

Cytokine profile in minor salivary glands from patients with bronchial asthma

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Background: T lymphocytes are important components of the bronchial inflammatory cell infiltrate in asthma. Because lymphocytes activated in the respiratory tract recirculate to remote glandular and mucosal sites, we previously studied the histologic features of minor salivary glands (MSGs) in bronchial asthma and found an airway-like inflammation with T-lymphocyte infiltration, the presence of mast cells that were often degranulated, and basement membrane thickening but no eosinophil infiltration.

Objective: We sought to investigate the cellular infiltration and cytokine profile in MSGs from untreated asthmatic subjects, steroid-treated asthmatic subjects, and control subjects and to compare these values with those found in bronchial biopsy specimens.

Methods: The cellular infiltration was studied by using immunohistochemistry. Cytokine messenger (m)RNA expression for IL-4, IL-5, and IFN- γ was determined by using in situ hybridization and cytokine immunoreactivity with immunohistochemistry.

Results: A significant increase in CD4 and IL-4 mRNA⁺ cells was observed in MSGs from asthmatic patients (both untreated and steroid-treated subjects) when compared with control subjects, which correlated with the clinical severity of asthma (FEV₁ and Aas score). In contrast to the bronchi, no IL-5 mRNA expression was observed in MSGs, and no difference was observed for MSG IFN- γ mRNA between the groups. At the level of MSG protein expression, the 3 cytokines were seen, with a significant increase in IL-4 protein expression in steroid-treated asthmatic subjects compared with untreated asthmatic subjects and control subjects, but there were no differences between the groups in IL-5 and IFN- γ protein expression.

Conclusion: The cytokine mRNA expression pattern observed in the MSGs of asthmatic subjects was different from that found in the bronchi, suggesting a different local immune regulation. (*J Allergy Clin Immunol* 2000;106:687-96.)

Key words: Major salivary gland, asthma, cytokines, common mucosal system

Abbreviations used

APAAP: Alkaline phosphatase-antialkaline phosphatase

ICAM: Intercellular adhesion molecule

MSG: Minor salivary gland

VCAM: Vascular cell adhesion molecule

Bronchial asthma is characterized by chronic inflammation underlying the two main features of the disease, bronchial constriction and bronchial hyperresponsiveness. The role of T lymphocytes in the pathogenesis of asthma has been emphasized in recent years. T lymphocytes have been demonstrated to be activated in the bronchial mucosa of asthmatic subjects¹ and to be increased in asthma.^{2,3} Through secretion of cytokines, CD4⁺ T lymphocytes actively participate in the inflammatory processes found in asthma. IL-3, IL-5, and GM-CSF are predominantly involved in eosinophil and mast cell differentiation and activation,⁴⁻⁶ whereas IL-4 allows IgE synthesis.⁷ The cytokine profile of asthma has been extensively studied, and a predominant T_H2-type profile with IL-4 and IL-5 production and messenger (m)RNA expression is observed in the lung from allergic,^{8,9} as well as nonallergic, patients.¹⁰

The mucosal system comprises a series of specialized lymphoid tissues, particularly at the lung level, which are critical for the dispersal and targeting of naive and memory lymphocyte populations that are required for immune surveillance. Subsets of memory T cells have been shown to home and recirculate constitutively, albeit at very low levels, through the mucosal sites.¹¹ Animal experiments have shown that precursors originating from bronchus-associated lymphoid tissue migrate to the intestinal mucosa and other mucosal-associated tissue, such as the salivary glands.^{12,13} Sensitization of bronchus-associated lymphocytes may thus be responsible for the induction of an immune response in the minor salivary glands.¹⁴ Indeed, in a previous study we demonstrated an airway-like inflammation in minor salivary glands (MSGs) from asthmatic patients. The main pathologic abnormalities were T-lymphocyte infiltration, the presence of mast cells that were often degranulated, basement membrane thickening, and endothelial cell intercellular adhesion molecule (ICAM) 1 expression. In contrast, no eosinophil infiltration or vascular cell adhesion molecule (VCAM) 1 expression was found.¹⁵ To gain further insight into the mechanisms involved in these abnormalities, we initiated the present study to

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determine whether a particular cytokine profile was observed in MSGs obtained from both asthmatic subjects treated with steroids and those not treated compared with control subjects. Messenger RNA expression and secretion of IL-4, IL-5, and IFN- γ were evaluated, as well as the cellular infiltration.

METHODS

Patients

Twenty-five patients with asthma were included in this study (13 female and 12 male subjects). Asthma was defined according to the criteria of the American Thoracic Society.¹⁶ All patients exhibited a history of episodes of dyspnea and wheezing and had a reversible airway obstruction characterized by a 20% increase in FEV₁ after the inhalation of 200 μ g of albuterol. Sensitivity to allergens was evaluated by skin prick tests comprising a series of common environmental allergens. Total (Phadebas, paper radioimmunosorbent test; Pharmacia Diagnostics, Fairfield, NJ) and specific IgE levels (Phadebas RAST, Pharmacia Diagnostics) were measured. Thirteen patients were allergic, and 12 were nonallergic. All patients had normal sinus and chest x-ray findings. The 25 patients were distributed into two groups according to their treatment at the time of study. The first group included 8 asthmatic subjects who received only intermittent inhaled short β_2 -agonists taken as needed and were defined as untreated asthmatic subjects. The second group consisted of 17 asthmatic subjects who were treated with inhaled steroids (mean daily dose, 1330 $\pm$ 191 μ g of beclomethasone or equivalent), associated with oral steroids in 6 patients (mean daily dose, 20.0 $\pm$ 5.6 mg of prednisone equivalent), who were defined as steroid-treated asthmatic subjects. The clinical severity of asthma was assessed according to the Aas scoring system (1 = very mild forms; 5 = incapacitating disease requiring medications).¹⁷ The grading was based on events that took place over a 1-year period and considered both the symptoms (number and duration of asthma episodes, total duration of symptoms, and presence or absence of symptom-free intervals between attacks) and the requirements for medication. It did not take into account the pulmonary function status of the patients. In addition, all patients performed lung function tests. Forced vital capacity and FEV₁ were determined by using an MSR spirometer (E. Jaeger, Würzburg, Germany). Samples from 7 of these patients were also used in a previous study.¹⁵ We also studied 14 nonallergic control subjects taking no medications. Control subjects had no history of allergic disease and negative skin prick test and RAST results. None of the patients had clinical criteria of Sjögren syndrome¹⁸; all serologic test results for antinuclear antibody, DNA, anti-Sjögren syndrome A, and anti-Sjögren syndrome B were negative; and there was no hyper γ -globulinemia. Control and asthmatic subject characteristics are summarized in Table I.

MSG biopsy

The lower lip was everted, and local anesthesia was performed by injection of 2% xylocaine. Two incisions 2 mm long and 1 to 2 mm deep were performed at the junction point of the secretory duct and the mucosa, and one or two MSGs were excised. The incisions were covered with coagulating powder (thrombase; Houde laboratory, Puteaux, France). In all cases healing was satisfactory. None of the patients reported undue discomfort either during or after the biopsy procedure. This protocol was approved by the ethical committee of the Centre Hospitalier et Régional Universitaire de Lille (CP 93/02).

MSGs were immediately fixed in 4% paraformaldehyde and paraffin embedded for in situ hybridization studies. In 16 patients, 8 untreated asthmatic subjects, 11 steroid-treated asthmatic subjects, and 13 control subjects, an additional MSG was available,

which was fixed in paraformaldehyde and snap-frozen in liquid nitrogen to study both the cytokine expression and cellular infiltration by using immunohistochemistry.

Bronchial biopsy specimens

Among the subjects, 8 asthmatic patients (4 untreated and 4 treated) and 7 control subjects had bronchial biopsies performed at the same time in the context of another study. Fiberoptic bronchoscopy was performed in the conditions previously described.¹⁹ In that previous study, acetone-fixed sections from unfixed frozen bronchial biopsy specimens were used to determine the cellular infiltration, particularly eosinophil infiltration. Results were expressed as mean cell counts $\pm$ SEM per millimeter of basement membrane in a submucosal zone 125 μ m deep. Additional biopsy specimens were fixed in 4% paraformaldehyde and embedded in paraffin. The latter biopsy specimens were used in this study to determine cytokine mRNA expression.

MSG pathologic examination

Paraffin sections were stained with hematoxylin-eosin and May-Grünwald-Giemsa stains for mast cells and eosinophils. Sections were examined, and a Chisholm focus score was determined, providing a semiquantitative assessment of inflammation. Details of the method have been described elsewhere,²⁰ but briefly, this standardized score is the number of inflammatory cell aggregates containing 50 or more lymphocytes or plasma cells in each 4-mm² area of salivary gland. Grading standard is used to quantify the lymphocytic infiltrate as follows: 0, no lymphocytic infiltrate; 1, slight lymphocytic infiltrate (1-20 cells/4 mm²); 2, moderate lymphocytic infiltrate (20-50 cells/4 mm²); 3, severe lymphocytic infiltrate (>50 cells/4 mm²); 4, very severe lymphocytic infiltrate with more than one focus per 4 mm².

Immunohistochemistry

Reagents. Anti-CD4 antibody was purchased from Becton Dickinson (Pont de Claix, France). EG2, which recognizes the activated form of eosinophil cationic protein, was obtained from Pharmacia (St Quentin en Yvelines, France). Rabbit anti-mouse Ig, monoclonal alkaline phosphatase-antialkaline phosphatase (APAAP), and control IgG antibodies were from Dako (Trappes, France). Alkaline phosphatase activity was developed by using Fast Red TR/naphtol ASMX tablets (Sigma, St Quentin Fallavier, France). Mouse monoclonal antitrypsin antibody was purchased from Chemicon (Temecula, Calif). For cytokine staining, rat mAbs against human IL-4 (clone MP4-25D2) and IL-5 (clone JES1-39D10) were purchased from Pharmingen (San Diego, Calif). Anti-human IFN- γ antibody (MD2 clone F14) was from Hycult biotechnology (Uden, The Netherlands). These antibodies have been previously shown to be suitable for immunocytochemistry.^{10,21}

Single staining. Cryostat sections were cut from frozen MSG biopsy specimens onto slides coated with 0.1% poly-L-lysine (Sigma, St Quentin Fallavier, France) and dried overnight at room temperature. Slides were incubated in 0.3% triton for 20 minutes. For all antibodies except IL-4 and IL-5, immunocytochemistry was performed by using a modified APAAP method, as previously described.²² For IL-4 and IL-5, immunocytochemistry was performed by using a modified avidin-biotin complex method, as previously described.¹⁰ Briefly, sections were incubated with PBS containing 1% hydrogen peroxide, and endogenous biotin was quenched by using a Vector laboratory kit (Peterborough, UK). Sections were preincubated with rabbit serum and incubated overnight with the cytokine antibodies in PBS containing 0.1% saponin (Sigma, St Quentin Fallavier, France). Sections were treated with the ABC Vectastain Elite kit (Vector) and developed by using diaminobenzidine tetrahydrochloride-nickel (Vector). Substitution of the primary anti-

TABLE I. Characteristics of the asthmatic and control subjects

	Untreated asthmatic subjects (n = 8)	Steroid-treated asthmatic subjects (n = 17)	Control subjects (n = 14)
Sex ratio (F/M)	3:5	10:7	4:10
Age, mean $\pm$ SEM	33.3 $\pm$ 4.9	47.2 $\pm$ 3.1	47.1 $\pm$ 4.8
Positive prick test responses*	5	8	0
Total IgE, IU/mL†	755 $\pm$ 370	610 $\pm$ 202	93 $\pm$ 37
FEV ₁ , % predicted	84.5 $\pm$ 6.3	65.1 $\pm$ 3.6‡	104.5 $\pm$ 3.2
Aas score	1.75 $\pm$ 0.3	3.35 $\pm$ 0.3§	NA

NA, Not applicable.

*Defined as positive when the wheal size was superior or similar to that of the positive control (histamine).

†Normal total IgE values are below 100 IU/mL.

‡Significantly different from untreated patients ($P < .003$) and from control subjects ($P < .0001$).

§Significantly different from untreated patients ($P < .001$).

TABLE II. MSG cellular infiltrate in asthmatic and control subjects

	Untreated asthmatic subjects (n = 8)	Steroid-treated asthmatic subjects (n = 17)	Control subjects (n = 14)
Lymphocytic infiltrate	7/8	16/17	3/14
Chisholm score* of 0	1/8	1/17	10/14
Chisholm score of 1	2/8	2/17	3/14
Chisholm score of 2	2/8	7/17	0/14
Chisholm score of 3	3/8	6/17	0/14
Chisholm score of 4	0/8	1/17	0/14
Mean Chisholm score	1.8 $\pm$ 0.3	2.2 $\pm$ 0.2	0.20 $\pm$ 0.1†
Eosinophils	2/8	0/17	0/14
Degranulated mast cells	5/8	11/17	0/14

*The standardized Chisholm score is the number of focal inflammatory cell aggregates containing 50 or more lymphocytes or plasma cells in each 4-mm² area of salivary gland: 0 = no infiltrate; 1 = 1 to 20 cells/4 mm²; 2 = 20 to 50 cells/4 mm²; 3 = >50 cells/4 mm²; 4 = more than one focus per 4 mm²

†Significantly different from untreated and steroid-treated asthmatic subjects ($P < .0001$).

body with an irrelevant antibody of the same species was used as a negative control. The sections were counted in duplicate blinded to the subjects' clinical status. Results were expressed as the number of positive cells per field at 250 $\times$ magnification.

Double staining. Double staining was performed by using anti-cytokine and antitryptase antibodies. Sections were processed as described for the anticytokine antibodies until the step of overnight incubation. Then sections were incubated with the antitryptase antibody for 30 minutes at room temperature and washed in PBS. Biotinylated rabbit anti-rat and rabbit anti-mouse antibodies diluted in PBS 15% rabbit serum were simultaneously added to the sections for 30 minutes. Sections were washed in TRIS-buffered saline, incubated with APAAP, washed again in TRIS-buffered saline, and developed with Fast Red, resulting in red staining. Sections were then incubated with avidin-biotin-peroxidase complex for 30 minutes, washed in PBS, and developed by using diaminobenzidine tetrahydrochloride-nickel, resulting in a brown-black color. Sections were counterstained with hematoxylin and mounted in glycerol (Dako, Trappes, France). With this method, tryptase-positive mast cells stained red, IL-4- or IL-5-containing cells stained black, and double stained cytokine-positive mast cells stained maroon.

In situ hybridization

In situ hybridization was performed as previously described.^{23,24} Briefly, after deparaffinization, sections were hybridized with ³⁵S-labeled riboprobes (2 $\times$ 10⁶ cpm per section) in hybridization buffer containing 50 mmol/L dithiothreitol (Promega, Madison, Wis). Sections were hybridized overnight at 50°C and washed in high-stringency conditions (50°C, 0.1 $\times$ standard saline citrate). A peripheral blood T-lymphocyte clone obtained from a patient with the hyper-

IgE syndrome, which expressed mRNA for IL-4 and IL-5, was used as a positive control. PHA-stimulated blood mononuclear cells were used as positive controls for IFN- γ . For negative controls, cytospin preparations were hybridized by using sense probes for the relevant cytokines. Additional sections were treated with RNase A before the prehybridization step with antisense probes. Specific hybridization was recognized as clear dense deposits of silver grains in the emulsion fluid overlying cells in the tissue preparations. Cells were identified as dense, discrete, well-circumscribed areas of silver grains. When hybridized cells were in close proximity, their numbers were determined by visualizing the number of individual nuclei by using dark-field illumination. The cells expressing mRNA for each lymphokine were enumerated with an eyepiece graticule. Slides were counted in a blinded manner. For each condition, at least 3 sections were hybridized with the probes. Results were expressed as the number of positive cells per field. Entire sections were evaluated. The within-observer coefficient of variation was less than 5%.

Statistical analysis

Statistical analysis was performed by using the Statview 5 software. Data were expressed as mean $\pm$ SEM. Differences within the 3 groups were evaluated by using a one-way ANOVA. Statistically significant differences between groups were subsequently analyzed by using the post hoc *t* test for multiple comparisons Bonferroni *t* test. Comparisons between bronchial and MSG data were performed by using a paired *t* test. Correlations were performed by using linear regression analysis. As indicated in the software, only *P* values of less than .0167 were considered significant for the Bonferroni tests, whereas *P* values of less than .05 were considered significant for the other tests.

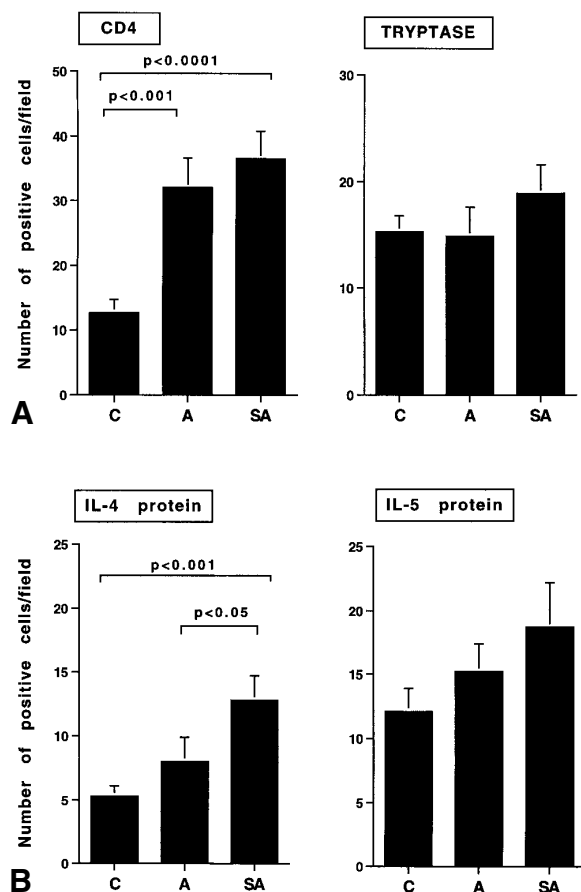


FIG 1. Immunocytochemical pattern of MSG CD4⁺ T-cell and mast cell infiltration (**A**) and of IL-4 and IL-5 immunoreactivity (**B**) in untreated asthmatic subjects (A), steroid-treated asthmatic subjects (SA), and control subjects (C). Results are expressed as the mean number of positive cells $\pm$ SEM per field. Statistical analysis was performed by using the Bonferroni *t* test for multiple comparisons. When differences were statistically significant, *P* values are indicated.

RESULTS

MSG pathologic examination

The importance and the composition of the cellular infiltrate in the subjects are shown in Table II. An inflammatory infiltrate was present in almost all asthmatic patients (23/25 patients) and in 3 control subjects, although with a low Chisholm score of 1. There was a significant difference in Chisholm scores between control subjects and asthmatic subjects irrespective of treatment but not between allergic and nonallergic asthmatic subjects (1.82 ± 0.24 and 2.3 ± 0.23 , respectively). Few and only intra-vascular eosinophils were found in two untreated asthmatic subjects who had high levels of circulating eosinophils ($>2000/\text{mm}^2$). Mast cells were present in all groups but often as degranulated cells in asthmatic patients without differences according to the treatment and whether the patients were allergic. In control subjects mast cells were also present, although as nondegranulated cells.

Immunohistochemistry analysis

Cellular infiltration. Cellular infiltration is shown in Fig 1, A. The cellular infiltrate was further examined by using immunohistochemistry. There was an increase in the number of CD4⁺ T lymphocytes in untreated asthmatic subjects (32.0 ± 4.6 per field) and in steroid-treated asthmatic subjects (36.5 ± 4.3 per field) compared with control subjects (12.6 ± 2.1 per field, $P < .001$). Lymphocytes appeared either as lymphoid aggregates or scattered in the MSGs (Fig 2, a). There was an inverse correlation between the numbers of CD4⁺ cells and FEV₁ values in the asthmatic group ($r = 0.52$, $P < .01$; Fig 3). No difference was observed between the number of CD4⁺ cells found in allergic (34.6 ± 3.9 per field) and the number found in nonallergic asthmatic subjects (34.5 ± 5.4 per field). There was no significant difference in tryptase-positive mast cells between untreated asthmatic subjects (14.9 ± 2.7 per field), treated asthmatic subjects (18.9 ± 2.7 per field), and control subjects (15.3 ± 1.5 per field) and between allergic (17.9 ± 2.8 per field) and nonallergic asthmatic subjects (16.0 ± 2.5 per field). Mast cells were scattered in MSGs (Fig 2, b). No EG2⁺ eosinophil cells were found, except intravascularly, in two patients, as already described in the pathologic examination section.

Cytokine protein production. Cytokine protein production is shown in Fig 1, B. The number of cells showing IL-4 immunoreactivity was increased in steroid-treated asthmatic subjects (12.8 ± 1.9 cells per field) compared with untreated asthmatic subjects (8.0 ± 1.9 cells per field, $P < .05$) and control subjects (5.3 ± 0.8 cells per field, $P < .001$). The positive IL-4-immunoreactive cells were scattered in MSGs (Fig 2, c). For IL-5⁺ cells, there was no significant difference between untreated asthmatic subjects (15.2 ± 2.2 cells per field), steroid-treated asthmatic subjects (18.7 ± 3.5 cells per field), and control subjects (12.1 ± 1.8 cells per field, $P = \text{not significant}$). There was a significant correlation between the numbers of tryptase-positive cells and IL-4⁺ ($r = 0.49$, $P < .005$) and IL-5⁺ cells ($r = 0.60$, $P < .0003$). Few cells were positive for IFN- γ in the untreated asthmatic subjects (0.20 ± 0.1 cells per field), steroid-treated asthmatic subjects (0.46 ± 0.17 cells per field), or control subjects (0.41 ± 0.13 cells per field; $P = \text{not significant}$, data not shown). Again, no differences were observed between allergic and nonallergic asthmatic subjects, irrespective of the cytokine type (IL-4, 11.7 ± 1.8 and 9.0 ± 2.4 per field; IL-5, 18.5 ± 2.6 and 14.9 ± 4.2 per field; IFN- γ , 0.5 ± 0.2 and 0.09 ± 0.04 per field).

Double-staining experiments. Double-staining experiments for IL-4 protein immunoreactivity showed that most IL-4 product was contained in mast cells ($63.7\% \pm 5.9\%$), and the rest was localized to small lymphocyte-like cells ($36.3\% \pm 5.9\%$, mean for 6 different asthmatic patients; Fig 2, d). In control subjects IL-4 protein was only localized to mast cells. When compared with total tryptase-positive cells, 38% of mast cells were found to stain with anti-IL-4 antibody in asthmatic subjects and in

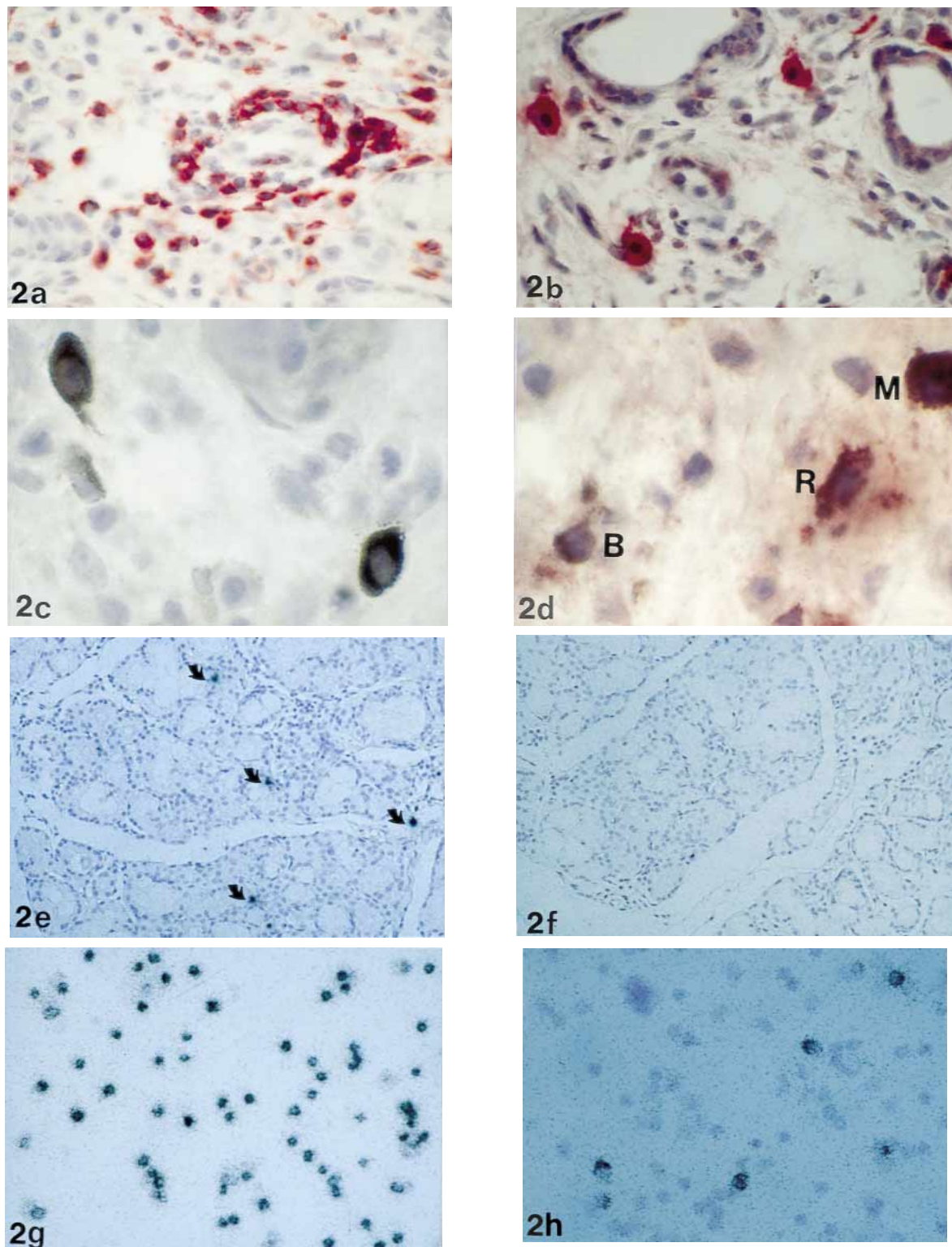


FIG 2. Photomicrographs of MSG sections. Example of CD4⁺ (a, magnification 400 $\times$) and tryptase staining (b, magnification 400 $\times$; positive cells in red) and of IL-4 protein staining (c, magnification 1000 $\times$; positive cells in black) in MSGs from asthmatic patients. Double staining of MSGs from an asthmatic patient with IL-4 and tryptase at 1000 $\times$ magnification is shown in d. Single IL-4⁺ cells stain brown-black (B), single tryptase-positive cells stain red (R), and double IL-4/tryptase-positive cells stain maroon (M). MSG section from an asthmatic patient hybridized with an antisense (e, arrows indicate positive cells) and sense (f) probes for IL-4 at 250 $\times$ magnification is also shown. Positive controls are peripheral T-cell clones from a subject with hyper-IgE syndrome hybridized with an antisense probe for IL-4 (g) and PHA-stimulated mononuclear cells hybridized with an antisense probe for IFN- γ (2 hours) at 400 $\times$ magnification. Positive cells appear as a well-circumscribed area of silver grains.

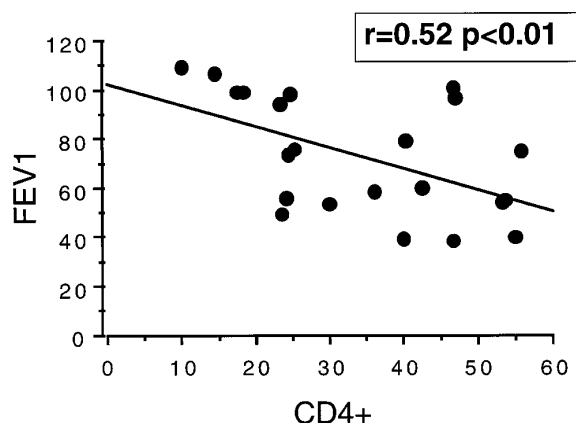


FIG 3. Linear regression analysis of the number of CD4⁺ cells in MSGs versus FEV₁ values in asthmatic patients.

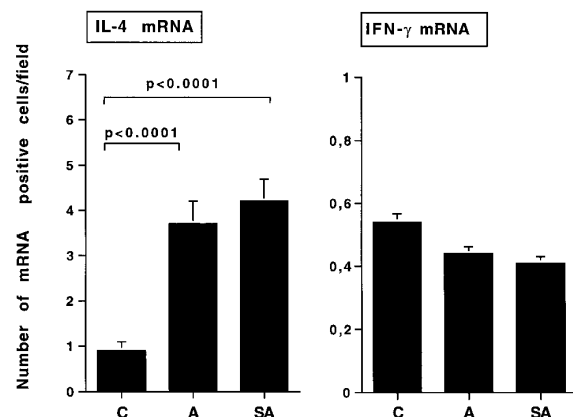


FIG 4. IL-4 and IFN- γ mRNA expression of MSGs in untreated asthmatic subjects (A), steroid-treated asthmatic subjects (SA), and control subjects (C). Results are expressed as the mean number of positive cells $\pm$ SEM per field. Statistical analysis was performed by using the Bonferroni *t* test for multiple comparisons. When differences were statistically significant, *P* values are indicated.

control subjects. For IL-5, only mast cells contained the cytokine product, and all mast cells were positive for it in both asthmatic and control subjects.

In situ hybridization experiments

Cytokine mRNA expression in MSGs. Cytokine mRNA expression in MSGs is shown in Fig 4. The number of cells expressing mRNA for IL-4 was increased in untreated asthmatic subjects (3.7 ± 0.5 cells per field) and in treated patients (4.2 ± 0.5 cells per field) compared with control subjects (0.9 ± 0.2 cells per field; $P < .0001$ and $P < .0001$, respectively). There was no difference between allergic (3.8 ± 0.6 mRNA⁺ cells per field) and nonallergic asthmatic subjects (4.3 ± 0.4 mRNA⁺ cells per field). A correlation was observed between the number of IL-4 mRNA-expressing cells and the severity of the disease (Aas score; $r = 0.41$, $P < .05$) in asthmatic subjects and the Chisholm score ($r = 0.50$, $P < .001$). The

positive IL-4 mRNA cells were scattered in MSGs and were not predominantly found in the biggest focal inflammatory cell aggregates (Fig 2, *e*). Positive and negative controls are shown in Fig 2, *f*, *g*, and *h*. Few cells expressed IFN- γ mRNA in both the asthmatic patients (0.41 ± 0.13 cells per field) and the control subjects (0.54 ± 0.3 cells per field). Concerning IL-5, no mRNA expression was observed in either the asthmatic or the control subjects.

Cytokine mRNA expression in bronchial biopsy specimens from asthmatic and control subjects. To directly compare the results obtained in MSGs with the bronchial level in the same subjects, we evaluated IL-4 and IL-5 mRNA expression, as well as cytokine protein expression and CD4 and eosinophil infiltration, in 8 asthmatic subjects (4 steroid-treated and 4 untreated subjects) and 7 control subjects in whom bronchial biopsy specimens were available concomitantly to MSG. As previously described in the literature, increased numbers of IL-4 and IL-5 mRNA⁺ cells were found in bronchial biopsy specimens from asthmatic subjects irrespective of treatment ($P < .002$), as well as increased numbers of IL-4 immunoreactive cells ($P < .05$) and eosinophils ($P < .001$) compared with control subjects. The results of the comparative evaluation in MSG and bronchial biopsy specimens for the 15 subjects are shown in Table III. Significant differences between MSG and bronchial biopsy specimens were observed in asthmatic subjects in terms of eosinophils and IL-5 mRNA expression ($P < .01$). No differences were observed between MSG and bronchial biopsy specimens from control subjects.

DISCUSSION

In this study we have confirmed our previous findings of the presence of MSG pathologic abnormalities in asthmatic patients mimicking some of those observed in the bronchial mucosa in asthma, particularly the presence of infiltrating T lymphocytes, but a lack of eosinophils. To better understand the mechanisms underlying these abnormalities, local cytokine expression (ie, IL-4, IL-5, and IFN- γ) has been evaluated at the level of mRNA and protein.

IFN- γ and IL-4 have antagonizing effects. Although IL-4 favors B-cell isotype switching toward IgE production^{7,25} and inhibits IFN- γ at the transcriptional level,²⁶ IFN- γ suppresses class switching to IgE production induced by IL-4.^{25,27} Few IFN- γ mRNA⁺ cells were found in MSGs from asthmatic or control subjects. This is in line with different studies that have shown a preferential T_H2-type cytokine profile at sites of allergic tissue reactions with little or no IFN- γ .^{8,28,29} In MSGs a general increase in the number of cells expressing IL-4 mRNA and immunoreactive protein was observed in asthmatic subjects compared with control subjects, with no difference of expression between allergic and nonallergic asthmatic subjects. This is in line with previous data showing similar increases in allergic and nonallergic asthma.¹⁰

In recent years, an increasing variety of cell types have

	Bronchi	Minor Salivary Gland	Gut
T-Lymphocytes	++	++	++
Eosinophils	++	0	++
Mast cells	+	++	++
IL-5 protein	++	++	++
IL-5 mRNA	++	0	?
IL-4 protein	++	++	?
IL-4 mRNA	++	++	++
IL-3 protein	++	?	++
IL-3 mRNA	++	?	?
ICAM-1	++	+	?
VCAM-1	++	0	?
Eotaxin	++	?	++

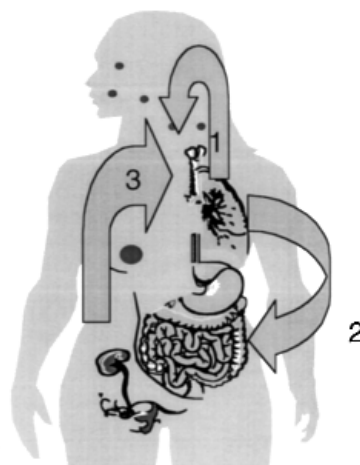


FIG 5. Summary of the data obtained in different mucosal sites in asthmatic patients in the context of lymphocyte recirculation. The mucosal immune system is composed of the lymphoid tissues associated with mucosal surfaces in the respiratory (bronchus-associated lymphoid tissue) and gastrointestinal (gut-associated lymphoid tissue) tracts and other immune mucosal sites, such as salivary glands. The concept of the common mucosal immune system suggests that activated lymphocytes can migrate from one mucosal site to another one. Therefore one can hypothesize that the sensitization of bronchus-associated lymphocytes in asthma may be responsible for the induction of an immune response in other mucosal sites (see diagram; ie, salivary glands [arrow 1] and gut [arrow 2]). Similarly activated lymphocytes sensitized at the gut level are able to migrate to the bronchial level (like in Crohn's disease, arrow 3). The table indicates the biologic characteristics of bronchi, MSGs, and gut tissue in asthma. An airway-like inflammation has been reported in the gut of asthmatic patients⁵¹; an accumulation of lymphocytes and mast cells, but not eosinophils, has been reported in MSGs in asthma (see "Discussion" section for details).¹⁵

TABLE III. Comparison of the cellular infiltration and cytokine expression in MSG and bronchial biopsy specimens from asthmatic patients and control subjects

	Control subjects		Asthmatic subjects	
	MSG specimens	Bronchial specimens	MSG specimens	Bronchial specimens
CD4 ⁺ cells	14.45 ± 3.8	21.63 ± 1.7	30.58 ± 6.1	35.13 ± 7.3
Eosinophils	0	1.45 ± 0.6	0	20.7 ± 5.5*
IL-4 mRNA	0.97 ± 0.9	0.49 ± 0.6	3.5 ± 1.5	5.68 ± 3.0
IL-5 mRNA	0	0.33 ± 0.6	0	4.02 ± 2.5*
IL-4 protein	4.6 ± 0.5	5.2 ± 1.0	11.9 ± 3.0	16.6 ± 3.3
IL-5 protein	11.6 ± 2.3	3.7 ± 0.4	16.1 ± 5.1	6.2 ± 2.5

Values are expressed as mean ± SEM for 8 asthmatic subjects, including 4 treated and 4 untreated patients and 7 control subjects.

*Significantly different from MSGs ($P < .007$ for eosinophils and $P < .002$ for IL-5 mRNA).

been shown to produce IL-4. Besides T lymphocytes, mast cells, basophils, and eosinophils can release IL-4, sometimes as a preformed mediator after IgE-dependent stimulation.³⁰⁻³⁶ The respective importance of these different cells in the IL-4 production observed in asthma is controversial. In some studies T lymphocytes have been shown to be the main source of IL-4,⁹ whereas in others mast cells appear to be the predominant cell source.³⁴ Recent studies have shown that these discrepancies are in fact related to the technique used for IL-4 identification. Indeed, immunohistochemistry illustrates mainly mast cell cytokine protein (related to the preformed storage of these molecules), whereas in situ hybridization shows mainly cytokine mRNA expressed by T cells. The failure to colocalize IL-4 protein (as opposed to mRNA) to T cells is attributed to the inability of T cells to store and concentrate sufficient intracellular amounts of this

cytokine to produce positive immunostaining.^{37,38} In the present study, when considering IL-4 immunoreactivity, there was a good correlation with the number of tryptase-positive mast cells in MSGs, suggesting that mast cells were an important cell source. This was confirmed by double-staining experiments of MSG sections from asthmatic subjects, which showed that mast cells accounted for 63.7% of the IL-4 immunoreactivity with few small lymphocytic-like cells staining for IL-4 (<10% of total T cells). Concerning IL-4 mRNA expression, several elements are in favor of a lymphocytic origin. First, there was a significant correlation between IL-4 mRNA and the Chisholm score defined on lymphocyte infiltration. Second, IL-4 mRNA⁺ cells were small round-shaped cells. Third, counterstaining of positive IL-4 mRNA MSG slides with Alcian blue Safranin staining, which is specific for mast cells, showed that mast cells were not the IL-

4 mRNA⁺ cells, and lymphocytes are the only other cells present in the MSG infiltrate able to express IL-4. The failure to colocalize IL-4 mRNA to mast cells may be linked to the absence of local antigen stimulation, whereas lymphocytes that recirculate can be activated at the bronchial level. All these results suggest that IL-4 immunoreactivity reflected mainly the mast cell origin while IL-4 mRNA probably reflected the lymphocyte origin to a greater extent. Steroids have been reported to downregulate IL-4 mRNA expression at the bronchial level in asthmatic subjects when evaluated in the same patients.³⁹ In MSGs from asthmatic subjects, there was no difference in IL-4 mRNA expression between untreated and steroid-treated patients, although treated patients had significantly more severe levels than untreated patients. Therefore one cannot exclude the possibility that steroids might have downregulated MSG IL-4 mRNA to levels similar to those observed in untreated patients.

No eosinophils were found in MSGs except in two patients in whom they were present in MSG microvessels, whereas this cell type was present at the bronchial level, showing a clear dichotomy between the two sites. One explanation might be that eosinophils cannot enter MSGs because of a lack of endothelial molecule adhesion. Studies have shown that IL-4 enhances the *in vitro* expression of the adhesion molecule VCAM-1 on the surface of endothelial cells.^{40,41} Because VCAM-1 participates in selective eosinophil adhesion through a very late antigen 4-dependent mechanism, IL-4 may account, at least in part, for selective eosinophil recruitment at sites of allergic tissue reactions.^{42,43} In a previous study we showed that in MSGs from asthmatic subjects, the adhesion molecule ICAM-1 was expressed on endothelial cells but not E-selectin and VCAM-1.¹⁵ Thus although IL-4 was present in MSGs, no VCAM-1 expression was induced. This dissociation has also been observed in *in vivo* studies, which showed that IL-4 was not a major factor in antigen-induced VCAM-1 expression⁴⁴ and that intradermal injection of IL-4 in monkeys induced no VCAM-1 expression on the endothelium.⁴⁵ Although the absence of VCAM-1 does not allow eosinophil endothelium transmigration into MSG tissue through the very late antigen 4/VCAM-1 pathway, eosinophils could theoretically use the other pathway of interaction, which is ICAM-1/lymphocyte function antigen-1 dependent⁴⁶ because ICAM-1 is expressed in MSGs from asthmatic patients. Hence an essential stimulus is probably missing. Among eosinophil-active factors, IL-5 has major importance. Indeed, IL-5 promotes eosinophil adhesion to vascular endothelium through the ICAM-1/lymphocyte function antigen-1 pathway⁴⁶ and also promotes the terminal differentiation of committed eosinophil precursors, activates mature eosinophils, and prolongs their survival.^{6,47} Although present, IL-5 immunoreactivity was exclusively located to mast cells but not to lymphocytes, as is the case for IL-4. Moreover, no IL-5 mRNA expression was evidenced, reinforcing the idea that lymphocytes found in MSGs from asthmatic patients do not express and produce IL-5. The fact that no eosinophil was attracted in

MSGs, although IL-5 was present in the environment through degranulated mast cells, suggests that mast cell-derived IL-5 is not sufficient *per se* to attract eosinophils. This might be due to insufficient levels of IL-5 or to a lack of certain additional signals required for eosinophil infiltration, such as chemokine expression, as shown with eotaxin for IL-5 transgenic mice.⁴⁸ Further experiments are needed to test this hypothesis.

An increased CD4⁺ T-lymphocyte infiltration was evidenced by immunohistochemistry in MSGs from asthmatic subjects compared with control subjects, which correlated with the bronchial obstruction assessed by FEV₁. Several studies have shown the importance of CD4⁺ T cells in asthma, and in particular, it has been shown that a systemic treatment with humanized anti-CD4 mAbs improves the peak expiratory flow of asthmatic subjects⁴⁹ and that there is a relationship between bronchoalveolar lavage-activated CD4 cells and FEV₁ in asthmatic subjects.⁵⁰ The pathologic similarities observed between MSGs and the bronchial mucosa from asthmatic subjects, as well as the correlations observed between the clinical severity of asthma and MSG CD4⁺ cells and MSG IL-4 mRNA expression, suggest that the infiltrating T lymphocytes found in MSGs might be the consequence of a homing process involving the common mucosal system, particularly T lymphocytes activated in the bronchi. If this hypothesis is true, such inflammatory reactions should also be observed in other mucosal areas, such as the gastrointestinal tract. Indeed, in a previous study we demonstrated the presence of an inflammatory infiltrate in the intestinal mucosa obtained from asthmatic patients but not from control subjects.⁵¹ In addition to lymphocytes, eosinophils were also evidenced in the intestine, where eotaxin is constitutively expressed. In the hypothesis of a homing process involving the common mucosal system, lymphocytes found at the MSG site should reflect those found at the bronchial level. A diagram summing up the data of the present and previous studies in the context of mucosal lymphocyte recirculation is presented in Fig 5. However, lymphocytes from the MSG site, although expressing IL-4, did not express IL-5 mRNA. This was not related to a lack of IL-5 mRNA expression related to steroid treatment in the patients because IL-5 mRNA was detected in bronchial biopsy specimens from untreated and steroid-treated patients. Although other cells than lymphocytes (particularly eosinophils) have been shown to express IL-5 mRNA, double-staining experiments have shown that lymphocytes represent the main IL-5-expressing cell in bronchial asthma.³⁷ The results obtained in the present study suggest that in MSGs, lymphocyte IL-4 mRNA expression was dissociated from IL-5 mRNA expression, which has been already observed in other studies.^{52,53} The induction of IL-5 mRNA expression by T cells might need additional costimuli not present in MSGs but found at the bronchial level.

In conclusion, although an infiltration of CD4⁺ T lymphocytes was found in MSGs from asthmatic patients, possibly through lymphocyte recirculation from the

bronchi, the analysis of cytokine mRNA expression showed some differences with that of the bronchus, suggesting different local immune regulations.

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