

Eotaxin and CCR3 are up-regulated in exacerbations of chronic bronchitis

Background: Eosinophils and T lymphocytes represent constant features in the airways of subjects with exacerbated chronic bronchitis. Eotaxin is the most potent and selective eosinophil chemoattractant which can also attract lymphocytes. The aim of the study was to evaluate the expression of eotaxin and its receptor, CCR3, in bronchial airways during exacerbation of chronic bronchitis.

Methods: By immunohistochemistry we studied eotaxin and CCR3 expression in the lamina propria of 14 subjects with acute exacerbation of chronic bronchitis, 20 asthmatics, and 8 healthy subjects. We determined the cell types expressing the CCR3 receptor by colocalization experiments. We finally studied the relationship between eotaxin and CCR3 and eosinophils and T lymphocytes.

Results: The number of eotaxin⁺ and CCR3⁺ cells was significantly higher in exacerbated chronic bronchitis ($P < 0.003$ and $P < 0.002$) and asthma ($P < 0.002$ and $P < 0.0001$) when compared to healthy subjects. CCR3 was mainly expressed by eosinophils and to a lesser extent by CD4⁺ and CD8⁺ lymphocytes. In exacerbated chronic bronchitis the number of CCR3⁺ cells was strongly correlated to the number of eosinophils ($P < 0.0002$, $r = 0.85$) and to the number of CD4⁺ lymphocytes ($P < 0.05$, $r = 0.57$).

Conclusion: Our study suggests that eotaxin and CCR3 are up-regulated and could be involved in the eosinophil and CD4⁺ lymphocyte recruitment into the airways which occur during acute exacerbations of chronic bronchitis.

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Increased eosinophil and T lymphocyte numbers in the airways represent major features, not only in subjects with bronchial asthma (1, 2), but also, as more recently demonstrated, in exacerbated chronic bronchitis (ECB) (3, 4).

Eotaxin is a protein of 73 amino-acids belonging to the C-C family of chemokines. It is a potent and selective eosinophil chemoattractant in animals and humans (5–9). Eotaxin was also identified as a potent activator of eosinophils capable of inducing superoxide generation and the release of granule proteins. Human eotaxin binds with high affinity to CC chemokine receptor (CCR)3. CCR3 is the only eotaxin receptor identified to date. CCR3 belongs to the large family of seven transmembrane G-protein coupled receptor and it is highly expressed on eosinophils (10). More recently, CCR3 has been found on a subpopulation of Th2 lymphocytes (11). CCR3 expressed on Th2 cells is functionally reactive to eotaxin, in that it induces intracellular calcium flux and chemotactic migration of these cells *in vitro* (12).

In addition, it has been demonstrated that eotaxin and CCR3 expression is increased and related to

eosinophil infiltration into airways of patients with asthma (13–15), suggesting a critical role for this pathway in airway eosinophilia.

To investigate the potential role of eotaxin and CCR3 in the inflammatory cell recruitment during exacerbation of chronic bronchitis, we analyzed:

1 the expression of eotaxin and CCR3 in the bronchial mucosa of patients with ECB compared to asthmatic and normal subjects;

2 the expression of CCR3 by inflammatory cells;

3 the relationship between eotaxin and CCR3 expression and the number of eosinophils and T lymphocytes infiltrated into the airways.

Material and methods

Subjects

The study population comprised: 14 subjects with acute exacerbation of chronic bronchitis, 20 subjects with bronchial asthma and 8 healthy controls. Subjects with chronic bronchitis had a history of cough and sputum production on most days of the month for at least 3 months a year during the previous 2 years. None of the patients had past history of asthma or allergic rhinitis, and a significant reversibility of airflow obstruction ($>15\%$) after inhalation of

400 µg salbutamol via a metered-dose inhaler. All patients were nonatopic. All patients were smokers or ex-smokers with a history of smoking equivalent to at least 20 pack-year.

Acute exacerbation of chronic bronchitis was defined as a worsening of symptoms (increased dyspnea associated with increased cough and sputum production) that led the subject to seek medical attention within the week preceding the study (16).

The diagnosis of asthma was made according to internationally accepted criteria (17). Subjects with asthma had a history of intermittent wheeze, chest tightness, cough, and sputum production either spontaneously, after allergen exposure, or after exercise together with repeated and reversible airflow obstruction. All the asthmatics had an increased airway responsiveness to methacholine (cumulative concentration producing a fall in forced expiratory volume in 1 s (FEV₁) of 20% from baseline (PC₂₀) < 16 mg/ml). All the asthmatics were lifetime non-smokers and atopic, as defined by positive skin prick tests to common aeroallergens. Respiratory symptoms of the asthmatic subjects were controlled with β_2 -adrenergic drugs on demand.

None of the subjects of both diseases had taken inhaled or oral corticosteroids for at least 6 weeks prior to the study.

Normal controls were not taking any form of medication, had no history of cigarette smoking or asthma or other allergic diseases, had negative skin-prick tests, and methacholine PC₂₀ > 64 mg/ml.

The study conformed to the Declaration of Helsinki. The study of asthmatic group was approved by the University of Parma Ethical Committee. Written informed consent was obtained from each subject.

Fiberoptic bronchoscopy

Fiberoptic bronchoscopy was performed as previously described (18). Subjects were pre-medicated intramuscularly with 0.5 mg atropine sulfate and 10 mg diazepam, 30 min before the procedure. Local anesthesia was obtained by 2% lignocaine 15 min before bronchoscopy. An additional aliquot of 2% lignocaine was aerosolized into upper airways and applied topically to the pyriform sinuses and vocal cords to prevent coughing and as a local anesthesia. No other drugs were administered before or during bronchoscopy.

Fiberoptic bronchoscopy was introduced through the nose or transorally (Olympus BF, Type 1T 10; Olympus Optical Co., Tokyo, Japan). Using alligator forceps 3–5 mucosal biopsy specimens were taken from the subcarinae in the middle and lower lobes of the right lung.

Processing of bronchial biopsies and staining procedure

Mucosal biopsies were immediately transferred into ice-cooled acetone, containing the protease inhibitors iodoacetamide (20 mM) and phenylmethylsulfonylfluoride (PMSF) (2 mM) for fixation, stored at –20°C for 24 h, and then processed into the water-soluble resin, glycolmethacrylate (GMA) resin (Polysciences, Northampton, UK) for embedding.

Two-micrometer sections were cut by ultramicrotomy, floated on 0.2% (v/v) ammonia solution for 1 min, picked up onto polyL-lysine-coated slides, and dried at room temperature for 1–4 h. Mouse monoclonal antibodies (mAbs) directed against the following human antigens have been used: eotaxin (6H9, LeukoSite Inc., Cambridge, USA); CCR3 (7B11, LeukoSite Inc); eosinophil cationic protein (EG2, Pharmacia, Uppsala); mast cell tryptase (AA1, DAKO, Glostrup, DK); CD4 (MT310, DAKO); CD8 (DK25, DAKO); neutrophil elastase (NP57, DAKO).

Immunostaining applied to GMA-embedded tissue has been previously described (19). Slides were pre-treated with a solution of 0.1% (w/v) sodium azide and 0.3% (v/v) hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. After three 5-min washes in Tris-buffered saline (TBS) pH 7.6, blocking medium,

consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 1% bovine serum albumin, was applied for 30 min. Sections were then incubated with the primary antibody for 16–20 h overnight at room temperature. After overnight incubation, sections were washed in TBS. Bound antibodies were then labeled with biotinylated antimouse IgG (Vector Laboratories, CA, USA) during a 2 h incubation and made visible using a streptavidin-biotin-horseradish-peroxidase complex detection system (Dako, Glostrup, DK). Aminoethylcarbazole was applied as chromogen, resulting in a red reaction product. Sections were counterstained with Mayer's hematoxylin. Appropriate control sections were similarly treated either with the primary antibody omitted or replaced by an unrelated mouse mAb.

Assessment and quantification of immunohistochemical staining

Light microscopy was performed using a LEICA microscope (Leica DMLB, Wetzlar, Postfach, Germany) at a magnification of 10 × 100. Cells were expressed per mm² lamina propria. For each biopsy, all positively stained cells were counted in a zone 100 µ deep in the bronchial mucosa along the length of the epithelial basement membrane. The length of epithelial basement membrane and the lamina propria area were calculated using an image processing and analysis system (LEICA Q500C, LEICA Cambridge Ltd, Cambridge, UK).

The analysis system was also used to carry out comparison of sequential sections. CCR3 immunoreactivity in five biopsies from the asthmatic group and five biopsies from the chronic bronchitis group was paired with adjacent sections separately stained for ECP, CD4, CD8, tryptase and neutrophil elastase. At least three fields at ×400 magnification were compared for each pair of adjacent sections to enable CCR3 to be localized to specific cell types. The results were expressed as the percentage coexpressed by each cell type.

Statistical analysis

Non-parametric statistical tests (Mann–Whitney *U*-test) were used in the analysis, and median values and range are presented in the text and the accompanying figures and tables. Correlation coefficients were obtained by Spearman's rank-order method. In all cases a value of *p* < 0.05 was considered significant.

Results

Demographic and functional characteristics of subjects

The demographic and functional characteristics of the study population are reported in Table 1. The mean ages of asthmatic patients and normal subjects were similar, but ECB patients were significantly older than these two groups (*p* < 0.001 and *p* < 0.001). The pulmonary function of patients with ECB as assessed by FEV₁ ranged from 43 to 133% of predicted values. The FEV₁ of asthmatics ranged from 64 to 148%. Asthmatic patients were classified as mild to moderate. The pulmonary function of normal subjects was within normal range.

Biopsy findings

Bronchoscopy and bronchial biopsies were performed successfully and they were well tolerated in all subjects.

Eotaxin and CCR3 immunoreactivity was evident in the biopsies of all subjects. The distribution of eotaxin staining did not significantly differ between ECB and

Table 1 Clinical and demographic characteristics of the study subjects (mean $\pm$ SD).

	Exacerbated chronic bronchitis	Asthmatics	Controls
n°	14	20	8
Age (year)	54.50 $\pm$ 11.6	28.65 $\pm$ 11.65	23.5 $\pm$ 1.06
Sex (males)	13	16	3
Atopy (n° of subjects)*	0	20	0
FEV ₁ (% predicted)†	86.1 $\pm$ 26.6	105.7 $\pm$ 21.25	110.3 $\pm$ 11.04
PC20 (mg/ml)‡	> 64	3.79 $\pm$ 3.13	> 64

*As determined by positive skin prick tests (weal diameter > 3 mm) to at least two common inhalant allergens.

† Forced expiratory volume in one second.

‡ Cumulative provocative concentration of methacholine causing a 20% drop in FEV₁.

asthma. The chemokine was mainly localized on epithelial and endothelial cells and on cells diffusely localized in the submucosa. CCR3⁺ cells were localized in the epithelium and in the lamina propria. No significant staining was seen in any of the immunohistochemical control sections.

Eotaxin⁺ and CCR3⁺ cells were significantly higher in the lamina propria of both ECB and asthmatic subjects when compared to controls ($p < 0.002$ and $p < 0.003$ in ECB; $p < 0.0001$ and $p < 0.002$ in asthma). No significant differences were observed between asthma and ECB (Fig. 1).

Examination of serial sections was performed in a subgroup of five EBC and five asthmatic subjects chosen because of their impressive CCR3 receptor immunoreactivity. Comparison of adjacent sections showed that CCR3 was strongly expressed by eosinophils and to a lesser extent by CD4⁺ and CD8⁺ lymphocytes. No co-localization of CCR3 on mast cells and neutrophils was seen. In the five ECB and five asthmatic biopsies studied, a median of 87.2% (67.8% to 100%) and 85% (72.8% to 100%), respectively, of CCR3⁺ cells were eosinophils and a median of 78.4% (58.9% to 92%) and 63.8% (45% to 88.2%), respectively, of eosinophils were positive for CCR3 (Table 2). Less than 1% of CCR⁺ cells were CD4⁺ and CD8⁺ lymphocytes and *vice versa*. Representative examples of sequential sections demonstrating localization of CCR3 to eosinophils and lymphocytes are shown in Figs 2 and 3.

In both ECB and asthmatic subjects there was an increase in the number of eosinophils compared to controls. No differences in the number of CD4⁺ and CD8⁺ lymphocytes among the groups were seen (Table 3).

The number of eotaxin⁺ cells was significantly correlated to the number of CCR3⁺ and eosinophils in asthma ($p < 0.0005$, $r = 0.71$; $p < 0.05$, $r = 0.46$) but not in ECB although a trend towards correlation was apparent ($p < 0.08$, $r = 0.47$; $p < 0.06$, $r = 0.49$). No significant correlations between eotaxin⁺ cells and CD4⁺ or CD8⁺ cells were found in both ECB and asthma.

In ECB, the number of CCR⁺ cells was strongly correlated with the number of eosinophils ($p < 0.0002$,

$r = 0.85$) (Fig. 4). A significant correlation was present in asthma also ($p < 0.001$, $r = 0.73$). In ECB, the number of CD4⁺ lymphocytes was significantly correlated with the number of CCR3⁺ cells ($p < 0.05$, $r = 0.57$) (Fig. 4). CCR3⁺ cells were not correlated with CD8⁺ cells in both ECB and asthma.

No significant correlations could be drawn between CCR3/eotaxin expression and airway obstruction in

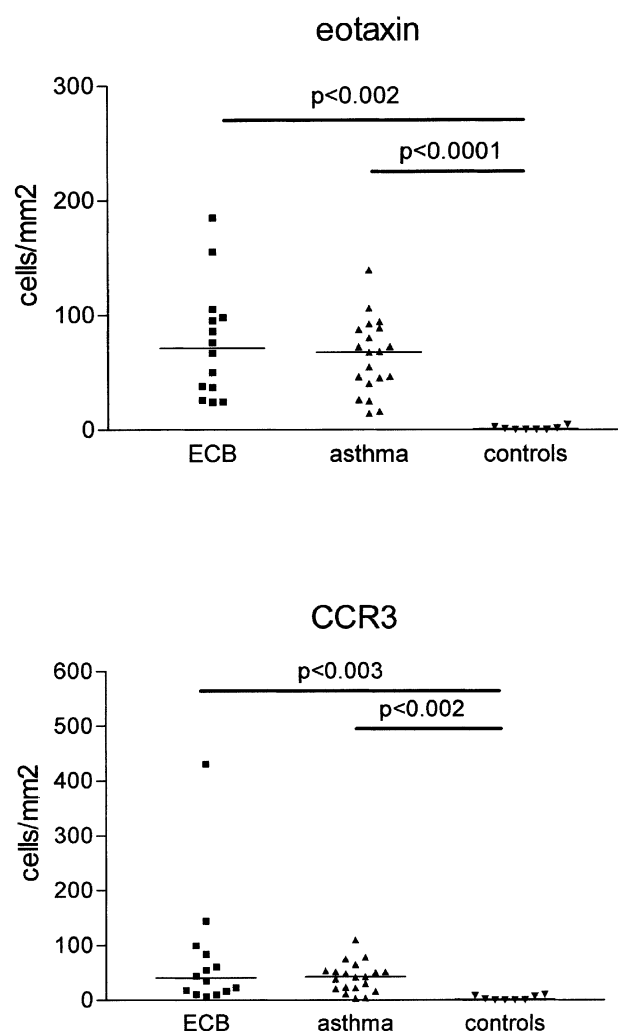


Figure 1. Eotaxin and CCR3 in the lamina propria of subjects with acute exacerbations of chronic bronchitis (ECB), asthmatics and healthy subjects. Statistical analysis: Mann-Whitney *U*-test.

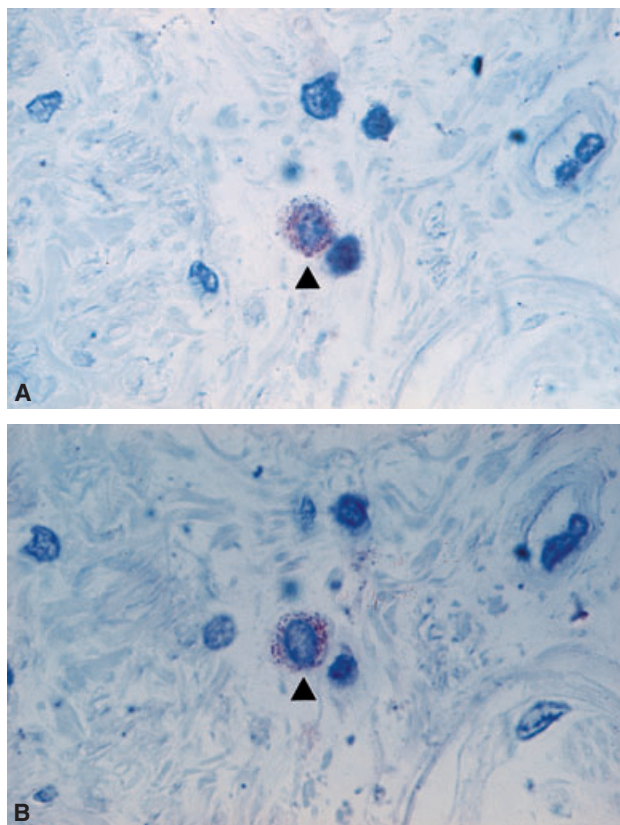


Figure 2. Sequential 2 μ m sections of bronchial mucosa from an exacerbated chronic bronchitis subject stained with MoAb EG2 (Pharmacia) to human eosinophil cationic protein (2a), and MoAb 7B11 (LeukoSite) to human CCR3 (2b). The arrows show cells stained with both antibodies, indicating the colocalization of CCR3 receptor to activated eosinophils.

either groups, nor could significant correlations be drawn between the two proteins and bronchial hyperreactivity (expressed as PC₂₀ methacholine), in the asthmatics.

Discussion

Our results show that: (a) the cellular expression of eotaxin and CCR3 is higher in ECB and asthmatic subjects when compared to controls; (b) CCR3 is mainly expressed by eosinophils and to a lesser extent by CD4⁺ and CD8⁺ lymphocytes; (c) the number of

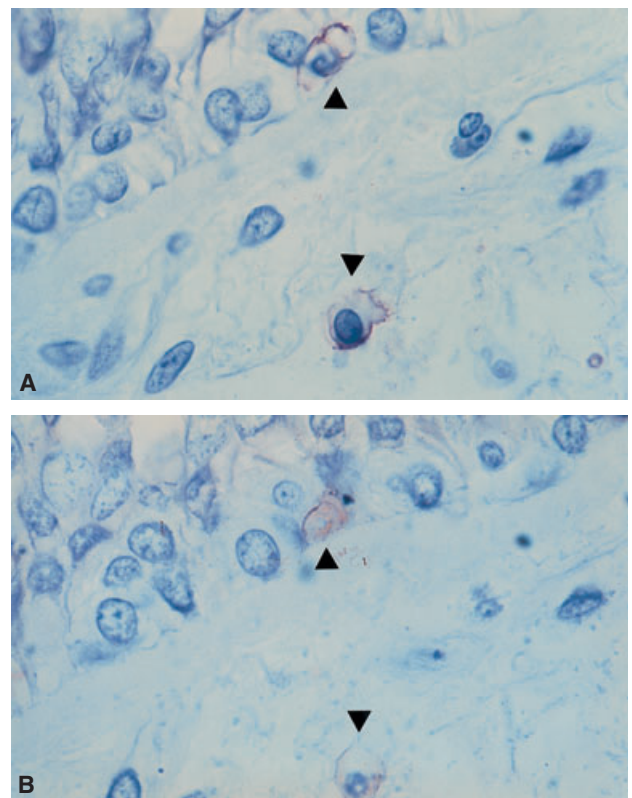


Figure 3. Sequential 2- μ m sections of bronchial mucosa from an exacerbated chronic bronchitis subject stained with MoAb DK25 (Dako) to human CD8 (3a), and MoAb 7B11 (LeukoSite) to human CCR3 (3b). The arrows show cells stained with both antibodies, indicating the colocalization of CCR3 receptor to CD8⁺ lymphocytes.

CCR3⁺ cells is significantly related to the number of eosinophils in ECB and asthma. Only in ECB subjects, CCR3 expression is significantly related to the number of CD4⁺ lymphocytes.

The present study showed for the first time an increased expression of eotaxin and CCR3 in the airways of ECB patients with respect to normal subjects. Eosinophils were also increased in ECB. The latest data is in agreement with the findings by Sietta et al. showing that airway eosinophilia, which is usually considered a characteristic of asthma, may also be present in subjects with exacerbations of chronic bronchitis (3, 4).

Using adjacent sections, we showed that most CCR3

Table 2 Assessment of CCR3 positive cells that were activated eosinophils (EG2) and EG2 positive for CCR3 in five subjects with acute exacerbation of chronic bronchitis and five asthmatics.

Patient (n)	CCR3 ⁺ cells coexpressing EG2		EG2 ⁺ cells coexpressing CCR3	
	Exacerbated chronic bronchitis (%)	Asthmatics (%)	Exacerbated chronic bronchitis (%)	Asthmatics (%)
1	92.4	89.9	82.3	45
2	87.2	85	76.8	63.8
3	100	79.3	58.9	56.7
4	67.8	72.8	78.4	88.2
5	83.4	100	92	68.6
Median (range)	87.2 (67.8–100)	85 (72.8–100)	78.4 (58.9–92)	63.8 (45–88.2)

Table 3 EG2 and T cell subsets in the lamina propria of exacerbated chronic bronchitis, asthma and normal subjects

	EG2 (cells/mm ²)	CD4 (cells/mm ²)	CD8 (cells/mm ²)
Exacerbated chronic bronchitis	72.52 (5.11–489.1)*	73.46 (2.20–545.7)	114.1 (35.4–241.8)
Asthma	90.00 (24.0–346)**	63.85 (18.4–212.4)	73.5 (28.0–215.0)
Normal subjects	4.50 (0.0–16.4)	35.00 (0.00–144.8)	69.5 (0.00–182.0)

Median (range), *P*-value Mann–Whitney *U*-test

*ECB vs. normal subjects *P* < 0.0005

** Asthmatics vs. normal subjects *P* < 0.0001

staining was localized on eosinophils and most eosinophils expressed CCR3 in both ECB and asthmatic groups. However, in ECB a higher percentage of eosinophils express CCR3 when compared to asthma. This can be explained by the acute recruitment of eosinophils occurring during exacerbations of ECB.

CCR3⁺ cells were significantly correlated with eosinophils in both ECB and asthma subjects. These data strongly support the hypothesis that eotaxin and CCR3 play a key role in the selective recruitment of eosinophils not only in asthma but also in chronic bronchitis during exacerbations. In the ECB group, in contrast with the asthma group, eotaxin did not significantly correlate with eosinophils and CCR3⁺ cells, although a trend towards correlation was apparent.

In our study, the number of CD4⁺ lymphocytes was significantly correlated with CCR3⁺ cells in ECB. These findings are in accordance with a recent report by Gerber et al. (20). In tissues from contact dermatitis, nasal polyp and ulcerative colitis, they showed that T lymphocytes are recruited into the inflamed tissue together with eosinophils by way of CCR3. The lack of a significant correlation between CD4⁺ and CCR3⁺ cells in the asthma group is probably because most of the asthmatic subjects analyzed were affected by mild asthma with a low infiltration of CD4⁺ T lymphocytes into the airways.

In a previous study, we found that VLA-4 was significantly related to the number of sputum eosinophils and to the degree of disease in asthmatics (21). CCR3 is considered an important molecule in the selective recruitment of eosinophils into the asthmatic airways as is VLA-4. Interestingly, Kitayama et al. suggest that CCR3 and VLA-4 molecules share common mechanisms of regulation. Most specifically, they demonstrated that chemokines produced by TNF- α and IFN- γ -stimulated endothelium can act through CCR3 to enhance the avidity of VLA-4 on eosinophils to bind VCAM-1 on the endothelium (22).

In addition, there is now plenty of evidence to suggest that IL-5 plays a prominent role in eosinophil recruitment (9). We know that exacerbated chronic bronchitis is characterized by airway eosinophilia. Interestingly enough, however, in subjects with exacerbated chronic bronchitis a lack of IL-5 expression has been shown as compared to asthmatic

subjects (23). It was observed that the degree of airway eosinophilia was similar in asthma and in exacerbations of chronic bronchitis, but only in asthma it was associated with an increased expression of IL-5 protein in the bronchial mucosa; this invites speculation that different mechanisms inducing eosinophilia may be involved in these two inflammatory diseases. Recently, studies of the kinetic release of eotaxin (24) have found that release of this cytokine in BAL fluid of asthmatic patients peaked at 4 h. Interestingly, IL-5 has been found to have different kinetics of release. Following allergen challenge, levels of IL-5 increase gradually in BAL fluid, peaking later than eotaxin after challenge (25), which suggest that eotaxin initiates eosinophil recruitment, whereas IL-5 maintains lung eosinophilia. In asthma airway eosinophilia is a constant feature of the disease suggesting the presence of a persistent mechanism, possibly involving the production of IL-5 protein. By contrast in chronic bronchitis, airway eosinophilia is present only during the exacerbation of the disease. This suggests the presence of a stimulus that is triggered when symptoms worsen and eotaxin could represent the trigger that initiates the acute recruitment of eosinophils.

According to previous studies, we found an increased amount of eotaxin and CCR3 expression in bronchial asthma. Indeed, increased eotaxin mRNA expression has been found both in bronchial biopsies of mild asthmatics and in BAL cells obtained six hours after asthmatics were exposed to allergen challenge (24, 26, 27). Moreover, soluble eotaxin has been found in the serum of asthmatic patients and concentrations of this chemokine correlated with the severity of the disease, particularly during acute asthma (28). Increased CCR3 mRNA expression, predominantly localized to eosinophils, has also been found in the bronchial biopsies of asthmatic patients (24).

Ying et al. (13) found that CCR3 and eotaxin, proteins, and mRNAs are significantly correlated with bronchial hyperreactivity. We did not find any relationship between immunohistochemical and functional data, probably because in our study most asthmatics had a mild form of the disease.

In conclusion, our study shows that the bronchial expression of eotaxin and its receptor, CCR3, is increased in exacerbated chronic bronchitis. Eotaxin

and CCR3 could be involved in the recruitment of eosinophils and CD4⁺ lymphocytes occurring during acute exacerbations of chronic bronchitis.

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