# Endothelin Production and Effects of Endothelin Antagonism During Experimental Airway Inflammation

FINN FINSNES, OLE H. SKJØNSBERG, THEIS TØNNESSEN, ODDVAR NÆSS, TORSTEIN LYBERG, and GEIR CHRISTENSEN

Department of Pulmonary Medicine, Institute for Experimental Medical Research, Research Forum, and Department of Pathology, University of Oslo, Ulleval Hospital, Oslo, Norway

**Endothelin (ET) has strong bronchoconstrictor properties, stimulate mucus secretion and mucosal edema, and may also exert proinflammatory effects. Therefore, ET may play a pathogenic role in inflammatory airway diseases such as bronchial asthma. The production and localization of ET and the effect of blocking ET receptors was investigated in rats during airway inflammation induced by intratracheal instillation of dextran (Sephadex). We observed a considerable increase in the concentration of ET in bronchoalveolar lavage fluid (BALF) during the early phase of inflammation, with an increase from 2.2 ± 0.6 pg/ml in controls to 40.0 ± 6.7 pg/ml at Day 1, declining to 5.3 ± 1.1 pg/ml at Day 14. Correlated with the ET response in BALF was a considerable increase in total cell count (r = 0.61), eosinophils (r = 0.83), and neutrophils (r = 0.81). Plasma ET was not elevated. Immunohistochemical analyses revealed ET-like staining in the bronchial epithelium. Treatment with the ET receptor antagonist bosentan inhibited the increase in eosinophils in BALF and reduced the inflammatory reaction in the lung tissue. In summary, a considerable increase in the ET concentration in BALF was demonstrated during the acute phase of an experimental eosinophilic airway inflammation, coinciding with an increased ET-like immunostaining in the bronchial epithelium. Treatment with an ET receptor antagonist inhibited the inflammatory response in BALF and in the tissue. Finsnes F, Skjønsberg OH, Tønnessen T, Næss O, Lyberg T, Christensen G. Endothelin production and effects of endothelin antagonism during experimental airway inflammation.** AM J RESPIR CRIT CARE MED 1997;155:1404–1412.

Endothelin (ET), originally derived form cultured endothelial cells, is a 21-amino-acid peptide that is the most potent vasoconstrictor substance yet known (1). This peptide has also been shown to have very strong bronchoconstrictor properties (2). In addition, several other effects of ET may be relevant to the pathophysiology of pulmonary diseases. A recent report indicates that ET induces mucus secretion from isolated submucosal glands (3). Furthermore, ET seems to increase vascular leakage and may therefore induce mucosal edema (4, 5). Interestingly, these effects are also key features of inflammatory airway diseases such as bronchial asthma.

The first part of the present study was undertaken to investigate whether airway inflammation is associated with increased ET production *in vivo*, and to localize the histologic site of mature ET within lung tissue. Moreover, we wanted to examine whether the ET response changes during the course of inflammation. These issues were studied by using intratracheal instillation of Sephadex beads, which induce an inflammation in rat airways. This model shows similarities to bronchial asthma, since Sephadex beads act as antigens and provoke an eosinophilic inflammation in the lower airways (6). Animals were evaluated in groups during the acute phase of inflammation at Days 1 and 2, as well as later in the process at Days 7 and 14, when the inflammation ceased. We performed bronchoalveolar lavage (BAL) to determine the concentration of ET in the bronchoalveolar fluid (BALF), and studied the extent of airway inflammation by total and differential cell count in the fluid. The lungs were removed for histologic examination and for immunohistochemical analyses to reveal the localization of ET.

Cytokines and inflammatory mediators contribute to development of the characteristic eosinophilic inflammation observed in bronchial asthma, which in turn is accompanied by mucosal edema, increased mucus production, and bronchoconstriction. Products of the metabolism of arachidonic acid are believed to play an essential role in this inflammatory process. Stimulated by the enzyme phospholipase $A_2$ ($PLA_2$), arachidonic acid is metabolized to potent inflammatory mediators such as prostaglandins, thromboxanes, and leukotrienes. An *in vitro* study (7) has shown that ET may activate $PLA_2$. In addition, ET has been shown to stimulate arachidonate-15-lipoxygenase activity and oxygen radical formation in the rat lung (8). Thus, if present during the inflammatory reaction, ET may participate in and reinforce the inflammatory process. However, whether ET is of importance during the development of inflammatory airway diseases is not yet known. Therefore, the second part of the present study was undertaken to investigate whether blocking of endothelin receptors inhibits the inflammatory response. Blocking of the ET receptors was achieved with bosentan, a recently developed antagonist of both $ET_A$ and $ET_B$ receptors (9).

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Correspondence and requests for reprints should be addressed to Finn Finsnes, M.D., Institute for Experimental Medical Research, Ulleval Hospital, 0407 Oslo, Norway.

## METHODS

### Experimental Procedure

Forty-eight male Wistar rats, aged 11 wk and with an average weight of 320 g, were used. They were kept at constant temperature and allowed food and water *ad libitum*. All interventions were performed between 9:00 and 12:00 A.M. The experiments were approved by the Norwegian Ethics Committee for Animal Research, and were performed according to National Institutes of Health (NIH) guidelines.

The animals were anesthetized with a mixture of 30% $O_2$, 70% $N_2O$, and 4% halothane (Fluothane®; Zeneca, Macclesfield Cheshire, UK). Atropine sulfate (Atropin®; Nycomed Pharma, Oslo, Norway), 1.0 ml/kg body weight, was injected intraperitoneally immediately before the experiments to reduce saliva production. Sephadex (G-200 Superfine; Pharmacia, Uppsala, Sweden) was dissolved in phosphate buffered saline (PBS) (5.0 mg/ml) and given intratracheally through a cannula in a volume of 1.0 ml/kg body weight. Sephadex consists of dextran, to which rats have an endogenous hypersensitivity (6).

Blood samples were collected from the abdominal aorta before the trachea and lungs were dissected free and the heart removed. Immediately thereafter a modified polyethylene tube (BC-5C; Olympus, Tokyo, Japan), was inserted into the trachea and ligated to the right stem bronchus, distal to the upper lobe. The guide wire was then removed and the tube attached to a 5-ml syringe. Three milliliters of PBS were instilled and gently aspirated. The lavage procedure was repeated twice with another 2 ml PBS. Immediately after BAL, the lungs were distended *in situ* to total lung capacity and fixed by instillation of 4% phosphate buffered formaldehyde at pH 7.4. Subsequently, the lungs were removed for histologic and immunohistochemical analyses. A control group consisting of six animals received an equal volume of PBS intratracheally. These animals were evaluated at Day 1. To exclude any interference by intratracheal PBS instillation on the endpoints, three rats were compared with the control group in a pilot study. No differences between the two groups were observed.

To investigate whether an increase in ET production in the airways occurred during the course of inflammation, we induced airway inflammation in 24 rats. The animals were divided into four groups and killed after 1, 2, 7, or 14 d. In order to examine whether blocking of ET receptors could inhibit the inflammatory response, 18 animals were subjected to treatment with the ET receptor antagonist bosentan (Hoffman-La Roche, Basel, Switzerland). Bosentan is a new orally active nonpeptide compound with mixed antagonist properties for $ET_A$ and $ET_B$ receptors (9). In six animals, bosentan was given intravenously (30 mg/kg body weight) 1 h before intratracheal Sephadex instillation, with repeated injections after 8 and 16 h. These animals were killed after 24 h (Day 1). In 12 animals bosentan was administered by gastric gavage 2 h prior to Sephadex instillation in a dose of 100 mg/kg body weight. The treatment was repeated every 12th hour. These animals were divided into two groups and killed after 1 or 2 d.

### Morphologic and Immunohistochemical Studies

Blocks of lung tissue were embedded in paraffin, and 5-μm-thick sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for assessment of histologic changes. For immunohistochemistry, antisera were raised in Chinchilla brown rabbits against synthetic ET-1 (Sigma Chemical, St. Louis, MO) linked to keyhole limpet hemocyanin with glutaraldehyde. Primary immunization (200 μg of ET-1 in 0.5 ml 0.9% NaCl) was done with Freund's complete adjuvant, and subsequent immunizations (100 μg ET-1) were done with Freund's incomplete adjuvant. Antiserum code K-45 showed the highest anti-ET-1 titer and was selected for further study of ET-like immunoreactivity in rat lungs. Sections were also stained with rabbit antisera against ET and preproendothelin-1 (prepro-ET-1) (Peninsula, Belmont, CA) at a dilution of 1:1,000, using the alkaline phosphatase antialkaline phosphatase (APAAP) method as described by Cordell and associates (10). Briefly, sections were dewaxed in xylene and incubated with the primary antibody at 4° C overnight, followed by sequential incubation (30 min) with a 1:40 dilution of mouse antirabbit IgG (Dako, Glostrup, Denmark) and a 1:40 dilution of rabbit antimouse IgG (Dako). The antisera against ET do not distinguish between the three isoforms of the peptide or between ET and its precursor, big ET. The antibody against prepro-ET-1 has no cross-reactivity with big ET or mature ET. All reagents were diluted in phosphate buffered saline (PBS), pH 7.5, containing 1.0% bovine serum albumin (BSA). After incubation with APAAP complexes (Dako, 1:40, 30 min), antibodies bound to ET were visualized by staining with the alkaline phosphatase substrate Fast Red. Background alkaline phosphatase activity in both control and Sephadex-exposed tissue was evaluated by omitting the primary (anti-ET) antibody. Controls with a normal (nonimmune) rabbit serum and irrelevant antibodies (rabbit antihuman growth hormone) in the primary step were also included. All sections were counterstained with hematoxylin.

The histologic sections were screened by two observers in a blinded manner. The extension of the inflammatory region around the Sephadex beads was graded separately in each tissue, with at least 10 regions being examined.

### Analysis of BALF and Plasma

The lavage fluid was collected into prechilled tubes containing ethylenediamine tetraacetic acid (EDTA) and kept on ice until centrifuged at 800 × *g* for 10 min at 4° C. The cell pellet from the BALF was resuspended in PBS and the total number of white blood cells were counted in a Bürker hemocytometer. Slides were prepared with a cytocentrifuge (Cytospin; Shandon Southern Ltd., Runcorn, UK) at 100 × *g* for 5 min. The slides were stained with Diff-Quick® (Baxter Diagnostics AG, Düdingen, Switzerland), and at least 400 nonepithelial cells were counted to determine the proportion of pulmonary alveolar macrophages, eosinophils, lymphocytes, and neutrophils.

Total protein in unconcentrated BALF was measured with a colorimetric method, using reagents from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) containing Coomassie Brilliant Blue as indicator.

The blood samples were collected directly into prechilled tubes containing EDTA (4.1 mM final concentration) and centrifuged at 800 × *g* for 10 min at 4° C. Plasma and the supernatant from the BALF were immediately stored at −70° C until analyzed. ET was determined as described by Tønnessen and coworkers (11), using an ET-1-21-specific $^{125}I$ assay system (RPA 555) from Amersham International (Cardiff, UK). Prior to ET analysis, samples were extracted in duplicate from 1 ml plasma and 2 ml BALF, respectively. This assay system is specific for ET-1-21, with 100% cross-reactivity with ET-1, 144% cross-reactivity with ET-2, and 52% cross-reactivity with ET-3, and has no cross-reactivity with big ET. The ET assay has a limit of detection equivalent to 1.6 pg/ml. For samples with ET concentrations below this limit, the values were set to 1.6 pg/ml.

### Statistical Analysis

All values are expressed as means ± SEM. Statistical analyses were done with scientific statistical software (SigmaStat, Jandel Scientific GmbH, Ekrath, Germany). The groups were compared with the Kruskal-Wallis test followed by the Student-Newman-Keuls test for multiple comparisons. Calculation of correlation coefficients for the ET concentration in BALF versus cell counts was done with Spearman's method (12). A value of $p < 0.05$ was considered statistically significant.

## RESULTS

### Effects of Intratracheal Sephadex Instillation on Lung Morphology and Bronchoalveolar Cell Profile

Figure 1A shows normal tissue morphology in control animals at Day 1 after intratracheal PBS instillation. In contrast, Sephadex challenge induced a peribronchial and peribronchiolar inflammation. At Day 1, a focal bronchiolitis and alveolitis occurred, with an accumulation of inflammatory cells in close connection with the Sephadex beads (Figure 1B). This infiltration of inflammatory cells was dominated by eosinophils, neutrophils, and macrophages. At Day 2 the eosinophilic and neutrophilic inflammation was less pronounced, but there was still a substantial number of eosinophils in the peribronchial and peribronchiolar tissue as well as in the alveoli. Later, at Days 7 and 14, there were areas with interstitial inflammation dominated by eosinophils, and formation of granulomas with giant cells in the inflammatory regions.

Instillation of Sephadex caused a considerable increase in the number of inflammatory cells in the BALF. Compared with con-

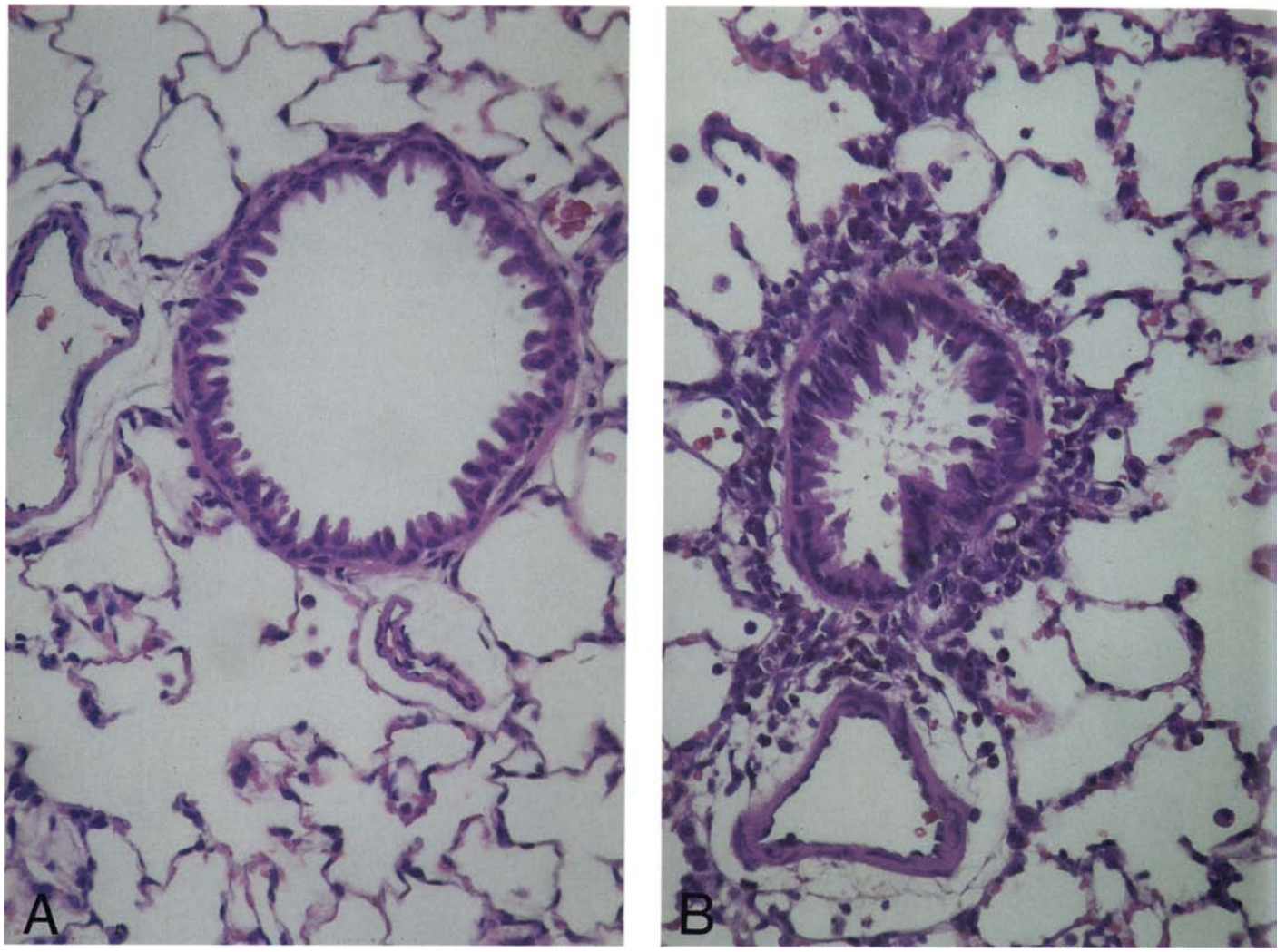


*Figure 1.* Normal tissue morphology in control animals (*A*). Intratracheal Sephadex instillation caused a focal peribronchial inflammation at Day 1 (*B*). (H&E stain; original magnification *both panels* ×100.)

trols, a threefold increase in total cell count at Day 1 was seen (Figure 2). At Day 2 the inflammation was less pronounced, but there was still a twofold increase in total cell count in the BALF. Later, at Days 7 and 14, the total cell counts showed a further decline and were not significantly different from controls.

The differential cell count in BALF from control animals showed 97% macrophages and 0.2% eosinophils (Table 1). Intratracheal Sephadex installation promoted a pronounced increase in the number of eosinophilic granulocytes. At Day 1 the differential cell count demonstrated as much as 34% eosinophils. At Day 2 the percentage of eosinophils remained high in the Sephadex group (32%). At Day 7 the proportion of eosinophils had decreased to 28%, and at Day 14 a further decline to 10% occurred. Sephadex instillation caused an increase in BALF neutrophils as well. In controls there was less than 1% neutrophils. However, in Sephadex-challenged animals at Day 1, neutrophils accounted for 19% of the leukocytes, rapidly decreasing to 7% at Day 2. At Days 7 and 14 the percentage of neutrophils showed a further decrease and remained low, amounting to 3%. Consequently, the relative proportion of macrophages in the Sephadex-treated animals was strongly reduced, to 38% of the total cell count at Day 1 and to 48% at Day 2. Later, at Days 7 and 14, the proportions of macrophages were 63% and 83%, respectively (Table 1).

### Effects of Intratracheal Sephadex Instillation on ET Concentration in BALF and Plasma

Intratracheal Sephadex instillation caused a substantial increase in ET concentration in the BALF during the acute phase of inflammation (Figure 3). In the control animals, which were given intratracheal PBS, the ET concentration in BALF was very low, being below the limit of detection (1.6 pg/ml) in five of six animals. When set to the limit of detection, this gave a mean concentration of 2.2 ± 0.6 pg/ml for that group. In contrast to the low ET concentration in the control group, the ET concentration at Day 1 after Sephadex-induced airway inflammation was 40.0 ± 6.7 pg/ml. At Day 2 the concentration of ET was significantly lower, and amounted to 18.0 ± 3.2 pg/ml. However, the ET concentration was still considerably increased as compared with controls. As the acute airway inflammation had ceased at Day 7, the ET concentration fell further, to 6.4 ± 1.1 pg/ml. At Day 14, Et was detectable in five of six animals (5.3 ± 1.1 pg/ml), and the concentration tended to be lower than at Day 7, but still significantly increased as compared with controls.

In bosentan-treated, Sephadex-challenged animals, the ET concentration in BALF at Day 1 was significantly lower than in Sephadex-challenged animals not having received bosentan. The ET concentration was 17.2 ± 4.1 pg/ml after intravenous

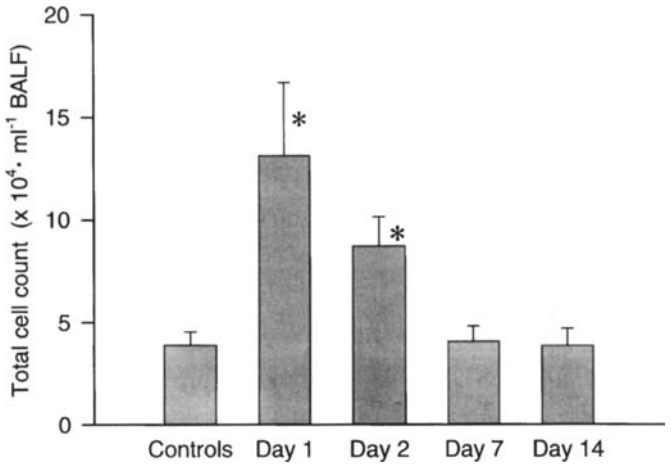


*Figure 2.* Total cell count in BALF (× $10^4$/ml) in controls (n = 6) and in animals receiving intratracheal Sephadex (n = 6 in each group), evaluated at Days 1, 2, 7, and 14 after challenge (*p < 0.05 compared with controls). Values are given as means ± SEM.

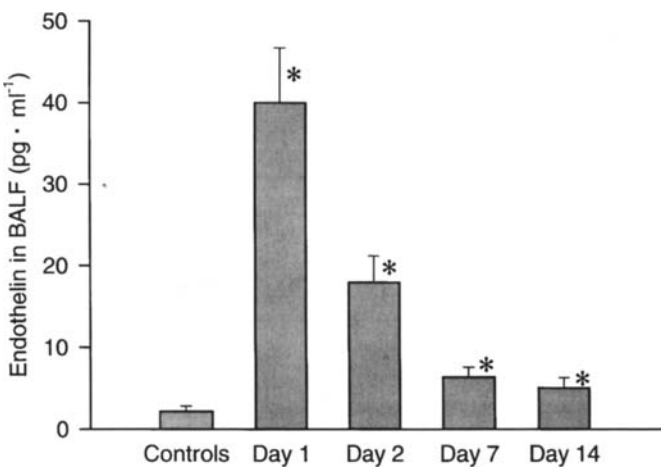


*Figure 3.* ET concentration in BALF (pg/ml) in controls (n = 6) and in animals receiving intratracheal Sephadex (n = 6 in each group), evaluated at Days 1, 2, 7, and 14 after challenge (*p < 0.05 compared with controls). Values are given as means ± SEM.

and 25.2 ± 1.9 pg/ml after oral treatment with the ET receptor antagonist. At Day 2 the ET level was 16.7 ± 3.5 pg/ml following oral bosentan treatment, not significantly different from that with Sephadex alone. However, the ET concentration in BALF was higher in all bosentan-treated animals than in controls.

The concentration of total protein in BALF from Sephadex-treated animals was increased. When correcting the ET concentrations for protein content in BALF, the ET-protein ratio showed virtually the same pattern as described earlier. The concentration of ET in BALF was 20.2 ± 9.7 pg · $ml^{-1}$ · $g^{-1}$ protein in controls (Figure 4). The ratio of ET to protein was 194.2 ± 64.3 pg · $ml^{-1}$ · $g^{-1}$ at Day 1 in Sephadex-challenged animals. At Day 2 it was significantly lower (102.6 ± 39.2 pg · $ml^{-1}$ · $mg^{-1}$), but was still increased as compared with controls. At Day 7 the ET/protein ration was 38.1 ± 10.6 pg · $ml^{-1}$ · $g^{-1}$, and at Day 14 the ratio further declined to a value of 26.8 ± 8.3 pg · $ml^{-1}$ · $g^{-1}$.

The ET concentrations were significantly correlated with the total cell counts in BALF (r = 0.61) (Figure 5A). An even higher correlation was found between ET concentration and the number of eosinophilic granulocytes (r = 0.83) and between ET and neutrophils (r = 0.81) (Figure 5B and C).

The plasma concentration of ET was 25.0 ± 1.7 pg/ml in the control group. There was no significant difference between plasma ET concentrations in the controls and the animals receiving Sephadex at Day 1 (25.7 ± 3.8 pg/ml), Day 2 (29.0 ± 4.8 pg/ml), Day 7 (24.1 ± 2.7 pg/ml), or Day 14 (21.2 ± 2.8 pg/ml).

**TABLE 1**

**DIFFERENTIAL CELL COUNT IN BALF AFTER INTRATRACHEAL SEPHADEX INSTILLATION**

| Group | Macrophages (%) | Eosinophils (%) | Lymphocytes (%) | Neutrophils (%) |
|---|---|---|---|---|
| Controls | 97.1 ± 0.5 | 0.2 ± 0.2 | 2.1 ± 0.5 | 0.6 ± 0.2 |
| Sephadex, Day 1 | 37.8 ± 2.1* | 34.2 ± 2.2* | 9.0 ± 1.2* | 19.0 ± 3.3* |
| Sephadex, Day 2 | 48.0 ± 2.9* | 32.1 ± 2.0* | 13.2 ± 2.2* | 6.7 ± 1.7* |
| Sephadex, Day 7 | 62.8 ± 5.0* | 27.8 ± 3.8* | 6.6 ± 0.8 | 2.8 ± 1.1 |
| Sephadex, Day 14 | 82.6 ± 3.1 | 9.7 ± 1.9* | 4.5 ± 1.3 | 3.2 ± 1.1 |

Values are given as means ± SEM.
* p < 0.05 compared with controls.

### Localization of Mature ET in the Airways

The immunohistochemical analyses of normal lung tissue revealed a weak staining of ET-like immunoreactivity in the blood vessel walls, mesothelial cells of the visceral pleura, and bronchial epithelium in most epithelial cells of conducting airways from the hilum to the periphery (Figure 6A). In the specimens from the Sephadex-treated animals at Days 1 and 2, we observed stronger staining of the cytoplasm of most epithelial cells. Moreover, within the epithelial lining, intensely stained cells occurred singly or in clusters (Figure 6B). These showed stronger staining from the apex to the base, and represented ciliated epithelial cells and Clara cells. The secretory and basal cells of the epithelium were not stained. Neither the inflammatory regions surrounding the Sephadex beads nor the alveoli were stained. However, Type II pneumocytes occasionally showed intense immunostaining. At Days 7 and 14 the staining of the bronchial epithelium was weaker than at Days 1 and 2, and not different from controls. The bosentan-treated animals showed increased

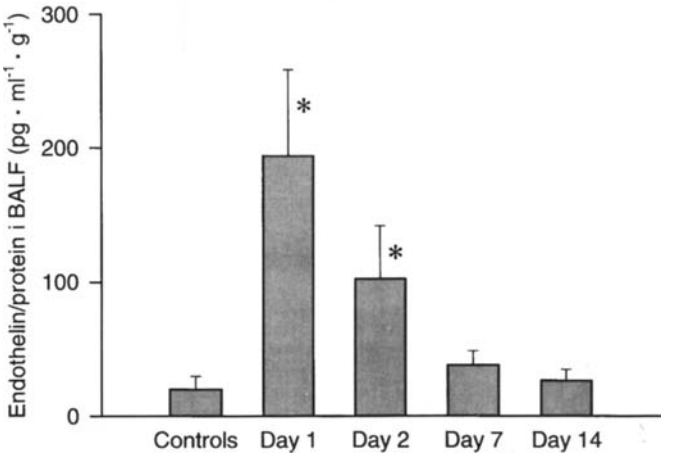


*Figure 4.* ET concentration adjusted for total protein content in BALF (pg · $ml^{-1}$ · $g^{-1}$) in controls (n = 6) and in animals receiving intratracheal Sephadex (n = 6 in each group), evaluated at Days 1, 2, 7, and 14 after the challenge (*p < 0.05 compared with controls). Values are given as means ± SEM.

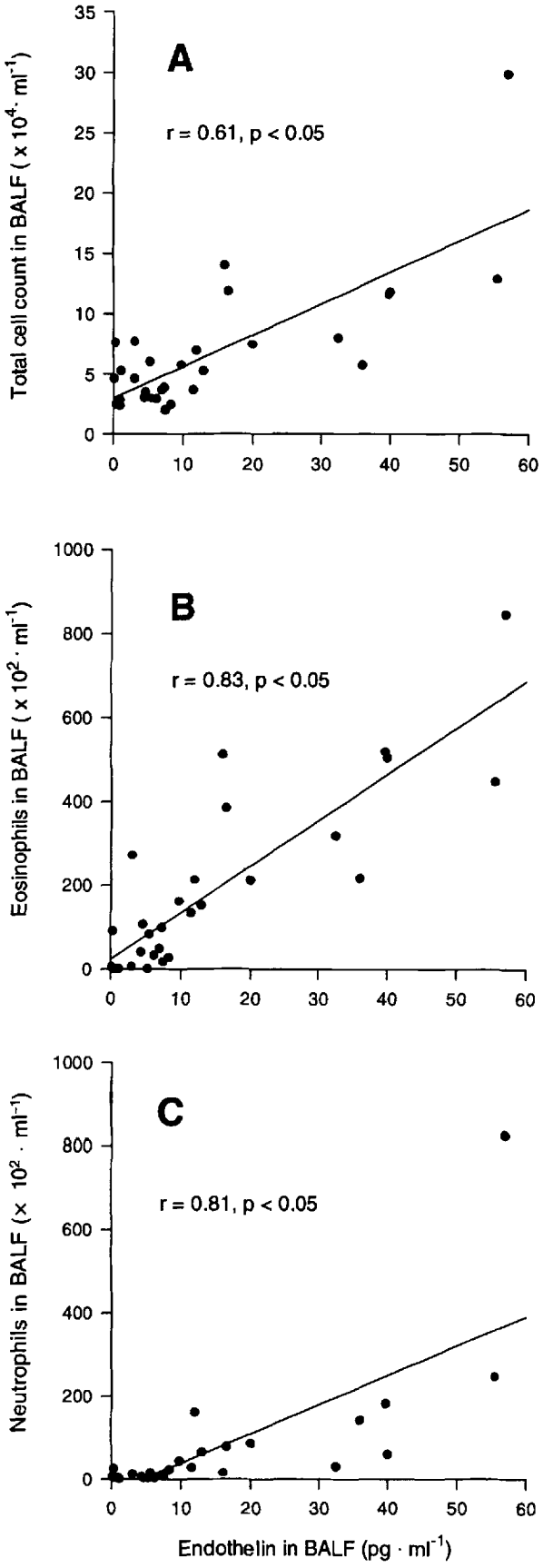


*Figure 5.* Relationship between ET concentrations in BALF (pg/ml) and total number of cells (*A*), eosinophils (*B*), and neutrophils (C) in BALF.

staining within the bronchial epithelium as compared with controls, and no difference was observed between these animals and Sephadex-challenged animals evaluated at Days 1 and 2. In both the controls and the Sephadex-challenged animals there were solitary alveolar macrophages showing intense immunostaining. Immunohistochemical analyses done with an antibody against prepro-ET-1 showed the same pattern of immunoreactivity.

### Effects of the ET Receptor Antagonist Bosentan on Bronchoalveolar Cell Profile and Lung Morphology

As described earlier, instillation of Sephadex caused a considerable inflammatory response, reflected by a threefold increase in total cell count in the BALF and a strongly affected differential count. Both intravenous and peroral treatment with bosentan abolished the increase in total cell count at Day 1 (Figure 7A). Similarly, at Day 2 there was a significant reduction in total cell count in animals given bosentan orally prior to Sephadex challenge.

The absence of an inflammatory cell increase in bosentan-treated animals was mainly due to a reduction in the number of eosinophils (Figure 7B). The early eosinophilic inflammation in the BALF at Day 1 after intratracheal Sephadex instillation (34% eosinophils) was significantly reduced to 10% and 13% when treatment with bosentan was given intravenously or orally, respectively. A significant reduction in the percentage of eosinophils was also observed at Day 2 following oral administration of bosentan.

Regarding the neutrophilic granulocytes, no significant differences were observed between the animals receiving Sephadex alone and those receiving Sephadex and bosentan. The relative proportion of macrophages, however, representing 38% of the bronchoalveolar cells at Day 1 in the Sephadex group, was significantly higher after bosentan treatment (i.e., 72% when the antagonist was given intravenously and 66% after oral administration). At Day 2 there were 48% macrophages in the Sephadex group and 71% in the Sephadex and bosentan group.

The extension of the inflammatory regions around the Sephadex beads in the lower airways was strongly reduced in all animals receiving treatment with bosentan intravenously. In these animals there was only a slight inflammatory reaction surrounding the beads (Figure 8B) as compared with animals that were not treated with bosentan (Figure 8A). When bosentan was administered orally, there was a tendency toward reduced inflammation at Day 1, but this finding was not evident.

## DISCUSSION

Intratracheal instillation of Sephadex caused a distinct eosinophilic peribronchial and peribronchiolar inflammation. In the later stage of the inflammation, granulomas also occurred. In accordance with previous studies (13), we found a substantial increase in total cell count in the BALF in the acute phase of inflammation, a high percentage of these cells being eosinophilic granulocytes (13). In this respect, Sephadex beads in the airways seem to show properties similar to those of inhaled antigens known to cause allergic asthma in humans. Our study confirms that intratracheal instillation of Sephadex is a reproducible method for studying eosinophilic airway inflammation in rats (6, 13).

A considerable increase in the concentration of ET in BALF was demonstrated, concomitant with the increase in total cell count and eosinophils in the BALF. When ET concentrations in BALF were corrected for total protein, the profound increase during the acute phase of inflammation and the gradually attenuated response showed a similar pattern. No increase in ET concentration was observed in plasma. Consequently, the in-

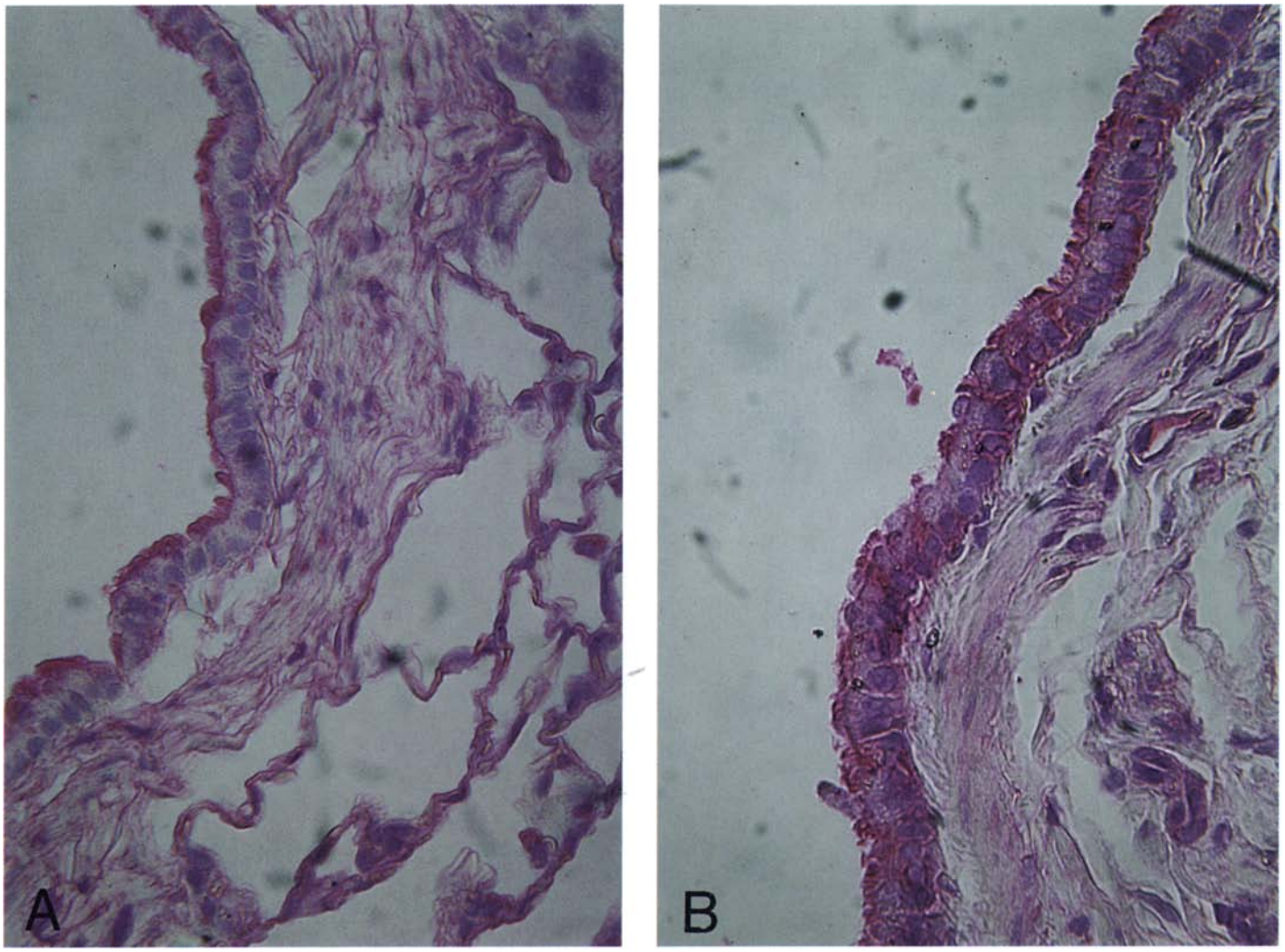


*Figure 6.* Immunohistochemical staining, using an antibody against ET-1, of lung tissue in a control animal (*A*). Lung tissue at Day 1 after Sephadex challenge (*B*) demonstrates a bronchial epithelial lining with singularly more strongly stained epithelial cells. The *red color* localized to the ciliae in *both panels* represents nonspecific staining. (APAAP method, hematoxylin counterstained; original magnifications *A* ×100, *B* ×160.)

creased ET concentration in BALF seemed not to be due to vascular leakage, but more likely to a *de novo* synthesis in the airways. Other investigators have shown an increased ET-like immunoreactivity in homogenized lung tissue in a similar model (14). In addition, increased ET concentrations in BALF have been demonstrated in patients with inflammatory pulmonary diseases such as asthma (15–18). Our study shows that the increase in the ET concentration peaks in the early phase of the inflammation, gradually leveling off during the following 2 wk. In addition, we found a strong correlation between the ET concentration and the number of inflammatory cells in BALF, both in terms of total cell count, eosinophils and neutrophils. Data demonstrating a time-related ET response during the course of an inflammatory process, and a correlation between this peptide and the number of inflammatory cells, have to our knowledge not previously been published.

*In vitro*, endothelial cells (1), vascular smooth-muscle cells (19), macrophages (20), and alveolar epithelial cells (21) have been shown to produce ET. In biopsies from humans, ET-like immunoreactivity has been detected in airway and glandular epithelial cells, Type II pneumocytes, and endothelial cells (22–24). Each of these cell types could therefore potentially participate in the increased production of ET during the Sephadex-induced airway inflammation *in vivo*. We could not show any increase in the expression of mature ET in endothelial or smooth-muscle cells in the Sephadex-treated animals, as determined by immunohistochemistry. Bronchial epithelial cells were the only subset of cells to exhibit an increase in ET-like immunoreactivity. Studies of asthmatic patients have revealed an increased expression of ET-like immunoreactivity in the epithelial layer (22, 23). However, in these studies no further localization within the epithelial lining was done. In our study, the most pronounced increase was seen in single ciliated epithelial cells and Clara cells, which were strongly stained from the base to the airway lumen. Other epithelial cells showed weaker staining or no staining at all. The basal cells and the secretory cells of the epithelium were not stained. Others have found immunostaining in secretory cells of rats (25), but we could not confirm that finding. In our material, some of the alveolar macrophages were stained, but the amount of stained cells did not obviously differ between controls and Sephadex-challenged animals. The localization of increased mature ET to the airway epithelial layer in our study is of particular interest, since these cells are the first to be exposed to allergens and other agents that can initiate airway inflammation and bronchial asthma.

The mechanism for the increased ET concentration during

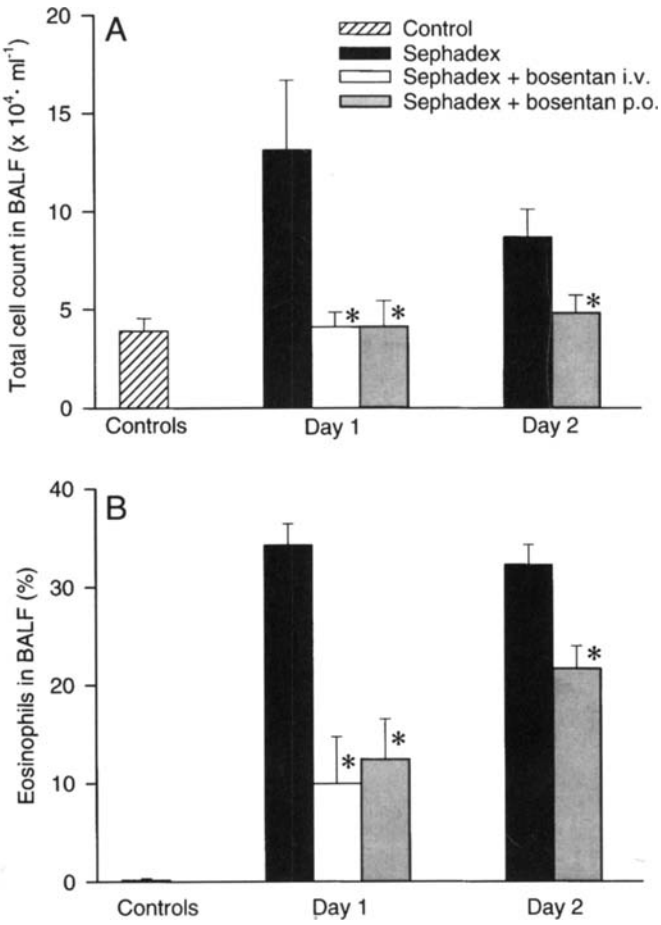


*Figure 7.* Effect of bosentan on total cell count (*A*), and eosinophils (*B*) in BALF from animals studied at Days 1 and 2 after intratracheal Sephadex instillation. *Significant differences in Sephadex + bosentan-treated groups compared with Sephadex alone ($p < 0.05$).

inflammation is unknown. The increased ET production might be triggered by cytokines involved in inflammation. This hypothesis is supported by *in vitro* studies reporting increased synthesis of ET-1 in cultured endothelial cells (26), cultured guinea-pig airway epithelial cells (27), and cultured human bronchial epithelial cells (28), promoted by such proinflammatory cytokines as interleukins (ILs) and tumor necrosis factor-α (TNF-α). Moreover, in a recent investigation (29), IL-1β and TNF-α induced an early peak in prepro-ET-1 mRNA and subsequently ET-1 peptide expression in cultured pulmonary endothelial cells of rats. Hence, there seems to be a link between proinflammatory cytokines and ET response, which could explain our findings.

In the second part of our study, we showed that treatment with the ET receptor antagonist bosentan abolished the increase in total cell count in BALF during Sephadex-induced airway inflammation. This effect was mainly due to a reduction in the number of eosinophils. Histologic examination demonstrated a lesser extent of inflammation after administration of bosentan intravenously. This antiinflammatory effect of bosentan has to our knowledge not previously been demonstrated. The inhibition of the inflammatory response was most striking after intravenous administration of bosentan.

The inhibition of the airway inflammation after bosentan treatment could potentially be a consequence of blocking the effects of ET on the arachidonic acid cascade. The metabolism of arachidonic acid is crucial in the inflammatory process, and the activation of $PLA_2$ is the initiating step. $PLA_2$ stimulates the metabolism of arachidonic acid to mediators with both chemotactic activity and strong bronchoconstrictor properties. ET has been shown to activate $PLA_2$ (7), and consequently, blocking of ET receptors could imply an inhibition of the arachidonic acid metabolism. Indeed, inhibition of $PLA_2$ is a main target for corticosteroids, which have been shown to be highly effective in the treatment of airway inflammation. Glucocorticosteroids have recently been shown to inhibit an increase in the ET concentration in BALF after intratracheal Sephadex instillation (30), weighing against a hypothesis of ET release as the initial step in inflammation. Bosentan treatment of Sephadex-challenged animals resulted in a reduction of the ET concentration in BALF at Day 1. This finding also indicates that ET production is triggered by inflammatory mediators, and that ET further promotes the inflammatory reaction. If, on the other hand, ET release precedes the inflammatory response, we would have to presume a positive feedback mechanism of released ET on ET production, which in turn is blocked by bosentan. The effects of ET and bosentan are probably not linked to changes in receptor density and distribution, since Goldie and colleagues, in a recent study, could not find any difference in ET receptor density in isolated asthmatic and nonasthmatic bronchi (31).

ET could also act as a chemoattractant in the airways, either directly or via release of proinflammatory substances involved in chemotaxis and recruitment of inflammatory cells to the airways. Consequently, bosentan could exhibit its antiinflammatory action by blocking these mechanisms. Indeed, recent studies have focused on the possible effects of ET with regard to leukocyte migration (5, 32). However, the results are not consistent. In a study done by Helset and coworkers (5), intravenous administration of ET-1 in rats caused blood neutropenia and accumulation of neutrophils in the pulmonary circulation, with leukocytes adhering to the vascular endothelium. This is an early step required for influx of leukocytes into tissues. Furthermore, 2 h after ET-1 infusion, an increase in alveolar wall cellularity was seen as well as an increase in macrophages and polymorphonuclear leukocytes in the alveoli. These findings support a hypothesis suggesting an attenuated inflammatory response of an ET antagonist through an inhibition of leukocyte migration. In a recent published work, Filep and colleagues (32) also found that infusion of ET-1 induced blood neutropenia in guinea pigs. This effect could be blocked by bosentan. However, the authors did not demonstrate influx of neutrophils from the circulation into the bronchoalveolar space or tissue. This does not necessarily contrast with a hypothesis of ET being involved in inflammatory cell migration into the airways, since the lack of cell influx could be due to the short time between ET-1 injection and tissue evaluation (20 min).

ET has the potential capacity to play a key role in effecting the functional consequences of airway inflammation, owing to its properties as a strong bronchoconstrictor (2) as well as its ability to increase vascular leakage (4, 5, 32) and mucus formation (3). Whether ET plays a role in the pathogenesis of the inflammation itself is not known, but the results of the present study indicate a central role for ET in this process. The results of the study also suggest a therapeutic potential for ET receptor antagonists. Further studies should be undertaken to investigate the role of ET receptor antagonists in bronchial asthma.

In summary, the present study has demonstrated a considerable increase in the ET concentration in BALF during the early phase of an experimental eosinophilic airway inflammation. The ET concentration correlated significantly with the total cell count and the number of eosinophils and neutrophils in BALF. The increased ET response also coincided with an increased ET-like immunoreactivity within the bronchial epithelial lining. Moreover, this investigation suggests an alternative approach in treating inflammatory disorders in the airways, since treatment with

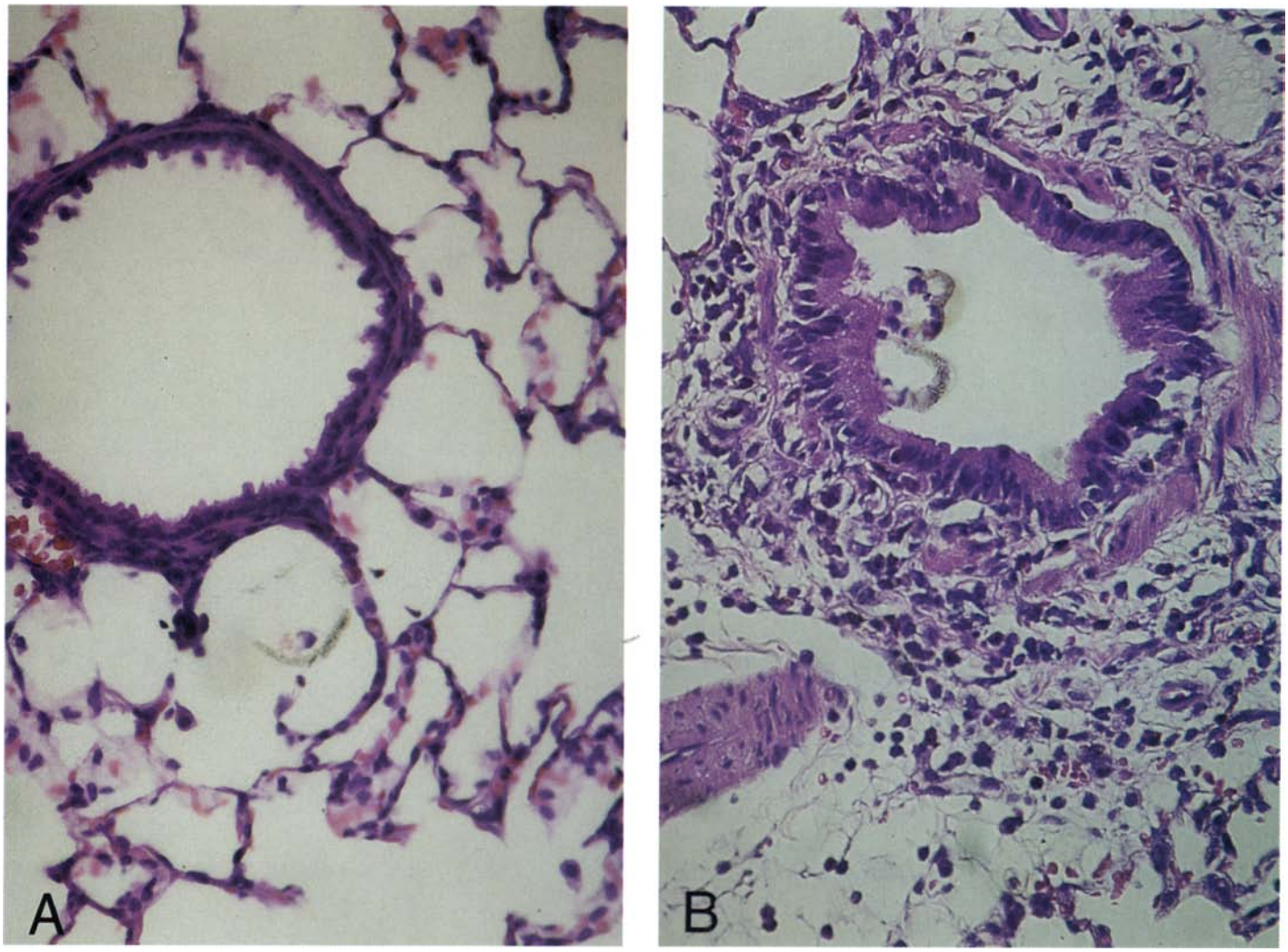


*Figure 8.* Effect of intravenous bosentan on histologic findings at Day 1 after intratracheal Sephadex challenge (*A*). For comparison, a representative picture is shown of the tissue inflammation at Day 1 without bosentan treatment (*B*). (H&E; original magnification ×100.)

the ET receptor antagonist bosentan inhibited the eosinophilic airway inflammation in BALF as well as in tissue.

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