

Involvement of IL-9 in the bronchial phenotype of patients with nasal polyposis

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Background: Nasal polyposis (NP) is frequently associated with asthma. In this disease, asymptomatic bronchial hyperresponsiveness (BHR) is thought to precede the development of asthma. IL-9 and its receptor have been reported as candidate genes for asthma and to be associated with BHR.

Objective: The objective of this study was to assess the contribution of IL-9 to the pathogenesis of BHR in NP by comparing the expression of IL-9 and its receptor in bronchial biopsy specimens from three groups of patients with NP: NP without BHR, NP with asymptomatic BHR, and NP with BHR and asthma.

Methods: Bronchial biopsy specimens were examined in terms of cellular infiltration and in terms of expression of IL-9 protein and mRNA as well as of its receptor by using immunohistochemistry and in situ hybridization.

Results: Patients with NP with asthma as compared with the two other groups exhibited an increased bronchial infiltration of basophils, eosinophils, and T cells that correlated with the asthma score. The two groups of patients with NP with BHR showed an increased expression in IL-9 protein and mRNA as well as an increase in the expression of IL-9R mRNA at the epithelial level. These modifications were inversely correlated with the airway responsiveness to methacholine, producing a 20% fall in FEV₁. There was a close association between IL-9⁺ cells, IL-5 mRNA expression, and eosinophil infiltration that correlated with each other.

Conclusions: These results suggest an important role for IL-9 in the pathogenesis of BHR and a causal relation between IL-9 and the development of bronchial eosinophilia in asthma.

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Key words: Nasal polyposis, bronchial hyperresponsiveness, asthma, interleukin-9, bronchial biopsy

Abbreviations used

APAAP: Alkaline phosphatase antialkaline phosphatase

BHR: Bronchial hyperresponsiveness

BM: Basement membrane

NP: Nasal polyposis

Nasal polyposis (NP) is a chronic eosinophilic inflammatory disorder of the nasal and paranasal sinuses associated with asthma in 50% of cases,¹ suggesting a causal relation. During the course of the disease, nasal polyposis can be isolated or associated with asymptomatic bronchial hyperresponsiveness (BHR) or with asthma. A possible evolutionary relation between these three stages has been discussed. Indeed, it has been suggested that asymptomatic BHR may precede the development of asthma.^{2,3} Therefore, this particular disease provides a unique opportunity to understand the events leading to BHR and asthma. The clinical pattern of NP associated with BHR with or without asthma is characterized by a bronchial cellular infiltration of T lymphocytes and eosinophils.⁴ These cells have been shown to actively participate to the pathogenesis of asthma as well as BHR in particular through the release of T_H2-type cytokines.⁵ Among T_H2-type cytokines, IL-9 is thought to have an important role in asthma and particularly in BHR. Support for this comes from studies in mice that identified a genetic defect altering IL-9 expression and baseline airway hyperreactivity⁶ as well as human studies linking the IL-9 gene locus with atopy.^{7,8} A potentially important role of IL-9 in the pathogenesis of asthma has been supported by the pleiotropic effects of IL-9 on various cell types implicated in allergic asthma, including T and B cells, mast cells, and eosinophils.⁹⁻¹³ Like all cytokines, IL-9 acts through cognate interactions with its own distinct receptor (IL-9R), which consists of a ligand-specific α -subunit and a common γ -chain that is shared with IL-2, IL-7, and IL-15.^{14,15} Interestingly, the IL-9R gene has also been linked to BHR and asthma.¹⁶ Studies in transgenic animals have shown the involvement of IL-9 in lung mast cell numbers, eosinophilia, mucus hypersecretion, subepithelial fibrosis, bronchial hyperresponsiveness, and IgE production.¹⁷⁻¹⁹ A recent study on IL-9 knockout mice in a model of asthma showed no effects on airway responsiveness and on the

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T_H2 phenotype,²⁰ whereas in asthma models with neutralizing antibodies, a significant inhibition of airway hyperresponsiveness²¹ and of the T_H2 phenotype²² was observed, consistent with a role for IL-9 in the pathogenesis of airway hyperreactivity and asthma in mice. In human beings, bronchial biopsies performed in asthmatic patients have shown an overexpression of IL-9 in the bronchi as compared with normal subjects,²³ but no study has evaluated the involvement of IL-9 and its cognate receptor in the pathogenesis of BHR. Therefore, IL-9 and IL-9R expression were evaluated in bronchial biopsy specimens from patients with NP exhibiting different phenotypes, that is, an asthma phenotype, a BHR phenotype (with or without asthma), or no BHR (isolated NP).

METHODS

Patients

Twenty-eight patients were referred to the Calmette Hospital for pulmonary evaluation of noninfectious nasal NP. Nasal polyps were identified in all patients by anterior rhinoscopy and sinus computed tomography scan. Nasal symptoms associated with nasal polyps (obstruction, anosmia, sneezing, rhinorrhea, itching) were scored from 0 to 3: 0 for no symptoms, 1 for mild symptoms, 2 for moderate symptoms, and 3 for severe symptoms, so that the maximal nasal score was 15. All patients were treated with nasal topical steroids (beclomethasone, 200 to 600 µg/d). Patients with infectious sinusitis (ie, with nasal purulence and positive bacteriology on nasal lavage fluid) and/or previous history of nasal surgery were excluded from the study.

Study design

The study protocol was approved by a local ethics committee, and informed consent was obtained from all the patients. Subjects attended the clinic on 2 different occasions, 2 days apart. On the first visit, subject characteristics were recorded and skin-prick tests for common inhalant allergens and spirometric measurements were performed. Serum total IgE was measured by radioimmunosorbent test (Pharmacia Diagnostics, Uppsala, Sweden). On the second visit, subjects underwent methacholine inhalation challenge and a fiberoptic bronchoscopy for bronchial biopsy (within 3 hours after methacholine challenge). Previous reports have demonstrated that methacholine inhalation does not alter the airway cellularity in healthy and asthmatic subjects.²⁴ Asthma was defined according to the criteria suggested by the American Thoracic Society (ATS).²⁵ The asthma severity was assessed according to the GINA classification.²⁶ A score of 1 was assigned to patients with intermittent asthma, a score of 2 to patients with mild asthma, a score of 3 to patients with moderate asthma, and a score of 4 to patients with severe asthma. Exclusion criteria for patients with asthma were lower respiratory tract infection or asthmatic exacerbation in the previous 8 weeks, current smoking habit, and treatment with inhaled or oral corticosteroids in the 4 months before the study. Asymptomatic BHR was defined as a methacholine PC₂₀ < 16 mg/mL in the absence of symptoms or previous history suggestive of asthma.²⁷ The patients were then divided into 3 groups: NP without BHR (n = 13), NP with asymptomatic BHR (n = 6), and NP with BHR and asthma (n = 9). Therefore, the BHR phenotype included the two groups with BHR and the asthma phenotype the group with BHR and asthma.

Pulmonary function tests and methacholine challenge

FEV₁ and maximal midexpiratory flow (FEF₂₅₋₇₅) were obtained from flow-volume curves through the use of a Medgraphics spirom-

eter (St Paul, Minn) and expressed as percentage of predicted normal values.²⁸ Methacholine challenge was performed by using a DeVilbiss 646 nebulizer²⁷ with methacholine concentrations ranging from 0.125 to 16 mg/mL. The provocative concentration (PC₂₀) was defined as the methacholine concentration necessary to decrease FEV₁ by 20% from baseline values

Fiberoptic bronchoscopy and bronchial biopsy specimens

The bronchoscopy procedure was performed at the same time of day (11 AM) according to published guidelines.²⁹ Half of the endobronchial biopsy specimens were immediately snap-frozen in isopentane and then stored in liquid nitrogen for immunohistochemistry studies. The other biopsy specimens were fixed immediately in 4% paraformaldehyde/PBS for 2 hours, washed in 15% PBS/sucrose 3 times, embedded in ornithine carbamyltransferase compound (Tissue-Tek, Miles, Elkhart, Ind), and then snap-frozen in isopentane cooled in liquid nitrogen for in situ hybridization (ISH) and shipped to Canada.

Immunohistochemistry

Reagents. Anti-CD4 antibody was purchased from Becton Dickinson (Pont de Claix, France). Anti-MBP antibody, which recognizes eosinophil major basic protein, was from Serotec (Cergy St Christophe, France). Anti-CD3, anti-CD8, anti-CD68 (for monocytes/macrophages), antineutrophil elastase (for neutrophils), rabbit anti-mouse Ig, monoclonal alkaline phosphatase antialkaline phosphatase (APAAP), and control IgG antibodies were from Dako (Trappes, France). Mouse monoclonal antitrypsin antibody was from Chemicon (Temecula, Calif). Monoclonal antibody BB1 was used to specifically stain human basophils.³⁰ Anti-CCR3 mab (7B11), from Leukosite Inc (Cambridge, Mass), was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, Bethesda, Md.^{31,32} Goat anti-IL-9 polyclonal was from R & D (Abingdon, United Kingdom). Rabbit anti-goat IgG biotin conjugate antibody and ExtrAvidin alkaline phosphatase conjugate were from Sigma (St Quentin Fallavier, France). Alkaline phosphatase activity was developed with the use of Fast Red TR/naphtol ASMX tablets (Sigma).

Cell infiltration immunostaining. Cryostat sections were cut from frozen biopsy specimens onto slides and fixed in acetone/methanol for 7 minutes. Immunohistochemistry was performed as previously described,³³ using a modified APAAP method for anti-human monoclonal antibody staining; the color was developed with the use of Fast Red. Substitution of the primary antibody with an irrelevant antibody of the same species was used as negative control.

Cytokine immunostaining. For cytokine immunostaining, cryostat sections were cut from frozen biopsy specimens onto slides coated with 0.1% poly-L-lysine (Sigma), fixed in 4% paraformaldehyde for 20 minutes, washed in PBS, and dried overnight at 37°C. Before immunostaining, slides were incubated in 0.3% Triton X-100 for 20 minutes. For IL-9, sections were incubated with a goat anti-human IL-9 polyclonal antibody and then with a rabbit anti-goat Ig biotin conjugate as secondary antibody, followed by ExtrAvidin alkaline phosphatase complex. The reaction was visualized with Fast Red alkaline phosphatase substrate.

In situ hybridization

Radiolabeled complementary RNA (cRNA) probes coding for IL-9 and its receptor (IL-9R) were prepared from complementary DNA (Genaera Corp, Plymouth Meeting, Pa), as previously described.³⁴ IL-5 mRNA was concomitantly evaluated.³⁵ S-labeled uridine triphosphate antisense (complementary to mRNA) and

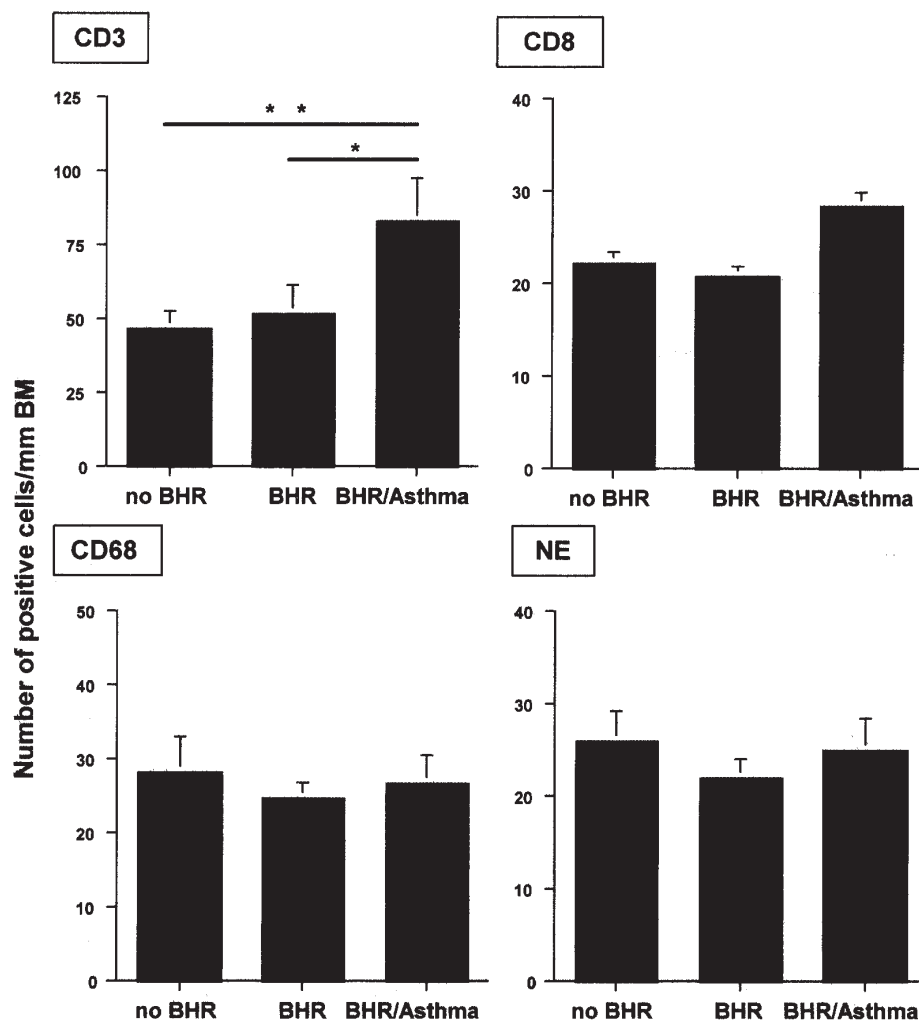


FIG 1. T-cell, monocyte/macrophage, and neutrophil infiltration in bronchial biopsy specimens from patients with NP. Cellular infiltration was assessed by using immunohistochemistry with the APAAP technique. Results are expressed as mean number of positive cells per millimeter of basement membrane in the three groups of patients. * $P < .05$, ** $P < .01$.

sense probes (identical to mRNA) were prepared. Cryostat sections 10 μ m thick were cut on 0.1% poly-L-lysine-coated slides, baked in an oven at 37°C, and stored at -80°C until further use. ISH was performed as previously described.³⁴ Specific hybridization was demonstrated by clear dense deposits of silver grains in the emulsion overlying the tissue sections.

Quantification and statistical analysis

Sections were coded and counted in a blinded, random fashion with an Olympus microscope with an eyepiece graticule at $\times 200$ magnification. At least 2 sections were stained, from which 6 to 8 fields were evaluated. Positively stained cells were counted to a depth of 125 μ m below the basement membrane (BM), and the results were expressed as the mean number of positive cells $\pm$ SEM per millimeter of BM. The numbers of positive cells in the three groups of patients were compared through the use of a 1-way ANOVA followed by a post hoc *t* test for multiple comparisons. Correlations were done with use of linear regression analysis. Results were considered statistically significant for *P* values of

$<.05$. Statistical analyses were performed with the use of a standard computer package (Statview 5 for MacIntosh).

RESULTS

Characteristics of the patients

The clinical and physiologic data of the patients are shown in Table I. Thirteen patients did not respond to metacholine challenge, 6 exhibited BHR but did not have asthma symptoms, and 9 had asthma symptoms associated with BHR. There was no significant differences in total IgE, nasal score, and FEV₁ (%) among the three groups, whereas FEV₂₅₋₇₅ (%) and PC₂₀ were significantly different in the two groups with BHR as compared with the isolated NP group. The asthmatic group included mainly patients with intermittent and mild asthma because of the steroid exclusion criteria.

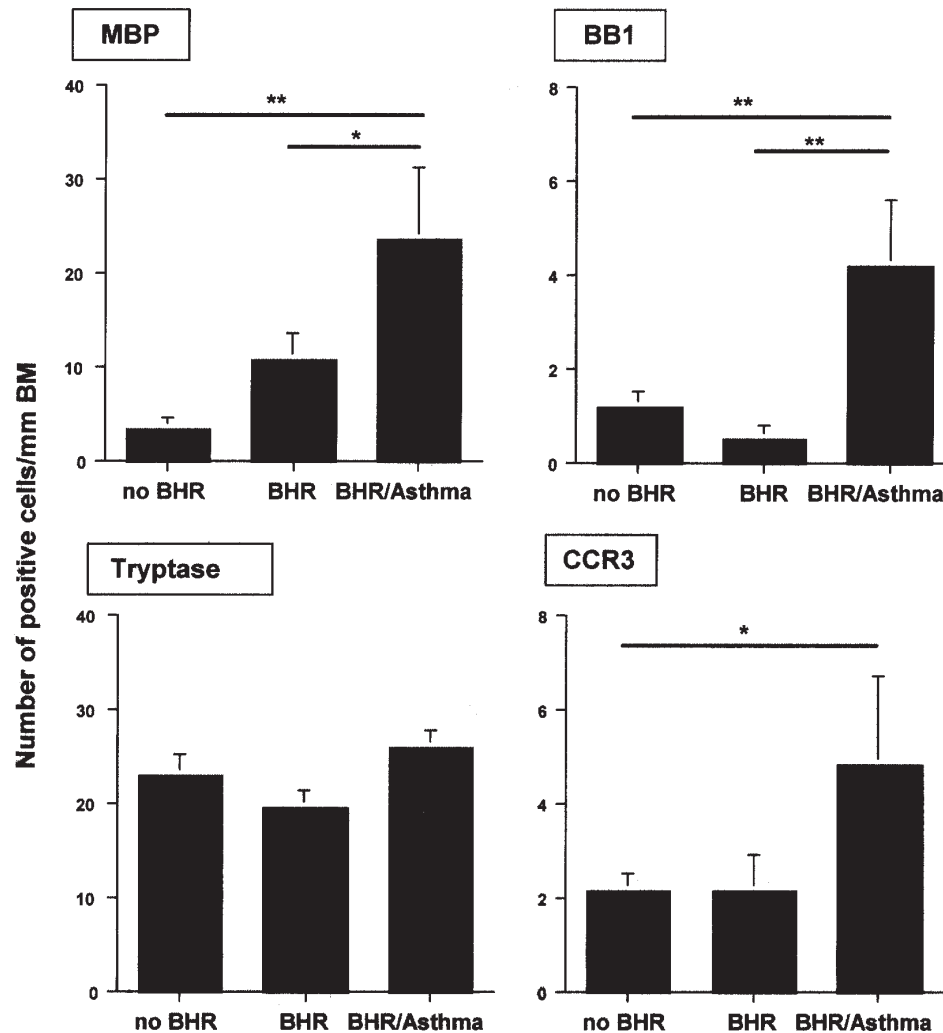


FIG 2. Infiltration of eosinophils, basophils, and mast cells and expression of chemokine receptor CCR3 in bronchial biopsy specimens from patients with NP. These different parameters were assessed by immunohistochemistry. Results are expressed as in Figure 1. * $P < .05$, ** $P < .01$.

T-lymphocyte but not monocyte/macrophage and neutrophil infiltration in bronchial biopsy specimens is associated with the asthma phenotype (Fig 1)

T-lymphocyte immunostaining showed an increased infiltration of CD3⁺ T cells in the BHR/asthma group (83.3 ± 14.1) as compared with both the BHR (51.7 ± 7.6) ($P < .05$) and the isolated NP groups (47.0 ± 5.3) ($P < .01$). A similar pattern was observed for CD4⁺ cells (data not shown), whereas there was no significant difference for CD8⁺ T-cell infiltration. The number of CD3⁺ cells significantly correlated with the asthma score in the asthma group ($r = 0.80$, $P \leq .05$). In contrast, there were no modifications in monocyte/macrophage and neutrophil numbers among the three groups.

Eosinophils and basophils but not mast cells were increased in the asthma group

Similar to T cells, immunostained eosinophils and basophils were significantly increased in the BHR/asthma group (23.7 ± 7.5 and 4.2 ± 1.4 , respectively) as compared with the BHR (10.8 ± 2.2 and 0.5 ± 0.25) ($P < .05$ and $P < .01$) and the isolated NP groups (3.5 ± 1.2 and 1.1 ± 0.29) ($P < .01$) (Fig 2). However, eosinophil and basophil numbers did not correlate with the asthma score. There was no significant difference between the BHR and isolated NP group for eosinophils and basophils. There was no variation of mast cell numbers, whatever the group. As the chemokine receptor CCR3 is expressed on these three cell types,^{31,35,36} its expression was assessed in the three groups. The number of CCR3-immunostained cells was

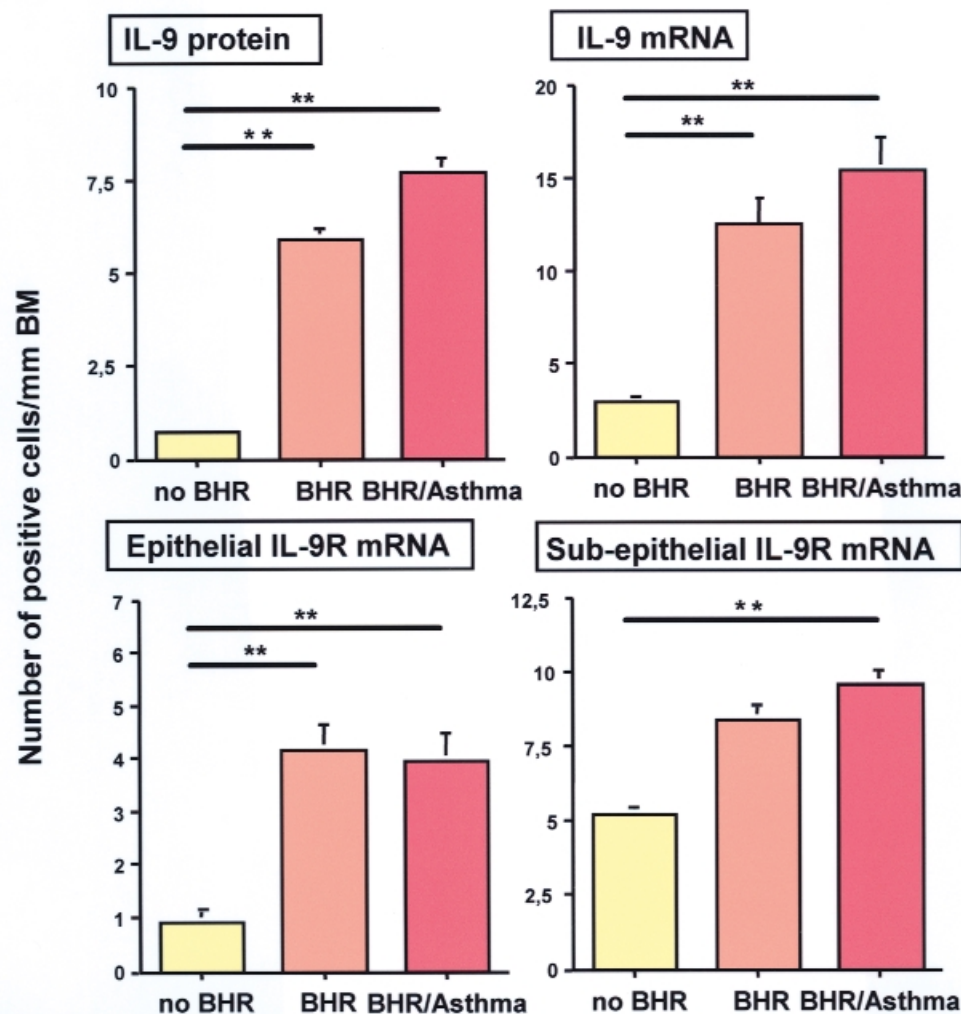


FIG 3. Expression of IL-9 and its receptor in bronchial biopsy specimens from patients with NP. IL-9 protein was assessed by immunohistochemistry; IL-9 and IL-9R mRNA expression was evaluated by in situ hybridization. Results are expressed as in Figure 1. ** $P < .01$.

significantly increased in the asthma group (4.84 ± 1.9) as compared with the isolated NP group (2.2 ± 0.36) ($P < .01$ for both) but not with the asymptomatic BHR group (2.2 ± 0.8). A positive correlation was observed between CCR3 expression and the numbers of basophils ($r = 0.6$, $P < .01$) but not with eosinophils.

IL-9 and epithelial IL-9R expressions were associated with BHR phenotype

IL-9 protein-positive cells were significantly increased in the two groups exhibiting a BHR (7.7 ± 1.9 for the BHR/asthma group and 5.8 ± 1.4 for the BHR group) as compared with the isolated NP group (0.7 ± 0.2) ($P < .01$ for both) (Fig 3). Similarly, IL-9 at the mRNA level was increased in the group with asthma (15.4 ± 1.8) and in the group with BHR (12.5 ± 1.2) as compared with the isolated NP group (2.9 ± 0.4) ($P < .001$ for both). Consistently, IL-9 protein-positive cells inversely correlated with the PC_{20} ($r = -0.70$, $P < .001$),

as did IL-9 mRNA-positive cells ($r = -0.76$, $P < .001$). IL-9 mRNA-positive cells correlated with IL-9 protein-positive cells ($r = 0.72$, $P < .001$). There was no significant difference between atopic and nonatopic patients in IL-9 mRNA expression. Respective values were 1.75 ± 0.7 and 3.35 ± 0.3 for the isolated NP group, 12.3 ± 1.1 and 12.9 ± 2.4 for the group with BHR, and 15.3 ± 2.7 and 15.6 ± 1.8 for the asthma group. The expression of IL-9R mRNA in the epithelial layer was also increased in the two BHR groups (BHR: 4.1 ± 0.47 ; asthma: 3.9 ± 0.53) as compared with NP alone (0.9 ± 0.21) ($P < .001$ for both) and showed a negative correlation with the PC_{20} ($r = -0.70$, $P < .001$). Subepithelial IL-9R mRNA-positive cells, reflecting the bronchial subepithelial infiltrating cells, were significantly increased only in the asthma group (9.6 ± 1.6 versus 5.2 ± 0.63 for the isolated NP group) ($P < .005$) but not in the asymptomatic BHR group (8.4 ± 0.9) and correlated with MBP+ eosinophils ($r = 0.78$, $P < .001$).

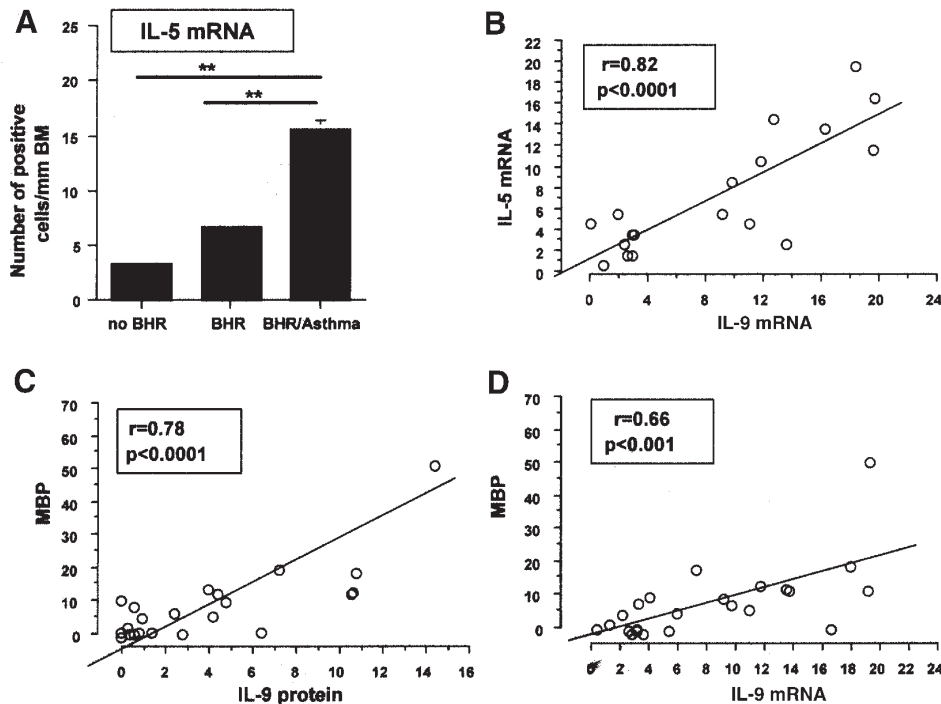


FIG 4. Relation between IL-9, IL-5 mRNA expression, and eosinophil infiltration in bronchial biopsy specimens from patients with NP. **A**, Expression of IL-5 mRNA in the three groups, $**P < .01$. **B**, Correlation between IL-9 and IL-5 mRNA expression. **C**, Correlation between IL-9 protein expression and eosinophil infiltration. **D**, Correlation between IL-9 mRNA expression and eosinophil infiltration.

TABLE I. Characteristics of patients

	NP without BHR	NP with asymptomatic BHR	NP with BHR and asthma
No. of patients	13	6	9
Age (y)	40.8 ± 3.2	48.5 ± 4.3	39.3 ± 3.4
Sex ratio (M/F)	10/3	5/1	5/4
Atopy*	4	3	6
IgE (kU/L)	124 ± 46	177 ± 58	235 ± 74
Nasal score†	7.6 ± 0.8	6.0 ± 0.9	7.8 ± 1.1
Asthma score‡	—	—	2 ± 0.4
FEV ₁ (%)	106.6 ± 2.0	94.3 ± 5.8	95.2 ± 7.9
FEF ₂₅₋₇₅ (%)	101.2 ± 5.4	66.6 ± 10.1§	66.3 ± 7.4§
PC ₂₀ (mg/mL)	>16	4.8 ± 1.4§	2.4 ± 1.2§

Results are expressed as mean ± SEM.

*Positive skin prick test results to more than one aeroallergen.

†Nasal score included assessment of nasal obstruction, anosmia, sneezing, rhinorrhea, and itching. Each nasal symptom was scored 0 for no symptoms, 1 for mild symptoms, 2 for moderate symptoms, and 3 for severe symptoms.

‡Asthma was scored 1 for patients with intermittent asthma, 2 for patients with mild asthma, 3 for patients with moderate asthma, and 4 for patients with severe asthma.

§ $P < .01$, different from NP without BHR.

Eosinophil infiltration and IL-5 mRNA expression were closely associated with IL-9 upregulation

In a previous study, we have shown an upregulation of IL-5 mRNA and protein in NP associated with asthma.³⁷ As IL-9 has been shown to induce indirect effects on eosinophils through IL-5,^{12,13} we analyzed IL-5 mRNA expression in the three groups of the cur-

rent study (Fig 4). Here, we confirm our previous results of an increase in IL-5 mRNA expression associated with the asthma phenotype (Fig 4, A). IL-5 mRNA expression correlated with eosinophil infiltration ($r = 0.76$, $P < .001$). When IL-9 expression was compared with eosinophil infiltration and with IL-5 mRNA expression, the three parameters correlated with each other, suggesting a causal relation (Fig 4, B, C, and D).

DISCUSSION

Bronchial T-lymphocyte and eosinophil infiltrations are key pathologic features of asthma.³⁸ In the current study, the patterns of bronchial eosinophil infiltration were similar to those reported in our previous study,³⁷ as was the finding of an increased IL-5 mRNA expression in patients with NP with asthma. In contrast, patients with isolated NP had higher numbers of lymphocytes in their bronchial mucosa in the current study than in our previous study.³⁷ Analysis of the two groups of isolated patients with NP in the two studies showed that the only difference consisted in a longer duration of nasal symptoms in isolated patients with NP from the present study (192 ± 40 months) than in the other one³⁷ (72 ± 22 months), perhaps reflecting the chronicity of the disease. Altogether, in patients with NP the asthma phenotype was associated with an increased T-cell and eosinophil infiltration of the bronchial mucosa as well as an upregulation of IL-5 mRNA expression.

In sharp contrast to the cellular infiltration, IL-9 protein and mRNA expression exhibited a totally different repartition profile in that they appeared overexpressed in both BHR and BHR/asthma groups as compared with the isolated NP group. Furthermore, IL-9 protein and mRNA inversely correlated with the PC₂₀ strengthening the hypothesis of a predominant role of IL-9 in the development of BHR in patients with NP. To our knowledge, this is the first study showing such a relation in human beings. These results are consistent with different animal studies in the literature showing a close relation between BHR and IL-9 protein levels in the lung.⁶ IL-9 overexpression in patients with NP with asthma and/or BHR was not related to the atopic status of the donors, since no difference in IL-9 expression was observed between atopic and nonatopic patients in each group.

IL-9 induces direct effects on epithelial cells through its binding to its receptor, such as the production of the chemokines RANTES and eotaxin,^{38,39} specialized in the recruitment of eosinophils. Interestingly, recent data have shown that epithelial cells, through cytokine stimulation (in particular IL-13), can have a direct effect on the induction of airways hyperresponsiveness.⁴⁰ Consistently, we showed in this study that epithelial IL-9R-positive cells were also significantly upregulated in the two groups of patients with NP exhibiting a BHR. Moreover, they inversely correlated with the PC₂₀, suggesting their involvement in the development of BHR. Altogether, epithelial cells might represent a primary target of the action of IL-9 resulting in the production of eosinophil chemoattractant factors and may directly participate in the promotion of BHR. IL-13 represents another cytokine that may directly participate in BHR.^{5,20,40} A possible interrelation between IL-9 and IL-13 is still under debate; more studies are needed to clarify this point.

In contrast with the direct action of IL-9 on epithelial cells, the effect of IL-9 on eosinophil recruitment is probably indirect. Eosinophils are known to express IL-9R, and in this study, the subepithelial expression of IL-

9R closely correlated with MBP⁺ cells but not with neutrophils and T cells, which also express this receptor.^{41,42} In particular, it has been shown that IL-9 acts on the differentiation and survival of eosinophils by increasing eosinophil reactivity to IL-5.^{12,13} These data are consistent with a recent study in which the effect of lung-specific inducible expression of IL-9 on eosinophil infiltration in a murine model of asthma has been shown to be dependent on IL-5.⁴³ To assess the relation between IL-9, IL-5, and eosinophils in vivo in our patients with NP, we decided to reevaluate in this study for IL-5 mRNA expression. There was a strong correlation between these factors; however, whereas IL-5 and eosinophil infiltration were associated with the asthma phenotype, IL-9 was associated with the BHR phenotype.

A major pathway involved in eosinophil recruitment is represented by chemokines and their receptors. The chemokine receptor CCR3 is expressed on eosinophils, basophils,^{31,35} T_H2 cells, and mast cells.^{36,44} The pattern of CCR3 expression observed in the current study is in agreement with our previous work in which eotaxin, the main CCR3 ligand, was increased in the group of patients with NP with asthma.³⁷ CCR3 expression correlated with basophil but not eosinophil infiltration of the bronchial mucosa, in contrast to another study performed on bronchial biopsy specimens from asthmatic patients.⁴⁵ This difference might be linked to the antibodies used to identify eosinophils, which was the EG2 in the other study, which recognizes activated eosinophils, whereas we used in the current study anti-MBP, which recognizes pan-eosinophils.

In conclusion, this study shows that in patients with NP, the asthma phenotype is associated with a bronchial infiltration of T cells, IL-5 mRNA⁺ cells, and eosinophils, whereas the BHR phenotype is closely associated with an increase in IL-9 and its receptor at the epithelial level. These results suggest that IL-9 and its receptor are involved in the pathogenesis of BHR and that their expressions may precede the appearance of eosinophils and the development of asthma.

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