveness. To determine the effects of orally administered L-arginine on airway hyperresponsiveness, we measured Rrs in animals that inhaled ACh. Base-line Rrs was not significantly different among the four experimental groups (data not shown). The value of PC150 in the water-ovalbumin group was significantly less than that in the water-vehicle group (table 1, P < .05). There was no significant difference in the value of PC150.
Oral administration of L-arginine significantly enhanced ovalbumin-associated increases in number of eosinophils (P < .001 vs. the water-vehicle group) and number of neutrophils (P < .0001 vs. the above groups). L-Arginine treatment did not affect findings obtained by the intratracheal administration of the vehicle alone.

The number of eosinophils infiltrating around the airways and goblet cells in the bronchial epithelium showed 9-fold and 4-fold increases, respectively, in the water-ovalbumin group as compared with the water-vehicle group (table 3). Oral administration of L-arginine significantly enhanced ovalbumin-associated increases in number of eosinophils (P < .01 vs. the L-arginine-vehicle group) and number of neutrophils (P < .01 vs. the above groups). L-Arginine treatment did not affect findings obtained by the intratracheal administration of the vehicle alone.

1-ArGINine potentiates eosINophilic airway inflammation. The number of eosinophils and neutrophils in BAL fluid showed 15-fold and 4-fold increases, respectively, in the water-ovalbumin group as compared with the water-vehicle group (table 2). Oral administration of L-arginine significantly enhanced ovalbumin-associated increases in number of eosinophils (P < .01 vs. the water-vehicle group) and the water-ovalbumin group; P < .01 vs. the L-arginine-vehicle group) and number of neutrophils (P < .05 vs. the above groups). L-Arginine treatment did not affect findings obtained by the intratracheal administration of the vehicle alone.

1-ArGINine increases IL-5 expression. The mean values of the protein levels of IL-5 and IL-2 in the lung tissue supernatants were greater in the water-ovalbumin group than in the water-vehicle group, although the differences did not attain statistical significance (table 4). Levels of IL-5 and IL-2 were significantly greater in the L-arginine-ovalbumin group than in the other groups (table 4, P < .01 vs. other groups).

A significant correlation was evident between number of eosinophils in BAL fluid in each mouse and protein levels of IL-5 in BAL fluid.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
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<tbody>
<tr>
<td>Water-vehicle</td>
</tr>
<tr>
<td>Water-ovalbumin</td>
</tr>
<tr>
<td>L-arginine-vehicle</td>
</tr>
<tr>
<td>L-arginine-ovalbumin</td>
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</tbody>
</table>

* P < .05 vs. water-vehicle group.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-vehicle</td>
<td>34.8 ± 3.54</td>
<td>34.1 ± 3.64</td>
<td>0.007 ± 0.007</td>
<td>0.71 ± 0.28</td>
</tr>
<tr>
<td>Water-ovalbumin</td>
<td>45.6 ± 7.37</td>
<td>42.5 ± 6.45</td>
<td>0.106 ± 0.047</td>
<td>3.01 ± 1.21</td>
</tr>
<tr>
<td>L-arginine-vehicle</td>
<td>43.1 ± 1.17</td>
<td>41.8 ± 1.15</td>
<td>0 ± 0</td>
<td>1.34 ± 0.51</td>
</tr>
<tr>
<td>L-arginine-ovalbumin</td>
<td>78.2 ± 9.68</td>
<td>50.2 ± 5.59*</td>
<td>11.4 ± 1.49*</td>
<td>16.6 ± 3.82*</td>
</tr>
</tbody>
</table>

* P < .0001 vs. water-vehicle group, P < .01 vs. water-ovalbumin group, P < .001 vs. L-arginine-vehicle group. * P < .05 vs. water-vehicle group, P < .001 vs. water-ovalbumin group, P < .01 vs. L-arginine-vehicle group. * P < .0001 vs. water-vehicle group, P < .01 vs. water-ovalbumin group, P < .001 vs. L-arginine-vehicle group. * P < .0001 vs. other groups.

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
<th>Goblet cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-vehicle</td>
<td>0.48 ± 0.34</td>
<td>0.16 ± 0.12</td>
<td>0.55 ± 0.37</td>
</tr>
<tr>
<td>Water-ovalbumin</td>
<td>4.30 ± 2.36</td>
<td>0.36 ± 0.14</td>
<td>2.16 ± 1.18</td>
</tr>
<tr>
<td>L-arginine-vehicle</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.28 ± 0.16</td>
</tr>
<tr>
<td>L-arginine-ovalbumin</td>
<td>18.5 ± 4.78*</td>
<td>0.59 ± 0.29*</td>
<td>13.0 ± 6.66*</td>
</tr>
</tbody>
</table>

* P < .01 vs. other groups.

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-5</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-vehicle</td>
<td>34.9 ± 1.34</td>
<td>23.4 ± 0.99</td>
</tr>
<tr>
<td>Water-ovalbumin</td>
<td>40.6 ± 5.30</td>
<td>25.9 ± 1.35</td>
</tr>
<tr>
<td>L-arginine-vehicle</td>
<td>34.2 ± 9.82</td>
<td>25.2 ± 1.22</td>
</tr>
<tr>
<td>L-arginine-ovalbumin</td>
<td>295 ± 99.5a</td>
<td>43.8 ± 7.51a</td>
</tr>
</tbody>
</table>

* P < .01 vs. other groups.

### Discussion

The present study demonstrated that p.o. administration of L-arginine significantly enhanced the eosinophilic airway inflammation and goblet cell proliferation associated with intratracheal instillation of allergen. The number of eosinophils in BAL fluid correlated significantly with the levels of IL-5 in lung tissue supernatants. The L-arginine treatment did not reverse allergen-associated airway hyperresponsiveness to inhaled Ach.

Inhaling NO reportedly can reverse methacholine-induced bronchoconstriction in guinea pigs (Dupuy et al., 1992), and inhibitors of NO synthesis have been demonstrated to induce airway hyperresponsiveness to histamine both in vitro and in vivo (Nijkamp et al., 1993). In addition, NO is recognized as a neurotransmitter of inhibitory nonadrenergic noncholinergic airway constrictor.
ergic nerves that induces relaxation in airways (Li and Rand, 1991). These effects of NO appear to be protective against airway hyperresponsiveness, a pathophysiologic hallmark of bronchial asthma. Inhalation of NO by patients with bronchial asthma has been shown to reduce bronchospasm induced by methacholine (Kacmanek et al., 1996). Furthermore, l-arginine, the precursor of NO, prevents airway hyperresponsiveness associated with intratracheal inoculation of para influenza virus in guinea pigs (Folkerts et al., 1995). Inhalation of NO gas has limited applicability to clinical use because special apparatus is required, so therapeutic efficacy, a regulator of the microvascular integrity and circulation. 

<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>Correlation coefficients between number of granulocytes in BAL fluid and expression of cytokine proteins</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IL-5 in Lung</td>
</tr>
<tr>
<td>Eosinophils in BAL fluid</td>
<td>0.869</td>
</tr>
<tr>
<td>Neutrophils in BAL fluid</td>
<td>0.581</td>
</tr>
</tbody>
</table>

Excess NO generated by inducible NO synthase has been implicated in the pathogenesis of various inflammatory diseases (McCartney-Francis et al., 1993; Miller et al., 1995; Takano et al., 1997b). Expression of inducible NO synthase has been demonstrated in the epithelial layer of biopsy specimens taken from asthmatic patients (Hamid et al., 1993), and asthmatics were found to exhale a greater amount of NO than healthy volunteers (Kharitonov et al., 1994). Furthermore, expression of inducible NO synthase is decreased strikingly by the corticoid inhalants commonly used in the treatment of inflammatory airways diseases such as bronchial asthma (Guo et al., 1995).

In our study, p.o. administration of l-arginine produced an increase in allergen-associated expression of IL-2, which correlated positively with the infiltration of neutrophils. In addition, l-arginine treatment markedly increased expression of IL-5 in the lung tissue supernatants associated with allergen exposure. Expression of IL-5 correlated significantly with infiltration of eosinophils. As far as we know, the present experiment represents the first demonstration of enhancing effects of l-arginine on production of IL-5 in vivo. Because IL-5 is recognized as a key mediator in bronchial asthma, affecting eosinophilic inflammation and airway hyperresponsiveness (Robinson et al., 1993; Foster et al., 1996; Takano et al., 1997a), the deterrent effects of l-arginine on our model are likely to involve the increased expression of IL-5. NO-generating compounds have been reported to induce NF-κB binding activity and subsequent production of tumor necrosis factor α, a pro-inflammatory cytokine (Lander et al., 1993). Future experiments should examine whether l-arginine or NO activates transcriptional factors other than NF-κB in the presence or absence of allergen.

To confirm the effects of l-arginine on NO production at the inflammatory site, we measured nitrate and nitrite in BAL supernatants. Unfortunately, the amount of NO produced in the four experimental groups was below the detection limit for the method of Schmidt and co-workers (1988). In the present study, airway inflammation with goblet cell proliferation was not induced by p.o. administration of l-arginine alone (l-arginine-vehicle group).

In conclusion, p.o. administration of l-arginine aggravated eosinophilic airway inflammation associated with allergen exposure via expression of IL-5, and l-arginine treatment did not show therapeutic efficacy on airway hyperresponsiveness associated with allergen. Oral administration of l-arginine, the precursor of NO, does not appear to show promise as an intervention in allergic asthma.

References
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