

Inflammation and Structural Changes in the Airways of Patients with Atopic and Nonatopic Asthma

KAWA AMIN, DÓRA LÚDVÍKSDÓTTIR, CHRISTER JANSON, OTTO NETTELBLADT, EYTHÓR BJÖRNSSON, GODFRIED M. ROOMANS, GUNNAR BOMAN, LAHJA SEVÉUS, and PER VENGE on behalf of the BHR Group*

Section of Human Anatomy, Department of Medical Cell Biology, Section of Respiratory Medicine and Allergology, Laboratory for Inflammation Research, Department of Medical Sciences, Asthma Research Center, University of Uppsala; and Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden

The aim of the present study was to compare the cellular pattern and structural changes in the airway walls of atopic and nonatopic patients with asthma. Bronchial biopsy specimens were obtained from 13 atopic subjects with asthma, nine nonatopic patients with asthma, and seven healthy control subjects and investigated using immunohistochemical methods. The number of eosinophils increased in both asthma groups, but significantly more in the atopic group. The number of mast cells increased similarly in the two asthma groups, whereas the number of neutrophils increased only in the nonatopic asthma group. The number of T-lymphocytes (CD3-, CD4-, CD8-, CD-25-positive cells) was higher in patients with atopic asthma compared with nonatopic asthma. Interleukin-4 (IL-4) and IL-5-positive cells were more frequently found in the atopic asthma group, whereas cells staining for IL-8 were more frequent in the nonatopic group. The degree of epithelial damage was significantly higher in the atopic asthma group compared with the control subjects and the nonatopic asthmatics. The tenascin and laminin layer was significantly thicker in the atopic group compared with the group of nonatopic asthmatics. In the atopic group, there was a significant negative correlation between epithelial integrity (defined as the relative length of intact epithelium) and the eosinophil count and also between the number of CD25-positive cells and epithelial integrity. The number of mast cells correlated positively with the thickness of tenascin- and laminin-positive layers. In conclusion, we provide evidence of different patterns of involvement of inflammatory cells in atopic and nonatopic patients with asthma. There were also structural differences in the bronchial mucous membrane between atopic asthma and nonatopic asthma. This suggests that there are differences in the extent of the immunopathologic response of these clinically distinct forms of asthma.

Asthma is a chronic inflammatory disease of the lower airways, characterized clinically by reversible airway obstruction and bronchial hyperresponsiveness (BHR). The mechanism

responsible for BHR in asthma is unknown. It is conceivable that there is a direct or indirect connection between the inflammatory reaction and BHR. The characteristic features of airway inflammation in asthma are leukocyte infiltration, epithelial sloughing, basement membrane thickening, edema and hyperplasia of mucus-secreting glands, and hypertrophy of bronchial smooth muscle (1).

Atopic asthma has been extensively investigated and shown to comprise structural changes in the airways and an inflammatory process characterized by the involvement of eosinophils and the T helper cell, type 2 (Th2) lymphocytes (2). Considerably less information is, however, available about the pathologic characteristics of nonatopic asthma (3). There has been discussion about whether atopic and nonatopic patients with asthma have two distinct inflammatory conditions. The few studies that have compared the inflammatory response in atopic and nonatopic asthma have reported both differences and similarities (3–5). We have previously reported that atopic and nonatopic subjects with asthma differ in two important ways. First, we have found that atopic patients with asthma have higher concentrations of exhaled nitric oxide (NO) compared with nonatopic patients with asthma who have exhaled NO concentrations no different from normal values (6). Second, atopic patients with asthma are more hyperresponsive to inhaled adenosine-5'-monophosphate (AMP) than nonatopic patients with asthma (7).

The aim of this study was to investigate whether atopic and nonatopic patients with asthma displayed differences in their cellular patterns of airway inflammation and whether such differences might be related to structural differences in the airway wall. For this reason, we have quantified the cellular involvement and structural changes by means of immunohistochemistry on tissue biopsies obtained from asthmatic patients with and without atopy and compared the findings with those from a healthy control group.

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Correspondence and requests for reprints should be addressed to Kawa Amin, Department of Genetics and Pathology, Rudbeck Laboratory 11C, University of Uppsala, SE-75185 Uppsala, Sweden. E-mail: kawa.amin@genpat.uu.se

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*The BHR Study Group consists of: Kawa Amin^a, Anders Ahlander^a, Eythór Björnsson^b, Gunnar Boman^b, Britt-Marie Eriksson^c, Björn Gudbjörnsson^d, Monika Hall^e, Göran Hedenstierna^e, Hans Hedenström^e, Lena Håkansson^f, Mariann Högmán^e, Christer Janson^b, Maria Lampinen^f, Kerstin Lindblad^f, Dóra Lúdvíksdóttir^b, Otto Nettelbladt^b, Godfried M. Roomans^g, Lahja Sevéus^g, Ulrike Spetz-Nyström^b, Gunnemar Stålenheim^b, Sigrídur Valtydóttir^d, Charlotte Woschnagg^f, Per Venge^e.

^aSection of Human Anatomy, Department of Medical Cell Biology, ^bSection of Lung Medicine and Allergology, ^cSection of Infectious Diseases, ^dSection of Rheumatology, ^eSection of Clinical Physiology, ^fLaboratory for Inflammation Research, Department of Medicine, Uppsala University, ^gPharmacia & Upjohn Diagnostics, Uppsala, Sweden.

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METHODS

Subjects

Bronchial biopsies were collected from 29 nonsmoking adults (Table 1), divided into the following groups: healthy control subjects (n = 7), atopic patients with asthma (n = 13), and nonatopic patients with asthma (n = 9) (Table 1). The participants formed part of a larger group included in an ongoing study of the pathophysiology of BHR (6).

Patients with asthma. All the patients had a clinical diagnosis of asthma, current asthma symptoms, and increased responsiveness to inhaled methacholine, defined as the provocative concentration of methacholine causing a $\geq 20\%$ reduction in FEV₁ (PC₂₀) ≤ 32 mg (Table 1) (6).

The patients with a clinical diagnosis of asthma were being followed as outpatients at the Department of Respiratory Medicine and Allergology at our hospital. Prior to inclusion in the study, the participants received a questionnaire on the occurrence of airway symptoms in the past 12 mo. The questionnaire was based on the European Community Respiratory Health Survey questionnaire (8). In all the

patients the initial FEV₁, measured during the selection period, was more than 70% of the predicted value. All the investigated subjects were nonsmokers and had been free from respiratory infections for at least 6 wk before the study, and none had a history of cardiovascular disease.

All the atopic patients with asthma had a positive skin prick (≥ 3 mm) for at least one common allergen, whereas the nonatopic patients with asthma all had a negative skin prick test. All but four atopic patients and two nonatopic patients with asthma were on regular treatment with inhaled glucocorticosteroids (budesonide or beclomethasone 200 to 800 μ g/d) and inhaled β_2 -agonists as required. The average use of inhaled glucocorticosteroids was similar in the two asthma groups (415 versus 400 μ g/d).

Control subjects. Control individuals were healthy subjects responding to a request for volunteers; none had asthmatic symptoms or was receiving antiasthmatic treatment. All had a negative skin prick test.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the Faculty of Medicine at the University of Uppsala.

Laboratory Procedures

Definition of atopy. Skin prick tests were carried out using a standardized allergen extract (SoluPrick; ALK, Copenhagen, Denmark) against the following allergens: birch, timothy grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*), cat, dog, horse, house dust mite (*Dermatophagoides pteronyssinus*), *Cladosporium*, and *Alternaria*. Atopy was defined as a skin reaction to one or more of the allergens with a mean diameter of ≥ 3 mm and no dermatographism (9).

Methacholine tests. In this study, an automatic, inhalation dosimeter Spira Elektro 2 (Respiratory Care Centre, Hameenlinna, Finland) (10) was used with a nebulization onset of 50 ml and a nebulization duration of 1.0 s. The dosimeter output was 16 ± 5 μ l per breath. Methacholine was inhaled in five breaths, followed by lung function measurements during the next 3 min. The provocation was continued by doubling the concentrations of methacholine until the FEV₁ had decreased by 20% or the highest concentration (32 mg/ml) was reached. Baseline values were measured after an inhalation of saline. The methacholine provocation was performed on average 5.8 mo before the bronchoscopy. All the asthmatic subjects were hyperresponsive to methacholine. The PC₂₀ values from these provocations are given in Table 1. All the control subjects were negative to the methacholine provocation (PC₂₀ values ≥ 32 mg/ml).

Symptom and peak flow diaries. Symptom and peak flow diaries were kept during a 17-d period starting on the day of the methacholine challenge. The peak expiratory flow rate (PEFR) (best of three measurements) was recorded four times daily with a Mini-Wright Peak Flow Meter (Clement Clarke, London, UK). Minimum requirements were at least two recordings a day, one of which was in the morning. Peak flow variability was calculated by dividing the difference between the highest and lowest daily peak expiratory flow (PEF) reading by the daily mean PEF value. The results are expressed as the percentage daily of variability (vPEF%). In the symptom diary, the subjects stated on awakening whether or not they had had: breathing difficulties during the previous night, wheezing in the chest, attacks of

breathlessness or attacks of coughing during the previous 24 h. Each affirmative answer was given a score of 1, and a symptom score was calculated (6).

Bronchoscopy. The patients were given 10 mg of diazepam (Stesolid; Dumex, Copenhagen, Denmark) orally and 0.5 mg of atropine (Atropine; NM Pharma, Stockholm, Sweden) subcutaneously 30 min before the investigation. The upper airways were anaesthetized with lidocaine hydrochloride (Xylocaine; Astra, Södertälje, Sweden). Using a flexible fiber bronchoscope (Olympus P 20D) with a FB 15C 2.0 mm forceps (Olympus, Tokyo, Japan), two biopsies were taken in the right lung in the upper lobe bronchus immediately after the division from the main bronchus. The specimens were examined immediately by light microscopy to ensure the presence of a complete mucosa and fixed as described subsequently. The patients were instructed to take their regular asthma sprays on the morning of the bronchoscopy.

Immunohistochemistry

Monoclonal antibodies. The eosinophils, neutrophils, and mast cells were identified with monoclonal antibodies on frozen sections. To ensure a reliable count of neutrophils, two different antibodies, human neutrophil lipocalin (HNL) and myeloperoxidase (MPO), were used for identification. Six different primary monoclonal antibodies to HNL (11) were obtained from Pharmacia Upjohn Diagnostics (Uppsala, Sweden) and were added as a cocktail at a final concentration of 0.01 mg/ml for each frozen section. The specificity of these antibodies had been tested by ELISA and confirmed using BIAcore (Pharmacia Upjohn Biosensors, Uppsala, Sweden). Primary monoclonal MPO-7 antibody was obtained from Dako (M 748; Glostrup, Denmark). The concentration of the MPO antibody was 0.002 mg/ml in frozen sections. The monoclonal antibodies EG1 (eosinophil cationic protein [ECP]) and EG2 (eosinophil cationic protein/eosinophil protein-X [ECP/EPX]) (Pharmacia Upjohn, Diagnostics AB) were used for the identification of eosinophils. The working concentration of these antibodies was 0.0003 mg/ml for frozen sections. The antitrypsin antibody 1, AA1 (M 7052; Dako, Glostrup, Denmark), for the identification of mast cells, was used at a final concentration of 0.04 mg/ml. For the localization of interleukins 4 and 5 (IL-4 and IL-5), mouse anti-human IL-4 and rat anti-human IL-5 were used at 0.001 mg/ml in frozen sections. Both antibodies came from PharMingen (8 D 4-8 for IL-4 and JES1-39 D 10 for IL-5, San Diego, CA). Monoclonal mouse anti-IL-8 (Pharmacia & Upjohn, Diagnostics AB) was used on frozen sections at a concentration of 0.002 mg/ml. A panel of monoclonal antibodies including anti CD3 (A 0452), CD4 (M 0716), CD8 (M 7103), and CD25 (IL-2 receptor) (M 0731) was used to identify various types of T lymphocyte. The lymphocyte-specific antibodies were purchased from Dako (Glostrup, Denmark) and used on frozen sections at the following concentrations: anti-CD3 at 0.007 mg/ml, anti-CD4 and anti-CD8 at 0.004 mg/ml, and anti-CD25 at a concentration of 0.004 mg/ml.

Frozen sections. One of the bronchial biopsy specimens taken from the upper lobe was frozen immediately in melting propane previously cooled in liquid nitrogen. Frozen biopsies were kept in liquid nitrogen until sectioned. The samples were attached to the specimen holder of a cryostat microtome (Microm, HM 500 M; Heidelberg, Germany) in a drop of OCT compound (Tissue-Tek; Miles, Elkhart, IN) and cut in sections with a thickness of 4 μ m. After drying in air at room temperature, the sections were wrapped in aluminum foil and stored at -70° C until they were used for immunohistochemistry.

Granulocytes and T cells in cryosections. The sections were thawed and then fixed with undiluted Ortho Permeafix (Ortho Diagnostics, Raritan, NJ) for 40 min at room temperature before incubation with eosinophil and neutrophil markers. In preliminary experiments it was found that fixation and permeabilization with the commercial reagent, Ortho Permeafix, produced comparable results in immunocytochemistry to the paraformaldehyde saponin method but resulted in improved structural preservation. For incubation with lymphocyte markers, the sections were fixed in acetone at -20° C for 10 min. Sections were incubated with monoclonal antibodies at room temperature in a humid chamber for 30 min and the incubation was terminated by washing in phosphate-buffered saline (PBS) with 0.2% bovine serum albumin (BSA) (A 7030; Sigma, Stockholm, Sweden). The antigen-antibody complex was visualized by using an alkaline phosphatase/anti-alkaline phosphatase (APAAP) kit with fast red

TABLE 1

PATIENT CHARACTERISTICS*

	Healthy Controls (n = 7)	Atopic Asthma (n = 13)	Nonatopic Asthma (n = 9)
Age, yr	30 (22–44)	38 (19–63)	43 (17–62)
Sex, M/F	2/5	4/9	2/7
Inhaled steroids	0/7	9/13	7/9
FEV ₁ , % pred	98 (77–120)	96 (72–132)	85 (72–97)
FVC, % pred	95 (78–109)	104 (86–141)	87 (79–96)
Symptom score	0.1	2.3 (0–4)	1.5 (1–2)
PEF variability, %	5 (3–9)	11 (5–22)	11 (5–20)
PC ₂₀ , mg/ml	—	6.1 (0.07–32)	11.0 (0.9–32)

Definition of abbreviations: PC₂₀ = provocation concentration that reduces FEV₁ by 20%; PEF = peak expiratory flow.

* Values are mean (range).

substrate (K670; Dako), according to manufacturer's instructions. In the negative control, the primary antibody was omitted. After washing, the samples were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) for 6 min. The cover glasses were mounted with Dako Fluorescence Mounting Medium.

Cytokines in cryosections. For localization of the cytokines IL-4, IL-5, and IL-8, the sections were either fixed in undiluted Permeafix (Ortho Diagnostics), as previously described, or treated using the paraformaldehyde-saponin method described by Sander and coworkers (12). The frozen sections were incubated with antibodies to cytokines overnight in a refrigerator. The incubation was terminated by washing three times in PBS with 0.2% BSA. For IL-4 and IL-8, the APAAP visualization kit for mouse monoclonal antibodies was used as previously described. A linking rabbit anti-rat antibody (Z 455; Dako) was used before incubation with APAAP complex for rat monoclonal antibodies, also from Dako (D 0488). The incubation time for the antibody and the complex was 30 min at room temperature. The reaction product was visualized with the fast red substrate to alkaline phosphatase with an incubation time of 20 min. The sections were counterstained and mounted with cover slips as described above.

Tenascin and laminin in cryosections. Frozen sections were thawed and fixed in acetone at -20°C for 10 min before the addition of the monoclonal antibodies to tenascin (M 0636) and laminin (M638; Dako). Sections were incubated with monoclonal antibodies at room temperature in a humid chamber for 30 min, and the incubation was terminated by washing in PBS with 0.2% BSA. The sections were counterstained and mounted as described earlier. In the negative control, the primary antibody was omitted.

Plastic-embedded Tissue for Morphology

For morphologic studies, light microscopy was carried out on glycol methacrylate (GMA) (Agar Aids, Stansted, UK) embedded tissue as described previously by Britten and coworkers (13). Sections with a thickness of 2 μm were cut with glass knives on an LKB Historange Microtome (LKB Instruments, Bromma, Sweden).

Microscopic Evaluation of Sections

All the specimens were coded and examined by the microscopist without knowledge of the diagnosis. The number of labeled cells in the tissue was counted manually at a magnification of $\times 40$. One field of approximately 0.8 mm^2 was selected at random. This field did not include glands and muscle cells. One field was analyzed in each section. The number of sections that could be obtained in a particular biopsy was not sufficient in all cases for incubation with all the antibodies. The number of subjects investigated in each group with a particular antibody may therefore occasionally be lower than the number of biopsies in that group. The evaluation of the sections were carried out with a Nikon (Tokyo, Japan) Eclipse E800 microscope. Fuji 200 film was used for the colorprints.

The epithelial integrity of the bronchial wall of the subjects from the different groups was assessed by light microscopy in plastic sections stained with Mayer's hematoxylin. One section per biopsy was used. Epithelial integrity was estimated using a $\times 10$ objective and side-arm light-microscopic attachment that allowed the image of the section to be projected onto a computer screen. Measurements were carried out with a Bit Pad Two data tablet (Summagraphics Corp., Seymour, CT) with a Synoptics (Cambridge, UK) Synapse framestore and software package after calibration with the aid of a stage micrometer. The total length of the basement membrane and the length of intact epithelium in the section through each biopsy was determined. Epithelial integrity was defined as the length of basal membrane with intact epithelium divided by the total length of the membrane.

The measurement of the thickness of the tenascin and laminin layers (in μm) was performed in 4- μm -thick immunolabeled frozen sections using a $\times 40$ objective and a computerized image analysis system as described previously. One section per biopsy was used. Measurements were carried out on 100 randomly selected sites per section.

Statistics

All the statistics were calculated using nonparametric tests. Comparisons between the three groups were performed with the Kruskal-Wallis test. If a significant difference was found with this test, comparisons be-

tween two groups were performed with the Mann-Whitney U-test. For correlation within a group, Spearman's rank correlation test was used. A p value of less than 0.05 was regarded as statistically significant.

RESULTS

There were no statistically significant differences between atopic and nonatopic patients with asthma in terms of lung function, symptom score, peak flow variability or PC_{20} (Table 1).

Inflammatory Cells and Cytokines

Both atopic and nonatopic patients with asthma had a higher number of eosinophils (EG1- and EG2-positive cells) and mast cells compared with the control subjects. The number of neutrophils (HNL- and MPO-positive cells) was higher in patients with nonatopic asthma compared with the control group (Table 2). Atopic patients with asthma had a higher number of eosinophils and a lower number of neutrophils than nonatopic patients with asthma, whereas no significant difference in the number of mast cells was found between atopic and nonatopic asthmatics (Table 2).

The total number of T lymphocytes (CD3-positive cells) and the number of CD- and CD8-positive cells were higher in both the atopic and nonatopic asthma group compared with the healthy control group. The atopic patients with asthma had a higher number of CD25-positive cells than the control group. When the atopic and nonatopic groups of asthmatics were compared, the total number and the number of all subtypes of T lymphocyte were significantly higher in the atopic group (Table 2).

The number of cells expressing cytokines in the two patient groups is shown in Table 2. Atopic and nonatopic patients with asthma had higher numbers of cells expressing IL-4, IL-5, and IL-8 than control subjects. In atopic asthmatics, IL-4 and IL-5 were predominantly expressed in eosinophils and mast cells (Figure 1). The number of cells expressing IL-4 and IL-5 was higher in atopic asthmatics than nonatopic ones, whereas the nonatopic asthma group had a significantly higher number of cells expressing IL-8 (Figure 1, Table 2). IL-8 was expressed in bronchial epithelial cells and neutrophils in nonatopic asthmatics and in eosinophils in atopic asthmatics.

The number of EG2-positive cells correlated positively with the number of CD25-positive ($r = 0.66$, $p = 0.14$) and CD4-

TABLE 2
NUMBER OF GRANULOCYTES, T LYMPHOCYTES, AND CELLS EXPRESSING CYTOKINE IN BRONCHIAL BIOPSIES*

Positive Cells	Healthy Controls ($n = 7$)	Atopic Asthma ($n = 13$)	Nonatopic Asthma ($n = 9$)
EG1	0 (0–1)	66 (52–99) [§]	11 (8–48) ^{‡§}
EG2	0 (0–1.5)	78 (66–89) [§]	11 (11–33) ^{†**}
MPO	28 (26–30)	33 (22–33)	49 (45–50) ^{§§}
HNL	24 (12–24)	22 (11–22)	45 (33–45) [¶]
AA1	6 (3–7)	89 (66–150) [§]	100 (89–111) [§]
CD3	14 (11–15)	130 (100–150) [§]	45 (32–45) [¶]
CD4	8 (6.5–10)	93 (77–110) [§]	29 (22–29) [¶]
CD8	5 (5–6.5)	28 (23–45) [§]	12 (9–15) [¶]
CD25	0 (0–0)	17 (15–18) [§]	0 (0–0) [¶]
IL-4	0 (0–0.5)	33 (22–33) [§]	9 (8–9) ^{§¶}
IL-5	0 (0–1)	35 (31–44) [§]	20 (9–22) ^{¶§}
IL-8	0 (0–5)	11 (3–11) [†]	35 (9–38) ^{‡**}

Definition of abbreviations: AA1 = antitrypsin antibody 1; CD = clusters of differentiation; EG = eosinophil granulocyte; HNL = human neutrophil lipocalin; IL = interleukin; MPO = myeloperoxidase.

* Values are expressed as median (interquartile range).

^{†§§} $p < 0.05$, 0.01, 0.001 compared with control subjects.

^{**¶} $p < 0.05$, 0.01, 0.001 compared with patients with atopic asthma.

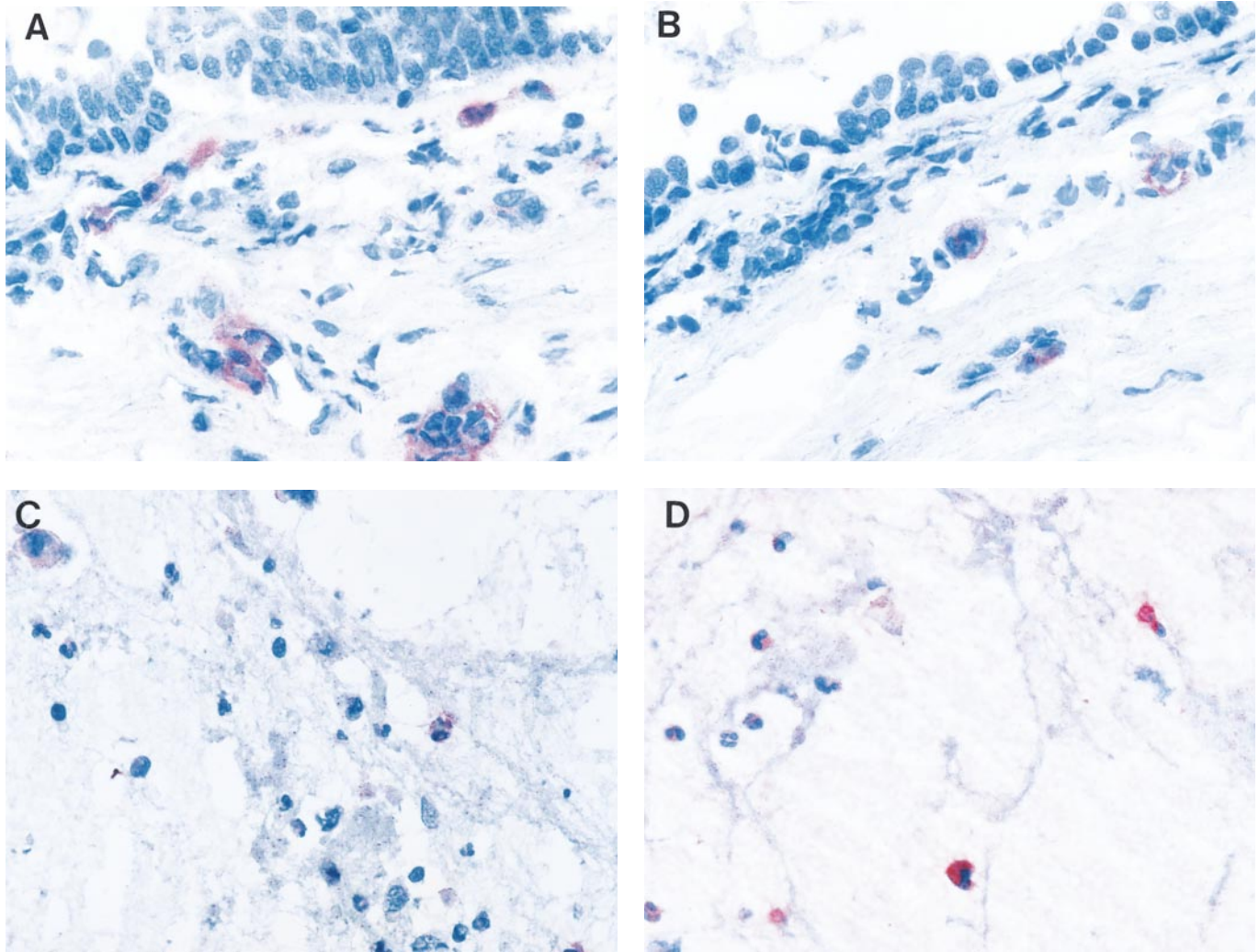


Figure 1. Cryostat sections of bronchial biopsies for IL-5 in atopic asthma (A) and IL-5 in nonatopic asthma (B). The figure shows that many IL-5-positive cells are present in the tissue of patients with atopic asthma, whereas only a few are present in the tissues of patients with nonatopic asthma. Immunohistochemical staining for IL-8 in atopic asthma (C) and nonatopic asthma (D). The figure shows that many IL-8-positive cells are present in the tissue of patients with nonatopic asthma, whereas there are only a few in the tissues of patients with atopic asthma. Mayer's hematoxylin. Original magnification: $\times 320$.

positive cells ($r = 0.60$, $p = 0.029$) in the atopic asthma group. Similar results were obtained when EG1-positive cells were used in the calculations. A positive correlation was found between the number of neutrophils identified with HNL and the

number of cells expressing IL-8 ($r = 0.70$, $p = 0.01$) in patients with nonatopic asthma.

Epithelial Damage

The epithelial integrity was lower in atopic patients with asthma than in nonatopic patients with asthma and in control subjects, whereas no significant difference was found between the group of nonatopic asthmatics and the control group (Figure 2). In the damaged area, the cylindrical ciliated epithelial cells were absent, whereas the layer of cuboidal basal cells was often intact. Eosinophils were frequently found in the area of epithelial damage (Figure 3).

Correlations between the Numbers of Inflammation Cells and Structural Changes in the Epithelium

In the group of atopic patients with asthma, a significant negative correlation was found between epithelial integrity and the eosinophil count and epithelial integrity and CD25-positive cells (Table 3). In the atopic patient group, epithelial integrity was also negatively correlated with the total number of CD3- and CD4-positive cells but not with the number of CD8-posi-

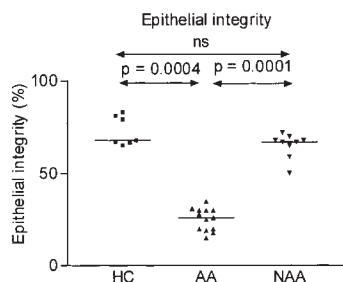


Figure 2. The comparison of the epithelial integrity (length of basal membrane with intact epithelium divided by the total length of the membrane) in the healthy control subjects (HC), patients with atopic asthma (AA), and patients with nonatopic asthma (NAA).

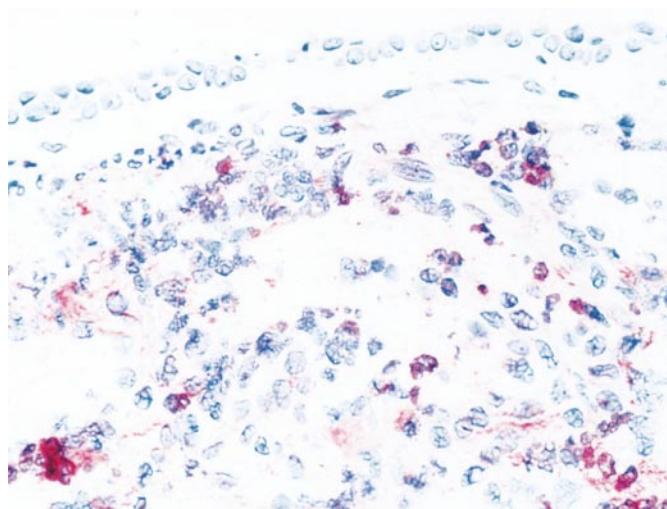


Figure 3. Resin (plastic) sections of a bronchial biopsy with EG2. Eosinophil accumulation in areas of reduced epithelial integrity is shown in a specimen from a patient with atopic asthma. Mayer's hematoxylin. Original magnification: $\times 320$.

tive cells. In nonatopic patients with asthma, no significant correlation between cell numbers and epithelial integrity was found (Table 3).

The tenascin- and laminin-positive layers in atopic patients with asthma were thicker than in nonatopic asthmatic patients and healthy control subjects (Figure 4).

A positive correlation was found between the thickness of the tenascin layer and the number of mast cells in the atopic asthma group, whereas no significant correlation was found between mast cell count and tenascin layer thickness in nonatopic patients with asthma (Table 3). In the group of atopic patients with asthma, a significant positive correlation was found between the thickness of the laminin layer and the mast cell count (Table 3). In nonatopic patients with asthma, there was a positive correlation between the number of CD3-positive cells and the thickness of the laminin layer (Table 3).

DISCUSSION

The main results of this study are the findings of differences in the pathologic characteristics of patients with atopic and nonatopic asthma, despite the fact that both groups of asthmatics had respiratory symptoms, peak flow variability, and BHR of similar severity. The cellular pattern of inflammation in atopic patients with asthma was characterized by a high number of eosinophils, mast cells, and T lymphocytes, whereas nonatopic patients with asthma mainly displayed a high number of neu-

TABLE 3
CORRELATION (r VALUES) BETWEEN INFLAMMATORY CELL NUMBERS AND STRUCTURAL CHANGES

Cells	Epithelial Integrity		Tenascin Layer		Laminin Layer	
	Atopic Asthma	Nonatopic Asthma	Atopic Asthma	Nonatopic Asthma	Atopic Asthma	Nonatopic Asthma
EG1-positive	-0.80*	-0.53	0.39	0.29	0.32	-0.026
EG2-positive	-0.77†	-0.65	0.33	0.30	0.24	-0.028
MPO-positive	-0.53	-0.04	0.07	0.28	-0.02	0.28
HNL-positive	-0.37	-0.02	0.12	0.32	-0.12	0.13
AA1-positive	-0.15	-0.48	0.62‡	0.30	0.65‡	-0.20
CD3-positive	-0.60†	0.41	0.41	-0.61	0.45	0.60‡
CD4-positive	-0.57†	0.16	0.45	-0.03	0.47	-0.11
CD8-positive	-0.31	0.20	0.23	-0.32	0.35	-0.12
CD25-positive	-0.75†	0.41	0.39	-0.20	0.38	-0.28

For definition of abbreviations, see Table 2.

* $p < 0.001$.

† $p < 0.01$.

‡ $p < 0.05$.

trophils and mast cells. Epithelial damage was mainly found in patients with atopic asthma. Furthermore, we found that the tenascin- and laminin-containing layers in the airways were thicker in atopic patients with asthma than nonatopic patients with asthma.

The number of eosinophils (EG1- and EG2-positive cells) was higher in both the atopic and nonatopic asthma group than in the healthy control group. In the present study, the eosinophilic inflammation was more pronounced in the atopic group than in the nonatopic asthma group. This is in contrast with the findings of Bentley and coworkers, who reported a similar increase in EG2+ cells in allergic and nonallergic asthmatics (14). The discrepancy between the present study and the study by Bentley and coworkers (14) is surprising as the two investigations have a similar design. Our results are, however, in accordance with results from an epidemiologic study in which atopic subjects with BHR were found to have higher levels of serum ECP and higher number of blood eosinophils than nonatopic subjects with BHR (15).

One clear distinction between atopic and nonatopic patients with asthma in the present study was the finding of increased numbers of neutrophils in the latter group. The low number of neutrophils in atopic patients with asthma is in agreement with previous reports (16, 17), whereas the high number in nonatopic patients with asthma is a novel and potentially important finding. To ascertain the results, we used antibodies to two different granule proteins of the neutrophil, i.e., MPO and the recently discovered protein HNL. Both antibodies produced identical results. The consequence of the increased presence of neutrophils in the lungs of nonatopic

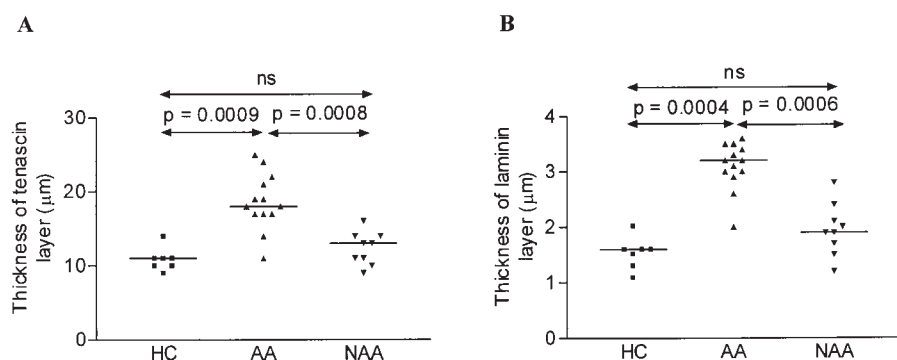


Figure 4. The comparison of the structural changes in the airways of the healthy control subjects (HC), patients with atopic asthma (AA), and patients with nonatopic asthma (NAA) as indicated by the thickness of the (A) tenascin and (B) laminin layers in the subepithelium.

asthmatics is uncertain, as these patients had no structural alterations in the mucosa of the lung, although neutrophils are highly destructive cells containing an abundance of cytotoxic and proteolytic molecules. The notion that neutrophils are of importance in asthma is supported by the work of Wenzel and coworkers, who found that the numbers of neutrophils were increased in the airways of patients with severe asthma compared with patients with moderate asthma (18).

The number of mast cells was similarly increased in patients with atopic and nonatopic asthma. In a previous study, we found that patients with atopic asthma had an increased responsiveness to AMP compared with nonatopic asthmatics (7). The bronchoconstriction induced by AMP is primarily mediated by mast cells (19). Our hypothesis was therefore that atopic asthmatics would have a higher number of mast cells than patients with nonatopic asthma (7). This hypothesis was not verified by the present study. One explanation for the difference in AMP responsiveness could be that mast cells of patients with atopic asthma are in a more activated state than mast cells of patients with nonatopic asthma.

The numbers of T lymphocytes (CD3-, and CD4-positive cells) were higher in atopic patients with asthma compared with nonatopic patients with asthma. Cells possessing the IL-2 receptor (CD25-positive cells) were virtually found only in the bronchial mucosa of atopic asthmatics. This result is in contrast to Bentley and coworkers, who found a higher number of CD4⁺ cells in nonallergic compared with allergic asthmatics and a similar increase in CD25⁺ cells in both groups of asthmatics compared with healthy control subjects (14). The T cell may play a major role in regulating the inflammatory response in asthma, possibly via a distinct pattern of cytokine release. In accordance with previous studies, we found a positive correlation between CD25- and EG2-positive cells in subjects with atopic asthma (20).

IL-4 and IL-5-positive cells were found in higher numbers in atopic than in nonatopic asthmatics, whereas IL-8-positive cells were more common in nonatopic patients with asthma. In accordance with Bradding and coworkers, IL-4 and IL-5 were predominantly located in eosinophils and mast cells (20). IL-8, on the other hand, was predominantly localized in eosinophils in atopic asthmatics, but in epithelial cells and neutrophils in nonatopics. Other studies have found high concentrations of IL-8 in epithelial cells and eosinophils in atopic asthmatics (21, 22). Our findings suggest that there are quantitative differences in the cellular and cytokine profiles between atopic and nonatopic asthmatics.

Epithelial shedding and loss of integrity are recognized features in the biopsy specimens of asthmatics (2). The degree of epithelial damage was higher in atopic asthmatics than in the other groups. In the present study, no significant difference in epithelial integrity was found between healthy control subjects and nonatopic asthmatics. The same pattern was found when examining the thickness of the tenascin- and laminin-positive layer. Patients with atopic asthma had evidence of increased basal membrane thickness, but this was not found in the group with nonatopic asthma. Previous studies have demonstrated increased thickness in the layers expressing tenascin (23) and laminin (24) in seasonal and occupational asthmatic patients, but to our knowledge no previous reports have investigated tenascin and laminin layer thickness in patients with nonatopic asthma.

Epithelial damage by activated eosinophils has been proposed as one of the major pathophysiologic mechanisms in asthma (25, 26). Activated eosinophils release cationic granule proteins, which are highly toxic to the respiratory epithelium (27). When major basic protein (MBP) or ECP was applied *in vitro* to human epithelial cells, they were found to cause direct

damage to the cells in a dose- and time-dependent manner (28, 29). In the present study, we found a negative correlation between the number of eosinophils and epithelial integrity in the group of atopic asthmatics, which supports this notion. We also regularly found a high number of eosinophils in areas of epithelial damage. The numbers of lymphocytes and their activation marker CD25 were also correlated to epithelial damage. This indicates that these cells play a role in the direct damage of the epithelial cells, and cytokines such as tumor necrosis factor- α (TNF- α) and interferon gamma (IFN- γ) have been shown to cause damage to bronchial epithelial cells cultivated *in vitro* (30). An alternative explanation of this relationship is that activated lymphocytes of the Th2 type are involved in the attraction and activation of eosinophils in the lungs of atopic asthmatics.

The finding of positive correlations between the thickness of the tenascin and laminin layers and mast cell numbers in atopic asthmatics is intriguing and could suggest that mast cells play a role in this process. Indeed, the activities of mast cells have been implicated in the development of fibrotic disorders (31). The relationship between mast cells and the structural alterations found in our patients, however, is not a simple one, as nonatopic asthmatics had only minor changes in their tenascin and laminin layers, although they had similarly increased numbers of mast cells in atopic asthmatics. One speculation is that mast cells and eosinophils cooperate in these processes, as eosinophil products such as ECP and transforming growth factors have been shown to interfere with fibroblast growth and the production of matrix proteins (32, 33).

The majority of the patients in the present study underwent treatment with inhaled corticosteroids, which is known to decrease the number of inflammatory cells. It was, however, impossible for ethical reasons to suspend the steroid treatment during the study. There were no differences in the dose of inhaled steroids between the two groups of asthmatic patients. It therefore seems unlikely that the differences found in this study between atopic and nonatopic asthma can be explained by differences in the anti-inflammatory treatment of the patients.

In conclusion, the cellular patterns for eosinophils, neutrophils, T lymphocytes, and cytokines differed between atopic and nonatopic patients with asthma. In atopic asthmatics, the airway inflammation was characterized by high numbers of eosinophils, mast cells, and T lymphocytes, whereas nonatopic asthmatics mainly displayed high numbers of neutrophils and mast cells. There were also distinct structural alterations in the airway mucosa in patients with atopic asthma that were not found in the group of nonatopic asthmatics. Our findings suggest that there are differences in the extent of the immunopathologic response of these clinically distinct forms of asthma.

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