

Adhesion molecules and their ligands in nasal polyps of aspirin-hypersensitive patients

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Background: Chronic inflammation with tissue eosinophilia plays a key role in the pathogenesis of asthma and nasal polyps in patients with aspirin hypersensitivity.

Objective: To evaluate the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and their ligands (the integrins lymphocyte function-associated antigen 1 and very late-activation antigen 4 [VLA-4]) in nasal polyps of patients with aspirin hypersensitivity compared with aspirin-tolerant individuals.

Methods: Immunohistochemical studies were performed using a peroxidase method and monoclonal antibodies on 6- μ m-thick cryostat sections cut from frozen polyps collected during elective surgery from 21 aspirin-sensitive and 23 aspirin-tolerant patients.

Results: The mean $\pm$ SD values of the semiquantitatively evaluated immunoexpression of ICAM-1, VCAM-1, and VLA-4 were significantly increased in patients with aspirin hypersensitivity compared with aspirin-tolerant patients (1.7 ± 0.8 vs 0.9 ± 0.8 , $P < .003$; 1.8 ± 0.8 vs 0.8 ± 0.8 , $P < .001$; and 2.2 ± 0.7 vs 1.3 ± 0.7 , $P < .001$, respectively), whereas the mean $\pm$ SD values of the expression of lymphocyte function-associated antigen 1 did not differ significantly (2.4 ± 0.5 vs 2.2 ± 0.9 ; $P = .57$). We found a correlation between the immunoexpression of VCAM-1 and its ligand VLA-4 in all studied tissue samples ($r = 0.4$; $P < .02$).

Conclusions: In nasal polyps of aspirin-hypersensitive patients, up-regulation of the adhesion molecules ICAM-1 and VCAM-1 and the integrin VLA-4 may play an important role in the development of chronic eosinophilic inflammation.

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INTRODUCTION

Nasal polyps emerge as pale-gray, semitranslucent protuberances in the area of the ethmoid bone and middle turbinate. Clinically, nasal polyps should often be regarded as a manifestation of other systemic diseases. Patients with cystic fibrosis, primary ciliary dyskinesia, and Churg-Strauss syndrome are known to experience recurrent rhinosinusitis and nasal polyposis.¹ Bronchial asthma is another disease comorbid with nasal polyposis. Up to 40% of cases of nasal polyps are associated with intrinsic asthma,² and 8% to 36% of patients with nasal polyps have aspirin intolerance. The association of nasal polyps and asthma with bronchoconstriction precipitated by aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs), nowadays called the “aspirin triad,” has been described by Widall et al³ and further studied by Samter and Beers.⁴ The mechanisms underlying aspirin sen-

sitivity are thus far not completely defined. The inhibitory action of NSAIDs on prostaglandin biosynthesis is considered to be one of the possible explanations of asthma exacerbations in aspirin-intolerant asthmatic patients. Most NSAIDs are potent inhibitors of cyclooxygenase-1 and cyclooxygenase-2.⁵ In individuals with aspirin hypersensitivity, disruption of the synthesis of prostaglandins E_2 and I_2 and overproduction of cysteinyl leukotrienes seem to be crucial in the pathogenesis of bronchial and nasal symptoms.^{6,7} This original hypothesis by Szczeklik et al^{8,9} was confirmed in several further studies. Tissue eosinophilia is a characteristic finding in nasal polyps associated with aspirin sensitivity. Activated eosinophils with decreased apoptosis ratios¹⁰ contribute to nasal polyp abnormalities by basic protein deposition, which can induce epithelial disruption and basement membrane denudation. Activated eosinophils produce several inflammatory mediators and cytokines, including, but not restricted to, interleukin 3 (IL-3), IL-4, IL-5, IL-13, RANTES (regulated upon activation, normal T cell expressed and secreted), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Bachert et al¹¹ proposed that the abundance of eosinophils in nasal polyps could be explained by a migration increase or prolonged survival of these cells. The recruitment of eosinophils from peripheral blood into polyp tissue is governed by adhesive interactions between leukocytes and endothelial cells. Adherence of cells to the endothelium involves adhesion molecules such as intercellular adhesion

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molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells and their ligands on leukocytes: lymphocyte function-associated antigen 1 (LFA-1) and very late-activation antigen 4 (VLA-4).^{12,13}

The aim of this study was to evaluate the expression of adhesion molecules (ICAM-1 and VCAM-1) and their ligands (LFA-1 and VLA-4) in nasal polyps of patients with aspirin hypersensitivity compared with aspirin-tolerant patients. We hypothesize that the results of this study will add to our understanding of chronic inflammation in the aspirin triad because the mechanisms of eosinophil migration in aspirin-sensitive patients have not yet been determined.

MATERIALS AND METHODS

Study Participants

Twenty-one aspirin-sensitive patients (14 women and 7 men; mean $\pm$ SD age, 54.9 $\pm$ 11.9 years) and 23 aspirin-tolerant patients (9 women and 14 men, mean $\pm$ SD age, 52.9 $\pm$ 14.1 years) were included in the study. Patients' medical histories, demographic characteristics, and clinical data were collected. In the aspirin-sensitive group, 16 patients (76%) also had asthma, in contrast to the aspirin-tolerant group, in which only 4 asthmatic individuals (17%) were found ($P < .001$) (Table 1). Patients with aspirin hypersensitivity had significantly more polypectomies than aspirin-tolerant patients (3.6 vs 1.8; $P = .04$). Aspirin hypersensitivity or tolerance had to be confirmed using oral or inhaled aspirin challenge, which was performed (as previously described by Dahlen and Zetterstrom¹⁴) at least 6 months before the polypectomy. Skin prick tests with a set of 8 allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, grass, trees, weeds, and feathers) were performed on the volar surface of the forearm using 1 lancet for 1 prick.^{15,16} All wheal sizes were measured as the mean of the longest diameter and the midpoint of the perpendicular diameter. A positive reaction was defined as a wheal at least 3 mm greater than the reaction to the saline control. Atopy was defined as having a positive skin prick test reaction to at least 1 tested allergen.

Nasal polyp samples were collected during routine, elective endonasal surgery. The tissue samples were frozen and

stored at -70°C until immunochemical analysis. None of the patients had received systemic or topical corticosteroids or leukotriene antagonists for at least 4 weeks before surgery. None had had any clinical demonstrable ear, nose, or throat infections during the month preceding surgery or a history of chronic bronchitis, cystic fibrosis, or Wegener granulomatosis. Informed consent was obtained from all the participants, and the ethics committee of the Medical University of Łódź approved the study protocol.

Immunohistochemical Analysis

Immunohistochemical studies were performed on 6- μm -thick cryostat sections cut from each frozen polyp. The sections were mounted onto glass slides (Superfrost Plus; Erie Scientific Co, Portsmouth, NH). One section from each polyp was stained with hematoxylin-eosin for light microscopic examination. Immunostaining was then performed at room temperature using a peroxidase method (EnVision+ Kits; DakoCytomation, Glostrup, Denmark) with horseradish peroxidase and diaminobenzidine. The monoclonal antibodies used and their specific reactivities are listed in Table 2. All the monoclonal antibodies were diluted in a ratio of 1:50.

The sections were air-dried and fixed in acetone for 10 minutes. Endogenous peroxidase was blocked for 5 minutes using 0.03% hydrogen peroxide containing sodium azide. One of a series of mouse monoclonal antibodies prepared in Tris-buffered saline containing 1% bovine serum was applied to each slide for 40 minutes in a moist chamber. After washing twice in Tris-buffered saline, the sections were incubated with labeled polymer horseradish peroxidase for 30 minutes, and then prepared diaminobenzidine substrate-chromogen solution was applied for 1 minute. After a final washing with water, the sections were counterstained with hematoxylin and coverslipped.

Tissue Control for Immunostaining

For each monoclonal antibody and for each sample, a positive control was processed using cryostat sections of surgically removed tonsil. Furthermore, the following negative controls were used: (1) omission of primary antibody and (2) incubation with appropriately diluted mouse IgG (DakoCytomation) as a first layer. The samples were prepared using the same

Table 1. Basic Characteristics of the Study Group

	Aspirin-sensitive patients (n = 21)	Aspirin-tolerant patients (n = 23)	P value
Age, mean $\pm$ SD, y	54.89 $\pm$ 11.86	52.95 $\pm$ 14.07	.98*
Sex, F:M, No.	14:7	9:14	.07†
Asthma, No.	16	4	<.001‡
Allergy, No.§	4	9	.09‡
Polypectomies, mean range, No.	3.63 (1–17)	1.76 (1–7)	.04*

* By Mann-Whitney *U* test.

† By χ^2 test.

‡ By Fisher test.

§ At least 1 positive skin prick test reaction (≥ 3 mm) to 1 of the tested allergens.

Table 2. Monoclonal Antibodies Used in the Study and Their Specificity

Monoclonal Antibody	Specificity	Source
ICAM-1 (CD54+)	Some monocytes, endothelial cells, lymphocytes, fibroblasts, and epithelial cells	Novocastra Laboratories Ltd, Newcastle upon Tyne, England
VLA-4	Fibroblasts, monocytes, and lymphocytes	DakoCytomation, Glostrup, Denmark
VCAM-1 (CD106+)	Endothelial cells	DakoCytomation
LFA-1, α -chain (CD11a+)	Granulocytes, monocytes, and lymphocytes	DakoCytomation

Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late-activation antigen 4.

method as described in the “Immunohistochemical Analysis” subsection. Specificity of labeling was shown by lack of staining in these samples. The extent and intensity of immunoreactivity of ICAM-1, VLA-4, VCAM-1, and LFA-1 was evaluated semiquantitatively in a blinded manner by 2 independent readers as follows: 0 = none, 1 = weak and patchy, 2 = moderate, and 3 = strong and extensive. The results are expressed as means of measurements of 6 randomly selected high-power fields (median $\pm$ 95% confidence interval).

Statistical Methods

Differences between groups were tested using unpaired *t* tests preceded by evaluation of normality and the Levene test (Statistica 4.3; StatSoft Inc, Tulsa, OK). The Mann-Whitney *U* test, χ^2 test, and Fisher test were used where appropriate. *P* < .05 was considered statistically significant.

RESULTS

Examination by light microscopy of sections stained with hematoxylin-eosin confirmed the diagnosis of nasal polyps in all the patients. When present, ICAM-1 immunoreactivity was distributed on some epithelial cells and in the submucosa on inflammatory cells and fibroblasts (Fig 1), whereas LFA-1 immunoreactivity was limited to inflammatory cells (Fig 2). Immunoreexpression of VCAM-1 was present on endothelial cells, especially of the small vessels (Fig 3). Immunoreactivity of VLA-4 was noted only in the submucosa on inflammatory cells and fibroblasts (Fig 4).

Semiquantitative data on the immunoexpression of ICAM-1, VLA-4, VCAM-1, and LFA-1 in the aspirin-sensitive and aspirin-tolerant groups are given in Table 3. The mean $\pm$ SD values of the immunoexpression of ICAM-1, VCAM-1, and VLA-4 were significantly increased in aspirin-sensitive patients compared with aspirin-tolerant patients (1.7 ± 0.8 vs 0.9 ± 0.8 , *P* < .003; 1.8 ± 0.8 vs 0.8 ± 0.8 , *P* < .001; and 2.2 ± 0.7 vs 1.3 ± 0.7 , *P* < .001), whereas the mean $\pm$ SD values of the expression of LFA-1 did not differ significantly (2.4 ± 0.5 vs 2.2 ± 0.9 ; *P* = .57). We found a correlation between the immunoexpression of VCAM-1 and its ligand VLA-4 in all the studied tissue samples (Spearman *r* = 0.4; *P* < .02) but not in a subgroup of aspirin-sensitive patients (*r* = 0.4; *P* = .06) and aspirin-tolerant patients (*r* = -0.4; *P* = .09). We did not find a correlation between ICAM-1 and LFA-1 expression in all samples (*r* = 0.1; *P* = .51) or in aspirin-sensitive patients (*r* = 0.3; *P* = .21) and aspirin-tolerant patients (*r* = 0.1; *P* = .53) (Table 4).

DISCUSSION

The results of this study show that expression of the adhesion molecules ICAM-1 and VCAM-1 and the ligand VLA-4 is up-regulated in nasal polyps of patients with aspirin hypersensitivity compared with aspirin-tolerant individuals. There was no difference in LFA-1 expression between the studied

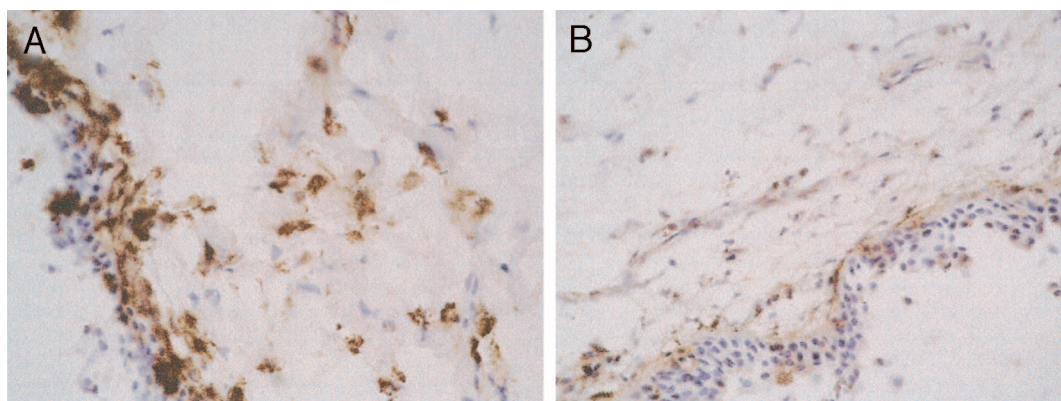


Figure 1. Immunoreexpression of intercellular adhesion molecule 1 (brown staining) on some epithelial cells and on inflammatory cells and fibroblasts in the submucosal area in a representative section of polyp tissue from aspirin-sensitive (A) and aspirin-tolerant (B) patients (original magnification $\times 400$).

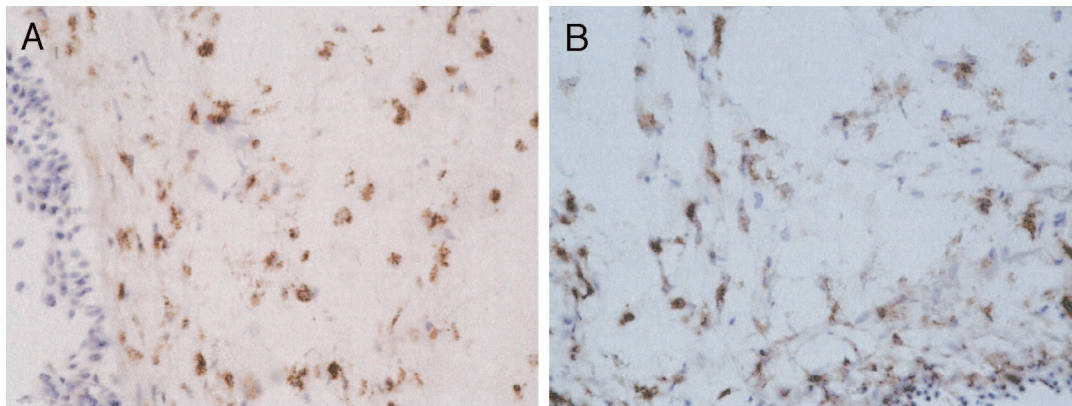


Figure 2. Immunorexpression of lymphocyte function–associated antigen 1 (brown staining) on inflammatory cells in the submucosal area in a representative section of polyp tissue from aspirin-sensitive (A) and aspirin-tolerant (B) patients (original magnification $\times 400$).

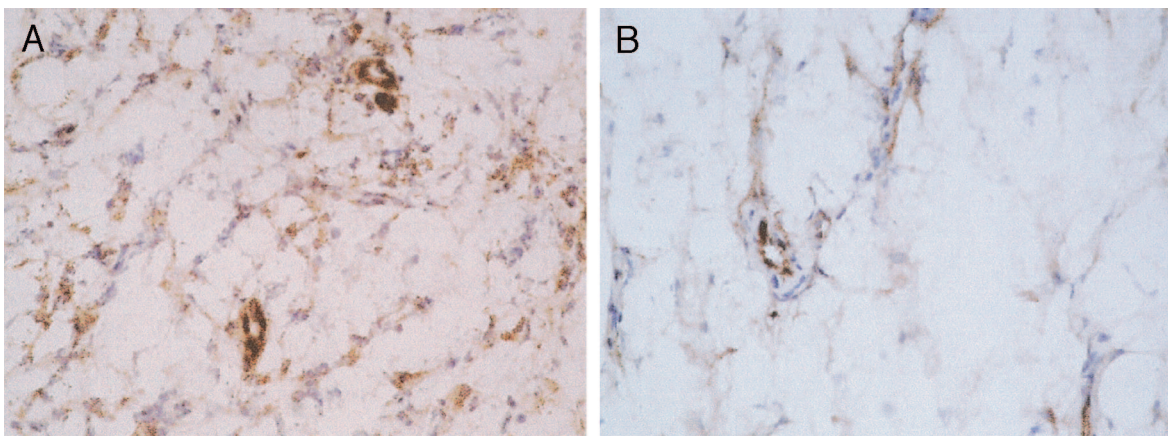


Figure 3. Immunorexpression of vascular cell adhesion molecule 1 (brown staining) on endothelial cells of the small vessels in the aspirin-sensitive group (A) and on fewer endothelial cells in the aspirin-tolerant group (B) (original magnification $\times 400$).

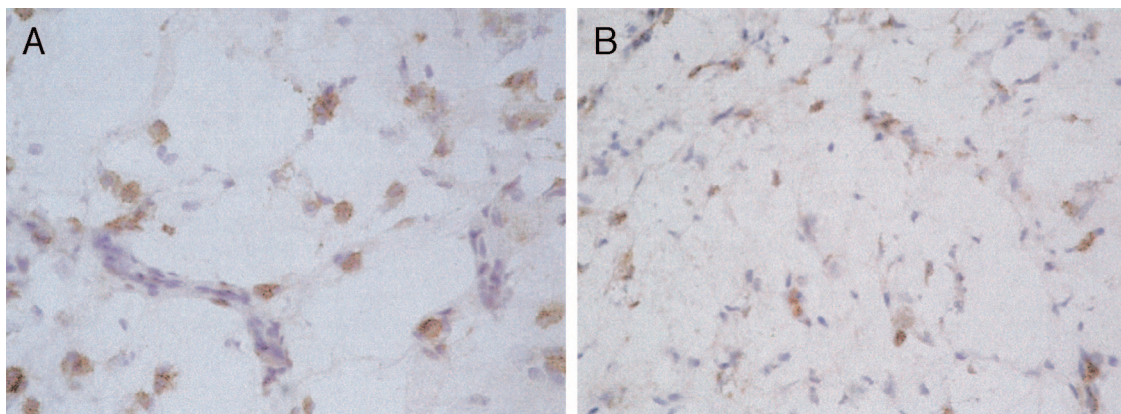


Figure 4. Immunorexpression of very late–activation antigen 4 (brown staining) on inflammatory cells and fibroblasts in the submucosal area in a representative section of polyp tissue from aspirin-sensitive (A) and aspirin-tolerant (B) patients (original magnification $\times 400$).

groups. We found a correlation between the immunorexpression of VCAM-1 and the ligand VLA-4 but not between ICAM-1 and LFA-1.

In histopathologic studies of nasal polyps, use of other reference tissue (from healthy individuals or from the same patients after polyp removal) can give us an idea about

Table 3. Semiquantitative Data on the Immunoexpression of ICAM-1, VLA-4, VCAM-1, and LFA-1 in the Study Groups*

	Aspirin-sensitive patients (n = 21)	Aspirin-tolerant patients (n = 23)	P value
ICAM-1	1.71 ± 0.84	0.89 ± 0.81	<.003
LFA-1	2.38 ± 0.45	2.22 ± 0.86	.57
VCAM-1	1.83 ± 0.78	0.83 ± 0.83	<.001
VLA-4	2.22 ± 0.65	1.30 ± 0.66	<.001

Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late-activation antigen 4.

* Data are given as mean ± SD.

Table 4. Correlation Between the Immunoexpression of Adhesion Molecules and Their Ligands in Nasal Polyps of Aspirin-Sensitive and Aspirin-Tolerant Patients

Group	ICAM-1 vs LFA-1		VCAM-1 vs VLA-4	
	Spearman r	P value	Spearman r	P value
Aspirin sensitive (n = 21)	0.28	.21	0.41	.06
Aspirin tolerant (n = 23)	0.13	.53	0.36	.09
Total (n = 44)	0.1	.51	0.37	<.02

Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late-activation antigen 4.

average levels of adhesion molecules and their ligands. If we take into consideration the history of recurrent polyposis due to chronic inflammation, these tissue samples cannot be regarded as a "healthy" reference. Taking nasal mucosa samples from healthy individuals raises some ethical questions. The aim of this study was to compare the expression of ICAM-1, VCAM-1, VLA-4, and LFA-1 in aspirin-hypersensitive and aspirin-tolerant patients. That is why aspirin-tolerant patients with polyposis were used as a control group. Counting the eosinophils in the tissue samples may have been helpful to examine the correlation of eosinophil numbers and adhesion molecules; however, such a correlation has already been established in nasal polyps.¹⁷ In future studies, the difference in the number of vessels between groups should be evaluated, and staining with von Willebrand factor could be used to allow normalization of the adhesion molecule stain to the number of vessels.

To our knowledge, this is the first study to demonstrate that the expression of 2 major adhesion molecules—ICAM-1 and VCAM-1—is significantly higher in polyp tissue samples of patients with aspirin hypersensitivity compared with aspirin-tolerant patients. Demoly et al¹² evaluated ICAM-1 immuno-reactivity in nasal polyp and turbinate biopsy samples from patients with sinusitis and chronic allergic or nonallergic rhinitis without a history of aspirin intolerance. They found no difference in the pattern of expression of ICAM-1 between

those 2 groups of patients. Intercellular adhesion molecule 1 is known to be constitutively expressed on vascular endothelial cells, and it can be induced by inflammatory mediators on other extravascular cells.¹⁸ Montefort et al¹⁹ found increased expression of ICAM-1 and VCAM-1 in nasal mucosal biopsy specimens of patients with perennial allergic rhinitis compared with control subjects. In contrast, Lee et al¹⁸ showed that ICAM-1 and VCAM-1 were expressed basally in patients with seasonal allergic rhinitis and nonallergic controls, but only VCAM-1 was significantly up-regulated after allergen challenge. They also found an increase in the number of submucosal eosinophils after challenge, which correlated with VCAM-1 expression. The VLA-4/VCAM-1 adhesion pathway seems to be the most important mechanism for specific eosinophil migration.

We do not suggest that the primary up-regulation of adhesion molecules is responsible for the development of eosinophilic inflammation in aspirin-sensitive individuals; however, this hypothesis has been proposed by some researchers.¹¹ It is more likely that increased expression of ICAM-1 and VCAM-1 is secondary to inflammation induced by aspirin, but in our opinion it should be regarded as one of the main reasons for evolution of the acute phase into chronic eosinophilic inflammation. It has been widely accepted that in aspirin-sensitive patients, after the ingestion of aspirin or other NSAIDs, inhibition of cyclooxygenase, overproduction of leukotrienes, and inhibition of prostaglandin E₂ synthesis are responsible for the development of nasal and bronchial symptoms. Kowalski et al²⁰ found that epithelial cells from aspirin-intolerant patients generate significantly less prostaglandin E₂ compared with controls. Furthermore, an increase in 15-hydroxyeicosatetraenoic acid (an arachidonic acid metabolite) generation was observed only in epithelial cells from aspirin-sensitive patients after incubation with aspirin. The results of this study suggest that epithelial cells from the upper and lower respiratory tract are target cells for aspirin to induce an adverse reaction. It has been proved that 15-hydroxyeicosatetraenoic acid stimulates mediator release from mast cells²¹ and is a potent regulator of cell survival.²² Another interesting finding in nasal polyps of aspirin-sensitive patients was an increased number of CD45+RO cells, a subset of activated T cells.²³ A significant positive correlation between CD45+RO cells and the number of eosinophils was confirmed, suggesting a possible role for these cells as a source of chemotactic and survival factors for eosinophils.²³ Activated eosinophils produce and release granule proteins, reactive oxygen species, and lipid mediators, including leukotriene C₄, IL-4, IL-5, and eotaxin.²⁴ Hamilos et al¹⁰ demonstrated increased amounts of GM-CSF, IL-5, and RANTES in nasal polyp tissue. They suggest that local production of these cytokines explains the accumulation of numerous eosinophils in nasal polyps. Secretion of GM-CSF by eosinophils has been shown to be a mechanism for autocrine stimulation promoting eosinophil survival in the polyp tissue.²⁵ Decreased delayed programmed cell death (apoptosis) of

inflammatory cells, including eosinophils, is a common finding in nasal polyps from aspirin-sensitive individuals.²³

Endothelial adhesion molecules are up-regulated by IL-1 β , IL-4, and tumor necrosis factor α released from resident cells (mast cells and T lymphocytes,²⁶ presumably CD45+RO), but activated eosinophils should also be regarded as an important source of cytokines that affect their own migration pathway. Interleukin 4 up-regulates the expression of ICAM-1 and VCAM-1; furthermore, IL-5 and eotaxin are important chemoattractant factors for eosinophils. The highest levels of IL-5 were observed in patients with eosinophilic diseases, such as asthma and aspirin hypersensitivity.²⁷

In patients with aspirin sensitivity, why does the eosinophilic inflammation run in a protracted course, despite the avoidance of aspirin and cross-reacting drugs?²⁸ To our knowledge, activated eosinophils seem to be able to produce mediators that induce the release of the next generation of eosinophils from bone marrow,²⁹ their migration, and their activation³⁰ and that can decrease apoptosis.²³ Once the loop of chronic inflammation in aspirin-sensitive patients is induced, it is difficult to stop. The vicious cycle we suggest, that activated eosinophils attract more eosinophils into the polyps, does not explain how polyps start and why oral corticosteroids, a potent inducer of eosinophil apoptosis, cannot break this cycle indefinitely but only temporarily in most patients with polyps. Something may be initiating, or reinitiating, the process after corticosteroid therapy. For example, there could be a causative agent (eg, allergen, infection, or other unknown) stimulating a local T_H2 lymphocyte response and attracting eosinophils.

In conclusion, we found that expression of the adhesion molecules ICAM-1 and VCAM-1 and the ligand VLA-4 is up-regulated in nasal polyps of patients with aspirin hypersensitivity compared with aspirin-tolerant patients. We speculate that the introduction into therapy of selective inhibitors of specific adhesion molecules, such as ICAM-1 and VCAM-1 or anti-IL-4 and anti-IL-5 monoclonal antibodies, since IL-4 and IL-5 are the main cytokines that regulate eosinophil migration and survival, could be effective tools in breaking the loop of chronic eosinophilic inflammation in aspirin-sensitive patients. Further studies that include patients with aspirin-intolerant and aspirin-tolerant asthma are needed to evaluate this hypothesis.

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