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Sun Ying,* Qiu Meng,* Kyriaki Zeibecoglou,* Douglas S. Robinson,* Alison Macfarlane,* Marc Humbert,*† and A. Barry Kay²*

Atopic (AA) and nonatopic (NAA) asthma are characterized by chronic inflammation and local tissue eosinophilia. Many C-C chemokines are potent eosinophil chemoattractants and act predominantly via the CCR3. We examined the expression of eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), MCP-4, and CCR3 in the bronchial mucosa from atopic (AA) and nonatopic (intrinsic; NAA) asthmatics and compared our findings with atopic (AC) and nonatopic nonasthmatic controls (NC). Cryostat sections were processed for immunohistochemistry (IHC), in situ hybridization (ISH), and double IHC/ISH. Compared with AC and NC, the numbers of EG2⁺ cells and the cells expressing mRNA for eotaxin, eotaxin-2, RANTES, MCP-3, MCP-4, and CCR3 were significantly increased in AA and NAA (p < 0.01). Nonsignificant differences in these variants were observed between AA and NAA and between AC and NC. Significant correlations between the cells expressing eotaxin or CCR3 and EG2⁺ eosinophils in the bronchial tissue were also observed for both AA (p < 0.01) and NAA (p = 0.01). Moreover, in the total asthmatic group (AA + NAA) there was a significant inverse correlation between the expression of eotaxin and that of the histamine PC20 (p < 0.05). Sequential IHC/ISH showed that cytokeratin⁺ epithelial cells, CD31⁺ endothelial cells, and CD68⁺ macrophages were the major sources of eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4. There was no significantly different distribution of cells expressing mRNA for these chemokines between atopic and nonatopic asthma. These findings suggest that multiple C-C chemokines, acting at least in part via CCR3, contribute to bronchial eosinophilia in both atopic and nonatopic asthma. *The Journal of Immunology, 1999, 163: 6321–6329.

I t is widely accepted that a subgroup of asthmatic patients are not demonstrably atopic (the so-called intrinsic variant of the disease) (1). Intrinsic asthmatics are skin test negative to extracts of common allergens, and there is no evidence of allergen-specific serum IgE Abs. Serum total IgE concentrations are within the normal range. There is also no clinical or family history of allergy. There has been debate about the relationship of this variant of the disease to atopy (2). Although the onset of the disease may be triggered by viral infection, such as a respiratory influenza-like illness, some authors have suggested a role for IgE and atopy in so-called intrinsic asthma (3). These data indicate that intrinsic asthmatics may be allergic to an as yet undetected allergen and that these patients might benefit from allergen-free environments, as previously demonstrated in atopic asthma (4). Our previous work indicated that although intrinsic asthma has some clinical characteristics similar to those of extrinsic asthma, it does not appear to be a distinct immunopathologic entity (5–8). Thus, in a series of bronchial biopsy studies using in situ hybridization (ISH)¹ and immunohistochemistry (IHC), alone or in combination, both variants of the disease were characterized by an allergic-like bronchial mucosal inflammation, including eosinophils, IL-4⁺ and IL-5⁺ Th2-type cells, RANTES, MCP-3, and FceRI⁺ cells (5–8). The only difference seemed to be a strong macrophage signal, i.e., there were increased numbers of CD68⁺ cells and cells mRNA⁺ for GM-CSF receptor α-chain in intrinsic asthma compared with extrinsic asthma (9).

In the present study we have extended these observations by measuring the expression of eotaxin, eotaxin-2, MCP-4, and CCR3 in bronchial biopsies from intrinsic and extrinsic asthma. Unlike RANTES, MCP-3, and MCP-4, which ligate several C-C chemokine receptors, eotaxin and eotaxin-2 bind exclusively to CCR3. The two C-C chemokines are also selective in their biological activity on eosinophils and basophils, although CCR3 may also be transiently expressed on Th2 cell lines. For these reasons we have tested the hypothesis that the range of C-C chemokines, including eotaxin and eotaxin-2, as well as the eotaxin receptor, CCR3, is

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¹ Abbreviations used in this paper: ISH, in situ hybridization; FEV₁, forced expiratory volume in 1 s; BAL, bronchoalveolar lavage; IHC, immunohistochemistry; MCP, monocyte chemoattractant protein; AA, atopic asthma; NAA, nonatopic asthma; AC, nonasthmatic atopic controls; NC, nonatopic nonasthmatic controls; RAST, radioallergosorbent; APAAP, alkaline phosphatase-antialkaline phosphatase.
similar in both atopic and nonatopic asthma, indicating a common mechanism of local eosinophilia in both subgroups.

Materials and Methods

Study patients

The study was approved by the ethics committees of the Royal Brompton Hospital (London, U.K.) and the Hochgebirgsklinik (Davos Wolfgang, Switzerland). Each participant gave informed consent before entering the study. Bronchial biopsy specimens were obtained from 10 atopic asthmatics (AA), 10 nonatopic asthmatics (NAA), 10 nonasthmatic atopic controls (AC), and 10 nonasthmatic nonatopic controls (NC) as previously characterized (5). The patients studied were recruited from the Royal Brompton Hospital (London, U.K.) and the Hochgebirgsklinik. Asthmatic subjects had a clear history of asthma, requiring intermittent inhaled β2-agonist therapy, and documented reversible airway obstruction (20% improvement in forced expiratory volume in 1 s (FEV1) either spontaneously or after administration of inhaled β2-agonist) and, a histamine PC20 provocation test result ≥6 mg/ml in the previous 2 wk. Astopy was defined as a positive skin prick test (wheel at 15 min >3 mm in diameter in the presence of positive histamine and negative diluent controls) to more than one of the extracts of the common local aeroallergens (i.e., mixed grass, tree pollen, mixed moults, cat and dog dander, and house dust mite). By use of the CAP system (Pharmacia Diagnostics, Uppsala, Sweden) all atopic patients demonstrated a positive radioallergosorbent test (RAST) test (>0.70 IU/ml) to one or more common allergens. Nonatopic subjects were defined by negative skin test to a wide range of local common aeroallergens in the presence of a positive histamine-positive control. The nonatopic subjects also exhibited a serum IgE concentration within the normal range and negative RAST test to common aeroallergens. For all subjects, total serum IgE and RAST measurements were performed in the same laboratory. All subjects were nonsmokers and had not taken oral (2 mg) or inhaled (2 wk) glucocorticoids before the study. Exclusion criteria included age <18 years or >65 years, FEV1 <60% of the predicted value on the study day, evidence of acute or chronic infection, pregnancy, or any chronic medical illness other than asthma.

Fiberoptic bronchoscopy

The bronchoscopy procedure was performed using an Olympus model IT30 bronchoscope (Olympus, Tokyo, Japan) at the same time of day (9 a.m.) in all the subjects. Bronchoscopy was performed in all subjects following premedication with 2.5 mg of salbutamol by nebulizer, 0.6 mg of atropine, and 5–10 mg of midazolam administered i.v. Biopsy was taken from the right middle and lower bronchi using Olympus alligator forceps (model FB15C). All asthmatics were given an additional 2.5 mg of nebulized atropine, and 5–10 mg of midazolam administered i.v. Biopsies were taken at the same time of day (9 a.m.) using an Olympus model IT30 bronchoscope (Olympus, Tokyo, Japan) at the same time of day (9 a.m.) in the Hochgebirgsklinik. Asthmatic subjects had a clear history of asthma, requiring intermittent inhaled β2-agonist therapy, and documented reversible airway obstruction (20% improvement in forced expiratory volume in 1 s (FEV1) either spontaneously or after administration of inhaled β2-agonist) and, a histamine PC20 provocation test result ≥6 mg/ml in the previous 2 wk. Astopy was defined as a positive skin prick test (wheel at 15 min >3 mm in diameter in the presence of positive histamine and negative diluent controls) to more than one of the extracts of the common local aeroallergens (i.e., mixed grass, tree pollen, mixed moults, cat and dog dander, and house dust mite). By use of the CAP system (Pharmacia Diagnostics, Uppsala, Sweden) all atopic patients demonstrated a positive radioallergosorbent test (RAST) test (>0.70 IU/ml) to one or more common allergens. Nonatopic subjects were defined by negative skin test to a wide range of local common aeroallergens in the presence of a positive histamine-positive control. The nonatopic subjects also exhibited a serum IgE concentration within the normal range and negative RAST test to common aeroallergens. For all subjects, total serum IgE and RAST measurements were performed in the same laboratory. All subjects were nonsmokers and had not taken oral (2 mg) or inhaled (2 wk) glucocorticoids before the study. Exclusion criteria included age <18 years or >65 years, FEV1 <60% of the predicted value on the study day, evidence of acute or chronic infection, pregnancy, or any chronic medical illness other than asthma.

In situ hybridization (ISH)

Sections (6 μm) were freshly cut from frozen bronchial biopsies. For identification of eosinophils, basophils, eotaxin, and CCR3 protein product, IHC of bronchial biopsies was performed using alkaline phosphate-antialkaline phosphatase (APAAP) method as described previously (6, 7, 12). Rabbit anti-mouse Ig, APAAP, and control IgG1 were purchased from Dako (High Wycombe, U.K.). EG2, a mAb against human activated eosinophils, was purchased from Pharmacia (Uppsala, Sweden). A mouse anti-human eosinxin mAb (2G6) and anti-human CCR3 mAb (7B11) were provided by Drs. C. MacKay, P. Ponath, and W. Newman (Stanford University, Stanford, CA) was inserted into pGEM-3 (7). The Bluescript vector containing 600 bp of human CCR3 cDNA was provided by Drs. J. Van Damme and G. Opdenakker (Rega Institute for Medical Research, University of Leuven, Leuven, Belgium) (7, 15). Riboprobes were prepared from cDNA for these chemokines and CCR3 as previously described (6, 7, 12). Briefly, riboprobes (antisense or sense) were synthesized in the presence of ATP, GTP, CTP, and [35S]UTP and appropriate RNA polymerases (T7, SP6, or T3), respectively. Permeabilization, prehybridization, and hybridization protocols were described previously (6, 7, 12). Incubation in N-ethylmaleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the [35S]UTP-labeled probes. Furthermore, the experiments were performed under very high stringency condition (hybridization at 50°C and posthybridization washing at 60°C, 0.1 × SSC) to minimize nonspecific hybridization. Negative controls employed hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, U.K.) before hybridization with the antisense probe. For autoradiography, slides were dipped into K-5 emulsion (Ilford, Basildon, U.K.) and exposed at 4°C for 2 wk in absolute darkness in a desiccated environment. The slides were developed (D-19 developing solution, Eastman Kodak, Rochester, NY), rinsed, and counterstained with Harris hematoxylin. Dense deposits of silver grains on autoradiographs were present over cells expressing chemokine mRNA. Slides were counted in duplicate, blind to the patients’ clinical status, using an eyepiece graticule, as previously described (6, 7, 12). The results were expressed as the total number of positive cells per field (per 0.202 mm2) of submucosa. The coefficient of variability of the duplicate counts obtained from all slides was <5%.

Immunohistochemistry

Sections (6 μm) were freshly cut from frozen bronchial biopsies. For identification of eosinophils, basophils, eotaxin, and CCR3 protein product, IHC of bronchial biopsies was performed using alkaline phosphate-antialkaline phosphatase (APAAP) method as described previously (6, 7, 12). Rabbit anti-mouse Ig, APAAP, and control IgG1 were purchased from Dako (High Wycombe, U.K.). EG2, a mAb against human activated eosinophils, was purchased from Pharmacia (Uppsala, Sweden). A mouse anti-human eosinxin mAb (2G6) and anti-human CCR3 mAb (7B11) were provided by Drs. C. MacKay, P. Ponath, and W. Newman (Stanford University, Stanford, CA) was inserted into pGEM-3 (7). The Bluescript vector containing 600 bp of human CCR3 cDNA was provided by Drs. J. Van Damme and G. Opdenakker (Rega Institute for Medical Research, University of Leuven, Leuven, Belgium) (7, 15). BB1, a mAb recognizing a human basophil granular protein, was a gift from Dr. A. F. Walls (Southampton General Hospital, Southampton, U.K.). This mAb did not react with lymphocytes, monocytes, platelets, neutrophils, eosinophils, mast cells, or any other cell type or tissue structure (17). The optimal concentrations of all Abs used were determined in pilot experiments. Briefly, the slides were incubated with 20% human serum in PBS for 20 min, then incubated with monoclonal anti-human eotaxin (2G6, 1/50), or monoclonal mouse anti-CCR3 (7B11, 1/50) in 20% human serum/ PBS overnight at room temperature. Slides then were treated with rabbit anti-mouse IgG (1/30, 30 min) and APAAP (1/30, 30 min), respectively. For immunostaining of BB1+ basophils, the slides were pretreated with 0.1% saponin (Sigma)/PBS for 30 min, incubated with BB1 Ab (1/10) overnight at room temperature, then processed as described above. Positive cells stained red after development with Fast Red (Sigma). Omission or substitution of the primary Ab with an irrelevant Ab of the same species

Table I. Clinical characteristics of AA, NAA, AC, and NC* and normal controls

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (F/M)</th>
<th>Sex (F/M)</th>
<th>Blood Eosinophils (%)</th>
<th>FEV1 (%)</th>
<th>Histamine PC20 (mg/ml)</th>
<th>Serum (total IgE) (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>25.9 (22–55)</td>
<td>24/8</td>
<td>2.9 (0.8–5.7)</td>
<td>77.5 (55.0–110.0)***</td>
<td>1.0 (0.02–3.0)</td>
<td>230.5 (73.0–570.0)*</td>
</tr>
<tr>
<td>NAA</td>
<td>25.0 (42–62)***</td>
<td>4/8</td>
<td>0.0 (1.4–14.2)</td>
<td>75.5 (55.0–112.0)****</td>
<td>1.0 (0.12–6.8)</td>
<td>160.0 (77.0–808.0)*</td>
</tr>
<tr>
<td>AC</td>
<td>25.5 (21–33)</td>
<td>2/8</td>
<td>3.7 (1.1–8.2)</td>
<td>103.5 (91.0–1123.0)***</td>
<td>&gt;16</td>
<td>162.0 (77.0–808.0)*</td>
</tr>
<tr>
<td>NC</td>
<td>20.5 (18–28)</td>
<td>4/6</td>
<td>1.5 (0.1–5.0)</td>
<td>98.5 (82.0–119.0)</td>
<td>&gt;16</td>
<td>210.0 (4.0–47.0)</td>
</tr>
</tbody>
</table>

* Results are expressed as median, and range is given in parentheses.

**p < 0.01, AA vs NC/NAA; ***p < 0.05, AA vs AC/NCC; ****p < 0.01, AA vs AC/NCC; +, p < 0.01, AA vs NC; #, p < 0.05, AC vs NC.
was used as a negative control. All the slides were counted in duplicate under blind conditions to the patients’ clinical status. Results were expressed as the total number of positive cells per field (0.202 mm²) of submucosa. The coefficient of variability of the duplicate counts obtained from all slides was <5%. No immunoreactivity was observed in sections stained with omission of the primary Ab or substitution of this Ab with an irrelevant Ab of the same species.

Sequential IHC and ISH

To identify the cell sources of eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 mRNA, sequential IHC/ISH was employed as previously described (6, 12). Briefly, cell phenotypes were first identified by IHC using the APAAP technique and phenotype-specific murine mAb. The mAb used were directed against human epithelial cells (cytokeratin, clone MNF 116), endothelial cells (CD31, clone JC/70A), macrophages (CD68, clone EBM11), mast cell tryptase (clone AA1), neutrophil elastase (clone NP57; Dako), T cells (CD3; Becton Dickinson, Oxford, U.K.), and eosinophil cationic protein (EG2). Sections from five atopic asthmatics and five nonatopic asthmatics with high expression of eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 RNA were chosen. After developing with Fast Red for cell phenotypes, ISH was performed using digoxygenin-labeled riboprobes (6, 12) specific for these C-C chemokines. The mRNA-positive signals were visualized using nitro blue tetrazolium/bicinchononic acid (Sigma) as the chromogen (6, 12). The numbers of positive cells expressing phenotypic markers, chemokine mRNA, or both were counted in the epithelial area and submucosa in whole sections. The results were expressed as the percent distribution of cells expressing eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 mRNA and the percentage coexpressed by each cell type within the epithelium and submucosa of biopsies.

Statistical analysis

Data were analyzed with the aid of a commercially available statistical package (Minitab for Windows, Minitab release 9.2, Minitab, State College, PA). The Mann-Whitney U test was then used for intergroup comparison (with Bonferroni’s correction). Correlation coefficients were obtained by Spearman’s rank-order method. A p value <0.05 was accepted as statistically significant.

Results

Patient population

Details of the study population are shown in Table I. As expected, atopic asthmatics had significantly elevated total serum IgE compared with NAA and NC (p < 0.01). Both AA and NAA had significantly lower FEV₁ and histamine PC₂₀ than those of AC and NC. Unlike NC, AA, NAA, and AC had relatively high blood eosinophil counts (Table I). Compared with AA, AC, and NC, the nonatopic asthmatics were significantly older. The total serum IgE in NAA was slightly higher than in NC, although it was significantly lower than in AA and AC. These observations are consistent with previous findings (5–7).

EG2⁺ eosinophils and BB1⁺ basophils

The numbers of EG2⁺ eosinophils in the bronchial mucosa from both atopic and nonatopic asthmatic patients were significantly elevated compared with those in both nonasthmatic control groups.

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EG2⁺ eosinophils and BB1⁺ basophils

The numbers of EG2⁺ eosinophils in the bronchial mucosa from both atopic and nonatopic asthmatic patients were significantly elevated compared with those in both nonasthmatic control groups.
Although the number of BB1\(^+\) basophils was \(\sim 10\)-fold less than that of EG2\(^+\) eosinophils, the numbers of BB1\(^+\) basophils in bronchial biopsies from asthmatics (AA and NAA) were significantly elevated compared with control values (AC + NC; \(p < 0.05\); Fig. 1). There were no statistically significant differences between AA and NAA, although the mean numbers of BB1\(^+\) basophils in AA were slightly higher than those in NAA.

**Eotaxin and eotaxin-2**

Bronchial biopsies from asthmatic patients had elevated numbers of cells encoding mRNA for eotaxin, which significantly increased compared with those from both the atopic and nonatopic control subjects without asthma (\(p < 0.001\); Fig. 2). No significant differences between either AA and NAA or AC and NC were observed. Immunohistochemistry revealed increases in the numbers of eotaxin-immunoreactive cells in the bronchial mucosa of asthmatics compared with nonasthmatic controls (\(p < 0.05\); Fig. 1). There were no statistically significant differences between AA and NAA, although the mean numbers of BB1\(^+\) basophils in AA were slightly higher than those in NAA.

**C-C chemokines and CCR3 in asthma**

The numbers of CCR3-expressing cells (mRNA and protein) were significantly elevated in asthmatics compared with AC and NC (Fig. 5). However, nonsignificant differences between either AA and NAA or AC and NC were observed. In the asthmatic group (AA + NAA), numbers of CCR3 (mRNA and protein)-positive cells were directly correlated with EG2\(^+\) eosinophil numbers (\(r = 0.8; p < 0.001\)) and inversely with histamine PC\(_{20}\) (\(r = -0.51; p < 0.05\)).

**Cell sources of eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4**

Cells expressing mRNA for C-C chemokines were determined by double IHC/ISH. In the epithelium, up to 70% of mRNA-positive cells were colocated to cytokeratin\(^+\) epithelial cells. The remainders of the mRNA\(^+\) cells were CD68\(^+\) macrophages, CD3\(^+\) T cells, or tryptase\(^+\) mast cells (Fig. 6). In the submucosa, CD31\(^+\) endothelial cells, CD68\(^+\) macrophages, and CD3\(^+\) T lymphocytes were the major cell sources expressing these C-C chemokines (Fig. 7). No significant differences in the various cell types expressing mRNA for these chemokines in either epithelium or submucosa were observed between AA and NAA (\(n = 5\) for each group). The percentages of each cell type coexpressing mRNA for eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 in the epithelium and the submucosa are summarized in Tables II and III.
Discussion

In this study we compared the expression of C-C chemokines and CCR3 between atopic and nonatopic asthmatics and control subjects. We confirmed our previous findings on MCP-3 and RANTES (7) and extended the observations to include other eosinophil chemotactic chemokines. Thus, to our knowledge, this is the first systematic comparison of expression of eotaxin, eotaxin-2, and MCP-4 in the pathogenesis of intrinsic asthma, although elevated expression of eotaxin and MCP-4 has been shown to be a feature of allergic asthmatics (12, 18–20). We have also studied the cell source of eosinophil chemotactic chemokines in both intrinsic and extrinsic asthma. Our findings suggest that multiple C-C chemokines may contribute to eosinophil, and possibly basophil, recruitment to the asthmatic airway.

Whether these mediators are elaborated by the same mechanisms and through the same pathways in atopic and nonatopic asthma is presently unclear. Walker and colleagues have suggested that intrinsic and extrinsic asthma might be regarded as distinct immunopathological entities, since they found evidence that atopic asthma was characterized by a cytokine profile typical of Th2-type response in bronchoalveolar lavage (BAL) fluid, while intrinsic asthma was associated with isolated, elevated expression of IL-5 (21). They postulated that the absence of production of IL-4 explained the lack of IgE elevation in these patients. On the other hand, studies that have examined C-C chemokine as well as cytokine expression in atopic and nonatopic asthma have provided evidence for similarities, rather than differences, in the immunopathogenesis of these clinically distinct forms of the disease.

For example, elevated numbers, compared with controls, of activated T cells, eosinophils (22), high affinity IgE receptor-bearing cells (8), IL-5 and IL-4 mRNA, and protein product-positive cells have been shown to be features of the bronchial mucosa of both atopic and nonatopic asthma (5, 6). Furthermore, no differences in the number of mRNA-positive cells for IL-13 (23), RANTES, and MCP-3 were detected (7).

Using mAb EG2 and BB1, the numbers of eosinophils and basophils were evaluated in the bronchial biopsies from asthmatics at baseline and from controls. Immunohistochemistry showed that the numbers of EG2+ eosinophils were significantly higher in asthmatics (AA and NAA) than controls (AC and NC; Fig. 1). Although the number of EG2+ eosinophils was slightly higher in NAA than in AA, there were no significant differences between

![FIGURE 5](http://www.jimmunol.org/) The numbers of CCR3 mRNA and protein positive measured by ISH and IHC in bronchial biopsies (per 0.202 mm²) from AA, NAA, AC, and NC. In each group, n = 10.

![FIGURE 6](http://www.jimmunol.org/) Comparison of distribution of mRNA+ cells for eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 in epithelial area within bronchial biopsies from AA and NAA using sequential IHC/ISH. In each group, n = 5.
AA and NAA. This is consistent with previous findings (5–7). Unlike eosinophils, BB1⁺ basophils were undetectable in the biopsies from either AC or NC, suggesting that BB1⁺ basophils are not infiltrated into tissue in the normal situation. Compared with controls (AC + NC), small, but statistically significant, numbers of BB1⁺ basophils were detected in bronchial mucosa from asthmatics (AA + NAA; p < 0.05; Fig. 1). The number of BB1⁺ basophils in AA was higher than that in NAA, but the difference was not statistically significant. Interestingly, the number of BB1⁺ basophils was much less than that of EG2⁺ eosinophils in bronchial mucosa. The reason is unclear. BB1 is an mAb directed against a basophil granule constituent with a molecular mass of 124 kDa (17). Thus, completely degranulated basophils, if present, may be undetected.

In general, chemokine expression was elevated in both asthmatic groups compared with nonasthmatic controls. There was a trend for more pronounced expression in nonatopic patients compared with atopic asthmatics correlated with eosinophil infiltration and clinical features (12, 18, 19). In this study, eotaxin mRNA and protein product-positive cells significantly correlated with EG2⁺ eosinophils and histamine PC20 in asthmatics (AA + NAA). There was also a significant correlation between eotaxin-2 mRNA⁺ cells and EG2⁺ eosinophils (see Results). Both eotaxin and eotaxin-2 activate and attract eosinophils, basophils, and Th-2 cells (24), but not other leukocytes, and appear to act exclusively via CCR3 receptor (25). In terms of structure, however, eotaxin and eotaxin-2 are distant, sharing only 39% identical amino acids, while there is a 43% homology for MCP-4, 39% for MCP-3 and eotaxin, and 32% for RANTES (25). In addition, MCP-3, MCP-4, and RANTES have chemotactic activity for eosinophils and basophils (26, 27). Thus, it is likely that several chemokines contribute to eosinophil recruitment in allergic inflammation. Eotaxin-deficient mice had reduced eosinophils in BAL fluid early, but not late, after allergen challenge (28). Using the allergen-induced human skin late-phase reaction as a model, we found that that expression of eotaxin and MCP-3 was associated with the early 6-h recruitment of eosinophils, while eotaxin-2, RANTES, and MCP-4 appear to be involved in later 24-h infiltration of eosinophils (29). These observations suggest that different mechanisms may guide the early vs late eosinophilia in allergic inflammation. Taha et al. (20) showed that eotaxin-2 and MCP-4 mRNA increased in bronchial biopsies from atopic asthmatics compared with nonasthmatics. This is consistent with previous findings (5–7). Unlike eosinophils, BB1⁺ basophils were undetectable in the biopsies from either AC or NC, suggesting that BB1⁺ basophils are not infiltrated into tissue in the normal situation. Compared with controls (AC + NC), small, but statistically significant, numbers of BB1⁺ basophils were detected in bronchial mucosa from asthmatics (AA + NAA; p < 0.05; Fig. 1). The number of BB1⁺ basophils in AA was higher than that in NAA, but the difference was not statistically significant. Interestingly, the number of BB1⁺ basophils was much less than that of EG2⁺ eosinophils in bronchial mucosa. The reason is unclear. BB1 is an mAb directed against a basophil granule constituent with a molecular mass of 124 kDa (17). Thus, completely degranulated basophils, if present, may be undetected.

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![FIGURE 7. Comparison of distribution of mRNA⁺ cells for eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 in submucosa within bronchial biopsies from AA and NAA using sequential IHC/ISH. In each group, n = 5.](http://www.jimmunol.org/)

Table II. **Percentages of each cell type coexpressing chemokine mRNA for eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 within the epithelium of bronchial biopsies from AA (n = 5) and NAA (n = 5)**

<table>
<thead>
<tr>
<th>Cell Markers</th>
<th>Eotaxin mRNA⁺ Cells (% of each cell type)</th>
<th>Eotaxin-2 mRNA⁺ Cells (% of each cell type)</th>
<th>RANTES mRNA⁺ Cells (% of each cell type)</th>
<th>MCP-3 mRNA⁺ Cells (% of each cell type)</th>
<th>MCP-4 mRNA⁺ Cells (% of each cell type)</th>
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<td>AA</td>
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<td>49 ± 7</td>
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<tr>
<td>CD68⁺ macrophages</td>
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<td>31 ± 8</td>
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<td>22 ± 4</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>CD3⁺ T cells</td>
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<td>28 ± 9</td>
<td>25 ± 6</td>
<td>19 ± 9</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Tryptase⁺ mast cells</td>
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<td>28 ± 3</td>
<td>7 ± 7</td>
<td>16 ± 11</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>EG2⁺ eosinophils</td>
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<td>7 ± 4</td>
<td>6 ± 6</td>
<td>14 ± 9</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Elastase⁺ neutrophils</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 2</td>
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<tr>
<td>CD31⁺ endothelial cells</td>
<td>5 ± 5</td>
<td>5 ± 5</td>
<td>4 ± 4</td>
<td>4 ± 4</td>
<td>7 ± 4</td>
</tr>
</tbody>
</table>

* A combination of ISH (using digoxigenin-labeled riboprobes for each chemokine) and IHC (employing mAb against several phenotype markers as indicated) was used. The results were expressed as mean ± SEM.
mucosa of atopic asthmatics and that eotaxin mRNA+ cells significantly correlated with eosinophils. BAL fluid from asthmatic patients contained chemotactic activity for eosinophils that was attributable in part to both eotaxin and MCP-4 (18). However, a combination of eotaxin, MCP-4, and RANTES accounted for only about 50% of the eosinophil chemotactic activity in the BAL, suggesting the existence of other eosinophil chemotactic factors (18). In view of this multiplicity of potential influences on eosinophils, it is perhaps surprising that we found a direct correlation between eotaxin (but not RANTES, MCP-3, or MCP-4) mRNA expression and eosinophil numbers, clinical features, and the degree of bronchial hyper-responsiveness. The specificity of eotaxin for eosinophils, as opposed to the multiple cell targets of RANTES, MCP-3, and MCP-4, may explain these positive clinical correlations.

Previous reports indicated that the various C-C chemokines are expressed by many cell types, including epithelial cells, endothelial cells, macrophages, T lymphocytes, muscle cells, fibroblasts, platelets, Langerhans cells, dermicitic cells, and eosinophils themselves (30–41). Here we showed by double IHC/ISH that cyto-keratin+ epithelial cells, CD31+ endothelial cells, CD68+ macrophages, and CD3+ T lymphocytes were the majority of cells expressing these C-C chemokines (Figs. 6 and 7 and Tables II and III). We also noted that some MCP-3 mRNA+ cells (~35%) were not colocalized to any of the cell types examined. In vitro, it had been reported that fibroblasts and platelets also express MCP-3 (42, 43). Thus, fibroblasts and platelets (for which phenotypic markers are unavailable) and other unknown cell types may contribute to the expression of MCP-3 in bronchial mucosa.

The mechanisms of multi-C-C chemokine gene overexpression in the bronchial mucosa from asthmatics are incompletely understood. A number of proinflammatory cytokines and mediators are probably involved. For example, IL-1 and TNF-α might up-regulate the expression of eotaxin (30, 32), MCP-4 (31), and other C-C chemokines in epithelial and endothelial cells in vitro. On the other hand, Th2 cell-derived cytokines probably contribute to eotaxin-mediated tissue eosinophilia, because adoptive transfer of Th2 cells into mice induced Ag-dependent lung eotaxin expression and eosinophilia (44). Also, IL-4, the prototypic Th2 cytokine, enhanced eotaxin expression by epithelial and endothelial cells and dermal fibroblasts in vitro (30, 45), and injection of IL-4 into rats induced eosinophil accumulation in skin that was partially mediated by endogenous production of eotaxin (46). Again, intratracheal mouse recombinant eotaxin into IL-5 transgenic mice induced α1 integrin-dependent bronchial hyper-responsiveness and eosinophil migration (47). IL-13, another Th2 cytokine, induced eotaxin expression on epithelium (48). In addition, the Th2 cytokines IL-4 and IL-13 both induce up-regulation of VCAM-1 on endothelium, which is probably involved in eotaxin-induced eosinophil accumulation. Finally, peptido-lipid mediators (i.e., leukotrienes C4, D4, and E4) as well as histamine may also regulate the expression of C-C chemokines. We have shown in preliminary observations that these agents can increase eotaxin expression on human endothelial cells in vitro (49), indicating that these mediators may contribute to the eosinophil influx by up-regulating eotaxin and other C-C chemokines.

CCR3 mRNA is mainly associated with eosinophils, with limited expression by macrophages and mast cells (12), although a recent report identified CCR3 on blood basophils (50). Heath et al. (16) have shown that >95% of the response of eosinophils to eotaxin, RANTES, MCP-3, and MCP-4 is mediated through CCR3 receptor and could be blocked completely with an anti-CCR3 mAb. On the other hand, basophils express CCR1-CCR4 (24). In addition, it is known that RANTES interacts with two more receptors, CCR1 and CCR4 (24, 51), and that MCP-3 binds to CCR1 and CCR2 (24, 52), while MCP-4 binds to CCR2 and CCR3 (24, 31, 53). Thus, these C-C chemokines may contribute to the infiltration of other inflammatory cells, including macrophages in the bronchial mucosa of asthmatics.

It is worth noting that the elevated airway expression of all the chemokines studied was demonstrably not a feature of atopy per se, but instead a feature specific to asthma. In addition, it is noteworthy that C-C chemokine mRNA expression was detectable in a proportion of the control subjects. Similar observations were found in animal model. For instance, eotaxin expression and eosinophil infiltrates were detectable in lamina propria of the jejunum from normal wild-type mice, while eosinophils were reduced in the jejunum in eotaxin-deficient mice (54). Thus, a basal physiological degree of chemokine expression in the airway may be required for the trafficking of patrolling leukocytes involved in host defense.

Thus, we present evidence that combined production of various eosinophil-active chemokines within the bronchial mucosa is a characteristic feature of bronchial asthma regardless of its atopic status. The mechanisms controlling the transcriptional regulation of C-C chemokines are still poorly understood. CCR3 seems to be the principal receptor for eosinophil responses to C-C chemokines, and blocking this receptor could be a promising therapeutic tool in the management of asthma.

Acknowledgments

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Table III. Percentages of each cell type coexpressing chemokine mRNA for eotaxin, eotaxin-2, RANTES, MCP-3, and MCP-4 within the submucosa of bronchial biopsies from AA (n = 5) and NAA (n = 5)

<table>
<thead>
<tr>
<th>Cell Markers</th>
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<td>NAA</td>
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<tr>
<td>CD31+ endothelial cells</td>
<td>40 ± 5</td>
<td>42 ± 6</td>
<td>31 ± 3</td>
<td>32 ± 5</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>CD68+ macrophages</td>
<td>10 ± 2</td>
<td>12 ± 2</td>
<td>31 ± 3</td>
<td>35 ± 3</td>
<td>24 ± 5</td>
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<tr>
<td>CD3+ T cells</td>
<td>8 ± 2</td>
<td>9 ± 2</td>
<td>6 ± 2</td>
<td>5 ± 1</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Tryptase+ mast cells</td>
<td>10 ± 3</td>
<td>11 ± 2</td>
<td>6 ± 2</td>
<td>9 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>EG2+ eosinophils</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>10 ± 4</td>
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<tr>
<td>Elastase+ neutrophils</td>
<td>4 ± 1</td>
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<td>2 ± 1</td>
</tr>
</tbody>
</table>

* A combination of ISH (using digoxigenin-labeled riboprobes for each chemokine) and IHC (employing mAb against several phenotype markers as indicated) was used. The results were expressed as mean ± SEM.
also thank Drs. G. Menz and R. Pfister (Hochgebirgsklinik, Davos Wolfgang, Switzerland) for recruiting some patients included in this study.

References


