

Topical azelastine reduces eosinophil activation and intercellular adhesion molecule-1 expression on nasal epithelial cells: An antiallergic activity

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Background: It is well known that allergen-specific nasal challenge (ASNC) is a fruitful tool with which to evaluate antiallergic activity exerted by a drug. Azelastine is a new antihistamine also available in topical form (i.e., nasal spray).

Objective: The aim of the study was to evaluate the effects of azelastine nasal spray on inflammatory changes after ASNC in both the early-phase reaction and the late-phase reaction.

Methods: The study had a double-blind, placebo-controlled, randomized, and parallel-group design. Twenty patients with pollen allergy were enrolled out of pollen season. ASNC was performed at baseline (T0) and after 1 week of washout (T7). At T7, 10 patients sprayed azelastine (1 puff) into their nostrils, and 10 patients used placebo. ASNC was performed after 30 minutes. The considered parameters (evaluated during early- and late-phase reactions) were: (1) clinical signs and symptoms, (2) cytologic assessment (neutrophils and eosinophils), (3) assay of mediators (eosinophil cationic protein and myeloperoxidase), and (4) expression of intercellular adhesion molecule-1 (ICAM-1) on nasal epithelial cells. We focused our attention on ICAM-1 because it is the natural ligand of leukocyte functional associated antigen-1 and Mac-1, expressed on eosinophils. In addition, ICAM-1 is expressed on epithelial cells only on allergen exposure (both natural and experimental).

Results: Placebo did not exert any modification on the considered parameters. After azelastine administration, significant decreases in total symptom score, eosinophilic and neutrophilic infiltration, and ICAM-1 expression were observed during both early- and late-phase reactions. Furthermore, serum eosinophil cationic protein levels decreased during the late-phase reaction, whereas myeloperoxidase was not affected by the treatment. These findings were confirmed by the powerful Koch's split-plot statistical analysis.

Conclusion: Azelastine exerts antiallergic activity, mainly affecting eosinophil function and downregulating ICAM-1 expression, on nasal epithelial cells. (*J Allergy Clin Immunol* 1996; 98:1088-96.)

Key words: Topical azelastine, antiallergic activity, allergic inflammation, adhesion molecules, allergen-specific nasal challenge, early phase, late phase

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It is now widely accepted that allergic rhinitis is sustained by an inflammatory response. After mast cell activation, release of mediators (such as histamine, arachidonic acid derivatives, and chemotactic substances) and infiltration of inflammatory cells (mainly neutrophils and eosinophils at the mucosal level) occur. Eosinophilic infiltrate may be regarded as the hallmark of allergic reactions. Several pathophysiologic mechanisms are involved in

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Abbreviations used

ASNC:	Allergen-specific nasal challenge
ECP:	Eosinophil cationic protein
EPR:	Early-phase reaction
ICAM-1:	Intercellular adhesion molecule-1
LFA-1:	Leukocyte functional associated antigen-1
LPR:	Late-phase reaction
mAb:	Monoclonal antibody

eosinophil recruitment; release of cytokines (i.e., IL-3, IL-4, IL-5, and IL-8) exerting chemoattractant and activating effect and adhesion molecules.¹

Eosinophils express on their surface the $\beta 2$ -integrin leukocyte functional associated antigen-1 (LFA-1), which is the counterreceptor of intercellular adhesion molecule-1 (ICAM-1). Epithelial cells do not constitutively express ICAM-1 but do express it early on allergen exposure. This may, at least in part, account for adhesion, because eosinophils express LFA-1 and Mac-1, particularly on allergen activation.²⁻⁴

Azelastine, a phthalazinone derivative, is an antiallergic drug with multiple activities.⁵ In fact, azelastine demonstrates histamine H_1 -receptor antagonist action and also inhibits histamine release from both human and animal mast cells in response to a variety of stimuli such as anti-IgE serum, antigen, calcium ionophores, and compound 48/80.⁶⁻⁹ By in vitro and in vivo studies, azelastine was demonstrated to prevent the activation of inflammatory cells (e.g., basophils, neutrophils, eosinophils, polymorphonuclear cells, and macrophages) and to inhibit the synthesis and/or release of different mediators (e.g., leukotrienes, $N\alpha$ -p-tosyl-L-methyl ester, superoxide anion, oxygen free radicals and platelet activating factor).^{6,7} In particular, azelastine inhibits antigen-induced production of leukotrienes (LTC_4 and LTD_4), which inhibits the 5-lipoxygenase pathway of arachidonic acid metabolism without affecting the cyclooxygenase pathway, because in vivo azelastine does not reduce prostaglandin D_2 levels on allergen challenge.¹⁰

In clinical trials, orally administered azelastine in doses up to 4 mg/day consistently relieved symptoms in patients with seasonal or perennial rhinitis.⁵ In addition, azelastine nasal spray was effective in alleviating symptoms in patients with seasonal allergic rhinitis.¹¹ Finally, azelastine exerts a significant protective effect on aspecific bronchial challenge.⁵

Because it was recently demonstrated that a single dose of oral azelastine is able to induce a significant reduction in sneezing and in recovered $N\alpha$ -p-tosyl-L-methyl ester, leukotrienes, and kinins in nasal lavages during the early-phase reaction (EPR) after allergen-specific nasal challenge (ASNC),¹⁰ this study was designed to evaluate the effects of a single dose of azelastine nasal spray on: (1) clinical events, (2) cytologic parameters (i.e., neutrophils and eosinophils), (3) mediator levels (i.e., eosinophil cationic protein [ECP] and myeloperoxidase), and (4) ICAM-1 expression on nasal epithelial cells during both the EPR and the late-phase reaction (LPR).

METHODS

Patients

We studied 20 patients, aged 18 to 51 years, outside the pollen season. Patients were divided into two groups, with 10 patients in each group. The study had a double-blind, placebo-controlled, randomized, parallel-group design and compared the effect of treatment with azelastine nasal spray or placebo on the early and late responses to nasal challenge with specific allergen.

A first challenge was performed in all subjects. A washout period of 1 week was observed after the first challenge. A second challenge was performed 30 minutes after a single dose of azelastine nasal spray (0.137 mg) or placebo was administered.

All patients had seasonal allergic rhinitis caused by only *Parietaria judaica* (Par j). All patients with allergy had a positive history (for at least 2 previous years), positive skin prick test results equal to or greater than +++ (3 mm or more; histamine = +++), and RAST (equal to or greater than class III) only for the specific allergen. All patients with allergy were free of symptoms during the study and had negative skin test responses to house dust mite or pet dander.

Results of local examination were negative in all subjects. At the time of admission, no subject had any upper or lower airway respiratory tract disease; they were not smokers. Patients with allergy were not treated with any kind of topical and/or systemic drug in the 6 weeks preceding the study and had not previously received specific immunotherapy. The study was performed according to the Declaration of Helsinki: informed consent according to the rules of the ethical committee of our department was obtained from each patient.

Allergens

The Par j extract (the same used for skin prick tests, kindly provided by Bayropharm-DHS, Bayer Group, Milan, Italy) stored as a lyophilized powder at 4°C, was reconstituted in the diluent (saline human albumin 0.03%) within 24 hours before the test.

The concentration was expressed as allergen units by

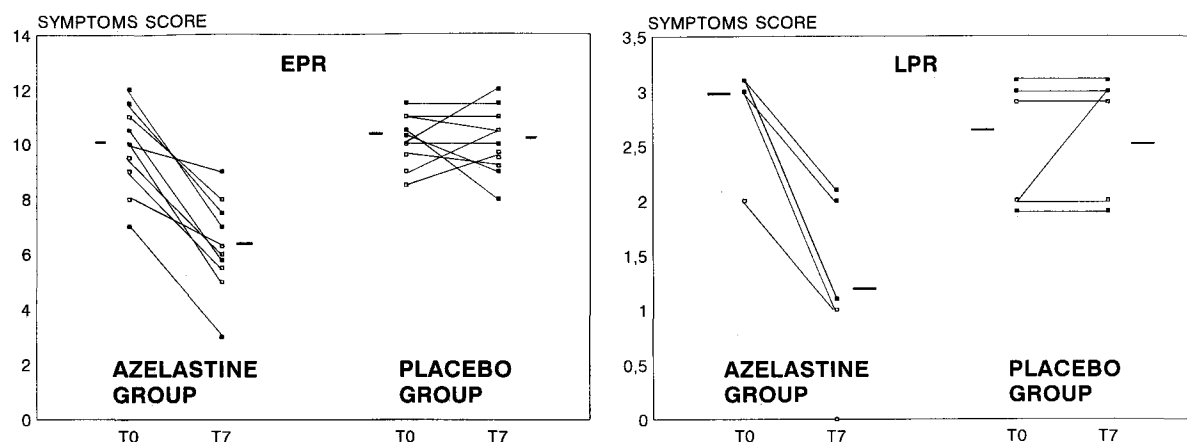


FIG. 1. Patients' total symptom scores after specific allergen nasal challenge, evaluated at T0 and T7. Median values are represented by thick bars.

RAST per milliliter (AUR/ml); seven different dilutions were used: 10, 25, 50, 100, 250, 500, and 1000 AUR/ml.

ASNC

Nasal challenge was performed according the method described by Naclerio et al.¹² and modified by us, as previously described.⁴ In brief, two preliminary 10 ml lavages with lactated Ringer's solution at 37°C were performed to remove secretions and establish a stable baseline. One-tenth milliliter of a solution of oxymetazoline hydrochloride, 0.05% (Nasivin, Bracco, Italy), was then applied. This drug is an α -adrenostimulator, which prevents excessive congestion of the nasal mucosa after allergen challenge and allows for the continuation of nasal lavages, guaranteeing nasal patency. In addition, oxymetazoline does not influence mediator release. Then, nasal lavage for cytologic assessment and mediator assay was performed as described below. Challenges with the vehicle used for the antigen (diluent, such as saline albumin 0.03%) were performed to rule out nonspecific hyperreactivity to the delivery system. No clinical reaction was observed after diluent challenge. Cytologic evaluation on nasal lavages performed as described below after diluent challenge invariably showed total inflammatory cell counts of less than 3 (as in a subject free of symptoms undergoing nasal lavage without any challenge). Then a series of challenges with increasing doses of antigen (as mentioned above) were begun at standard time intervals (usually 15 minutes) until a nasal clinical reaction was elicited, according to our previous reports.⁴

The initial allergen concentration used for nasal challenge was the same as that of the end-point skin test. Allergen solutions were blown into the nose by using a metered pump spray delivering one puff/volume ($90 \pm 2 \mu\text{l}$) in one nostril, with the patient holding his or her breath in full inspiration to avoid bronchial provocation.

After administering the spray, the patient was allowed to wipe his or her nose but not blow it.

Nasal lavages for cytologic examination and mediators (see below) were performed 30 minutes (i.e., EPR) and 6 hours (i.e., LPR) after clinical reaction. The same allergen dose was used for both nasal challenges (T0 and T7).

Clinical evaluation

The clinical reaction was assessed as total symptom score according to an arbitrary 4-point rating scale, from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe), increasing for severity in both nostrils. Nasal itching, sneezing, rhinorrhea, and congestion were considered (for sneezing evaluation 1 to 4 sneezes were graded as "mild," 5 to 8 as "moderate," and 9 or more as "severe"). Signs were separately evaluated by two physicians, and symptoms were spontaneously reported by the subjects. Patients were clinically evaluated hourly (up to the tenth hour).

A total symptom score after challenge higher than 7 was considered a positive response during the early phase, according to our previous reports,⁴ so allergen threshold dose was defined for each subject. Late phase was recognized when total symptom score was not less than 2, according to our previous reports.⁴

Cytologic assessment

Nasal secretions were obtained according to the following method. Each subject extended his or her neck backward while holding his or her breath; and 3 ml of lactated Ringer's solution, prewarmed to 37°C, was instilled into each nostril. Approximately 10 seconds after instillation, the fluid ($2 \pm 0.5 \mu\text{l}$) was expelled into a plastic tray and transferred to a 10 ml polypropylene tube. Then the lavages were centrifuged at 1400 g for 10 minutes, and each pellet was resuspended in phosphate-

TABLE I. Results of EPR after ASNC

Parameter	Time	Median change (minimum-maximum)		p Value
		Azelastine	Placebo	
Total symptom score	T0	10 (7-12)	10 (8-11)	NS
	T7	7 (3-10)	10 (8-12)	0.01
Neutrophils	T0	45 (28-80)	39 (22-70)	NS
	T7	28 (10-49)	37 (24-63)	0.01
Eosinophils	T0	13.5 (9-22)	12 (5-21)	NS
	T7	8.5 (4-15)	14 (6-19)	0.03
ICAM-1	T0	4 (2-4)	3.5 (3-4)	NS
	T7	2 (1-2)	3 (3-4)	0.001
Soluble ECP	T0	33 (20-43)	30 (19-42)	NS
	T7	29 (19-35)	30 (20-41)	NS
Soluble MPO	T0	88 (10-132)	93 (25-184)	NS
	T7	81 (41-119)	89 (12-185)	NS

NS, Not significant; MPO, myeloperoxidase.

TABLE II. Results of LPR after ASNC

Parameter	Time	Median change (minimum-maximum)		p Value
		Azelastine	Placebo	
Total symptom score	T0	3 (1-3)	3 (2-3)	NS
	T7	1 (1-2)	3 (2-3)	0.01
Neutrophils	T0	39 (18-63)	28 (19-63)	NS
	T7	20 (10-79)	26 (18-60)	0.01
Eosinophils	T0	17 (10-26)	16 (10-26)	NS
	T7	11 (8-20)	17 (12-25)	0.01
ICAM-1	T0	2 (1-3)	2 (1-3)	NS
	T7	1 (0-3)	2 (1-3)	0.05
Soluble ECP	T0	40 (27-50)	40 (25-52)	NS
	T7	25 (11-37)	41 (30-56)	0.01
Soluble MPO	T0	100 (48-170)	96 (23-163)	NS
	T7	85 (41-166)	87 (20-111)	NS

buffered saline solution (2 ml); cell suspensions were filtered to reduce the quantity of mucus, and cytospin slides were prepared with standard techniques.

Eosinophils, neutrophils, and epithelial cells were differentiated by May-Grünwald-Giemsa staining. The slides were read with an optic microscope (Leitz Laborlux Microscope, 250× focus; Leitz, Milan, Italy). The number of inflammatory cells (neutrophils, eosinophils) was considered as a total number of any cell type per each microscopic field: the data are expressed as a mean of 10 fields. Slides were examined by two investigators blinded to the identity of the samples.

Immunocytochemistry

An immunoenzymatic alkaline phosphatase-antialkaline phosphatase complex procedure, modified from the method of Cordell et al.,¹³ was used. Specimens were air-dried at room temperature for 30 minutes and sub-

jected to 1:50 dilution of purified CD54 monoclonal antibody (mAb) (1 mg/ml, 84H10, IgG₁; Immunotech, Marseille, France) or to 1:100 dilution of anti-cytokeratin mAb (aCK19, IgG₁; DAKO, Milan, Italy) as a marker of epithelial cells. After washing in phosphate-buffered saline (pH 7.6), samples were incubated with rabbit anti-mouse immunoglobulin, followed by alkaline phosphatase-antialkaline phosphatase complex. Afterward, specimens were incubated in substrate solution containing basic new fuchsin, naphthol AS-BI phosphate, and levamisole as an inhibitor of endogenous alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). In control samples either mAb or anti-mouse immunoglobulin was omitted. As negative isotype control for CD54 staining on epithelial cells, an anti-T-lymphocyte (CD3) mAb OKT3, IgG₁, (Ortho Diagnostic, Raritan, N.J.) at 1:20 dilution of the stock solution (provided by the manufacturer) was used.

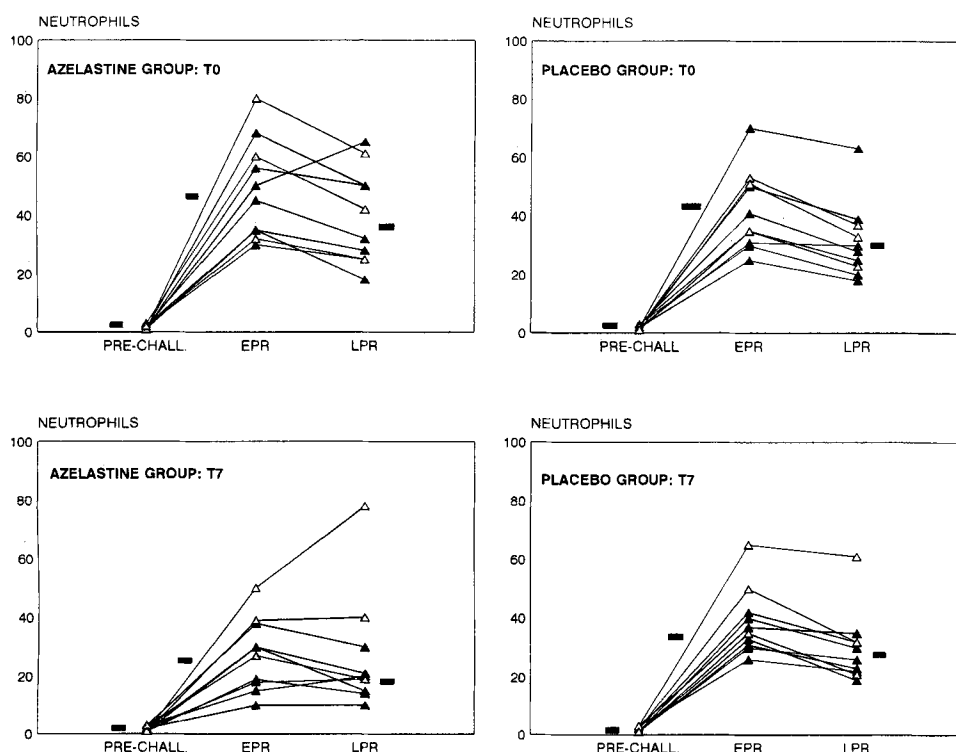


FIG. 2. Neutrophil counts at T0 and T7, before challenge (PRE-CHALL.) and after challenge (EPR and LPR). Median values are shown as horizontal thick bars.

The dilutions were established on the basis of previous titration experiments.

All preparations were counterstained with Carazzi's hematoxylin and examined by two investigators blinded to the identity of the samples. CD54 expression on epithelial cells was expressed according to a 4-point rating scale, from 0 to 4 where 0 = no positive cells, 1 = mild positivity on 25% of epithelial cells, 2 = mild positivity on 75% of epithelial cells, 3 = intense positivity on 75% of epithelial cells, and 4 = very intense positivity on all epithelial cells, according to our previous reports.²⁻⁴

Mediator dosage

ECP and myeloperoxidase levels in nasal lavages were assessed according to the methods described by Klementsson et al.¹⁴ and Venge et al.,¹⁵ by radioimmunoassay, with the use of commercially available kits (Kabi-Pharmacia Diagnostic, Milan, Italy).

Statistical analysis

Because the variables considered are not normally distributed, the Mann-Whitney test for unpaired samples was used to determine the median change between placebo and active treatment groups. Wilcoxon test for paired samples was used to compare the intragroup median changes between T0 and T7. In addition, a split-plot analysis according to Koch¹⁶ (no Geisser-

Grenhouse asphericity correction) with Dunn-Sidak correcting procedure^{17,18} was applied. This latter powerful test globally determines, for each parameter, whether administration of either azelastine or placebo modifies the response pattern to nasal challenge.

RESULTS

All subjects had a clinical EPR after nasal challenge. Six patients in the placebo group and five patients in the azelastine group also showed a clinical LPR (Fig. 1). No significant difference between the two groups was observed at T0 for all the considered parameters. The intergroup comparisons at T0 and T7 are summarized in Table I for EPR and in Table II for LPR.

Clinical scores

The azelastine-treated patients showed a significant improvement in total symptom score after specific challenge compared with patients in the placebo group (EPR, $p = 0.01$; LPR, $p = 0.01$) as shown in Fig. 1 and Tables I and II. The intergroup comparison (T0 vs T7) confirmed this effect for the azelastine group (EPR and LPR, $p = 0.01$). No difference was observed in the placebo group.

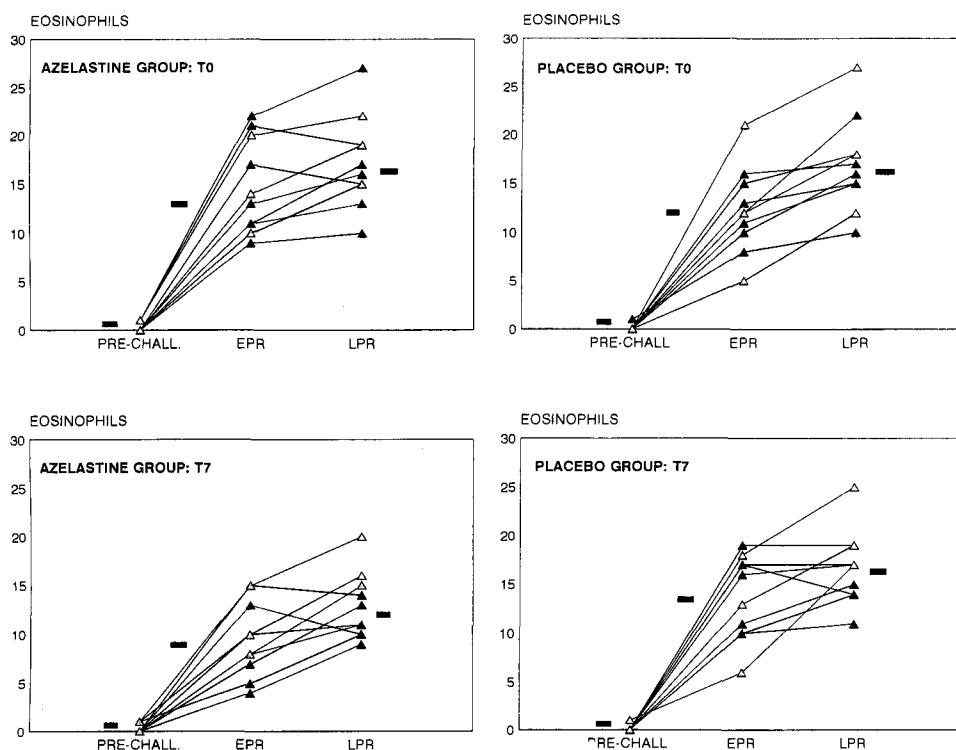


FIG. 3. Eosinophil counts at T0 and T7, before challenge (PRE-CHALL.) and after challenge (EPR and LPR). Median values are shown as horizontal thick bars.

Cytologic assessment

A significant reduction of neutrophilic (Fig. 2) and eosinophilic (Fig. 3) infiltration was observed only in the azelastine group both during EPR and LPR, when compared with the placebo group, as shown in Tables I and II.

ICAM-1 on epithelial cells

A significant decrease of ICAM-1 expression was evidenced in the azelastine group after challenge both during EPR ($p = 0.01$) and LPR ($p = 0.05$), as shown in Fig. 4 and reported in Tables I and II. The ICAM-1 expression decrease in the active group was also confirmed by the intragroup comparison (EPR, $p = 0.004$; LPR, $p = 0.01$).

Mediators

ECP in nasal lavage fluid decreased only during LPR in the azelastine-treated group (Fig. 5). This decrease was significant in both intergroup ($p = 0.01$) and intragroup ($p = 0.003$) comparisons. No significant change in myeloperoxidase levels was observed in the two groups.

Finally, the Koch's split-plot analysis, which globally assesses the effect of the drug on the nasal challenge response pattern (irrespective of LPR

occurrence), confirmed the quoted findings with high significance for symptom score, cellular infiltration, ICAM-1 expression, and ECP ($p < 0.001$). Furthermore, the Koch's test provided high reproducibility values (i.e., near 100%) of the experimental model for all parameters except myeloperoxidase.

No important adverse event was observed. Three actively treated subjects reported an alteration in taste.

DISCUSSION

It is well known that allergic rhinitis is characterized by inflammatory cell infiltrate, mainly consisting of leukocytes and mast cells. Mast cells, when activated by specific allergen, are able to release some mediators that induce immediate clinical events, such as histamine and substances with chemoattractant activity such as cytokines (i.e., IL-4, IL-5, IL-6, IL-8, and tumor necrosis factor- α).¹⁹⁻²¹ Then cytokines are synthesized, as well as secreted, in the active form. Cytokines upregulate adhesion molecule expression and their shedding, thus allowing further recruitment of inflammatory cells.²² The above described events are crucial steps in the pathogenesis of allergic

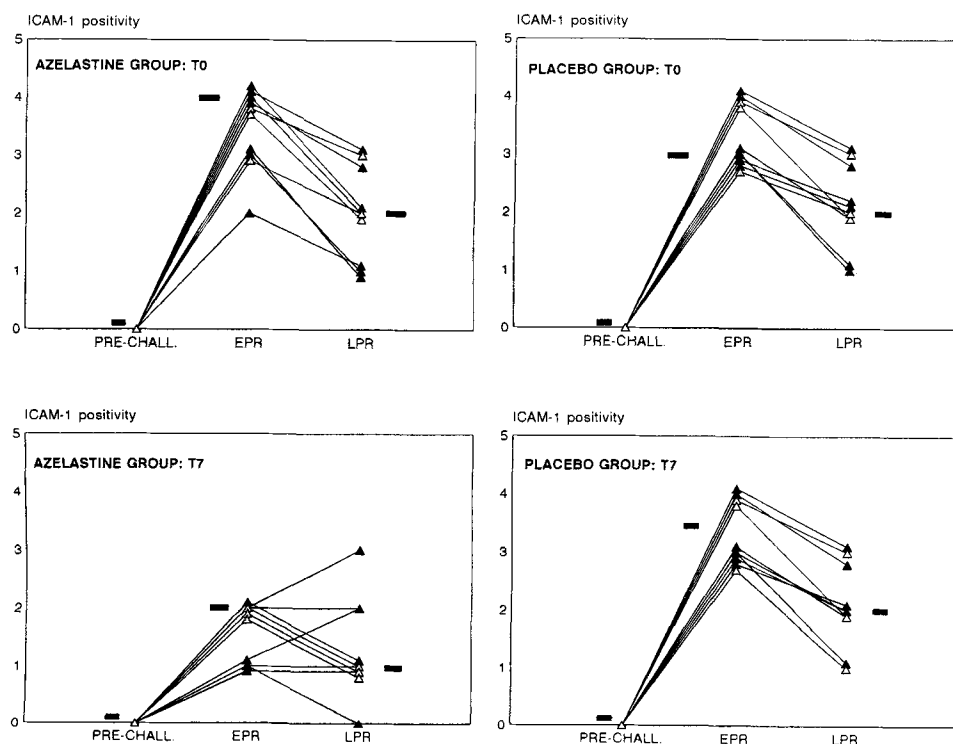


FIG. 4. ICAM-1/CD54 expression on nasal epithelial cells (evaluated by alkaline phosphatase-antialkaline phosphatase and expressed as positivity score, see text) at T0 and T7, before challenge (*PRE-CHALL.*) and after challenge (*EPR* and *LPR*). Median values are shown as horizontal thick bars.

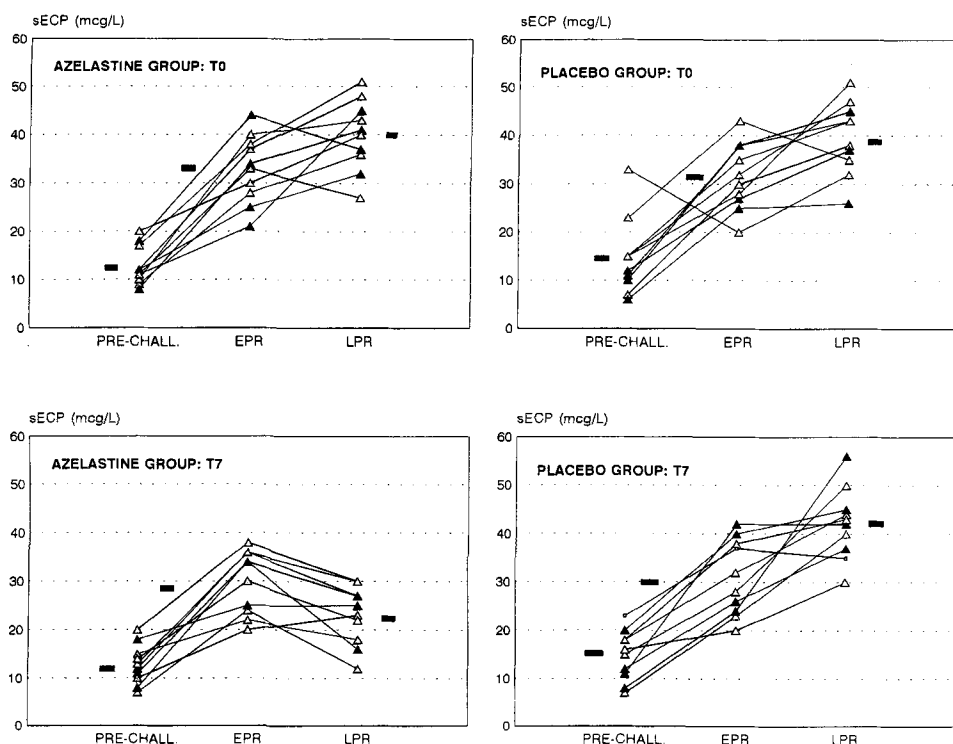


FIG. 5. ECP levels in nasal lavages at T0 and T7, before challenge (*PRE-CHALL.*) and after challenge (*EPR* and *LPR*). Median values are shown as horizontal thick bars.

inflammation. In addition, a neutrophilic infiltrate predominates in bronchial biopsy specimens of patients who died suddenly as a result of asthma attacks.²³

On the other hand, eosinophils are the principal inflammatory cells of allergic reaction, mainly mediating mucosal damage, because they release basic proteins able to produce typical epithelial desquamation.²⁴ In addition, eosinophils per se may induce inflammatory infiltration because ECP is able to induce ICAM-1 expression on nasal epithelial cells.²⁵ Moreover, IL-4 eosinophil priming induces a specific chemotactic response to IL-8.²¹ Tumor necrosis factor- α induces ICAM-1 expression on the epithelium.¹ In this regard, it has been demonstrated that epithelial cells (both nasal and conjunctival) of normal subjects do not express ICAM-1, whereas ICAM-1 expression on both nasal and conjunctival epithelial cells occurs early in patients with allergy on allergen-specific challenge, as well as during natural exposure to the allergen.^{1-4, 26} This molecule is important because it is the natural ligand of LFA-1, a β 2-integrin expressed on leukocytes.

Therefore it is possible to partially explain why inflammatory cell influx occurs as an early event: in fact, activated mast cells may promote and induce the early inflammatory phenomena through the above-mentioned proinflammatory substances. Because azelastine is a drug with multiple actions on allergic pathophysiologic mechanisms, we aimed at evaluating its antiallergic activity.

As expected, clinical parameters significantly improved in patients treated with azelastine. This phenomenon may be explained by its antihistaminic activity, as far as the early phase is concerned. Moreover, azelastine diminished clinical late phase (Fig. 1) and reduced the inflammatory infiltrate during both early and late phase. It is well known that late-phase phenomena are strictly related to inflammatory events and that they are more similar to allergic response occurring during natural exposure to the allergen.²⁷ A possible explanation of this antiallergic activity may be related to its activity of downregulation of ICAM-1 expression on epithelial cells during the early phase. In fact, if epithelial cells express fewer ICAM-1 molecules, inflammatory cell adhesion contact is likely to be reduced.

On the basis of both our results (Fig. 4) and evidence from the literature, it is possible to hypothesize that azelastine may induce downregulation of ICAM-1 expression in different ways: (1) by

acting on epithelial cells directly; (2) by acting on mast cells because these cells are able to release soluble factors, which upregulate ICAM-1 expression, and azelastine was demonstrated to reduce release of some mast cells products; (3) by acting indirectly because ECP may also upregulate ICAM-1 expression and a less abundant eosinophilic infiltrate results in lower ICAM-1 expression. This is consistent with the observed significant decrease of ECP recovered in nasal fluid, after specific allergen challenge, in azelastine-treated patients (Fig. 5). ECP is produced by eosinophils; however, its levels better correlate with the activation state than with the absolute numbers of cells. Neutrophil behavior is more variable. In the data obtained from this study, neutrophil influx in EPR was even more pronounced than in LPR, at variance with other reports.^{4, 28, 29} Myeloperoxidase levels, which reflect neutrophil activation, were highly dispersed and not affected by azelastine treatment. Taken together, these data strongly suggest a significant antiallergic effect of topical azelastine on local allergic inflammation induced by ASNC. The reduction of inflammatory cell counts, ICAM-1, expression on nasal epithelial cells, and ECP levels in the nasal fluid, which are closely related to activated cell recruitment, were dramatically reduced by the treatment.

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