

# Immunoreactive endothelin in bronchial biopsy specimens: Increased expression in asthma and modulation by corticosteroid therapy

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**Background:** The human endothelin (ET) family comprises three 21-amino-acid peptides, which are potent bronchoconstrictors and have a number of other biologic properties relevant to the pathophysiology of asthma.

**Objective:** We sought to compare the expression of immunoreactive ET in bronchial biopsy specimens from subjects with asthma treated only with inhaled  $\beta_2$ -agonists, subjects with asthma treated with  $\beta_2$ -agonists and corticosteroids, and control subjects without asthma.

**Methods:** Biopsy specimens were obtained by fiberoptic bronchoscopy and stained immunohistochemically with a specific ET antiserum. Epithelial ET expression was quantitated by using a computer-assisted system of image analysis. Numbers of inflammatory cells and depth of subepithelial collagen deposition were also determined.

**Results:** Immunoreactive ET was principally localized in the airway epithelium. The proportion of epithelium immunostained was significantly increased in the subjects with asthma not treated with steroids ( $35.4\% \pm 3.8\%$ ) compared with that of both the control subjects ( $16.2\% \pm 1.9\%$ ,  $p < 0.0001$ ) and the subjects with asthma treated with steroids ( $14.3\% \pm 2.0\%$ ,  $p < 0.0001$ ). The last two groups did not differ significantly from one another. There were no significant correlations between ET expression and either physiologic parameters or indexes of airway inflammation and remodeling.

**Conclusion:** Bronchial epithelial expression of immunoreactive ET is increased in subjects with asthma receiving treatment only with  $\beta_2$ -agonists but not in subjects with asthma also receiving corticosteroid therapy. These findings are consistent with the hypothesis that ET is implicated in the pathophysiology of asthma. (*J Allergy Clin Immunol* 1997; 100:544-52.)

**Key words:** Asthma, endothelin, bronchial biopsy, corticosteroids, inflammation, remodeling, collagen

The human endothelin (ET) family comprises three 21-amino-acid peptides denoted ET-1, ET-2, and ET-3. ET-1 was originally isolated and characterized as a

## Abbreviations used

|      |                           |
|------|---------------------------|
| BAL: | Bronchoalveolar lavage    |
| BSA: | Bovine serum albumin      |
| ET:  | Endothelin                |
| PBS: | Phosphate-buffered saline |

potent vasoconstrictor produced by vascular endothelial cells,<sup>1</sup> and the remaining two members were identified by screening of a human genomic DNA library.<sup>2</sup> The three peptides are encoded by separate genes<sup>2</sup> that have been assigned to different chromosomes,<sup>3</sup> but they are closely related. ET-2 and ET-3 differ from ET-1 by two and six amino acids, respectively. Each isopeptide is initially synthesized as a large precursor molecule, which is cleaved to yield 38 (ET-1), 37 (ET-2), or 41 (ET-3) amino acid intermediates known as big ETs. Final processing to the mature 21-amino-acid peptides results from the actions of specific ET-converting enzymes.<sup>4</sup>

The members of the ET family have a number of biologic effects that have suggested a role in the pathophysiology of asthma. All three isopeptides are potent bronchoconstrictors in isolated preparations of guinea pig<sup>5-7</sup> and human<sup>8</sup> airways and also exhibit in vivo bronchoconstrictor activity when administered to guinea pigs by either intravenous injection<sup>9</sup> or inhalation.<sup>10</sup> Other relevant properties include the ability to induce bronchial hyperreactivity,<sup>11</sup> mucus hypersecretion,<sup>12</sup> and inflammatory mediator release from leukocytes.<sup>13, 14</sup> Finally, ET-1 is mitogenic for airway smooth muscle cells<sup>15</sup> and fibroblasts,<sup>16</sup> as well as promoting collagen synthesis by fibroblasts,<sup>17</sup> and so may be involved in airway wall remodeling, which is also a characteristic pathologic feature of asthma.<sup>18</sup>

Members of the ET family exert their effects through an interaction with specific membrane receptors. In a variety of systems, two types of receptors have been characterized, which are denoted ET<sub>A</sub> and ET<sub>B</sub>.<sup>19</sup> The ET<sub>A</sub> receptor has a higher affinity for ET-1 or ET-2 compared with ET-3, whereas the ET<sub>B</sub> receptor has equal affinity for all three members. In human airways ET-1-induced bronchoconstriction is considered to be principally ET<sub>B</sub>-mediated,<sup>20</sup> whereas other properties such as the proinflammatory and mitogenic effects appear also to involve ET<sub>A</sub> receptors.<sup>21</sup>

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There are a number of potential sources of ET within the airways including bronchial epithelial cells,<sup>22</sup> vascular endothelial cells,<sup>1</sup> macrophages,<sup>23</sup> and mast cells.<sup>24</sup> However, the principal site of ET expression, as detected both by in situ hybridization and immunohistochemistry, is the bronchial epithelium.<sup>25</sup> In a previous study that used an arbitrary scoring system, we reported increased epithelial expression of ET in bronchial biopsy specimens from 17 subjects with asthma, some of whom were nonatopic and some of whom were receiving corticosteroid treatment.<sup>26</sup> In this study we have extended those earlier observations by using a more sensitive method of tissue processing/immunostaining and a computer-assisted system of image analysis. We have quantitated bronchial epithelial expression of immunoreactive ET in a group of subjects with atopic asthma receiving treatment with  $\beta_2$ -agonists alone, a separate group of subjects with atopic asthma receiving treatment with  $\beta_2$ -agonists and corticosteroids, and a group of control subjects without asthma. We have related these measurements to indexes of airway inflammation and remodeling.

## METHODS

### Subjects

Nine control subjects without asthma (3 male, 6 female; age,  $22.8 \pm 1.6$  years); five subjects with asthma treated only with inhaled  $\beta_2$ -agonists as required (1 male, 4 female; age,  $21.0 \pm 0.5$  years); and six subjects with asthma receiving treatment with  $\beta_2$ -agonists and inhaled, oral, or both types of corticosteroids (3 male, 3 female; age,  $30.5 \pm 5.9$  years) participated in this study. Subjects with asthma in the steroid-treated group had been receiving corticosteroids at the doses shown (or higher) for periods of at least 6 weeks. In addition, one subject in this group (number 6) was receiving an oral theophylline preparation. Control subjects were receiving no regular medication other than oral or injectable contraceptives. Subjects were all non-smokers and had not experienced recent symptoms of upper respiratory tract infection. The clinical and physiologic details of the subjects in the three groups, together with all medication taken at the time of the study, are summarized in Table I. The study was approved by the Southampton Joint University and Hospitals Ethics Committee, and all subjects gave their written consent after being fully informed about the nature and purpose of the study.

### Spirometry, bronchial reactivity, and atopic status

FEV<sub>1</sub> and bronchial reactivity to inhaled histamine were measured 1 to 4 weeks before bronchoscopy in all subjects. On this occasion, subjects with asthma were asked to withhold inhaled short-acting  $\beta_2$ -agonists for at least 6 hours. The baseline FEV<sub>1</sub> was recorded with a wedge bellows spirometer (Vitalograph Ltd., Buckingham, U.K.). To determine bronchial reactivity, subjects first inhaled 5 breaths of 0.9% saline solution from functional residual capacity to total lung capacity from an Inspiron nebulizer (CR Bard, Sunderland, U.K.) through a mouthpiece, and further measurements of FEV<sub>1</sub> were made after 1 and 3 minutes. Subjects then inhaled increasing doubling concentrations (0.03 to 16 mg/ml saline solution) of histamine (BDH, Lutterworth, Leicestershire, U.K.), and FEV<sub>1</sub> measurements were made at 1 and 3 minutes after each inhalation. These stepwise inhalations continued until either the FEV<sub>1</sub> had

fallen by at least 20% of the postsaline value or until the maximum concentration had been reached. The log<sub>10</sub> concentration of agonist was then plotted against the percentage change in FEV<sub>1</sub> from the postsaline value, and PC<sub>20</sub> was derived by linear interpolation between the last two data points.

To determine atopic status, skin prick testing was performed with the following common aeroallergens: *Dermatophagoides pteronyssinus*, cat allergen, mixed grass pollens, and dog allergen (Miles Inc., Hollister Stier, Elkhart, Ind.). Development of a wheal greater than or equal to 3 mm in diameter 15 minutes after exposure to one or more of these allergens in the presence of negative (0.9% saline solution) and positive (histamine acid phosphate) controls was considered a positive response. In addition, 10 ml of peripheral blood was obtained at the time of bronchoscopy for estimation of total serum IgE (normal range, <81 IU/ml).

### Fiberoptic bronchoscopy and bronchial biopsy

Fiberoptic bronchoscopy and bronchial biopsy were performed as described in our previously published protocol,<sup>27</sup> and in accordance with current National Heart, Lung, and Blood Institute recommendations.<sup>28</sup> Subjects received premedication consisting of nebulized salbutamol (5 mg) and ipratropium bromide (0.5 mg). Atropine (0.6 mg) was used intravenously as an antisecretory agent, and midazolam (4 to 10 mg; median, 6 mg) was administered to achieve mild sedation. Lignocaine (10% and 4% spray) was applied to the upper airways, and an Olympus BFIT20 fiberoptic bronchoscope (Olympus Company, Tokyo, Japan) was inserted through the nose or mouth. Additional aliquots of lignocaine (1%) were applied to the larynx and subsequently to the lower airways. Oxygen (28%) was administered through nasal cannulas throughout the procedure, and oxygen saturation was monitored continuously with a digital pulse oximeter (Pulsox-7; Minolta, Tokyo, Japan). Bronchial biopsy specimens were obtained from second- or third-generation airway carinas by using FB15C biopsy forceps (Olympus Company). Close clinical supervision was maintained throughout the procedure, and subjects were observed for a minimum of 4 hours afterwards.

### Sample processing and immunohistochemistry

For immunolocalization of ET, one biopsy specimen from each subject was fixed by immersion in a solution of 1% paraformaldehyde in phosphate-buffered saline (PBS: 0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.4). After 3 to 4 hours, specimens were transferred into PBS containing 0.01% (wt/vol) sodium azide as preservative and 15% (wt/vol) sucrose and stored at 4° C. To prepare tissue for cryosectioning, specimens were placed on small cork disks, embedded in OCT Compound mounting medium (Tissue Tek II; Miles Laboratories Inc., Naperville, Ill.), and frozen by immersion in melting dichlorofluoromethane (Arcton; ICI, Cheshire, U.K.) cooled with liquid nitrogen. Six-micrometer sections were cut, placed on poly-L-lysine-coated glass slides, and air-dried for 60 minutes. For assessment of epithelial integrity, sections were stained with hematoxylin and eosin. Immunohistochemistry was performed with an avidin-biotin-peroxidase technique. Endogenous peroxidase activity was first blocked by immersing slides for 30 minutes in 0.3% hydrogen peroxide in methanol. After washing in PBS, nonspecific binding was prevented by incubating with normal goat serum diluted 1:30 in PBS containing 0.1% bovine serum albumin (PBS-BSA) for an additional 30 minutes. Sections were then incubated overnight with a polyclonal rabbit ET antiserum raised by immunizing rabbits with synthetic

TABLE 1. Clinical and physiologic characteristics of the subjects studied

|                                             | Age<br>(yr) | Sex | Atopy | FEV <sub>1</sub><br>(L) | FEV <sub>1</sub><br>(percent<br>predicted) | PC <sub>20</sub><br>(mg/ml) | IgE<br>(IU/ml) | Treatment                  |
|---------------------------------------------|-------------|-----|-------|-------------------------|--------------------------------------------|-----------------------------|----------------|----------------------------|
| Control subjects                            |             |     |       |                         |                                            |                             |                |                            |
| 1                                           | 24          | M   | Y     | 4.20                    | 111.1                                      | >32                         | 700            | None                       |
| 2                                           | 20          | F   | N     | 3.95                    | 118.3                                      | >32                         | 17             | None                       |
| 3                                           | 19          | F   | N     | 3.80                    | 100.0                                      | >32                         | <10            | None                       |
| 4                                           | 23          | M   | Y     | 4.40                    | 97.8                                       | >32                         | <10            | None                       |
| 5                                           | 22          | F   | Y     | 4.05                    | 116.0                                      | >32                         | 22             | None                       |
| 6                                           | 19          | F   | Y     | 3.15                    | 90.5                                       | >32                         | <10            | None                       |
| 7                                           | 35          | F   | N     | 2.80                    | 105.7                                      | >32                         | <10            | None                       |
| 8                                           | 21          | M   | Y     | 6.50                    | 136.3                                      | >32                         | 175            | None                       |
| 9                                           | 22          | F   | Y     | 3.80                    | 105.6                                      | >32                         | 46             | None                       |
| Mean                                        | 22.8        |     |       |                         | 109.0                                      |                             |                |                            |
| SEM                                         | 1.6         |     |       |                         | 4.5                                        |                             |                |                            |
| Geometric mean                              |             |     |       |                         |                                            | >32                         |                |                            |
| Non-steroid-treated<br>subjects with asthma |             |     |       |                         |                                            |                             |                |                            |
| 1                                           | 21          | F   | Y     | 2.78                    | 77.4                                       | 3.49                        | 229            | S                          |
| 2                                           | 20          | F   | Y     | 3.17                    | 99.0                                       | 0.59                        | 727            | S                          |
| 3                                           | 20          | F   | Y     | 2.83                    | 91.3                                       | 0.29                        | 266            | S                          |
| 4                                           | 21          | F   | Y     | 4.04                    | 131.4                                      | 2.21                        | 275            | S                          |
| 5                                           | 23          | M   | Y     | 4.85                    | 105.9                                      | 0.32                        | 1230           | S                          |
| Mean                                        | 21.0        |     |       |                         | 101.0                                      |                             |                |                            |
| SEM                                         | 0.5         |     |       |                         | 9.0                                        |                             |                |                            |
| Geometric mean                              |             |     |       |                         |                                            | 0.84                        |                |                            |
| Steroid-treated<br>subjects with asthma     |             |     |       |                         |                                            |                             |                |                            |
| 1                                           | 21          | M   | Y     | 4.45                    | 100.5                                      | 3.35                        | 182            | P(10), S                   |
| 2                                           | 25          | M   | Y     | 3.10                    | 69.8                                       | 1.78                        | 267            | P(10), S                   |
| 3                                           | 41          | F   | Y     | 1.35                    | 50.2                                       | <0.03                       | <10            | BDP(1500), S               |
| 4                                           | 20          | M   | Y     | 5.00                    | 109.2                                      | 17.59                       | 72             | P(10), S                   |
| 5                                           | 21          | F   | Y     | 2.73                    | 85.4                                       | 0.71                        | 62             | P(10), S                   |
| 6                                           | 55          | F   | Y     | 1.12                    | 39.7                                       | NA*                         | 26             | BDP (2000),<br>P(10), T, S |
| Mean                                        | 30.5        |     |       |                         | 75.8                                       |                             |                |                            |
| SEM                                         | 5.9         |     |       |                         | 11.3                                       |                             |                |                            |
| Geometric mean                              |             |     |       |                         |                                            | <1.17                       |                |                            |

S, Salbutamol; P, prednisolone (daily dose in milligrams); BDP, beclomethasone dipropionate (daily dose in micrograms); T, theophylline. NA, not available.

\*In this subject measurement of PC<sub>20</sub> was not performed because her FEV<sub>1</sub> was less than 40% predicted.

mature ET-1. This antiserum recognizes all three ET isopeptides but does not cross-react with big ET-1. The antiserum was used at a final dilution of 1:1000 in PBS-BSA. After washing in PBS, sections were incubated successively with biotinylated goat antiserum to rabbit IgG (Vector Laboratories, Peterborough, U.K.) diluted 1:100 in PBS-BSA for 30 minutes and with freshly prepared avidin-biotin-peroxidase complex (Vectastain; Vector Laboratories) for 60 minutes. Peroxidase activity was revealed by using a glucose oxidase diaminobenzidine-nickel enhancement technique, which produces a black reaction product.<sup>29</sup> No counterstain was used. Sections were dehydrated and mounted in synthetic medium (Pertex; CellPath, Hemel Hempstead, U.K.). Control experiments were carried out with primary antiserum that had been adsorbed by overnight incubation at 4° C with synthetic human mature ET-1 at a final concentration of 10 nmol/ml diluted antiserum. Sections were photographed with a BX-60 microscope (Olympus).

For quantitation of inflammatory cell numbers and subepithelial collagen deposition, a separate biopsy specimen from

each subject was placed in ice-cooled acetone solution containing 20 mmol/L iodoacetamide and 2 mmol/L phenylmethylsulfonyl fluoride to inhibit protease activity and fixed overnight at -20° C. The following day these specimens were embedded in the water-soluble resin, glycol methacrylate (Polysciences, Northampton, U.K.). The processing of tissue into glycol methacrylate and the technique of immunohistochemistry as applied to such material have been described in detail previously.<sup>30</sup> Briefly, 2 µm sections were cut with an ultramicrotome (OM U3; Reichert, Vienna, Austria), floated onto 0.2% (vol/vol) ammonia solution for 1 minute, transferred to poly-L-lysine-coated slides, and air-dried at room temperature for 1 to 4 hours. Sections were pretreated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase activity. After three 5-minute washes in Tris-buffered saline (pH 7.6), blocking medium, consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 1% BSA, was applied for 30 minutes. Sections were then incubated with the following primary mouse mono-

clonal antibodies at previously determined dilutions: AA1 (Dr. Andrew F. Walls, University of Southampton) against mast cell tryptase to detect mast cells; EG2 (Pharmacia, Milton Keynes, U.K.) against eosinophil cationic protein to detect eosinophils; anti-CD3 (monoclonal antibody UCHT1, ICRF, London, U.K.) to detect total T lymphocytes; anti-CD4 (Becton-Dickinson, Abingdon, U.K.) and anti-CD8 (Dako Ltd., High Wycombe, U.K.) to detect T-lymphocyte helper and cytotoxic subsets, respectively; and anti-CD25 (Dako Ltd.). After overnight incubation at room temperature, bound antibodies were labeled with biotinylated rabbit anti-mouse F<sub>ab</sub> fragments (Dako Ltd.), and the procedure was completed with a streptavidin-biotin-peroxidase detection system (Dako Ltd.). Aminoethylcarbazole was used as a chromogen, and sections were counterstained with Mayer's hematoxylin.

### Quantitative analysis

Airway epithelial expression of immunoreactive ET in cryosections was quantitated by using a computer-assisted image analysis system (Seescan Symphony; Seescan, Cambridge, U.K.). For each biopsy specimen, the entire epithelium in three nonserial sections was assessed. The area of immunostaining above an optical density threshold, defined by interactive thresholding as equivalent to that of the background staining, was determined. The total epithelial area was also measured after manual delineation. A measurement of ET immunoreactivity was then calculated by expressing the area immunostained as a percentage of the total epithelial area. Epithelial integrity was also assessed in these sections by the Seescan Symphony image analysis system. The length of damaged epithelium was expressed as a percentage of the total length of epithelium measured along the basement membrane in each section. Measurements of ET expression and of epithelial damage were performed by an observer who was unaware of the clinical group from which the biopsy specimen was derived.

Quantitation of inflammatory cells and subepithelial collagen deposition was performed with sections from glycol methacrylate-embedded tissue. Numbers of positively staining cells in the entire area of subepithelial tissue present in each section, excluding glands and vessels, and in the airway epithelium were counted by using a Leitz Laborlux S microscope (Leica Ltd., Milton Keynes, U.K.) with the aid of an eyepiece graticule. A computer-assisted image analysis system (Apple Macintosh Quadra 700 computer; ColourVision 1.6 software; Improvision, Coventry, U.K.) was used to measure the area of subepithelial tissue and the length of airway epithelial basement membrane. Subepithelial and epithelial cell counts were then expressed per square millimeter of tissue area and per millimeter of basement membrane length, respectively. All measurements were performed in at least two nonserial sections per biopsy specimen by an observer who was unaware of the clinical status of the subject. For measurement of collagen thickness, 2  $\mu$ m sections were stained with toluidine blue. Photomicrographs ( $\times 1000$  magnification) of the subepithelial collagen layer were taken only in areas where the epithelium was seen to be clearly well-orientated, and multiple measurements were made with a digital micrometer (Digimatic Calipers, Mitutoyo, Japan). Average values for inflammatory cell numbers and collagen thicknesses were calculated for each biopsy specimen.

### Statistical analysis

Data for age, percent predicted FEV<sub>1</sub>, and ET immunoreactivity were expressed as means  $\pm$  SEM; data for epithelial damage, inflammatory cell numbers, and collagen measure-

ments were expressed as medians and ranges; and data for PC<sub>20</sub> was expressed as geometric means and ranges. Comparisons between groups were made by using a one-way analysis of variance with post hoc testing with Fisher's protected least significant difference test, except for PC<sub>20</sub> and epithelial damage, which were analyzed with the Kruskal-Wallis and Mann-Whitney U tests. Data for inflammatory cell numbers and collagen measurements were normalized by square-root transformation before analysis. Correlations were sought by using Pearson's and Spearman's tests for normally and nonnormally distributed data, respectively. A *p* value less than 0.05 was regarded as statistically significant. Statistical analysis was performed with StatView 4.02 software for Macintosh computers (Abacus Concepts, Berkeley, Calif.).

## RESULTS

### Subject characteristics

There were no statistically significant differences in age or sex distribution among the three groups. The percent predicted FEV<sub>1</sub> of the subjects with asthma treated with steroids ( $75.8\% \pm 11.3\%$ ) was lower than that of either the subjects with asthma not treated with steroids ( $101.0\% \pm 9.0\%$ ) or the control subjects ( $109.0\% \pm 4.5\%$ ) ( $p = 0.019$ ). All the control subjects had PC<sub>20</sub> histamine values within the accepted normal range ( $>32$  mg/ml), whereas both the subjects with asthma not treated with steroids ( $0.84$  [range,  $0.29$  to  $3.49$ ],  $p = 0.0005$ ) and the subjects with asthma treated with steroids ( $<1.17$  [range,  $<0.03$  to  $17.59$ ],  $p = 0.0005$ ) exhibited varying degrees of bronchial hyperreactivity. It was not possible to obtain a PC<sub>20</sub> measurement in the case of one individual in the steroid-treated group (subject number 6) because her FEV<sub>1</sub> measurement before use of a bronchodilator was less than 40% of predicted value. Bronchoscopy was completed without complications in all subjects.

### Immunoreactive ET

No immunoreactivity was detectable in the control sections in which the primary antiserum had been preincubated with synthetic ET-1 (Fig. 1, A). Immunoreactive ET was present in all biopsy specimens from control subjects and subjects with asthma (Fig. 1, B through D). The principal site of immunoreactivity was the bronchial epithelium in all three subject groups. The proportion of epithelial area exhibiting immunostaining for ET was significantly increased in the subjects not treated with steroids compared with the control subjects ( $35.4\% \pm 3.8\%$  vs  $16.2\% \pm 1.9\%$ ,  $p < 0.0001$ ) (Fig. 2). The area immunostained was significantly smaller in the subjects treated with steroids compared with the subjects not treated with steroids ( $14.3\% \pm 2.0\%$  vs  $35.4\% \pm 3.8\%$ ,  $p < 0.0001$ ) and was not significantly different from that of the control subjects ( $14.3\% \pm 2.0\%$  vs  $16.2\% \pm 1.9\%$ ,  $p = 0.59$ ). The coefficient of variability between different areas of the same section was less than 5%. There were no significant correlations between immunoreactive ET expression and physiologic parameters in any of the three groups.

In addition to the bronchial epithelium, expression of immunoreactive ET was evident in relation to airway

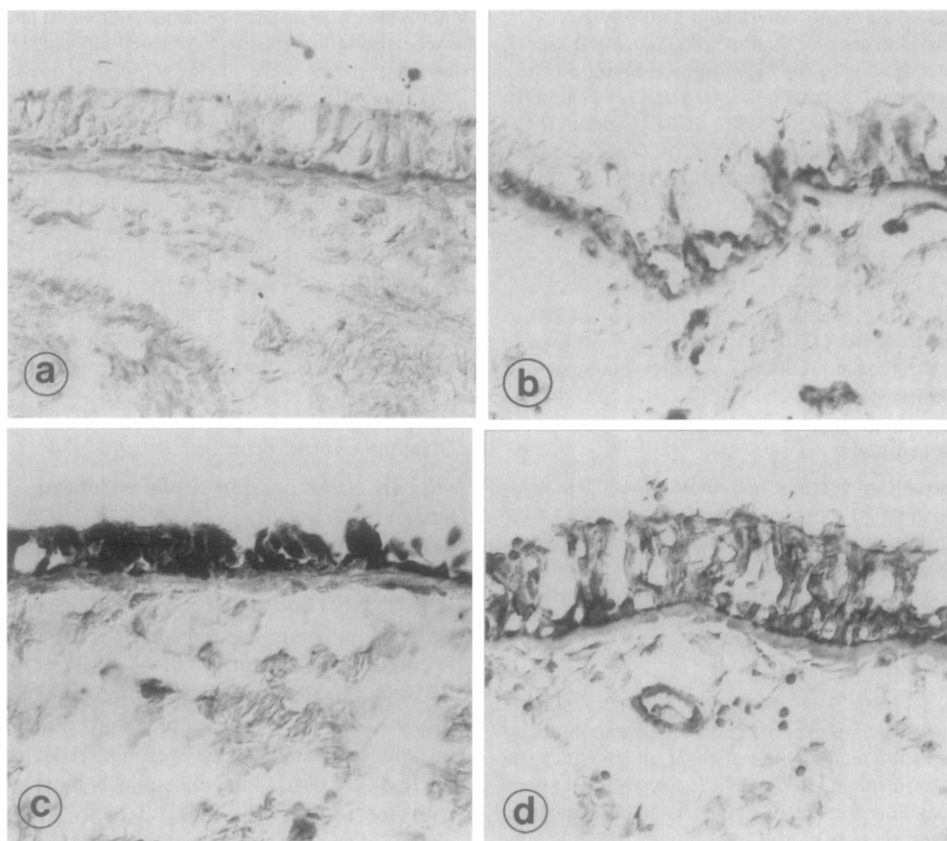


**FIG. 1.** Representative immunohistochemical staining patterns: **a**, control section in which ET antiserum had been preadsorbed by overnight incubation with ET-1; **b**, section from control subject without asthma; **c**, section from subject with asthma not treated with steroids; and **d**, section from subject with asthma treated with steroids. In each of three subject groups, the principal site of expression of immunoreactive ET is airway epithelium. Immunostaining of vascular endothelium is also evident in **d**.

glands and to the vascular endothelium. Vascular endothelial immunostaining was present in all biopsy specimens from the subjects with asthma not treated with steroids and in biopsy specimens from two subjects with asthma treated with steroids and one of the control subjects. Immunostaining of glands was evident in all biopsy specimens in which this tissue was present. Immunostaining was not obviously present in relation to macrophages or other inflammatory cells.

### Epithelial integrity

Bronchial epithelium, either intact or focally stripped, was present in all biopsy specimens. The stripping usually involved the suprabasal layer of cells, leaving the basal layer in situ. There was no statistically significant difference in the proportion of damaged epithelium between the control subjects (64.7% [range, 44.4% to 100%]), the subjects with asthma not treated with steroids (89.5% [range, 6.7% to 100%]), and subjects with asthma treated with steroids (46.3% [range, 15.4% to 100%];  $p = 0.34$ ). There were no significant correlations

between epithelial damage and either physiologic parameters or expression of immunoreactive ET in any of the three subject groups.

### Inflammatory cell numbers

Data for numbers of immunoreactive cells in bronchial biopsy specimens are presented in Table II. In the group of subjects with asthma not treated with steroids, the numbers of eosinophils in the epithelial compartment were significantly increased compared with those of the control subjects ( $p = 0.019$ ) and those of the subjects with asthma treated with steroids ( $p = 0.027$ ). The number of eosinophils in subepithelial tissue was also increased in the group of patients with asthma not treated with steroids. In the group of patients with asthma treated with steroids, the number of eosinophils was intermediate compared with those in the other two groups, although these changes just failed to achieve statistical significance (analysis of variance:  $p = 0.052$ ). Extracellular EG2 immunoreactivity, indicative of eosinophil degranulation, was only evident in biopsy speci-

mens from the subjects with asthma not treated with steroids. Numbers of other inflammatory cells did not differ significantly among the three groups. There were no significant correlations between inflammatory cell numbers and other parameters, other than in the case of the group of patients who were treated with steroids when there was an inverse correlation between percent predicted FEV<sub>1</sub> and numbers of subepithelial AA1<sup>+</sup> mast cells ( $r = -1.0$ ,  $p = 0.046$ ).

### Subepithelial collagen measurements

Biopsy specimens were unavailable or unsuitable for analysis in the case of two of the control subjects and one subject in the nonsteroid-treated group. In the remaining biopsy specimens, the subepithelial collagen layer could be clearly identified and was easily measured. The depth of this layer was significantly greater in the subjects with asthma not treated with steroids (20.4  $\mu$ m [range, 8.7 to 72.5  $\mu$ m]) than in either the control subjects (8.1  $\mu$ m [range, 4.0 to 12.0  $\mu$ m],  $p = 0.0096$ ) or the patients with asthma treated with steroids (9.9  $\mu$ m [range, 4.4 to 12.0  $\mu$ m],  $p = 0.014$ ). There were no significant correlations between the depth of this layer and either ET measurements or other parameters studied.

### DISCUSSION

Computer-assisted image analysis is becoming an increasingly popular tool for use in quantitative immunohistochemistry because it allows the operator to measure areas of interest within histologic sections both accurately and reproducibly. In this study we have used this technique to quantitate and compare *in vivo* expression of the peptide ET in bronchial biopsy specimens from three groups of subjects. We found that immunoreactive ET was principally localized in the bronchial epithelium. Compared with control subjects without asthma, the proportion of epithelial area immunostained was significantly increased in subjects with mild asthma treated only with  $\beta_2$ -adrenergic agents, whereas in subjects with asthma also receiving regular therapy with corticosteroids, ET expression did not differ significantly from control subjects.

The finding of increased ET expression in subjects with asthma not treated with steroids is consistent with a number of previous reports. In our own earlier study, by using an arbitrary scoring system and a less sensitive method of tissue processing, we were able to detect ET immunoreactivity in the bronchial epithelium in biopsy specimens from 11 of 17 subjects with asthma but in only 1 of 11 control subjects.<sup>26</sup> ET expression was also increased in the vascular endothelium and airway glands in subjects with asthma, although these changes did not reach statistical significance. However, nine subjects in the group with asthma in that study were receiving treatment with corticosteroids, and no clear relationship was detected between ET immunoreactivity and corticosteroid therapy. Increased concentrations of immunoreactive ET in bronchoalveolar lavage (BAL) fluid in

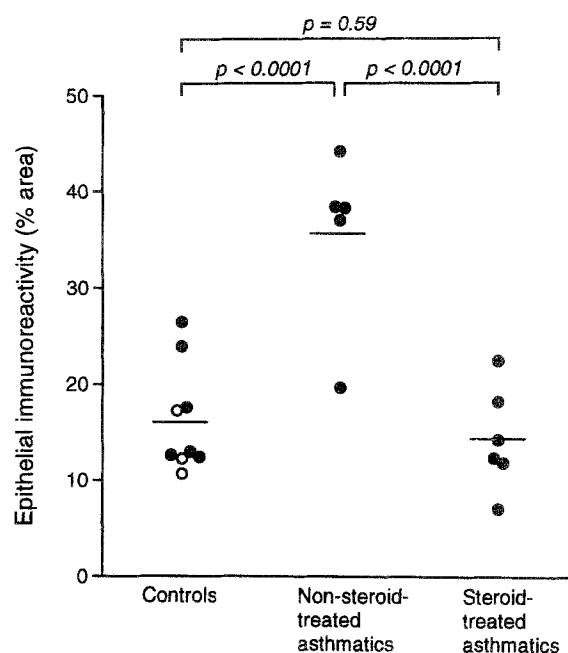


**FIG. 2.** Comparison of bronchial epithelial ET immunoreactivity in biopsy specimens from control subjects ( $n = 9$ ) (open circles, nonatopic; filled circles, atopic), subjects with asthma not treated with steroids ( $n = 5$ ), and subjects with asthma treated with steroids ( $n = 6$ ). Horizontal bars represent mean values.

subjects with asthma not treated with steroids have also been reported.<sup>31, 32</sup> In the former study we were able to detect an inverse correlation between BAL fluid ET concentration and percent predicted FEV<sub>1</sub> although there was no relationship to bronchial reactivity.

The possible mechanisms underlying the increased ET expression have not been directly addressed in this study. However, in cultured guinea pig airway epithelial cells, expression of ET-1 is increased by exposure to proinflammatory cytokines including IL-1, IL-2, IL-6, IL-8, tumor necrosis factor- $\alpha$ , and granulocyte-macrophage colony-stimulating factor.<sup>33</sup> Recently, the ability of IL-1 and tumor necrosis factor- $\alpha$  to increase ET-1 synthesis and release has been confirmed in primary cultures of human bronchial epithelial cells.<sup>34</sup> Expression of both of these cytokines is increased in asthma,<sup>35</sup> thus providing a possible mechanism that would explain the increase in ET immunoreactivity that we have observed.

In this study we have also documented a reduction in bronchial epithelial expression of immunoreactive ET in biopsy specimens from subjects with asthma receiving corticosteroid therapy. This finding is in agreement with reports that the *in vitro* synthesis and release of ET by the human pulmonary epithelial cell line A549<sup>36</sup> and by bronchial epithelial cells derived from subjects with asthma<sup>37</sup> are inhibited by corticosteroids. Furthermore, in a rat model of pulmonary inflammation, the increase in total lung ET content in response to Sephadex (Pharmacia) bead instillation was found to be reduced

**TABLE II.** Numbers of immunoreactive cells in bronchial biopsy specimens from the three subject groups

|                                             | AA1 <sup>+</sup>    | EG2 <sup>+</sup>   | CD3 <sup>+</sup>   | CD4 <sup>+</sup> | CD8 <sup>+</sup> | CD25 <sup>+</sup> |
|---------------------------------------------|---------------------|--------------------|--------------------|------------------|------------------|-------------------|
| <b>Epithelial cell counts</b>               |                     |                    |                    |                  |                  |                   |
| Control subjects                            | 0.9<br>(0-1.6)      | 0<br>(0-0)         | 10.0<br>(8.0-27.0) | 1.6<br>(0-4.7)   | 9.3<br>(0-16.5)  | 0<br>(0-0)        |
| Non-steroid treated<br>subjects with asthma | 1.6<br>(0.5-4.0)    | 1.2*<br>(0-2.4)    | 9.7<br>(0-15.8)    | 0<br>(0-0.7)     | 7.4<br>(0-7.5)   | 0<br>(0-0.3)      |
| Steroid-treated subjects<br>with asthma     | 0.8<br>(0-2.6)      | 0†<br>(0-0)        | 7.3<br>(0-24.0)    | 1.6<br>(0-11.4)  | 4.4<br>(0-10.4)  | 0<br>(0-0)        |
| <b>Subepithelial cell counts</b>            |                     |                    |                    |                  |                  |                   |
| Control subjects                            | 65.0<br>(25.7-117)  | 2.7<br>(0-21.0)    | 198<br>(160-521)   | 126<br>(52-408)  | 93<br>(48.5-270) | 6.1<br>(0-10.3)   |
| Non-steroid-treated<br>subjects with asthma | 28.0<br>(23.4-74.0) | 20.0<br>(2.4-87.0) | 169<br>(86-228)    | 117<br>(40-290)  | 80<br>(29.5-104) | 0.7<br>(0-18.5)   |
| Steroid-treated subjects<br>with asthma     | 30.0<br>(12.3-163)  | 8.2<br>(0-22.0)    | 122<br>(62.5-310)  | 94<br>(31-180)   | 51<br>(18.8-180) | 0<br>(0-5.7)      |

Data are expressed as median (range) and represent number of cells per millimeter of basement membrane length in the case of epithelial cell counts and number of cells per square millimeter in the case of subepithelial cell counts. Biopsy specimens were unavailable for two of the control subjects and one subject in the steroid-treated group of subjects with asthma. Comparisons between groups were performed using a one-way ANOVA on square root-transformed data.

\* $p < 0.05$  compared with control subjects.

† $p < 0.05$  compared with non-steroid-treated asthmatics.

by pretreatment with the glucocorticosteroid budesonide.<sup>38</sup> Concentrations of immunoreactive ET in BAL fluid have been reported to be significantly reduced in a group of five subjects with atopic asthma after a 15-day course of oral prednisolone in association with a reduction in airflow obstruction and a decrease in bronchial reactivity.<sup>32</sup> In our previous study BAL fluid ET levels in subjects receiving long-term treatment with corticosteroids were intermediate compared with those in subjects with asthma not treated with steroids and control subjects.<sup>31</sup> In that study the group of subjects with asthma treated with steroids included a number of individuals who, despite corticosteroid therapy, had persistent clinical symptoms and airway inflammation, which may explain the lack of a more marked reduction in ET levels.

To reduce the possibility of apparent differences in ET expression arising spuriously as a consequence of alterations in the amount of intact epithelium present, we also performed measurements of epithelial integrity. In this part of the study, we were unable to demonstrate significant differences in epithelial damage among the three subject groups or significant correlations between epithelial damage and ET expression. The wide range of measurements for epithelial integrity evident in all three subject groups and the occurrence of widespread epithelial damage in many of the control subjects suggest that at least a proportion of this apparent damage may have been artifactual and caused by the trauma of the biopsy procedure or the fixation process. This finding is in agreement with other reports indicating that extensive epithelial damage is a feature of many biopsy specimens obtained by fiberoptic bronchoscopy from healthy control subjects.<sup>39</sup> Our results are also consistent with previous studies in which no differences in epithelial integrity were detected between subjects with mild

asthma and control subjects,<sup>40</sup> although increased epithelial damage does appear to be a feature in subjects with more severe asthma.<sup>41</sup> In agreement with the findings of other investigators,<sup>40</sup> we were unable to demonstrate significant correlations between the degree of epithelial damage and either percent predicted FEV<sub>1</sub> or bronchial reactivity.

A role for ETs in the pathophysiology of asthma has been proposed on the basis of a number of biologic properties of these molecules. Most obviously, ET-1 is an extremely potent contractile agonist in isolated animal<sup>5-7</sup> and human<sup>5,8</sup> airways, with reported median effective concentration values in the range of 0.5 to 50 nmol/L. When administered to guinea pigs, either by intravenous injection<sup>9</sup> or by inhalation,<sup>10</sup> ET-1 also elicits bronchoconstriction, and we have recently confirmed the *in vivo* bronchoconstrictor properties of nebulized ET-1 in human subjects (Unpublished data). In addition to influencing airway smooth muscle tone, ET could potentially contribute to the pathophysiology of asthma in a number of other ways as summarized in the introduction to this study. In this study we were not able to demonstrate significant correlations between immunoreactive ET expression and either physiologic parameters or indexes of airway inflammation and remodeling. However, the relatively low number of subjects studied may have limited our ability to detect correlations, so that a role for ET in these processes cannot be excluded. This factor likely also contributed to the lack of clearer differences among the three groups in the case of some parameters. For example, the percent predicted FEV<sub>1</sub> measurements for the subjects with asthma not treated with steroids fell within a fairly narrow range and, although lower, were not significantly different from those of the control subjects. Similarly, the values for bronchial reactivity in the group of

subjects with asthma treated with steroids were not significantly greater than those in the group of subjects with asthma not treated with steroids. This last finding is consistent with the results of other investigators who have reported that although corticosteroid treatment improves airway hyperreactivity in the short term, it seldom returns to normal<sup>42</sup> and that persistent airway hyperreactivity may be present in subjects who have received treatment with corticosteroids for as long as 10 years.<sup>43</sup>

In summary, we have shown that immunoreactive ET in bronchial biopsy specimens is principally localized in the bronchial epithelium and that the proportion of epithelium immunostained is increased in subjects with mild asthma receiving treatment only with  $\beta_2$ -agonists but not in subjects with asthma also receiving corticosteroid therapy. These findings add to the increasing evidence that this potent peptide may be implicated in the pathophysiology of asthma. However, as is the case with many other putative mediators, studies with specific ET receptor antagonists or synthesis inhibitors will be required to determine the true importance of ET in this condition.

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