A two-year course of specific immunotherapy or of continuous antihistamine treatment reverse eosinophilic inflammation in severe persistent allergic rhinitis

Due anni di immunoterapia specifica o di trattamento antistaminico continuo determinano una regressione della flogosi eosinofila nella rinite allergica severa persistente

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Key words

Allergic rhinitis • Medical treatment • Immunotherapy • Eosinophilic infiltration

Parole chiave

Rinite allergica • Terapia medica • Immunoterapia • Infiltrazione eosinofila

Summary

Aim of the study was to evaluate the effect of a 2-year course of subcutaneous specific immunotherapy or continuous oral antihistamine treatment on the eosinophilic inflammation in nasal secretions of patients with severe persistent allergic rhinitis caused by house dust-mites. After informed consent, 31 rhinitis patients, sensitive to dust-mite antigens, were enrolled: 12 were randomly assigned to specific immunotherapy (group A), 11 to continuous oral antihistamine (cetirizine) treatment (group B), and 8 to an oral antihistamine (cetirizine) on demand (group C). Nasal scrapings were performed with a cotton-tipped swab and cells counted before and after 24 months of therapy. Intercellular adhesion molecule-1 and eosinophil cationic protein expression in cytological smears were assessed by immunohistochemistry. All patients completed the study. The percentage of inflammatory cell types was comparable in the 3 groups at the beginning of the study. Eosinophils, identified as cells expressing eosinophil cationic protein, significantly decreased dropping to zero after 2 years of treatment in groups A and B, while no change was observed in group C. Expression of intercellular adhesion molecule-1 also decreased significantly in groups A and B, but not in group C. This decrease was associated with a significant reduction in epithelial shedding. In the 2-year period studied, specific subcutaneous immunotherapy and continuous oral antihistamine treatment were found to be effective in reducing eosinophilic infiltration and adhesion molecule expression in the nasal mucosa of patients with persistent allergic rhinitis. Furthermore, immunotherapy was more effective in controlling epithelial disruption while antihistamines appeared to be more active in controlling nasal inflammation. Both treatments induced a significant decrease in intercellular adhesion molecule-1 expression in epithelial cells and also a dramatic reduction of eosinophil

Riassunto

Il presente studio si è prefisso l'obiettivo di valutare l'effetto di due anni di immunoterapia specifica o di un trattamento continuo con antistaminico orale sulla infiammazione eosinofila nella secrezione nasale di pazienti affetti da rinite allergica severa persistente causata da sensibilizzazione all'acaro della polvere. Previo consenso informato sono stati arruolati nello studio 31 pazienti rinitici sensibilizzati all'acaro della polvere: mediante scelta randomizzata 12 sono stati assegnati al gruppo A sottoposto ad immunoterapia specifica, 11 al gruppo B sottoposto a trattamento continuo con antistaminico orale (cetirizina) e infine 8 al gruppo C sottoposto a trattamento al bisogno con antistaminico orale (cetirizina). Lo scraping nasale è stato eseguito con cotone montato e la conta cellulare è stata effettuata al tempo 0 e 24 mesi dopo l'inizio del rispettivo trattamento. L'espressione di ICAM-1 e di ECP nei preparati citologici è stata valutata con metodiche immunoistochimiche. Tutti i pazienti hanno completato lo studio. La percentuale dei diversi tipi di cellule infiammatorie era analoga nei tre gruppi all'inizio dello studio. Gli eosinofili, identificati come cellule esprimenti l'ECP, si sono ridotti significativamente approssimandosi allo zero dopo due anni di terapia nei gruppi A e B, mentre la loro presenza non si è modificata in modo significativo nel gruppo C. Anche l'espressione di ICAM-1 si è ridotta significativamente nei gruppi A e B, ma non nel gruppo C. Tale riduzione è risultata associata ad una significativa diminuzione della desquamazione di cellule epiteliali. L'immunoterapia specifica sottocutanea o il trattamento continuo con antistaminico orale protratti per due anni si sono dimostrati parimenti efficaci nel ridurre l'infiltrazione eosinofila e l'espressione della molecola di adesione ICAM-1 nella mucosa nasale di pazienti affetti da rinite allergica persistente. Inoltre l'immunoterapia si è dimostrata più efficace nel controllare la desquamazione epiteliale, mentre l'antistaminico è risultato più attivo nel controllo dell'infiammazione nasale. Entrambi i trat-

cationic protein positive staining. These parameters can be considered useful means for controlling the state of persistent inflammation which is typical of persistent respiratory allergy. Nasal scraping was demonstrated to be a simple and safe procedure for monitoring some nasal inflammation parameters.

tamenti hanno indotto un significativo decremento dell'espressione di ICAM-1 sulle cellule epiteliali oltre che una drastica riduzione della positività per l'ECP. Questi parametri possono essere considerati validi indici nella valutazione della flogosi, tipica della allergia respiratoria persistente. Lo scraping nasale si è confermata tecnica semplice e sicura per il monitoraggio dei suddetti indicatori locali di flogosi.

Introduction

Allergic rhinitis is a very frequent chronic condition which affects the quality of life and is the cause of high economic costs. Extensive evidence indicates that allergic rhinitis is characterised by eosinophilic inflammation of the nasal mucosa. Activated eosinophils, expressing eosinophil cationic protein (ECP), and epithelial shedding through their mediators are recruited in the tissue intercellular adhesion molecule-1 (ICAM-1) and cause damage. At clinical level, allergic rhinitis is, at present, classified as intermittent, mild persistent and severe persistent [1], on the basis of symptoms. In severe persistent allergic rhinitis, inflammation is pronounced and frequently associated with asthma and other complications, involving high economic costs. Treatment of severe persistent rhinitis includes topical steroids, antihistamines and immunotherapy. However, no data are available to establish whether immunotherapy and antihistamine continuously administered are effective in controlling eosinophilic inflammation in severe persistent rhinitis. Eosinophils play an important role in the allergic inflammatory process and accumulate in the target organ especially during exacerbation phases of the disease [2]. Eosinophil infiltration, typical of the allergic process, is, in fact, the main cause of epithelial damage and shedding and of reticular layer thickening [3]. The major basic protein (MBP), eosinophil peroxidase and the ECP are some of the eosinophil granule proteins which can act as mediators causing pathophysiological changes of allergic rhinitis and nasal hyperreactivity [4]. ECP levels in nasal secretion are significantly higher in patients with allergic rhinitis than in controls [5] and increase in natural disease [6 7] as a consequence of eosinophil degranulation [8]. Therefore, the ECP is considered a consistent inflammatory marker of nasal eosinophilic inflammation [8-11].

ICAM-1, which can be considered as one of the main factors associated with the development of allergic rhinitis [12 13], can be highly represented on the epithelial nasal cells under the action of various substances, including inflammatory cytokines [14 15].

Using an ICAM-1 mRNA quantification system, Terada et al. [16] demonstrated that interleukin-5 (IL-5) induced ICAM-1 gene expression in the nasal mucosa of patients with nasal allergy, but not in the mucosa of patients with non-allergic rhinitis.

ECP and ICAM-1 can, therefore, be considered valuable markers of inflammation.

The mechanism of action of specific immunotherapy treatment (SIT), which is an effective approach to allergic rhinitis, is still not clear. In fact, the activity of SIT on eosinophilic inflammation has not yet been demonstrated, while it has been confirmed for continuous cetirizine treatment.

This study was aimed at evaluating a two-year course of treatment with specific immunotherapy or continuous cetirizine treatment respectively in two groups *vs.* a third group receiving antihistamine on demand only.

The number of eosinophils in nasal smears and the number of cells expressing ICAM-1 adhesion molecules were determined in order to evaluate eosinophilic inflammation.

Materials and methods

Patients and control subjects

A total of 31 patients (15 male, 16 female), age range 14-23 years (mean 18.2 ± 2.6 years) were enrolled, after obtaining informed consent. Demographic data in these patients were homogeneous (Table I).

All patients suffered from moderate/severe persistent allergic rhinitis due to house dust-mite monosensitization. The sensitization was assessed by skin prick test (wheal > 3 mm +++ or more) and RAST (Radioallergoassorbent Test) (class III or higher). Exclusion criteria were the presence of seasonal rhinoconjunctivitis, asthma, previous specific immunotherapy, habitual tobacco smoker, use of topical or oral drugs, anatomic alterations of the upper airways, immunologic deficiencies and systemic diseases (cardiac disease, diabetes, anaemia, renal or hepatic disorders). The levels of the major inhalant allergens (Der p1 and Der f1) were measured (detection kit purchased from Laboratory ALK-Abello, Milan, Italy) in the dust from the patients' houses at the beginning and end of the study.

Table I. Homogeneity of groups. Patient demographic data and Dpt house levels.

	Group A	Group B	Group C	p value
M/F	6/6	5/6	4/4	ns
Age (yrs)	14-24 17.9 ± 2.8	14-23 18.2 ± 2.5	14-23 18.1 ± 2.5	ns
Der p1 (µg/g dust) Baseline	0.51 ± 0.2	0.53 ± 0.4	0.52 ± 0.3	ns
End	0.48 ± 0.4	0.51 ± 0.3	0.51 ± 0.2	ns
Der p1 (µg/g dust) Baseline	2.73 ± 2.8	2.65 ± 3.2	2.68 ± 2.9	ns
End	2.98 ± 3.1	3.11 ± 2.9	2.71 ± 2.7	ns

Study design

Patients were randomly assigned to SIT (12 patients = group A), to continuous oral antihistamine treatment (11 patients = group B) or to oral anthistamine on demand (8 patients = group C). Subcutaneous (sc) SIT was given following the guidelines of the European Academy of Allergology and Clinical Immunology [17].

Group B received regular cetirizine treatment (10 mg/daily) with two months interruption (July and August). Group C received no treatment apart from cetirizine on demand.

Nasal scrapings were performed to assess inflammatory cells, ICAM-1 and ECP expression, before treatment and at the end of the 2-year treatment period.

Cytological assessment and immunohistochemical analysis

Nasal scrapings were carried out with a cotton tipped swab in both nasal cavities. Specimens were obtained from the middle third of the inferior turbinate. The swabs (one for each nasal cavity) were washed in a vial containing 2 ml of physiological solution, 0.9% NaCl, then carefully squeezed with a forceps to allow cell release in the liquid solution.

Four glass slides were prepared for each subject by cyto-centrifugation of 450 µl of cell suspension on each slide.

One slide was stained according to the May-Grünwald-Giemsa method.

The other slides for immunohistochemistry studies were fixed in acetone/methanol 1:1 (-20°C); preincubated for 30 minutes in a humid chamber with 3% H_2O_2 to inhibit the endogenous peroxidase and for 30 minutes with 3% bovine sero-albumin (BSA) to reduce the aspecific background staining.

Immunohistochemistry slides where treated with the following reagents: slide A was exposed to monoclonal anti ICAM-1, clone 15.2 (code sc-107) diluted 1:100 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); slide B was exposed to monoclonal anti ECP clone EG1 (code 10.91.95.01) diluted 1:100 (Pharmacia Diagnostics, Uppsala, Sweden); slide C served as the negative control, where the primary antibody was replaced by phosphate buffer 3% BSA.

After overnight incubation at 4 °C, the slides were carefully washed in PBS, and incubated with a biotin-conjugated secondary anti-mouse antibody (Biogenex, San Ramon, CA, USA) for 30 minutes at room temperature; washed in PBS, incubated with peroxidase-conjugated streptavidin (Biogenex, San Ramon, CA, USA) for 30 minutes and with 0.7 mg/ml 1,1 diaminobenzidine and 0.03% H_2O_2 for approximately 1 minute and checking stain development under the microscope; finally, counterstained with haematoxylin, washed in tap water, dehydrated and set in mounting medium (Eukitt, Kindler Gmbh, Freiburg, Germany).

Slides were examined by two independent investigators blinded to the identity of the samples: the agreement between observers was good (> 90% agreement).

The cell morphology assessment was carried out on the May-Grünwald-Giemsa-stained slides; 20 random fields (400x magnification) were analysed and the total number of cells, in each microscopic field, were counted. Cells were distinguished as: epithelial cells (recognized for the sheet aspect or the presence of cilia), and inflammatory cells (neutrophils,

eosinophils and basophils, lymphocytes, monocytes). Then the mean counts (± SD) were recorded for total cells, epithelial cells, and inflammatory cells.
Samples tested for ICAM-1 and ECP were evaluated analysing 20 random microscopic fields (400x magnification) and counting the number of positive cells out of the total number for each microscopic field. The average counts (± SD) were then recorded for total and positive cells.

Statistical analysis

Student t test was used in the statistical analysis of the results in order to determine the differences within and between groups (NS = not significant).

Results

All patients completed the study. Compliance to treatment (assessed on the basis of counting tablets in group B) was satisfactory both for group A and B. The levels of mite allergens in house exposure was comparable for the three groups (Table I). In group C, the demand for use of cetirizine was episodic (mean 1 tablet per week), and none of the patients, in this group, returned for treatment, on a regular basis. At the beginning of the study, the number of cells (group A 14.55 ± 4.4; group B 15.25 ± 3.21; group C 14.45 ± 3.39) and their distribution between different cells types were comparable (A *vs.* B NS, A *vs.* C NS, B *vs.* C NS), showing, in all groups, a relevant number (approximately 66%) of shedded epithelial cells and a mixed type of eosinophilic (approximately 6%)/neutrophilic (approximately 12%) inflammation (Table II).

Table II. Percentage of cellular types at beginning (T0) and end (T1) of study.

	Epithelial shedding cells %	Eosinophils %
Group A T0	66.15 ± 21.50	6.43 ± 0.79
Group A T1	49.55 ± 6.54	0.00
Group B T0	65.83 ± 20.30	6.74 ± 0.69
Group B T1	67.76 ± 22.00	0.00
Group C T0	66.71 ± 18.90	6.68 ± 0.72
Group C T1	65.93 ± 19.80	6.52 ± 0.65

Table III. Percentage of ICAM-1 and ECP positivity at beginning (T0) and end (T1) of study.

	ICAM-1 + %	ECP + %
Group A T0	11.73 ± 5.2	6.32 ± 3.5
Group A T1	4.18 ± 2.1	0.00
Group B T0	12.1 ± 3.2	6.18 ± 3.4
Group B T1	5.59 ± 0.6	0.1 ± 0.1
Group C T0	11.92 ± 4.6	6.23 ± 4.2
Group C T1	11.85 ± 5.1	6.12 ± 3.9

At the end of the study, a significant decrease in the total number of inflammatory cells was observed in group A (8.25 ± 3.2) and group B (7.0 ± 2.94), but not in group C (15.35 ± 4.46). In particular, eosinophils decreased both in group A and B ($p < 0.001$), but remained unchanged in group C (A *vs.* B NS, A *vs.* C $p < 0.001$, B *vs.* C $p < 0.001$) (Fig. 1). In group A, the decrease of eosinophils was associated with a reduction in the percentage of shedded epithelial cells ($p < 0.05$).
The percent values of ICAM-1 and ECP positivity at the beginning (T0) and at the end (T1) of the study are shown in Table III.
ICAM-1 decreased more in group A than in group B at the end of the treament, but the differences between the initial and final data are statistically significant in both groups ($p < 0.001$). ECP was absent in groups A and B at the end of the study ($p < 0.001$). Both ICAM-1 and ECP remained unchanged in group C (NS) (Fig. 2).
The intergroup comparison referring to the same parameters showed no significant difference referring to either ICAM-1 or ECP between the 3 groups at T0. At the end of the study, the differences, referring to ICAM-1, between groups A and C, B and C were statistically significant (A *vs.* C $p < 0.001$; B *vs.* C $p < 0.01$; A *vs.* B NS). For ECP, significant differences were detected between the same groups (A *vs.* C $p < 0.001$; B *vs.* C $p < 0.001$; A *vs.* B NS).

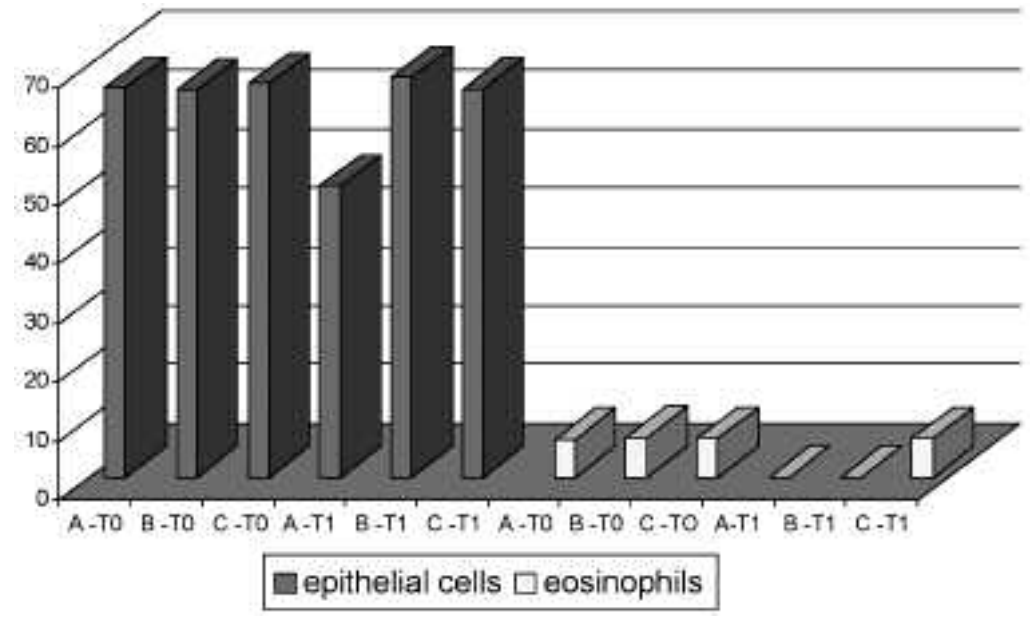

Fig. 1. Percentage of cellular types. The columns represent average cell counts of the main cellular type in 400 x microscopic fields (see methods).

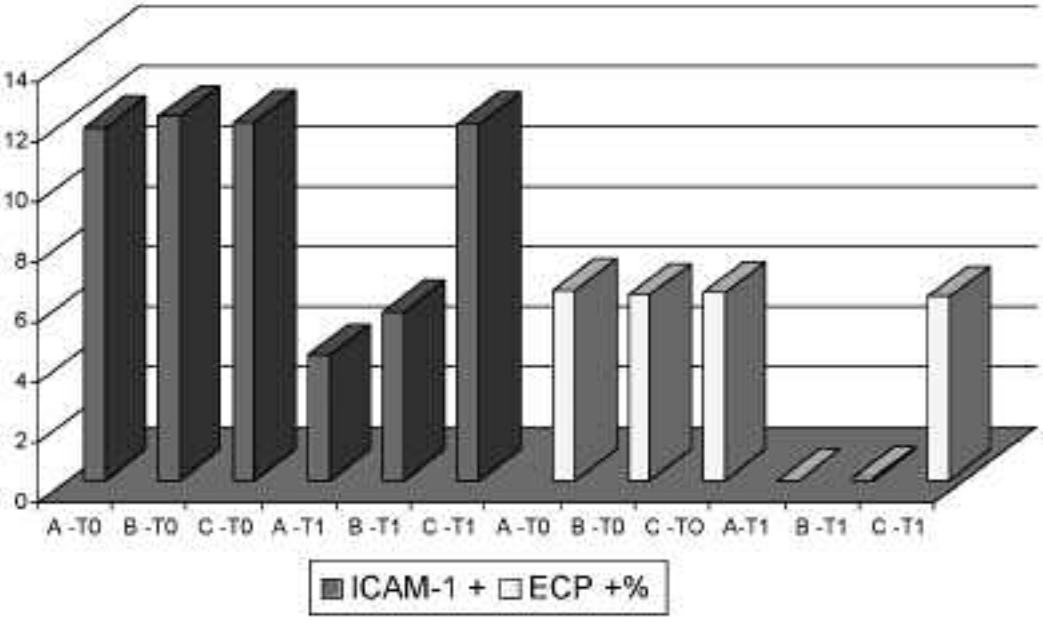

Fig. 2. Percentage of ICAM-1 and ECP positivity. The columns represent average cell counts of positive cells out of the total number in 400 x microscopic fields (see methods).

Furthermore, at the end of the study, the symptoms of allergic rhinitis (rhinorrhea, nasal obstruction, sneezing, hyposmia, sensation of ocular foreign body and lacrimation) were absent or only occasionally troublesome in 75% of group A, 78% of group B and 24% of group C, frequently troublesome in 25% of group A and 22% of group B, frequently or continuously troublesome in 76% of group C.

Discussion

Allergic rhinitis due to house dust-mite sensitization is a chronic inflammatory disease characterized by cell infiltration and respiratory epithelium disruption [18]. In fact, in genetically predisposed patients, the perennial exposure, following sensitisation, can sustain local inflammation and clinical symptoms of hyperreactivity [19]. At low allergen levels (< 2 μg/g dust), a minimal persistent inflammation can be demonstrated [12] both at conjunctival and nasal levels, even in asymptomatic patients. The infiltration of granulocytes, specifically the eosinophils, is typical of the late phase response [20] and eosinophils have been recognized as pro-inflammatory cells active during allergic reactions, through the release of granule proteins. Of these, the most important are: Major Basic Protein (MBP), Eosinophil Cationic Protein (ECP) which is an inflammatory cell activation marker [11], Eosinophil Derived Neurotoxin (EDN) and Eosinophil Peroxidase (EPO).

ECP, in particular, exerts toxic effects on the surrounding tissue [21 22] and probably affects the nasal mucosa by causing injury to ciliated cells and by accelerating the allergic reaction [23].

Since the infiltration by eosinophils and the release of ECP play a crucial part in allergic rhinitis [24], ECP concentration in the nasal secretion may be useful in monitoring chronic nasal inflammation in nasal allergic patients [25].

Intercellular adhesion molecule-1 (ICAM-1), which has an immunoglobulin-like structure, interacts with the β2 integrin LFA-1 [26 27]. In fact, it is involved in immune reactions requiring cell-to-cell contact [27 28] and is expressed on fibroblasts, endothelial cells, thymic epithelium and astrocytes; it is also present on resting T cells, B cells and monocytes [13 28-32].

Evidence of CD54 (ICAM-1) expression on the conjunctival epithelium following allergen-specific conjunctival challenge [33] gave rise, for the first time, to the hypothesis that this adhesion molecule may play a role in allergic inflammation by promoting the interactions between inflammatory cells and target organs [28 32].

Evaluation of the ICAM-1 epithelial expression in the nasal mucosa of patients with allergic rhinitis may be of interest for monitoring this disease through a non-invasive procedure such as nasal scraping, as proposed in the present investigation.

The sites of ICAM-1 expression in the nasal mucosa are vascular endothelial cells and the surface of eosinophils and lymphocytes [16]. While no significant cellular infiltrates are detectable, out of the pollen season, in the nasal secretion of allergic patients, moderate inflammatory cell infiltration and mild ICAM-1 expression are detectable, in subjects allergic to dust-mite [34]. In other words, pollen sensitive subjects do not constitutively express ICAM-1, on the nasal epithelium, out of the pollen season, while ICAM-1 is expressed, on the nasal epithelium, in mite-allergic patients who present a perennial inflammatory infiltrate due to the local release of cytokines from T lymphocytes and mast-cells [35].

ICAM-1 epithelial expression may be considered a sensitive marker, being restricted to allergic subjects; anti-inflammatory and anti-allergic approaches [36 37] may also be effective since they reduce the ICAM-1 expression [12] and the ECP level, in the nasal secretion.

In the present study, the ICAM-1 expression was analysed in flaking epithelial cells and the ECP positive granules in active eosinophils of nasal scrapings obtained from 3 groups of allergic patients, randomly assigned to specific immunotherapy (Group A), to continuous oral antihistamine treatment (Group B) or to oral antihistamines, on demand (Group C). Epithelial cells and inflammatory cells (mainly eosinophils) were found in all groups of patients suffering from mite allergen rhinitis.

The intragroup comparison showed that the percentage of epithelial cells decreased only in group A, after two years of treatment in the active groups, while the final percentage of epithelial cells was unchanged, and similar in groups B and C (Fig. 1).

At the end of the study, eosinophils were absent in groups A and B, but were unchanged in group C (Fig. 1).

We conclude that specific subcutaneous immunotherapy and continuous antihistamine treatment are both effective in reducing eosinophilic infiltration, in the nasal mucosa. Furthermore, immunotherapy was more effective in controlling epithelial disruption, while antihistamines appeared to be more effective in controlling nasal inflammation.

Both treatment regimens induced a significant decrease in ICAM-1 expression in the epithelial cells and also a dramatic reduction of ECP positive staining (Fig. 2) and these parameters can be considered useful for controlling the state of persistent inflammation, which is typical of perennial respiratory allergy. ICAM-1 and ECP values remained unchanged in the control group.

The decrease in ICAM-1 cannot be attributed to the reduction of epithelial cells. In fact, epithelial cells

decreased in group A and remained unchanged in group B, while ICAM-1 decreased in both groups.
Specific immunotherapy and oral antihistamine treatment exert comparable effects on chronic nasal immunophlogosis caused by house dust-mite sensitization.
The epithelial ICAM-1 expression and the ECP positive eosinophils, in the nasal mucosa of patients affected by mite allergic rhinitis, are interesting parameters for monitoring chronic local inflammation without discomfort for the patients. In fact, they can be evaluatued by a simple and safe procedure, such as nasal scraping, which can be performed, on a routine basis, in many subjects with allergic rhinitis.

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