Glucocorticosteroids Inhibit mRNA Expression for Eotaxin, Eotaxin-2, and Monocyte-Chemotactic Protein-4 in Human Airway Inflammation with Eosinophilia

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**References**

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Glucocorticosteroids Inhibit mRNA Expression for Eotaxin, Eotaxin-2, and Monocyte-Chemotactic Protein-4 in Human Airway Inflammation with Eosinophilia

Frode L. Jahnsen, Rolf Haye, Einar Gran, Per Brandtzaeg, and Finn-Eirik Johansen

How eosinophils are preferentially recruited to inflammatory sites remains elusive, but increasing evidence suggests that chemokines that bind to the CCR3 participate in this process. In this study, we investigated the transcript levels and chemotactic activity of CCR3-binding chemokines in nasal polyps, a disorder often showing prominent eosinophilia. We found that mRNA expression for eotaxin, eotaxin-2, and monocyte-chemotactic protein-4 was significantly increased in nasal polyps compared with turbinate mucosa from the same patients, or histologically normal nasal mucosa from control subjects. Interestingly, the novel CCR3-specific chemokine, eotaxin-2, showed the highest transcript levels. Consistent with these mRNA data, polyp tissue fluid exhibited strong chemotactic activity for eosinophils that was significantly inhibited by a blocking Ab against CCR3. When patients were treated systemically with glucocorticosteroids, the mRNA levels in the polyps were reduced to that found in turbinate mucosa for all chemokines. Together, these findings suggested an important role for CCR3-binding chemokines in eosinophil recruitment to nasal polyps. Such chemokines, therefore, most likely contribute significantly in the pathogenesis of eosinophil-related disorders; and the reduced chemokine expression observed after steroid treatment might reflect, at least in part, how steroids inhibit tissue accumulation of eosinophils. The Journal of Immunology, 1999, 163: 1545–1551.

Tissue eosinophilia is a characteristic feature of several chronic inflammatory airway disorders, and increasing evidence suggests that these cells can cause tissue damage by release of their cytotoxic granule proteins and lipid mediators (1). Mechanisms that control leukocyte extravasation to sites of inflammation are only partially understood, but a recently identified large family of chemotactic cytokines, or chemokines, probably controls several steps in the transmigratory process (2–6). In vitro studies have shown that chemokines presented on the surface of endothelial cells mediate integrin activation in rolling leukocytes within seconds (7, 8). This enables their strong adhesion through activated integrins that bind to endothelial ligands. When the leukocyte firmly adheres at the endothelial surface, concentration gradients of chemokines produced beneath the vessel wall supposedly direct the cell across the vascular wall and guide the subsequent migration within the tissue toward an inflammatory target.

Compared with classical chemoattractants that act on most leukocytes, chemokines show specificity for different leukocyte subsets (9). For example, a clear distinction can be made between chemokines that act on neutrophils (the CXC family) and eosinophils (the C-C family). Normodense eosinophils express high levels of the C-C chemokine receptor CCR3 (10, 11), which is expressed by eosinophils, basophils (12), and a T cell subset (13). Several chemokines of the CC family, including eotaxin, RANTES, monocyte-chemotactic protein-3 (MCP-3) and MCP-4, have been shown to attract and activate eosinophils via high affinity binding to CCR3 (14–18). However, only eotaxin binds selectively to this receptor, which supposedly explains that eotaxin is relatively selective in eosinophil recruitment (19). More recently, another CCR3-specific chemokine termed eotaxin-2 has been identified (20, 21), which functionally is quite similar to eotaxin (20–22).

In various eosinophil-associated disorders such as asthma and allergic rhinitis, increased levels of several eosinophil-activating chemokines have been detected in the inflammatory lesions (9, 23), suggesting that these mediators are involved in the pathogenesis. However, the relative roles of various chemokines remain poorly defined, and particularly the expression of eotaxin-2 has not as yet been examined.

Nasal polyps share several histopathological features with chronic allergic reactions such as bronchial asthma, including a prominent and often selective infiltration with eosinophils. Another common feature of eosinophil-related disorders is the remarkable anti-inflammatory effect of glucocorticosteroids, including reduction of airway eosinophilia (24–26). To investigate preferential chemokine association with eosinophil recruitment, we examined the transcript levels and chemotactic activity of CCR3-binding chemokines in nasal polyp tissue. The putative pathogenic involvement of the same chemokines was furthermore studied by measuring the effect of systemic glucocorticoid treatment on their mRNA levels in this inflammatory disorder.

Materials and Methods

Subjects

Nasal polyp patients included in this study (Table I) were part of a clinical trial evaluating whether preoperative systemic glucocorticosteroid treatment could facilitate the surgical procedure and reduce postoperative charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Norwegian Cancer Society, the Research Council of Norway, the Research Fund for Asthma and Allergy, and the Red Cross Research Fund for Children with Asthma and Allergy.

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3 Abbreviation used in this paper: MCP, monocyte-chemotactic protein.
Complications. All patients required surgical treatment for extensive nasal polyps and were randomly assigned to steroid treatment. The treatment group (n = 12) received 40 mg prednisone once daily for 7 days before surgery. The untreated group (n = 15) had not used systemic or topical steroids for at least 1 mo before admission. Patients with seasonal allergic rhinitis were subjected to surgery when being out of their allergen season. Blood samples were obtained from the treatment group the day before medication start and from all patients at the day of surgery. Polyp size was measured blind by the same investigator (F.L.J.). Eosinophils were numerically recorded per basement membrane length unit (1 mm) to a stromal depth of 300 μm by superimposing a grid (200 × 300 μm); all positive cells were counted in at least one section (>10 fields) from each specimen. Also, to determine the proportion of eosinophils and neutrophils relative to all leukocytes in nasal polyps, triple-stained cryosections were evaluated with regard to red, blue, and green cells by including at least 10 random fields; this approach provided a cell number sufficiently large to obtain a stable accumulative mean for the granulocyte-to-leukocyte ratio.

**Isolation and quantification of mRNA**

Two internal standards for competitive RT-PCR were employed. For this purpose, we constructed a synthetic DNA that contained cassettes with both forward and reverse PCR primer binding sites for chemokines (MCP-1, MCP-3, MCP-4, eotaxin, RANTES, lymphotactin, macrophage-inflammatory protein-1β), cytokines (IL-3, IL-13, IL-14, IL-16), adhesion molecules (VCAM-1, P-selectin), and the housekeeping gene GAPDH, using oligonucleotides essentially as described (29). The cassettes of forward and reverse primer binding sites were constructed separately and subcloned into pCR2 (Invitrogen, Leek, The Netherlands). The two cassettes were subsequently combined into pHQ1 that had been digested with EcoRI and PacI to remove the insert but leave the synthetic poly(A) tail (30). A standard for eotaxin-2 was constructed by subcloning the segment from the forward primer to an internal EcoRI site into pCR2, and a segment from the reverse primer to the internal EcoRI site into pBS(KS+) (Strategene, La Jolla, CA), the two segments were combined into pHQ1, as described above. Both standard DNAs were sequenced by the dyeoxy chain termination method with Sequenase (Amersham, Slough, U.K.). Total RNA was isolated by the guanidine/CsCl procedure from tissue specimens directly frozen in liquid nitrogen (see earlier) (30, 31). The two standard DNAs were linearized with HindIII and used as template for RNA synthesis according to the Megascript T7 protocol (Ambion, Austin, TX), and combined at equal molar ratios. Eight serial dilutions of 0.5 log intervals from 107 to 3.3 × 102 copies were used as template for cDNA synthesis together with a constant amount of 500 ng cellular RNA in a 20-μl reaction in 96-well plates. Synthesis of cDNA was primed by oligo(dT) and reverse transcribed with SuperscriptII, according to the manufacturer’s instructions (Life Technologies, Paisley, U.K.).

Primers, conditions, and product sizes for the PCRs are listed in Table II. All matching forward and reverse primers were designed with software from Genetics Computer Group (Madison, WI) and chosen from separate exons. All reactions were performed with Dynazyme (Finzymes Oy, Espoo, Finland) in 96-well plates containing 1 μl cDNA and 10 pmol primers in 25 μl reaction volume. Each PCR cycle included the following incubation temperatures for 1 min each: 95°C denaturing; annealing temperature as indicated; 72°C extension; and a final extension of 10 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualized with

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Untreated Patients</th>
<th>Treated Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>6/9</td>
<td>7/5</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>40 (19–67)</td>
<td>51 (39–68)</td>
</tr>
<tr>
<td>C-reactive proteina</td>
<td>&lt;5 (&lt;5–14)</td>
<td>&lt;5 (&lt;5–24)</td>
</tr>
<tr>
<td>Total blood leukocytes (10⁶/L)a</td>
<td>6.9 (4.7–13.3)</td>
<td>6.6 (5.0–7.9)</td>
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<tr>
<td>Blood eosinophils (10⁶/L)a</td>
<td>235 (70–1170)</td>
<td>275 (128–805)</td>
</tr>
<tr>
<td>Blood neutrophils (10⁶/L)</td>
<td>4.6 (2.2–8.3)</td>
<td>3.7 (2.8–4.4)</td>
</tr>
<tr>
<td>Aspirin intolerancec</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bronchial asthma</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Allergic rhinitsc</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

a Median (range).
b History of aspirin intolerance.
c History of allergic rhinitis and positive skin prick test.
ethidium bromide staining (Fig. 1). Relative band intensities were determined by densitometry on Polaroid 665 negatives (Kodak, Rochester, NY). All target mRNA levels were related to the level of GAPDH message in the same RNA sample to normalize for inaccuracies in quantifying the cellular RNA.

**Isolation of eosinophils from peripheral blood**

Peripheral blood was obtained from healthy individuals with normal eosinophil counts. Mononuclear cells were removed by Lymphoprep (Nycomed Pharma, Oslo, Norway) and erythrocytes were lysed by a NH4Cl solution, as previously described (32). Eosinophils were further purified by negative selection with anti-CD16-coated immunomagnetic microbeads and a magnetic cell separation (MACS) system (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturers’ directions. The purity of eosinophils was always >95%, with viability >98%, as assessed by ethidium bromide (2.5 μg/ml) staining.

**Chemotaxis assay**

Experiments were performed with a 96-well microchemotaxis chamber (Chemotx; Neuroprobe, Cabin John, MD), as recommended by the manufacturer. Purified eosinophils were washed twice in HBSS without Ca2+ and Mg2+, and then labeled with Calcein-AM (10 μM, 15 min, 37°C). The labeled cells were washed twice and resuspended in HBSS with 2% FCS (assay medium). Tissue supernatants, recombinant human eotaxin (PeproTech, London, U.K.), or recombinant human RANTES (R&D Systems, Abingdon, U.K.) diluted in assay medium were loaded into the wells (29 μl). The polycarbonate filter (5-μm pore size) was then installed, and 25 μl of labeled eosinophils (3 × 106 cells/ml) was loaded on top of the filter and incubated at 37°C for 60 min. Thereafter, the cells at the top of the filter were gently removed by flushing with media and wiping with a cell harvester. Cells that had migrated into the filter were assessed by recording the fluorescence signal in a Titertek Fluoroscan II fluorescence multiwell reader (Labsystems, Helsinki, Finland).

For blocking experiments, mAb 7B11 (10 μg/ml, IgG1; courtesy of Dr. Charles Mackay, LeukoSite, Cambridge, MA) directed against CCR3 (33) was added to the labeled cells before incubation.

**Statistics**

Wilcoxon matched pairs sign rank sum test was performed to compare mRNA levels and numbers of eosinophils at various mucosal tissue sites within patient groups. The Mann-Whitney two-tailed test was performed to compare untreated with steroid-treated patients and control subjects. Paired Student’s two-tailed t test was used to compare chemotactic activity of tissue supernatants with or without addition of blocking mAbs. Spearman’s rank correlation coefficient was used to assess the relationship between the percentage of tissue eosinophils and the chemokine mRNA levels.

**Results**

**Tissue eosinophils in untreated patients**

The fraction of leukocytes identified as eosinophils was significantly increased in nasal polyps compared with that in their lower turbinate (internal control) or normal control mucosa (Fig. 2). On average, 43% (range 0–80%) of all leukocytes were eosinophils in nasal polyps and lower turbinate mucosa from polyp patients untreated or after systemic steroid treatment and normal turbinate control specimens. Medians indicated by horizontal lines. Statistical analyses performed by Wilcoxon matched pairs sign rank sum test and the Mann-Whitney two-tailed test for paired and unpaired comparisons, respectively.

![FIGURE 1](Image)

**FIGURE 1.** Visualization of competitive quantitative RT-PCR. Varying numbers of standard RNA molecules were reverse transcribed together with a constant amount of cellular RNA (500 ng). The cDNA reaction mixture from the same cellular source was amplified with primers specific for GAPDH (upper panel) or eotaxin (lower panel). PCR products were separated by electrophoresis and visualized with ethidium bromide staining. When the ratio of band intensities equals one, the number of target RNA molecules matches the number of standard RNA molecules. In this example, 1 μg of cellular RNA contained 6 × 106 and 1.5 × 105 molecules of GAPDH and eotaxin mRNA, respectively, as assessed by densitometry.

![FIGURE 2](Image)

**FIGURE 2.** The fraction of eosinophils relative to all leukocytes in nasal polyps and lower turbinate mucosa from polyp patients untreated or after systemic steroid treatment and normal turbinate control specimens. Medians indicated by horizontal lines. Statistical analyses performed by Wilcoxon matched pairs sign rank sum test and the Mann-Whitney two-tailed test for paired and unpaired comparisons, respectively.
these samples, whereas a median of only 2% (0–10%) neutrophils was detected. The median number of eosinophils per mm basement membrane was 103 (range 0–375) in polyp tissue compared with 5 (0–281) and 0 (0–1) in lower turbinate (internal control) or normal control mucosa, respectively.

Expression of chemokine mRNA in untreated patients
Quantitative RT-PCR showed that mRNA for eotaxin, eotaxin-2, and MCP-4 were significantly increased in nasal polyps compared with lower turbinate mucosa from the same individuals and normal control mucosa (Fig. 3). All three chemokines displayed similar mRNA levels in turbinate mucosa of the patients (~1% of GAPDH levels), but in polyps the eotaxin-2 level was considerably higher (40% of GAPDH) than that of eotaxin (5% of GAPDH) and MCP-4 (6.5% of GAPDH). Furthermore, mRNA levels for eotaxin-2 and MCP-4 were significantly elevated in turbinate mucosa of the untreated patients compared with control mucosa from healthy individuals. RANTES displayed approximately the same transcript level as eotaxin and MCP-4 in the polyps, but the same magnitude of mRNA expression was also found in turbinate mucosa from both patients and controls (Fig. 3). For MCP-3, the mRNA levels were low (and in some samples undetectable) in all specimens tested (not shown). The cumulative or individual chemokine mRNA levels did not correlate significantly with the percentage of eosinophils in the polyps. However, virtually no eosinophils were found in the only polyp sample that displayed low mRNA levels for all chemokines tested (data not shown).

Effect of glucocorticosteroids on polyp size and eosinophils
By endoscopic inspection of both nasal cavities, an average of 50% (range 25–75%) reduction of polyp size was observed after glucocorticosteroid treatment. Steroids also significantly reduced the fraction of eosinophils in the polyps compared with untreated counterparts (Fig. 2). Moreover, steroids dramatically reduced (95%) the number of eosinophils in peripheral blood, but at the same time increased (90%) the number of circulating neutrophils (Table I); the latter effect accounted for the total increase of peripheral blood leukocytes (Table I).

Effect of glucocorticosteroids on chemokine mRNA expression
To examine whether glucocorticoids affected mRNA levels for CCR3-binding chemokines in polyp tissue, we performed quantitative RT-PCR on RNA isolated from tissue specimens of patients treated for 7 days with systemic steroids. Levels of mRNA for
FIGURE 4. Stimulation of eosinophil chemotaxis by nasal polyp fluid and its inhibition by blocking mAb against CCR3 (mAb 7B11). Tissue fluid obtained from nasal polyps with eosinophilia was used as attractant in microchemotaxis chamber. Purified labeled eosinophils, either untreated or pretreated with anti-CCR3, were examined for migration toward the supernatants. An optimal concentration of recombinant human eotaxin (200 ng/ml) served as positive control. The magnitude of migrated cells was expressed as arbitrary units measured in a fluorescence multiwell reader. Basal migration values are indicated by dotted line. * p = 0.01. Representative results from two independent experiments (mean ± SD of triplicate wells).

eotaxin, eotaxin-2, and MCP-4 in polyps after treatment were significantly reduced compared with untreated counterparts, being similar to those found in lower turbinate mucosa (Fig. 3). Also, mRNA levels for RANTES were significantly reduced in treated polyps compared with untreated counterparts (Fig. 3).

Chemotactic activity of polyp tissue fluid

Because nasal polyps from untreated patients expressed high mRNA levels for CCR3-binding chemokines, we wanted to examine whether polyp tissue fluid could activate this receptor. Tissue fluid isolated from polyps with eosinophilia demonstrated significant chemotactic activity toward purified eosinophils, and initial dose-response experiments showed that eosinophils migrated most effectively in response to undiluted fluid (data not shown). Interestingly, eosinophils migrated 3- to 5-fold more efficiently in response to undiluted polyp tissue fluid than to an optimal concentration (200 ng/ml) of recombinant human eotaxin (Fig. 4). This effect was significantly (p = 0.01), but not completely, inhibited by mAb 7B11 directed against CCR3, while the chemotactic effect of recombinant human eotaxin was entirely inhibited by this mAb (Fig. 4). This finding suggested that the polyp fluid also contained eosinophil chemotactic factors acting independently of CCR3. However, mAb 7B11 completely abrogated eosinophil migration in response to an optimal concentration (100 ng/ml) of recombinant human RANTES (data not shown), suggesting that the chemotactic activity of that chemokine was mediated only through CCR3 and not CCR1, although RANTES can bind to both receptors.

Discussion

In this study, expression of mRNA for CCR3-binding chemokines was quantified in nasal polyps obtained from patients with or without steroid treatment. In the untreated state, the transcript levels for eotaxin, eotaxin-2, and MCP-4 were significantly higher in the polyps than in uninvolved turbinate mucosa from the same patients or controls. Accordingly, polyp tissue fluid contained strong chemotactic activity for eosinophils, an effect shown to be partially mediated through CCR3.

The remarkable diversity of chemokines implies that they have a key role in controlling the characteristics of an inflammatory infiltrate, such as preferential accumulation of eosinophils in nasal polyps. Several chemokines bind CCR3 and therefore attract eosinophils, but only eotaxin and eotaxin-2 show selectivity for this receptor. Interestingly, mRNA levels for both these chemokines as well as MCP-4 were significantly increased in untreated nasal polyps compared with turbinate mucosa. Increased mRNA and protein expression of eotaxin has recently been detected in various human inflammatory lesions with increased eosinophil recruitment (15, 34–36), but to our knowledge this is the first study to report in situ mRNA expression for eotaxin-2. Notably, among the chemokines examined, eotaxin-2 showed the highest mRNA levels in polyp tissue (eight times higher than eotaxin), and it was 500-fold increased compared with the levels in turbinate mucosa of normal controls. In contrast to most other chemokines, eotaxin-2 mRNA expression has not been detected in normal organ blots (20).

Therefore, this chemokine most likely plays a crucial role in eosinophil-associated inflammation.

Levels of mRNA for eotaxin-2 and MCP-4 were also significantly higher in the lower turbinate of untreated patients compared with normal controls. Moreover, in the patient group, 6 of 12 specimens showed histologically slight to moderate inflammation. However, only two of these lower turbinate specimens showed accumulation of eosinophils. This suggested that the levels of these chemokines, although elevated, were below a critical threshold concentration necessary to attract eosinophils. Simultaneous expression of several CCR3-activating chemokines might be crucial to achieve the proper cues for eosinophil extravasation. Alternatively, this redundancy could ensure a threshold concentration of chemokines that is necessary to engage CCR3 on eosinophils. Interestingly in this respect, it has been shown that a subset of T cells bearing CCR3 accumulates in nasal polyps (37), supporting the notion that CCR3-binding chemokines are important in the pathogenesis.

Altogether, based on this study and previous reports (28, 38), accumulation of eosinophils in nasal polyps appears to involve multiple leukocyte adhesion and chemoattractant receptors and their ligands, as well as eosinophil survival factors such as IL-5 (39). The lack of significant correlation between chemokine mRNA levels and the percentage of tissue eosinophils is therefore not incompatible with the notion that CCR3-binding chemokines play an important role in this process; it rather reflects a complex biological situation in which leukocyte emigration and retention depend on an array of diverse factors.

Because Abs to eotaxin-2 and MCP-4 were unavailable in our laboratory, we could not examine protein expression of these chemokines in situ. Recent studies have detected mRNA and protein expression for eotaxin, MCP-4, and RANTES in many different cell types at sites of inflammation (15, 17, 34, 36, 40–42). Notably, resident cells such as epithelial cells, fibroblasts, and endothelial cells have been shown to produce significant levels of such chemokines after stimulation in vitro (17, 18, 36, 41, 42, 43–45). It is therefore likely that several cell types within nasal polyps are responsible for the increased transcript levels for eotaxin, eotaxin-2, and MCP-4. In agreement with previous studies, we found high levels of RANTES mRNA in untreated polyps (46). However, these levels were not different from those found in normal control samples, suggesting that RANTES plays a role in normal homeostasis rather than in the pathogenesis of nasal polyps.

Systemic glucocorticoids have prominent anti-inflammatory effects and are the most effective drugs in the treatment of eosinophil-related inflammatory diseases such as asthma and nasal polyps. In this study, we observed that such treatment often reduced the size of nasal polyps by more than 50% concurrently with a significant reduction of tissue eosinophils. The significant decrease of tissue eosinophils in polyps of steroid-treated patients could partly
be caused by the dramatic reduction (>95%) of blood eosinophils after treatment. However, it is well documented that glucocorticoids act locally in peripheral tissues, and it has been shown that topical administration of these drugs has a profound effect on the accumulation of eosinophils in both asthma and nasal polyposis (24–26, 47). Interestingly in this context, we found that mRNA levels for all tested chemokines were reduced in polyps of treated patients to levels similar to those detected in turbinated mucosa. Whether this result was caused indirectly via extracellular mediators that regulate chemokine production or by a direct effect of glucocorticoids on this production could not be determined. In favor of the latter possibility, glucocorticoids have been shown to inhibit directly cytokine-induced production of eotaxin, MCP-4, and RANTES in epithelial cells in vitro (41, 42, 45). Anyhow, the observed pronounced reduction of chemokine expression in nasal polyps after steroid treatment apparently defines an important mechanism for how steroids inhibit tissue accumulation of eosinophils.

We found that supernatants from polytissue expressed strong chemotactic activity for eosinophils. This effect was significantly inhibited by a blocking mAb specific for CCR3, demonstrating that polyps with eosinophilia contained high levels of CCR3-binding ligands. This finding was consistent with our mRNA data and strongly suggests that transcripts for CCR3-binding chemokines were translated into functional proteins. Interestingly, tissue fluid demonstrated 3–5 times more chemotactic activity than an optimal concentration of recombinant human eotaxin, implying the involvement of additional factors. Thus, engagement of multiple distinct receptors most likely has an additive or synergistic effect on eosinophil extravasation. The fact that anti-CCR3 did not completely abrogate the chemotactic activity supports the possibility that such additional chemotactic factors act independently of CCR3. In agreement with these observations, it was recently shown that classical chemoattractants such as C5a and FMLP had an additive effect on CCR3-mediated transendothelial chemotaxis of eosinophils (48). It would have been interesting to test the chemotactic activity of tissue fluid also from untreated polyps with only small numbers of eosinophils, as well as from steroid-treated polyps. However, the limited size of the tissue samples, especially after steroid treatment, and also the fact that polyps with few eosinophils are infrequent (3 of 15 in our study), rendered it impossible to obtain sufficient material for such experiments.

Together, our findings accord with a combinatorial role played by several CCR3-binding chemokines in tissue recruitment of eosinophils, and strengthen the supposed importance of these chemokines in the pathogenesis of eosinophil-related disorders such as nasal polyps and bronchial asthma.

Acknowledgments

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References


