IL-9 and c-Kit$^+$ mast cells in allergic rhinitis during seasonal allergen exposure: Effect of immunotherapy

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Background: IL-9 is an important stimulus for tissue infiltration by mast cells, a feature requiring concomitant activation of c-Kit.
Objectives: We assessed IL-9 expression and c-Kit$^+$ mast cells in the nasal mucosa of patients with allergic rhinitis during seasonal pollen exposure and observed the effects of allergen immunotherapy.
Methods: We studied 44 patients with seasonal rhinitis and asthma before and 2 years after a double-blind trial of grass pollen immunotherapy. Nasal mucosal IL-9$^+$ cells and c-Kit$^+$ mast cells were assessed by means of immunochemistry. Cell types expressing IL-9 protein were determined by means of dual immunofluorescence. IL-9 mRNA–positive cells were assessed by means of *in situ* hybridization, and their phenotype was determined by using sequential immunohistochemistry and *in situ* hybridization.
Results: Nasal mucosal c-Kit$^+$ mast cells were increased during the pollen season ($P = .0001$). IL-9 mRNA–positive cells also tended to increase ($P = .1$) and correlated with nasal EG2$^+$ eosinophils ($r = 0.47$, $P = .05$) and IL-5 mRNA-positive cells ($r = 0.54$, $P = .02$). The cell sources of IL-9 included T cells, eosinophils, neutrophils, and mast cells. When compared with placebo, successful pollen immunotherapy markedly inhibited seasonal increases in nasal mucosal c-Kit$^+$ mast cells ($P = .001$) and the seasonal expression of IL-9 mRNA–positive cells ($P = .06$). Immunotherapy also inhibited IL-9 protein expression from nonendothelial cell sources ($P = .0007$).
Conclusion: IL-9 is upregulated in the nasal mucosa during the pollen season and correlates with tissue infiltration by eosinophils. Successful pollen immunotherapy is associated with inhibition of seasonal increases in both nasal c-Kit$^+$ mast cells and eosinophils. This effect might be explained, at least in part, by the reduced local expression of IL-9. (J Allergy Clin Immunol 2005;116:73-9.)

Key words: *Allergy, IL-9, mast cells, eosinophils, cytokine*

Mast cells, as well as eosinophils and basophils, are important effector cells in human allergic diseases. They mediate immediate responses to allergen exposure triggered by specific IgE and promote allergic inflammation through the release of proinflammatory mediators, including histamine, leukotriene C_4, IL-4, and IL-13.[1,2] The number of mast cells is increased in tissue sites of allergic inflammation.[3-5]

Allergen immunotherapy is highly effective for seasonal allergic rhinitis[6]; clinical improvement correlates with inhibition of the seasonal recruitment of eosinophils and basophils to the nasal mucosa.[7,8] However, in a previous study we did not observe significant changes in mast cell numbers (tryptase positive) in the nasal mucosa of immunotherapy-treated patients.[8] Moreover, although reduction in IL-5 expression could account for the inhibition of tissue eosinophilia after immunotherapy,[7] the influence of immunotherapy on cytokines related to mast cell development has not been assessed in the nasal mucosa.

IL-9 has been identified as a mast cell growth-enhancing factor.[9] IL-9 also stimulates differentiation of mast cells,[10] which express specific receptors for IL-9.[11] Studies on transgenic mice indicate that IL-9 induces the accumulation of mast cells in mucosal tissues,[10,12] with this effect requiring the concomitant activation of the stem cell factor (SCF)/c-Kit pathway.[10] Further studies confirmed a role for IL-9 in many aspects of allergic airway disease,[13] including tissue eosinophilia, bronchial hyperresponsiveness,[14] IgE production,[15] and mucus hypersecretion.[16,17] The IL-9 gene has been localized to the human chromosome 5q35-q33 cluster gene region and has been proposed to contribute directly to the genetic link between this important locus and the asthma phenotype.[18] IL-9 expression is increased in atopic asthma[19,20] and is induced in the lung on local allergen challenge.[21] However, its expression in the airways after natural allergen exposure and its modulation by means of immunotherapy has not been evaluated.

Therefore we assessed IL-9 as a potential key cytokine driving mast cell infiltration of the nasal mucosa from patients with seasonal allergic rhinitis. We examined nasal

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Abbreviations used
MBP: Major basic protein
SCF: Stem cell factor (or c-Kit ligand)

biopsy specimens obtained from patients with severe seasonal allergic rhinitis during the peak pollen season compared with specimens obtained out of the pollen season for the presence of mast cells expressing c-Kit and for expression of IL-9 protein and mRNA. We also assessed the potential relationship between IL-9 expression and eosinophils in the nasal mucosa and analyzed the effects of 2 years of conventional grass pollen immunotherapy on c-Kit$^+$ mast cell numbers and IL-9 expression.

METHODS

Patients

The clinical features of the 44 patients recruited from the allergy clinic of the Royal Brompton Hospital, London, United Kingdom, have been described previously.[22] All subjects had a history of severe summer hay fever that was not controlled by antiallergic drugs and a positive skin response to timothy grass pollen. The study was performed with the approval of the ethics committee of the Royal Brompton Hospital and with the informed consent of all participants.

This was a randomized, double-blind, placebo-controlled, parallel-group study, as previously described.[22] After one summer of symptom monitoring and before randomization to immunotherapy or placebo injections, baseline nasal biopsies were performed at a time when patients were asymptomatic. After 2 years of treatment, a second biopsy specimen was taken at the peak of the grass pollen season from the 37 subjects remaining in the study.

Additional biopsy specimens were taken from 9 nonatopic control subjects at the same time as the second (peak seasonal) set of study biopsy specimens to control for the effects of seasonal pollen exposure in nonsensitive subjects.

Immunohistochemistry

Biopsy specimens were divided into 2 halves. One half was immediately mounted in OCT compound (VWR; Lutterworth, Leics, United Kingdom), snap-frozen by means of immersion in isopentane precooled in liquid nitrogen, and then stored at −80°C. Immunohistochemistry was performed on 6-μm cryostat sections fixed in acetone/methanol (60:40) by using the anti-mouse Vector kit (Vector laboratories, Inc, Burlingame, Calif), according to the manufacturer's protocol. Sections were incubated with purified mouse mAb to human IL-9 (clone MH9P3; gift from J. Van Snick, Ludwig Institute for Cancer Research, ICP-Brussels, Belgium) diluted at 2.5 μg/mL in diluent (DakoCytomation, Cambridgeshire, United Kingdom), followed by incubations with biotinylated horse anti-mouse IgG and avidin-biotin complexes–alkaline phosphatase. The reaction was developed with Fast Red substrate and counterstained with hematoxylin.

Positively stained cells were predominantly endothelial cells. Staining was completely abrogated by means of preincubation of the mAb with the specific epitope peptide (gift from Professor Van Snick). Since IL-9 mRNA was not found in endothelial cells,[23] the IL-9 protein was assessed both in the endothelial cells and separately in the nonendothelial cells. Double immunohistochemistry was performed to determine the phenotype of nonendothelial IL-9 protein–positive cells. Unless stated otherwise, the IL-9 data presented do not include IL-9$^+$ endothelial cells.

Immunostaining for c-Kit was performed with a polyclonal rabbit antibody to c-Kit (DakoCytomation) diluted 1:50 and goat anti-rabbit IgG (1:200) as a secondary antibody. Primary controls included isotypic mouse IgG1 (IL-9) and rabbit IgG (c-Kit).

In situ hybridization

The second half of each biopsy specimen was fixed in 4% paraformaldehyde for 2 hours and dehydrated in 15% sucrose-PBS for 1 hour and overnight before being mounted in OCT and snap-frozen as described above. Riboprobes, both antisense and sense, were prepared from cDNA for IL-9 (gift from J. C. Renauld, Ludwig Institute for Cancer Research, ICP-Brussels, Belgium). The cDNA was inserted into different pGEM vectors (Promega, Southampton, United Kingdom) and linearized with appropriate enzymes before transcription. Transcription was performed in the presence of sulfur 35 (^{35}S)-labeled uridine triphosphate and the appropriate T7 or SP6 RNA polymerases. *In situ* hybridization was performed on 6-μm cryostat sections on polysine slides (VWR). Sections were permeabilized with Triton X-100 in PBS, followed by proteinase K digestion. Sections were treated with iodoacetamide and N-ethylmaleimide and then acetic anhydride-triethanolamine to inhibit hybridization binding of ^{35}S. As a negative control, sections were either hybridized with the sense probe or treated with ribonuclease A solution before the prehybridization step with antisense probes.

Colocalization of IL-9 protein and mRNA to leukocytes

Double immunohistochemistry. The cytokine was colocalized to T cells (CD3), eosinophils (major basic protein [MBP]), mast cells (tryptase), and neutrophils (neutrophil elastase) by using double immunofluorescence on acetone-fixed sections to assess the respective contribution of infiltrating leukocytes to IL-9 expression. IL-9 protein was detected by using mouse MH9P3 antibody as stated above, and polyclonal antibodies were used to reveal T lymphocytes (rabbit anti-CD3; Abcam Ltd, Cambridge, United Kingdom), eosinophils (goat anti-MBP; Santa Cruz Biotechnology, Santa Cruz, Calif), mast cells (goat anti-tryptase, Santa Cruz), and neutrophils (goat anti-neutrophil elastase, Santa Cruz). IL-9 protein was detected by using FITC-labeled rabbit anti-mouse antibody, whereas biotinylated anti-rabbit (Vector) or anti-goat antibodies (Stratech Scientific, Ltd, Cambridge, United Kingdom) were used as secondary antibodies for leukocyte phenotyping, followed by Alexa Fluor 594-labelled streptavidin (Molecular Probes, Cambridge Biosciences, Cambridge, United Kingdom).

Sequential immunohistochemistry–in situ *hybridization*. Colocalization of IL-9 mRNA to infiltrating leukocytes was performed on paraformaldehyde-fixed sections by using immunohistochemistry with the phenotype-specific markers CD3 (T cells), MBP (eosinophils), or neutrophil elastase (neutrophils; DakoCytomation) and alkaline phosphatase–antialkaline phosphatase (DakoCytomation) developed with Fast Red (Sigma, St Louis, Mo). This leukocyte staining was followed by *in situ* hybridization with a ^{35}S-labeled IL-9 antisense probe. Only double-positive cells were counted.

Quantification

IL-9 protein, IL-9 mRNA, c-Kit$^+$ cells, and colocalization of IL-9 mRNA to leukocyte subsets were counted at 200× magnification along the entire length of the basement membrane at one grid depth (0.45 mm) with an Olympus BH2 microscope (Olympus Optica Company Ltd, Tokyo, Japan). On average, this involved counting cells in 4 fields, which is equivalent to 0.8 mm^2 (0.2-1.8 mm^2) of