

Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose

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Summary

Background Repeated inhalation of allergen at low-dose induces an increase in bronchial hyper-responsiveness, without any associated symptom. The concomitant events in the bronchus have not been described.

Objective We have studied the dynamic number of mast cells in the airways of patients with mild asthma before and after repeated inhalation of allergen at low-dose and the expression of nerve growth factor (NGF), which is reported to promote growth and survival of mast cells.

Methods Twelve patients with mild asthma to cat allergen were enrolled at random in a blind placebo-controlled study, and submitted to repeated low-dose allergen exposure (1/5 of the provocative dose). Mast cells were immunolocalized using an antibody against mast cell tryptase. NGF and its high affinity receptor, TrkA, were immunolocalized using anti-NGF and anti-TrkA antibodies, respectively. NGF mRNA was quantified by competitive polymerase chain reaction (PCR) after reverse transcription of total RNA extracted from bronchial biopsy. NGF protein levels were measured by ELISA in bronchoalveolar lavage (BAL) fluid.

Results Bronchial mast cell number was increased significantly after allergen exposure as compared with before. NGF expression in the bronchus was immunolocalized mainly to epithelial cells, but also to fibroblasts, blood vessels, and a few infiltrated cells. NGF mRNA levels in bronchial biopsies were increased significantly after allergen exposure. The high affinity receptor for NGF, TrkA, was immunolocalized to the infiltrated mast cell membrane.

Conclusion Our study shows that the increase in the number of mast cells and in the expression of NGF induced by allergen exposure in the bronchus of asthmatic patients is occurring before the onset of symptoms. In addition, our finding of the presence of the TrkA receptor on the membrane of the infiltrated mast cell *in situ* brings evidence of the mast cell as a target cell for the growth factor activity of NGF in the airways in asthma.

Keywords: neurotrophin, asthma, airway, mast cell, NGF, tryptase, allergy, bronchus, bronchoalveolar lavage, bronchial biopsy

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Introduction

An increase in the number of mast cells is a characteristic feature of the asthmatic airways [1–6]. This increase is observed even at an early stage of the disease [4]. In asthmatic patients, allergen challenge-inducing bronchoconstriction is followed by an increase in the number of infiltrated bronchial mast cells [7–9], as well as by an enhancement of haematopoietic cell development in the bone marrow [10]. However, the dynamic mast cell number in the asthmatic airways before the asthma attack has not been studied.

Mast cell numbers in tissues are regulated by factors involved in the development and infiltration of mast cell progenitors, in their maturation and acquisition of phenotype, as well as in their survival in the tissue [11–13]. The factors locally produced to attract mast cells or increase their survival, and involved in the increase in the number of mast cells in the bronchus of asthmatic patients are still unknown. Early studies suggest a role for nerve growth factor (NGF) in the growth and survival of mast cells, besides its effects on the development and survival of peripheral sympathetic and sensory neurones [for review, 14]. First, treatment of newborn rats with NGF induces an increase in the number of mast cells in several locations [15]. Second, administration of a neutralizing anti-NGF antibody reduces the number of mast cells in hepatic granulomas of *Schistosoma mansoni*-infected rats [16]. Moreover, NGF induces *in vitro* the development of mast cells from mouse bone marrow cells in the presence of interleukin (IL)-3 [17,18]. NGF also promotes the maturation of human mast cells by inducing expression of the mast cell protease chymase *in vitro* [19], and of an immature mast cell line (HMC-1) by inducing the expression of the high affinity receptor for IgE [20]. Furthermore, NGF increases survival of mast cells from rat peritoneal cavity *in vitro* by inhibiting their apoptosis [21–23]. The hypothesis of an involvement of NGF in the development, maturation and survival of mast cells in tissues has been put forward. It has been recently strengthened by the demonstration *in vitro* that cultured cord blood-derived mast cells, and immature mast cells from the HMC-1 cell line express the high affinity tyrosine kinase receptor for NGF, TrkA [19,24]. However, expression of the TrkA receptor by mast cells infiltrated in tissues, particularly in asthma *in situ*, has not been demonstrated yet. This would give the first evidence of mast cell as a target cell for the growth factor activity of NGF in the airways.

NGF levels are increased in serum of asthmatic patients as compared with healthy subjects [25], as well as in bronchoalveolar lavage (BAL) fluid of asthmatic patients after a segmental challenge to allergen at high, provocative dose [26]. We have studied NGF expression and mast cell

number in patients repeatedly exposed to cat allergen at low-dose, inducing an increase in bronchial hyper-responsiveness in the absence of asthma symptoms, as previously described by our group and by others [27–30]. We have studied in a blind placebo-controlled fashion, the dynamic number of mast cells in bronchial biopsies before and after exposure, and the expression of NGF by quantification of its mRNA in bronchial biopsies, and of the protein in BAL fluid. Furthermore, we have immunolocalized NGF in the bronchial mucosa and the TrkA receptor on the infiltrated mast cell.

Materials and methods

Patients

Twelve mild asymptomatic asthmatic patients were recruited in our study, and separated into two groups inhaling either allergen or saline. Inclusion criteria were:

- a history of cat allergic asthma with a positive skin-prick test to cat allergen (Stallergènes Pasteur, Fresnes, France);
- specific IgE levels higher than class 2 (Matrix, Abbott Laboratory, Chicago, IL, USA);
- a forced expiratory volume in one second (FEV₁) greater than 70% of the predicted value;
- a positive conventional bronchial challenge test to cat allergen (Aquagen SQ, ALK-Abelló, Hørsholm, Denmark), performed one month before inclusion, and leading to the calculation of the allergen dose for further challenge;
- a moderate non-specific bronchial hyper-responsiveness to methacholine.

The patients were free of medication for at least one month before the study, except for an occasional use of inhaled short-acting β_2 agonists, that were stopped at least eight hours before each test session. All were non-smokers, and none had a history of respiratory infection for at least 3 weeks before the study. During the course of the study, patients were not exposed to cat allergen, as assessed by measurement of airborne Fel d 1 at home as previously described [27], except one patient from the saline group who had to be excluded from the data analysis.

Study design

This was a blind placebo-controlled study in parallel groups, with inclusion at random. It was approved by the ethical committee of our hospital, and all patients gave their written informed consent. Subjects came on day (D)1 for a methacholine challenge test and on D2 for bronchial fibroscopy with bronchial biopsy sampling and BAL. Then, for five consecutive days (D5–D9) and three consecutive days the following week (D12–D14), the subjects inhaled either cat allergen at low-dose or saline. They came

back on D15 for a methacholine challenge test to verify the presence of an increased bronchial hyper-responsiveness, and on D16 for bronchial fibroscopy.

Repeated inhalation of allergen at low-dose or saline

On each of the eight inhalation days, the patients were exposed to either cat allergen at 1/5 of the dose that had induced the positive response at inclusion, or to saline. Inhalation was performed at the same time of the day (± 2 h). Inhalation was administered through an automatic inhalation-synchronized dosimeter jet nebuliser (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland). FEV₁ was recorded before and at 5, 10, 15, 30, and 60 min after allergen or saline inhalation. Patients recorded their peak expiratory flow values twice daily throughout the study.

No patient experienced any asthmatic symptoms after repeated cat allergen or saline inhalation. The mean variation of peak expiratory flow was 3.2% (range 0–12) after allergen and 3.5% (range 0–12) after saline (NS). FEV₁ did not vary by more than 5% in either group. An increase in bronchial hyper-responsiveness was observed after allergen inhalations, but not after saline [27].

Fiberoptic bronchoscopy

After premedication with diazepam (10 mg per os), nebulization with salbutamol, and local anaesthesia with 1% adrenaline-free xylocaine, a bronchial fibroscope (Olympus BF P30, Paris, France) was inserted through the nose into the trachea. Oximetry was recorded throughout the procedure. Six bronchial biopsies were taken from subsegmental divisions of the main bronchi in the lower lobes. BAL was carried out in the middle lobe by instilling four times 50 mL of sterile isotonic saline gently aspirated immediately after. Patients received oxygen during bronchoscopy; an intravenous access was prepared, and adrenaline was readily available. Oximetry monitoring continued for four hours after the fibroscopy, and spirometry was performed every hour for four hours.

Biopsies and BAL fluid processing

Two biopsies were fixed in 10% formaldehyde and embedded in paraffin; four biopsies were immediately transferred into 400 μ L TriReagent™ (Molecular Research Center, Cincinnati, OH, USA) for RNA extraction. The BAL fluid was immediately chilled on ice, and centrifuged at 1500 *g* for 15 min at 4 °C. Supernatant was aliquoted, lyophilized and kept frozen at – 80 °C, until assessment of NGF protein levels.

Immunohistochemistry

Serial 4 μ m biopsy sections were deparaffinized in xylene, step-rehydrated through graded alcohol, and washed with Tris-buffered saline (TBS, 20 mM Trizma base, 150 mM NaCl, pH 7.6). Sections were pretreated for 8 min with 0.1% pronase E (Sigma Chemical Co., St Louis, MO, USA) allowing antigen retrieval. Unspecific protein binding was blocked with fetal calf serum (FCS) (10% v/v) (Gibco BRL, Cergy Pontoise, France) and BSA (1% w/v) (Sigma) in TBS (blocking buffer) for 30 min at room temperature (20 °C). Sections were then incubated for 90 min with either mouse anti-mast cell tryptase monoclonal antibody (clone AA1; 1 : 50) (a generous gift from Dr A. Walls, Southampton University, UK), or rabbit anti-human NGF polyclonal antibody (1 : 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were washed in TBS, and incubated at room temperature for 30 min with an appropriate biotinylated anti-mouse or anti-rabbit IgG anti-serum (1 : 200) (Amersham, Les Ulis, France), then for 30 min with an Extravidin®-alkaline phosphatase conjugate (1 : 200) (Sigma). Finally, sections were incubated in Fast Red TR/Naphtol AS-MX (Sigma), and allowed to develop for 20 min at room temperature. Sections were counterstained with Mayer's haematoxylin (Fluka, Saint Quentin Fallavier, France), and mounted in gelatin-glycerol (1 : 1). For TrkA-mast cell tryptase double immunostaining, sections were first labelled with a rabbit anti-human TrkA polyclonal antibody (1 : 100) (Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation for 2 h at room temperature with a donkey biotinylated anti-rabbit IgG anti-serum (1 : 200) (Dako, Trappes, France), then for 45 min with fluorescein (1 : 300) (DTAF)-conjugated streptavidin (Immunotech, Marseille, France). The same sections were then stained for mast cell tryptase as described above, except for the secondary antibody that was a cyanine (CY3)-conjugated goat anti-mouse antibody (1 : 200) (Amersham). Double staining was analysed by confocal microscopy (Laser Scanning Confocal Imaging System, MRC-1024, Biorad, Ivry/Seine, France). Negative controls were performed by replacement of the primary antibody by its control isotype from the same species, as well as by blocking buffer alone. All antibodies were diluted in blocking buffer.

Quantification of mast cell numbers on bronchial sections

Slides were coded and examined under light microscopy (magnification of $\times 400$) (Zeiss Axiophot, Oberkochen, Germany) by two investigators, one of which was totally unaware of the study protocol. Mast cell tryptase-positive cells were counted on the whole section. Images were digitalized with a high performance charge-coupled device (CCD) camera (Cohu Inc., San Diego, CA, USA), and areas

were measured by an image analyser (Biocom Visiolab 100, Biocom, Les Ulis, France). Mast cells were counted in 3 different sections from each sample, and counts were averaged. Results were expressed as number of mast cells per mm².

Extraction of total RNA and reverse transcription (RT)

Total RNA was extracted from biopsies using TriReagent™ (Molecular Research Center). Isolated RNA was diluted in RNase free water. Total RNA was incubated with 0.5 µg random primers for 5 min at 70 °C, and allowed to cool down at room temperature. RNA was subsequently reverse transcribed in 1 × RT buffer (75 mM KCl, 15 mM MgCl₂, 10 mM DTT, 50 mM Tris, pH 8.5) containing 1 U/µL of the RNase inhibitor RNasin, 1 mM of each dNTP, and 10 U/µL RNase H minus-Moloney leukaemia virus reverse transcriptase (all reagents from Promega, Madison, WI, USA). The reaction was conducted for 1 h at 37 °C, and the reverse transcriptase was inactivated by heating at 98 °C for 5 min.

Quantification of NGF cDNA by competitive PCR

One µL of a 1 : 2 to 1 : 10 dilution of the RT product was amplified by PCR in 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3) containing 2 mM MgCl₂, 0.2 mM dNTP, 20 pmol of each primer:

sense primer: 5'-TGGTGCTGCCCCCTTCAACAG-3';

anti-sense primer: 5'-CCCAACCCACACGCTGACAC-3',

and 1 U Taq DNA polymerase (Promega) in a 50 µL final volume, for 35 cycles (60 s at 94 °C, 60 s at 59 °C, and 60 s at 72 °C), in the presence of increasing amounts of NGF competitor cDNA. NGF competitor cDNA has been constructed using a PCR strategy as previously described for stem cell factor [31,32], using the anti-sense primer described above, and a composite sense primer (5'-sense primer-GAGCAAGCGGTCATCATCC-3') leading to a 9 bp deletion in the NGF cDNA sequence.

Results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA quantified by competitive PCR [31,32]. Briefly, 1 µL of a 1 : 20 dilution of the RT product was amplified by PCR in 1 × PCR buffer containing 2.5 mM MgCl₂, 0.2 mM dNTP, 20 pmol of each primer (sense: 5'-GGTGAAGGTCGGAGTCAACGGA-3'; anti-sense: 5'-GAGGGATCTCGCTCCTGGAAGA-3') and 1 U Taq polymerase in a 50 µL final volume, for 30 cycles (60 s at 96 °C, 60 s at 60 °C, and 60 s at 72 °C), in the presence of increasing amounts of GAPDH competitor cDNA. GAPDH competitor cDNA was constructed by PCR using the anti-sense primer described above, and a composite sense primer (5'-sense primer-CCAGGGCTTT TAAGTCTGG-3') leading to a 25-bp deletion in the GAPDH cDNA sequence [31,32].

PCR product analysis

PCR products (15 µL) were denatured (99 °C, 10 min) in the presence of 50% deionized formamide, and resolved by electrophoresis on denaturing 50% urea-17% formamide-10% polyacrylamide gels. Gels were stained with ethidium bromide, digitalized under UV light using a high performance CCD camera (Cohu Inc.). Band densities were analysed using the public domain NIH Image program (written by W. Rasband at the US National Institutes of Health and available by anonymous FTP from zippy.nimh.nih.gov).

For determination of NGF cDNA, ratios of PCR products of NGF to NGF competitor cDNAs, and GAPDH to GAPDH competitor cDNAs were calculated for each concentration of competitor, and plotted against the concentration in order to calculate the initial concentrations of NGF and GAPDH cDNAs at ratio equals 1 [31,32]. Results were expressed as amol NGF cDNA/amol GAPDH cDNA.

NGF ELISA

Immunoreactive NGF was quantified in BAL fluid by a sensitive ELISA procedure (NGF Emax™ Immunoassay System, Promega), using a capture anti-human NGF polyclonal antibody, a detection rat anti-human NGF monoclonal antibody, revealed by an anti-rat horseradish peroxidase-conjugated antibody, and a 3,3',5,5'-tetramethylbenzidine liquid substrate system.

Statistical analysis

Differences between groups have been compared using a Mann-Whitney *U*-test. The significance level was set at 5%.

Results

Quantification of mast cell numbers

Before challenge with either allergen or saline, baseline number of tryptase-immunoreactive mast cells in bronchial biopsy sections were similar in the two groups of patients. An increase in the number of mast cells was observed after repeated exposure to cat allergen at low-dose, whereas no change was observed after saline (Fig. 1): median variations were +209% and -2.1% after allergen and saline, respectively (*P* < 0.05).

Immunolocalization of NGF

Immunolocalization of NGF was done on bronchial biopsy sections from patients of both groups. It showed in both

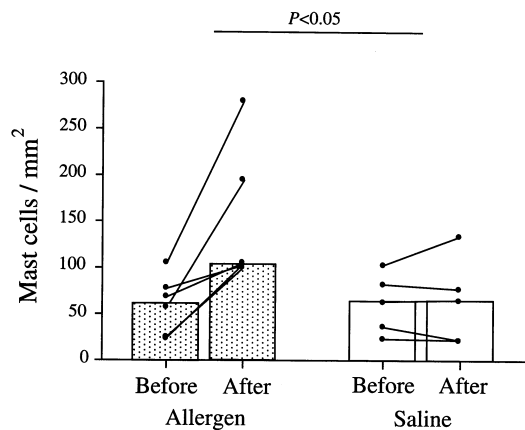


Fig. 1. Number of mast cells in the bronchus of asthmatic patients before and after repeated inhalation of allergen at low-dose or saline. Mast cells stained for tryptase were counted on sections of bronchial biopsies performed before and after inhalation of allergen or of saline. Individual values are represented as dots and the median values as blocks.

groups a strong NGF immunoreactivity in epithelial cells, slighter in fibroblasts, blood vessels, and a few infiltrated cells (Fig. 2).

Expression of NGF mRNA in bronchial biopsies

Before challenge with either allergen or saline, baseline NGF mRNA expression was similar in the two groups of patients. Expression of NGF mRNA was increased after exposure to cat allergen at low-dose, whereas no modification was observed after saline (Fig. 3): median variations were +201% and -27.5% after allergen and saline, respectively ($P < 0.05$).

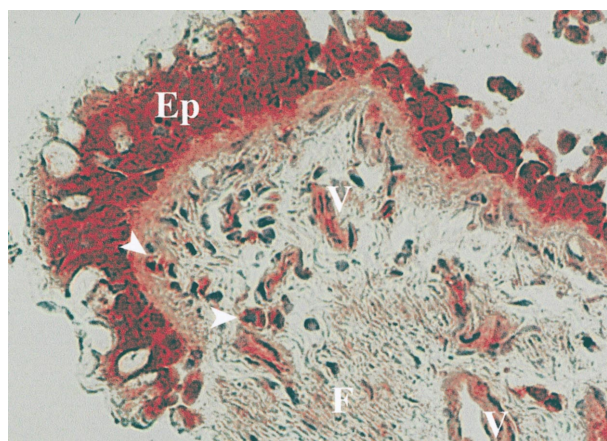


Fig. 2. Immunohistochemical localization of NGF protein in the bronchus. A bronchial biopsy section from a representative patient after repeated inhalation of allergen is shown. Note the staining of epithelial cells (Ep), blood vessels (V), fibroblasts (F), and infiltrated cells (arrow heads). Magnification: $\times 400$.

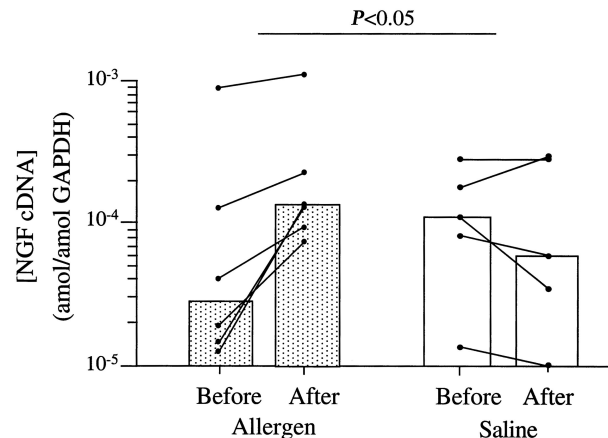


Fig. 3. NGF mRNA expression in bronchial biopsies before and after repeated inhalation of allergen at low-dose or of saline. NGF mRNA expression was assessed by competitive PCR after reverse transcription. Results are expressed as amol NGF cDNA/amol GAPDH cDNA. Individual values are represented as dots and the median values as blocks.

NGF protein levels in BAL fluid

Before challenge with either allergen or saline, baseline NGF protein in BAL fluid was similar in the two groups of patients. NGF protein was slightly increased after allergen exposure, although difference did not reach significance as compared with saline inhalation (Fig. 4): median variations were +25% and -2.3% after allergen and saline, respectively.

Expression of TrkA receptor by mast cells infiltrating the asthmatic bronchi

Double immunostaining with antibodies directed against mast cell tryptase and the tyrosine kinase receptor for NGF

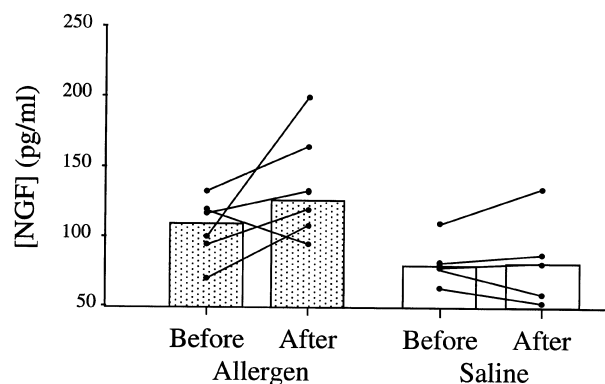


Fig. 4. NGF protein levels in BAL fluid from asthmatic patients before and after repeated inhalation of allergen at low-dose or of saline. NGF levels were measured by ELISA. Individual values are represented as dots and the median values as blocks.

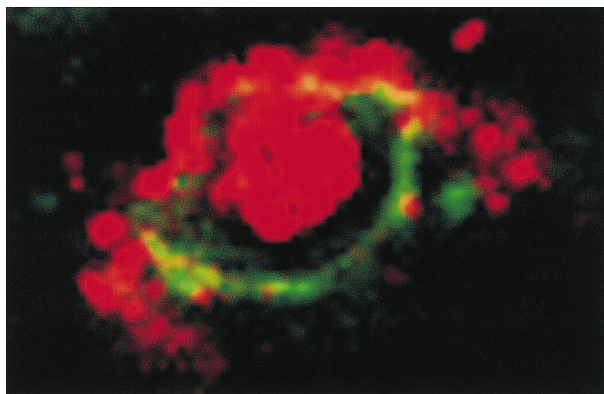


Fig. 5. Expression of the high affinity receptor for NGF (TrkA) by a bronchial mast cell. Mast cell was identified by immunostaining with a mouse monoclonal antibody to human mast cell granular tryptase, and a cyanin (CY3)-conjugated secondary antibody (red fluorescence); TrkA expression was localized to mast cell membrane using a rabbit polyclonal antibody to TrkA, and a biotinylated secondary antibody revealed by a streptavidin-fluorescein conjugate (green fluorescence). Staining was analysed by confocal microscopy. The photomicrograph shows a representative mast cell from a bronchial biopsy section obtained after repeated inhalation of allergen. Magnification: $\times 2000$.

TrkA was performed on bronchial biopsy sections, and analysed by confocal microscopy. Qualitative analysis showed colocalization of the granular mast cell tryptase and of the membrane-bound TrkA receptor, indicating expression of the tyrosine kinase receptor for NGF TrkA on mast cells infiltrating the bronchial wall of asthmatic patients (Fig. 5).

Discussion

We report here that allergen exposure at a non-provocative dose induced an increase in the number of bronchial mast cells, as well as an increase in NGF mRNA expression in the bronchus. Also, NGF protein levels were slightly enhanced in BAL fluid, but not significantly, indicating that increased expression of NGF was local within the bronchus. We have localized NGF protein expression in the bronchus mainly to epithelial cells, and more slightly to fibroblasts, blood vessels, and a few infiltrated cells. Finally, we also show that the infiltrated mast cell expressed the high affinity tyrosine kinase receptor for NGF, TrkA in the bronchus of the asthmatic patient.

We have exposed asthmatic patients to allergen at low-dose, inducing no asthma symptoms. However, exposure induced an increased bronchial hyper-responsiveness, as previously reported by our group [27] and others [28–30]. This experimental protocol provides an interesting model to

study the events occurring in the bronchus in asthma before the symptoms, at a stage where the inflammatory response to allergen aggression is low as compared with the repair processes occurring after the asthmatic bronchoconstriction [27–30]. In addition, this model also gives the opportunity to study the mechanisms underlying bronchial hyper-responsiveness.

An increase in the number of mast cells is reported in the bronchus of asthmatic patients [1–6], even at an early stage of the disease [4]. The mechanisms involved in this increase are still unknown. Experimentally, only provocative allergen exposure, inducing bronchoconstriction, is reported to increase bronchial mast cell numbers [7–9], and apparently only transiently [9,33]. We show here that exposure of asthmatic patients to allergen at low-dose, inducing no asthma symptoms in any patient, was also responsible for increasing mast cell numbers. This increased number of infiltrated mast cells may thus be considered an early event following subclinical allergen exposure. This emphasizes a possible early role of the mast cell in the pathophysiology of asthma, as well as of bronchial hyper-responsiveness. This possible role is confirmed by studies performed in mast cell-deficient mice, where an antigen challenge induced a lower increase in airway hyper-responsiveness than in mast cell non-deficient mice [34]. Hence, our results raise the possibility of a relationship between the increased bronchial hyper-responsiveness and the increased number of mast cells in patients after repeated exposure to allergen at low-dose.

The increase in the number of bronchial mast cells might result either from an increased development of mast cell progenitors in the bone marrow, from bronchial infiltration of the progenitors, and/or from modulation of their survival by local factors. An increased level of circulating haematopoietic progenitors has been reported in allergic rhinitis and asthma [35–37], and development of the progenitors in the bone marrow occurs after a positive bronchial provocation to allergen in asthmatic patients [10]. The final differentiation and maturation of these haematopoietic cells in tissues rely on locally produced growth factors that are still unidentified [38]. Additionally, the regulation of survival of the differentiated mast cells in tissues also depends upon secretion of local factors. Since NGF increases mast cell survival *in vitro* by inhibiting their apoptosis [21,22], we hypothesized NGF as a good candidate for this action in the airways.

The first evidence of a potential role for NGF in the pathophysiology of asthma was reported by Bonini *et al.* [25], showing increased serum levels of NGF in asthmatic patients as compared with healthy subjects. We show here a local increase in NGF mRNA in bronchial biopsies, and a trend towards an increase in NGF protein levels in BAL fluid, suggesting this dynamic expression occurs within the bronchus. We show additionally that NGF expression was

localized to epithelial cells, endothelial cells, fibroblasts and infiltrated cells of the bronchial mucosa. Our results of the expression of NGF *in vivo* in the bronchus of asthmatic patients confirms *in vitro* studies on cultured cells, showing the production of NGF by airway epithelial cells [39], airway fibroblasts [40], and immune/inflammatory cells [19,24,41–43]. Furthermore, our results bring new insight into the regulation of NGF expression in asthmatic airways in presymptomatic stages of the allergic reaction. Indeed, a previous report describes enhanced NGF levels in BAL fluid of asthmatic patients after a segmental challenge to allergen at high, provocative dose [26]. Their finding was in accordance with results from a mouse model of allergic pulmonary inflammation, where provocative allergen challenge also promotes an increase in the levels of NGF in BAL fluid and serum [44]. Our results strengthen the importance of these findings, since we here demonstrate increased local NGF expression as an early event preparing the bronchus to asthma attack, in addition to a consequence of the allergenic bronchoconstriction. Additionally, this increase in NGF expression has been related to bronchial hyper-responsiveness in animal models [44,45]. First, the administration of a neutralizing anti-NGF antibody prevents the development of allergen-induced non-specific airway hyper-responsiveness in sensitized and challenged mice [44]. This has recently been strengthened by the demonstration that administration of exogenous NGF induces airway hyper-responsiveness in the guinea-pig [45]. Therefore, the possibility of a relationship between the observed increase in NGF expression and the increase in non-specific bronchial hyper-responsiveness after repeated exposure to allergen at low-dose may be submitted. Taking into account the presumed role of mast cells in bronchial hyper-responsiveness, it is interesting to focus on a possible role for NGF in the increase in mast cell numbers.

In the hypothesis of an interrelationship between NGF expression in the bronchus and the number of infiltrated mast cells after allergen exposure, the first piece of evidence is given by the study of Aloe & Levi-Montalcini [15], showing that treatment of newborn rats with NGF induces an increase in the number of mast cells in several locations. It was further strengthened by the observation of a reduction in the number of mast cells in hepatic granulomas of *Schistosoma mansoni*-infected rats after administration of a neutralizing anti-NGF antibody [16]. The hypothesis of a role for NGF as a growth factor for mast cells has been confirmed *in vitro*, by studies showing that NGF induces the development of mast cells from mouse bone marrow cells in the presence of IL-3 [17,18]. Furthermore, NGF promotes the maturation of human mast cells: it increases the number of mast cells developed *in vitro* that express the mast cell protease chymase [19], and induces the expression of the high affinity receptor for IgE

by immature mast cells (HMC-1) [20]. Moreover, and this might be particularly relevant to increased tissue mast cell numbers, NGF increases mast cell survival *in vitro* by inhibiting their apoptosis [21–23].

In our study, the analysis of the possible interrelationship between NGF and the mast cell has led to the observation of the expression by the mast cell of the NGF high affinity tyrosine kinase receptor TrkA. This demonstration of TrkA expression on bronchial mast cells infiltrated in the bronchus in asthma is in agreement with recent reports of TrkA expression on cultured cord blood-derived mast cells, on mast cells from the HMC-1 cell line [19], and on cultured mast cells isolated from human lung parenchyma [24]. Our evidence of TrkA expression by mast cells in the bronchus of asthmatic patients *in vivo* strongly supports mast cells as a target cell for NGF actions. Together with the observation of an increased local expression of NGF, the increased number of bronchial mast cells raises the possibility of an involvement of NGF in the local development, maturation, and survival of mast cells in the bronchus in presymptomatic stages of allergic asthma.

In conclusion, our study shows an increased expression of NGF in the bronchus of asthmatic patients after repeated exposure to allergen at low subclinical dose. This was associated with an increase in the number of infiltrated mast cells. The demonstration of TrkA receptor expression on the membrane of mast cells infiltrated in the bronchus of asthmatic patients strongly supports mast cells as a target cell for NGF in the bronchus in asthma. Together with the evidence that injected NGF induces an increase in mast cell number in murine tissues [14,15], and that NGF activates mast cells and promotes their survival [21–23,46,47], our results suggest a role for the mast cell in the early events following allergen exposure, leading to the development of airway hyper-responsiveness in asthma before the onset of symptoms.

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