

Demonstration of mast cell chemotactic activity in bronchoalveolar lavage fluid collected from asthmatic patients before and during pollen season

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Background: Mast cells are versatile effector cells of primary importance in asthma and airway inflammation. During inflammation mast cells accumulate in the bronchial epithelium. The mechanism for this increase in mast cell number has not been defined.

Objectives: The aim of this study was to examine the presence of mast cell chemotactic activity in bronchoalveolar lavage (BAL) fluid taken before and at the end of 2 pollen seasons from patients with allergic asthma.

Methods: To measure mast cell chemotactic activity, we used a modified Boyden chamber and the human mast cell line HMC-1 or in vitro–developed mast cells as responder cells.

Results: A total of 27 patients were investigated, of which 8 exhibited mast cell chemotactic activity in their BAL fluid collected before season. A significant increase in the activity was found in 18 of 27 BAL fluids sampled at the end of the pollen season. No difference was found between patients treated with immunotherapy or placebo. The presence of stem cell factor could be detected in all BAL fluids analyzed. Blocking antibodies against stem cell factor or transforming growth factor- β partially blocked the activity in some of the BAL fluids. Treatment of the responder cells with pertussis toxin reduced the migratory activity in 13 of 14 BAL fluids collected during pollen season.

Conclusion: This study demonstrates the presence of mast cell chemotactic activity in BAL fluids from patients with allergic asthma, with a significant increase in activity during pollen season. The major part of this activity consisted of factors mediating their effect through G_i-protein coupled receptors. This activity may be responsible for the mast cell accumulation in the intraepithelial layer seen in allergic asthmatic patients. (*J Allergy Clin Immunol* 2000;105:455-61.)

Key words: Asthma, inflammation, mast cells, migration, stem cell factor, transforming growth factor- β

Abbreviations used

BAL: Bronchoalveolar lavage
HMC: Human mast cell
PKC: Protein kinase C
PTx: Pertussis toxin
SCF: Stem cell factor
TGF- β : Transforming growth factor- β

Mast cells are the principal effector cells in IgE-dependent hypersensitivity reactions. They are normally distributed throughout connective tissues and are particularly numerous beneath the epithelial surface in the respiratory system.¹ Mast cells live in tissues for several months and under normal conditions their numbers are relatively constant.

In allergic rhinitis and allergic asthma, an accumulation of mast cells within the epithelial layer of the mucosa, which normally has very few if any mast cells, has been reported after natural exposure to pollen or after provocation tests.²⁻⁷ Provocation studies showed that this increase occurs within hours after allergen challenge.⁸⁻¹⁰ One possible cause for this increase could be migration of mast cells within the tissue.

More recent work suggests that mast cells are not solely effector cells but also have important regulatory functions.^{11,12} We therefore believe it is important to study mast cell chemotactic activity in bronchoalveolar lavage (BAL) fluid, which may be involved in the regulation of the mast cell accumulation seen in allergic asthma.

To define chemotaxins involved in recruitment of mast cells in vivo, we examined the ability of BAL fluid collected from subjects with allergic asthma to induce mast cell migration in vitro. As responder cells we used the human mast cell line (HMC-1) and in vitro–developed mast cells. On the basis of their neutral protease contents, HMC-1 cells resemble the MC_T type of human mast cells, which correspond to the human mast cell found in the bronchial epithelium.¹³ This cell line has been used to identify several human mast cell chemotaxins, including stem cell factor (SCF), serum amyloid A protein, IL-8, C3a, and C5a.¹⁴⁻¹⁹ In this study we report that BAL fluid from patients with allergic asthma contains mast cell chemotactic activity that is significantly increased during pollen season.

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METHODS

Patient selection

The BAL fluid was collected from patients participating in 2 independent birch pollen studies during the seasons of 1995 (series A) and 1997 (series B). All patients were nonsmokers. The diagnosis was based on history of allergic symptoms during birch pollen season confirmed by positive skin prick test with standardized birch extract (ALK, Hørsholm, Denmark) and positive CAP at least class 2 (CAP-system, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). They all had seasonal rhinoconjunctivitis and asthma as defined by symptoms from the lower airways; cough, wheeze, changes in peak expiratory flow and a positive methacholine test ≤ 16 mg/mL. The patients had no perennial symptoms. If skin prick test to animal dander was positive, contact with pets was not allowed. All participating patients had mild asthma because only β_2 -agonists for the seasonal symptoms from lower airways was used as required. For the symptoms of rhinoconjunctivitis, symptomatic local medication, antihistamines and decongestants, were used as well as the oral short-acting antihistamine acrivastine (Semprex). The study of 1995 was an immunotherapy trial with patients randomized to placebo or immunotherapy treatment with birch pollen extract. They all reached maintenance dose before the start of the pollination period. The study was approved by the Ethics Committee of the University Hospital, Gothenburg.

BAL procedure

Premedication consisted of morphine-scopolamine and salbutamol 0.4 mg (Ventoline) 15 to 30 minutes before examination. Bronchoscopy was performed through the mouth with a fiberoptic bronchoscope (Olympus BF1 T) with use of local anesthesia with 2% xylocaine (Astra, Södertälje, Sweden). The bronchoscope was wedged in the middle lobe bronchus. One hundred milliliters of sterile saline solution at 37°C was instilled in 3 portions of 40, 30, and 30 mL, gently aspirated, and put on ice. Cells were pelleted at 400g for 10 minutes at 4°C. The supernatants from portion 1 "bronchial washing" were divided in 2-mL aliquots. The BAL fluid used in this study consisted of bronchial washings (portion 1).

Mast cell cultures

The human mast cell line HMC-1, established from a patient with mast cell leukemia,²⁰ exhibits a phenotype of mast cells.²¹ HMC-1 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% bovine calf serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 50 μ g/mL streptomycin, and 1.2 mmol/L α -thioglycerol.

Primary human mast cells were developed from umbilical cord blood cultured in the presence of 50 ng/mL SCF and 10 ng/mL IL-6 (Amgen, Thousand Oaks, Calif) as described.^{14,22}

Chemotaxis assay

Mast cell migration was examined with use of a 48-well microchemotaxis assay as described.¹⁴ The migration of mast cells was assayed by means of the leading front technique. Thirty microliters of the attractant, BAL fluid, was added to each well below the filter, and 50 μ L of the cell suspension (1.5×10^6 cells/mL) was added above the filter. After 150 minutes at 37°C and 5% carbon dioxide, the filters were fixed, stained with Mayer's-Hemalum solution, and mounted. The spontaneous migration of HMC-1 cells suspended in medium with 10% bovine calf serum served as control and was referred to as 100% migration. Cell chemotaxis was assayed as the migration of the 2 furthest migrating cells visible in focus of one high-power field (10×20). The migration distance on each filter was calculated as the mean of the readings of three different areas of the filter. The assay was always done in at least triplicate.

Measurements of cytokine level in BAL fluid

Measurements of transforming growth factor- β (TGF- β) and SCF concentrations in BAL fluid were performed by ELISA kits (R&D Systems Europe, Abingdon, UK).

Inhibition of activity of BAL fluid with use of anticytokine antibodies

Anticytokine antibodies (goat antihuman recombinant SCF and rabbit panspecific TGF- β) or isotype controls (R&D Systems) were added at 10 μ g/mL to the BAL fluids just before their addition to the well for chemotaxis assay.

Treatment with inhibitors

HMC-1 cells were treated with pertussis toxin (PTx) (0.5 μ g/mL), genistein (0.5 μ g/mL), or bisindolylmaleimide I (100 nmol/L) (Sigma, St Louis, Mo) as indicated. Inhibitor treatment was performed by incubating 2.5×10^6 cells/mL for 90 minutes at 37°C, 5% carbon dioxide with each inhibitor in complete medium and were then washed and resuspended at 1.5×10^6 cells/mL in complete medium before chemotaxis assays were performed.

Statistical analysis

For statistical analysis we used an ANOVA followed by multiple comparison by Fisher's method. An asterisk denotes $P < .05$, two asterisks $P < .01$, and three asterisks $P < .001$. Values presented are the means $\pm$ SEM.

RESULTS

Identification of mast cell chemotactic activity in BAL fluids

The presence of mast cell chemotactic activity in BAL fluids was analyzed with use of the human mast cell line HMC-1 or primary mast cells as a bioassay. Eight of 27 BAL fluids collected before pollen season exhibited mast cell chemotactic activity (Fig 1, A and B). Interestingly, all the BAL fluids collected during pollen season contained mast cell chemotactic activity (27/27). Of those, 18 showed a significant increase in activity during the pollen season compared with before the season. Similar results were obtained when primary mast cells were used as responder cells (Fig 1, C). Mast cell chemotactic activity in BAL fluid from patients that had been treated by immunotherapy did not differ compared with placebo control (Fig 1, A).

SCF and TGF- β in BAL fluids

It has been shown that SCF and TGF- β_1 can induce mast cell migration in vitro. We therefore analyzed the presence of SCF and TGF- β in the BAL fluids. SCF could be detected in all fluids tested (Table I). In contrast, the concentrations of TGF- β were below the detection limit of the assay.

To test the involvement of SCF and TGF- β in the migration of mast cells toward BAL fluids, we used blocking antibodies against SCF and TGF- β . The activity in 3 of 14 BAL fluids collected before season was affected by anti-SCF treatment (Fig 2, A), whereas 6 of 14 BAL fluids collected during season were affected (Fig 2, B).

Treatment with anti-TGF- β antibodies demonstrated results similar to those with treatment with anti-SCF (Fig

TABLE I. Measurements of SCF in BAL fluids collected from patients with allergic asthma before and during pollen season

Patient	Before season SCF (pg/mL)	During season SCF (pg/mL)
A1	21	42
A2	196	110
A3	92	382
A4	32	46
A5	27	134
A6	52	60
A7	82	60
A8	229	232
A9	39	102
A10	64	28
A11	12	53
A12	32	92
A13	129	227
A14	67	33

2, C and D). Of the BAL fluids collected before the pollen season, 4 of 14 had significantly attenuated activity, whereas 9 of 14 of the BAL fluids collected during the season were affected. Most of those affected by anti-SCF treatment were also affected by anti-TGF- β treatment.

Inhibition of mast cell migration to BAL fluids

To further analyze the nature of the mast cell chemotactic activity in BAL fluids, we treated the responder cells with different inhibitors. We chose to use PTx to inhibit G_i protein-coupled receptors, genistein for tyrosine kinase receptors, and bisindolylmaleimide I for the inhibition of protein kinase C (PKC).

PTx-treated responder cells demonstrated a significant reduction in the migratory response to 4 of 14 BAL fluids collected before the pollen season (Fig 3, A). When BAL fluids collected during season were tested, 13 of 14 of the fluids showed significantly reduced activity on the treated cells (Fig 3, B), indicating the presence of chemotaxins in BAL fluids mediating their signal through a G_i-coupled receptor.

Inhibition of tyrosine kinases by genistein treatment had minor effects on the migratory response to BAL fluids collected before season (Fig 3, C), whereas migration to 8 of 14 BAL fluids collected during season were significantly inhibited (Fig 3, D).

Inhibition of PKC by bisindolylmaleimide I treatment significantly reduced the migratory response to 2 of 14 of the BAL fluids collected before the pollen season (Fig 3, E), whereas 12 of 14 were significantly inhibited when BAL fluids collected during the pollen season were tested (Fig 3, F).

DISCUSSION

We demonstrate for the first time that mast cell chemotactic activity is present in BAL fluids collected from asthmatic patients. Furthermore, activity was sig-

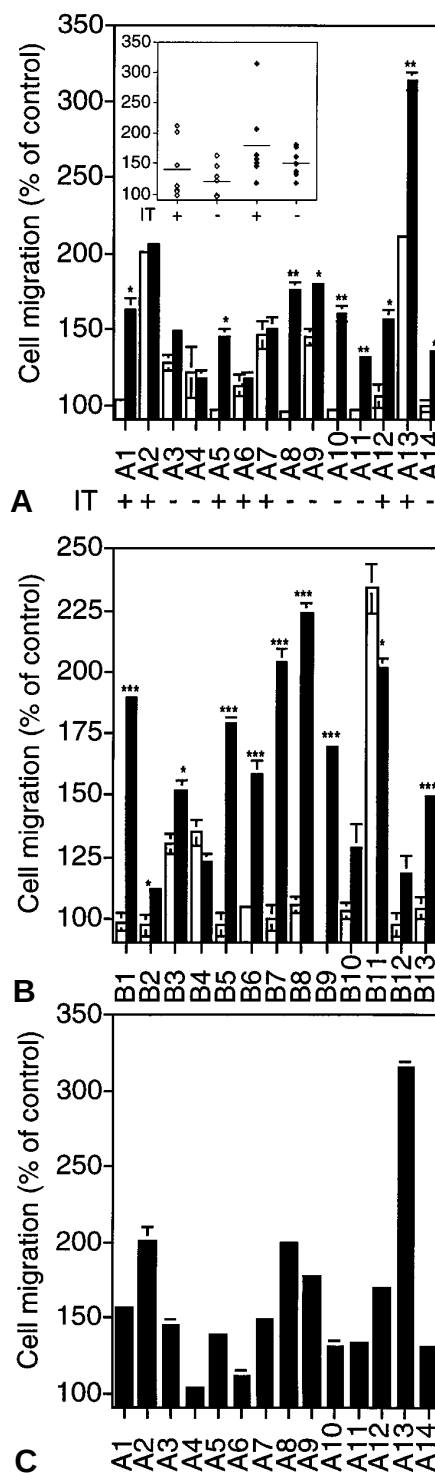


FIG 1. Mast cell chemotactic activity in BAL fluid. BAL fluids were collected either before (open bars) or during pollen season (filled bars) and tested on HMC-1 cells (A and B) or primary mast cells (C) (series A = 1995, series B = 1997). Migratory response is expressed as percentage of response to medium control. Inset in A, Side-by-side comparison between patients treated with immunotherapy or placebo. Results are from 3 experiments performed in triplicate. Results are given as mean $\pm$ SEM. Significant effect was obtained as indicated (asterisk, $P < .05$; two asterisks, $P < .01$; three asterisks, $P < .001$). IT, Immunotherapy.

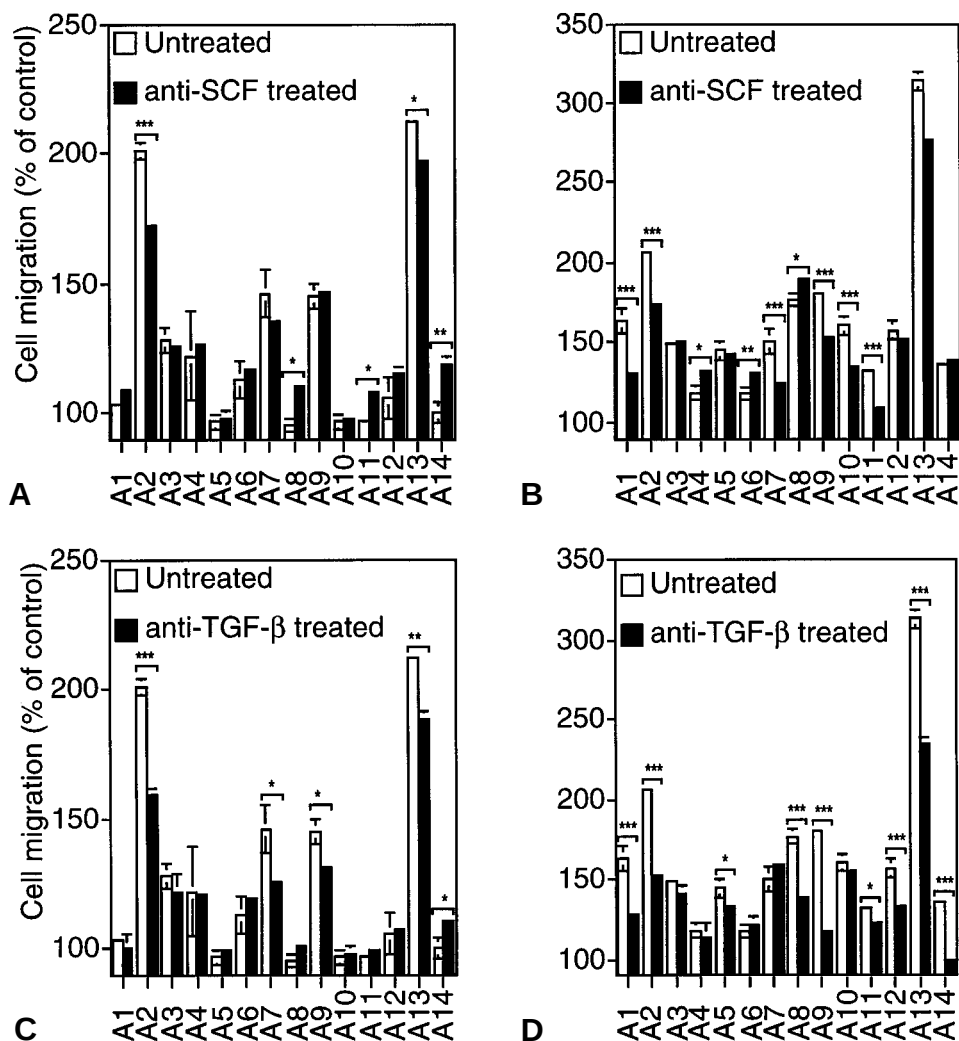


FIG 2. Blocking of mast cell chemotactic activity in BAL fluid by antibodies against SCF or TGF- β . BAL fluid was collected before (A and C) and during (B and D) pollen season and was treated with antibodies against SCF (A and B) or TGF- β (C and D). Migration assay was performed on HMC-1 cells. Results are from 3 experiments performed in triplicate. Results are given as mean $\pm$ SEM. Significant effect was obtained as indicated (asterisk, $P < .05$; two asterisks, $P < .01$; three asterisks, $P < .001$).

nificantly higher in BAL fluids collected during the pollen season compared with those collected before the pollen season. Asthma is a complex disease characterized by an inflammatory response with leukocyte infiltrates, bronchial hyperresponsiveness, airway edema, and tissue remodeling.²³ Mast cells are believed to be important for these responses.^{12,24,25} In bronchial asthma there is an increase in intraepithelial mast cells in the bronchial wall, and their number can be correlated with the degree of the clinical symptoms.^{6,24,26,27} The recruitment of mast cells into the intraepithelial cell layer is therefore likely to be of great significance for the pathophysiologic mechanisms of asthma. This study indicates that mast cell chemotactic activity present in BAL fluid may be responsible for the attraction of mast cells into the intraepithelial cell layer in allergic asthma.

Previous studies have shown that after natural pollen

exposure^{2,4,27,28} or experimental allergen challenges^{8-10,29} patients with allergic rhinitis or asthma have increased numbers of mast cells. In this study we observed that in the BAL fluids collected before the pollen season mast cell chemotactic activity could be detected in 8 of 27 patients, whereas all the BAL fluids contained chemotactic activity when collected during the pollen season. This significant increase in chemotactic activity during the pollen season suggests that IgE-mediated allergic inflammation in the airways leads to increased secretion of chemotactic factors. One possible source for these factors is epithelial cells that are known to have the capacity of generating a variety of different cytokines.³⁰

One of the defined human mast cell chemotaxins is SCF.¹⁴ Although we could detect SCF in all the BAL fluids measured (Table I), only 6 of 14 BAL fluids (collected during the pollen season) demonstrated significant decreased

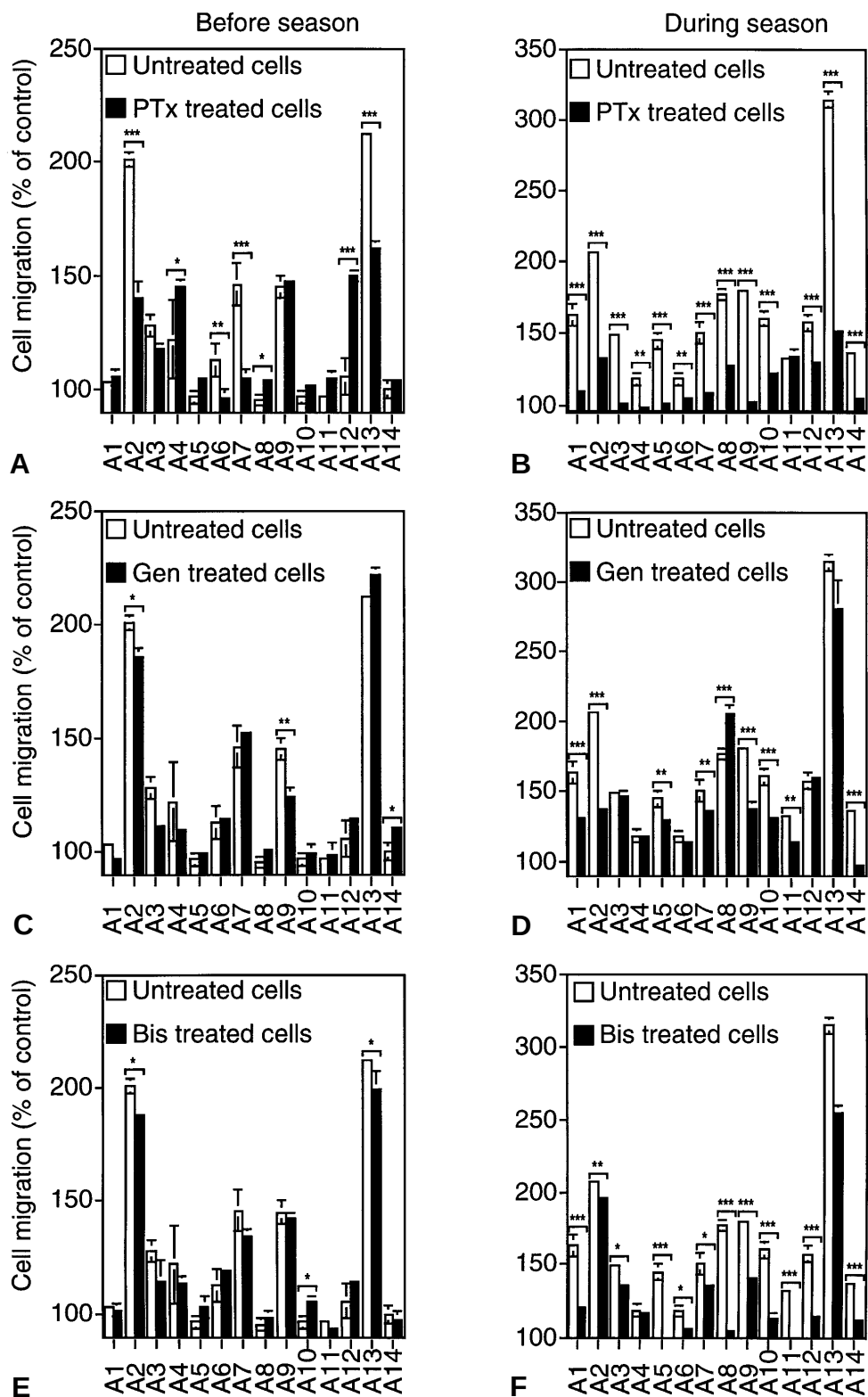


FIG 3. Analysis of signal transduction pathways involved in BAL fluid-mediated mast cell migration. HMC-1 cells were treated with PTx (A and B), genistein (C and D), or bisindolylmaleimide I (E and F) for 90 minutes before chemotaxis assay. Mast cell migration was tested with use of BAL fluids collected before (A, C, and E) or during pollen season (B, D, and F). Results are from 3 experiments performed in triplicate. Results are given as mean $\pm$ SEM. Significant effect was obtained as indicated (asterisk, $P < .05$; two asterisks, $P < .01$; three asterisks, $P < .001$).

chemotactic activity when antibodies against SCF were added. These results indicate that SCF is part of the chemotactic activity in BAL fluids but that other factors are of greater importance for the recruitment of mast cells in allergic asthma. SCF is not only a mast cell chemotaxin but is also important for other mast cell functions such as growth and differentiation, survival, adhesion, and activation.³¹ Thus SCF may play a role in the recruitment of mast cells but also in the perpetuation of chronic inflammation by acting as a mast cell survival and activating factor.

The most potent mast cell chemotaxin is TGF- β with a median effective dose of 10 fmol/L (unpublished data, Olsson et al). TGF- β_1 has been detected in BAL fluids from asthmatic patients and has been shown to increase on allergen challenge.³² In this study we were not able to detect any TGF- β in the BAL fluids. This difference could be explained by the fact that the authors concentrated their samples before analysis and we did not. However, TGF- β is active as a chemotaxin at concentrations far below the detection limit of the ELISA assay and could thereby still be a part of the mast cell chemotactic activity in BAL fluids. When anti-TGF- β antibodies were added to the BAL fluids, a significant decrease in migratory response was obtained in 9 of 14 of the BAL fluids sampled during pollen season. Thus TGF- β is one of a number of factors produced in vivo that can contribute to mast cell accumulation seen in asthmatic subjects.

One approach for delineating the different types of factors in BAL fluid mediating mast cell migratory responses is to block different receptor signaling pathways with use of inhibitors. In this study PTx was used to block the family of inhibitory guanine nucleotide-binding proteins (G_i). We, and others, have reported that factors mediating their effects through receptors sensitive to PTx treatment, such as C3a, C5a, serum amyloid A, and IL-8, are mast cell chemotaxins,¹⁵⁻¹⁹ and a number of these have been reported to be present in BAL fluid.³³⁻³⁵ The reduction in migratory response of PTx-treated cells to almost all BAL fluids tested indicates that factors mediating their response through PTx-sensitive signaling pathways are present in BAL fluid and are probably of major importance for mast cell migration in asthma.

BAL fluids exhibit a great complexity in their composition, so it is therefore not surprising that blocking with antibody to single cytokines or inhibitors are insufficient to completely block the activity of the fluid. Cytokines other than those studied here have been reported to attract human mast cells, and still more may be discovered. One complicating factor in the understanding of the control of cell migration is how different chemotaxins interact with each other. They could be additive or synergistic but may also counteract each other. We have found that platelet activating factor-induced mast cell migration is attenuated by C5a and SCF (Nilsson et al, unpublished data). This observation may explain migratory responses to some BAL fluids where an increase in the migratory response was obtained when the responder cells or antibodies were treated with inhibitors (eg, A8).

In conclusion, BAL fluids contain mast cell chemotactic activity that could be of importance for the mast cell hyperplasia observed in asthmatic subjects. Inhibition of mast cell migration could decrease the local mast cell hyperplasia and as a consequence reduce the destructive inflammatory process seen in asthmatic inflammation.

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