Early decrease in nasal eosinophil proportion after nasal allergen challenge correlates with baseline bronchial reactivity to methacholine in children sensitized to house dust mites

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Abstract. *Background:* Allergic rhinitis is induced by an IgE-mediated inflammation after allergen exposure of the membranes lining the nose which, in predisposed individuals, may constitute a risk factor for the occurrence of asthma.
Objective: To detect early changes in nasal inflammation after allergen exposure, 11 children [9.0 (7, 11) yrs], sensitized to house dust mites (HDM), with rhinoconjunctivitis and asthma and an age- and gender-matched control group (Ctr) were studied.
Methods: The following parameters were evaluated: i) pulmonary function; ii) bronchial reactivity to methacholine (MCh), expressed as Pd_{20}MCh; iii) nasal brushing (NB) 'at baseline' and, on a separate day, 30 min after nasal allergen challenge (NAC). On NBs, the following markers of inflammation were evaluated: a) neutrophil and eosinophil proportion, b) 'intact to degranulated eosinophil' ratio, and c) expression of intercellular adhesion molecule (ICAM)-1 and HLA-DR by nasal epithelial cells.
Results: 'At baseline', allergic children showed elevated nasal eosinophilia and increased ICAM-1 and HLA-DR expression ($p<0.05$), as compared to Ctr. In allergic children, nasal eosinophilia correlated with Pd_{20}MCh ($p=0.002$). The significant decrease in nasal eosinophilia observed after NAC ($p=0.002$) was associated with a significant decrease in the 'intact to degranulated eosinophil' ratio ($p=0.001$). Interestingly, correlations were still present between Pd_{20}MCh and 'post NAC' eosinophilia ($p=0.004$) or the NAC-induced decrease in eosinophilia ($p=0.010$).
Conclusions: In children sensitized to HDM, experimental allergen exposure is followed by an early depletion of nasal eosinophils. The correlation between allergen-induced changes in nasal eosinophilia and bronchial reactivity to MCh further supports the concept of a tight link between upper and lower respiratory tract involvement in respiratory allergy.

Key words: Nasal eosinophils, bronchial hyperreactivity, allergen challenge, childhood, rhinoconjunctivitis, allergic asthma, house dust mites.

Introduction

Allergic rhinitis, a disorder of the nose induced by an IgE-mediated inflammation upon allergen exposure, is an extremely common disease also in childhood, that affects school learning performance and represents a risk factor for the occurrence of asthma in predisposed individuals [1-4].

The mechanisms involved in the pathogenesis of allergic rhinitis have been clarified by using nasal challenge with allergen or pro-inflammatory mediators, followed by the measurement of cells and mediators released in the nasal fluid during the early and late-phase allergic reaction [5,6].

Experimental studies have shown that in the early-phase reaction that follows allergen challenge, mast cells activation via the high affinity receptor of IgE, with cell degranulation and the release of several mast cell-derived mediators [4-6] are able to induce nasal mucosal plasma exudation, nasal hypersecretion and congestion, recruitment and/or activation of inflammatory and parenchymal cells [5-8].

During the early-phase reaction, IgE receptor bearing inflammatory and parenchymal cells, leading to the more complex inflammatory response that characterizes the late-phase reaction [9,10] also releases a range of cytokines and chemokines. Indeed, in approximately 30 to 40% of the patients, 4 to 12 hours after the challenge a mixed inflammatory infiltrate is observed at the site of the allergic reaction characterised by the appearance of a great number of eosinophils, basophils, and activated CD4+ T-lymphocytes [8-10].

Eosinophils express various membrane molecules including the tetrameric high affinity receptors of the

Table 1. Demographic and clinical data of children.

No	Patients	Age (years)	Gender	Cumulative dose of inhaled allergen (BU/ml)
Atopic				
1	ME	7	M	14
2	BM	11	M	2
3	CM	12	M	14
4	MA	11	F	6
5	BA	9	F	6
6	AM	7	F	0
7	CE	7	F	6
8	FE	5	F	2
9	PA	8	M	2
10	MA	9	F	6
11	TM	14	M	2
Normal healthy controls				
1	SO	11	M	
2	RE	14	F	
3	CM	8	F	
4	SP	5	M	
5	SG	8	F	
6	AL	9	F	
7	PS	17	M	
8	BM	7	F	
9	RM	4	F	
10	EV	11	M	

IgE, FcεRI [11]. The binding of the allergen-IgE complex with the FcεRI on the eosinophil surface results in signal transduction which activates the cell to release preformed, granule-associated proteins, arachidonic acid-derived products, cytokines and oxygen free radicals [12]. Since an increased expression of FcεRI has been found on eosinophils of atopic patients, compared to non-atopic subjects [13], it is possible that a significant eosinophil activation may occur also in the very early events characterizing the reaction to allergen exposure.

In addition to mast cells and eosinophils, also epithelial cells appear to be activated after allergen challenge, directly through the high affinity IgE receptor or by mast cell derived mediators, with upregulation of adhesion molecules expression, such as intercellular adhesion molecule (ICAM)-1 and HLA-DR, possibly involved in the interaction with inflammatory cells [8, 14].

The aim of the study was to evaluate the early inflammatory changes in nasal epithelium that may be induced by nasal allergen challenge in a group of subjects, sensitised to house dust mites (HDM), with respiratory symptoms. Nasal brushings were collected before and 30 minutes after nasal allergen challenge and the proportion of eosinophils and neutrophils and the ICAM-1 and HLA-DR expression by nasal epithelial cells were measured. Possible correlations between changes in nasal inflammation indices and pulmonary function parameters were also evaluated.

Materials and methods

Patients

As a part of a study aimed at evaluating the effect of sublingual immunotherapy on upper airway inflammation, we enrolled 11 outpatients, 9.0 (7, 11.5) yrs of age, 6 male and 5 female. Demographic and clinical characteristics of the studied population are summarized in Table 1. All patients were sensitized to HDM and had experienced respiratory symptoms in at least the 2 previous years. Inclusion criteria were: sensitization to HDM assessed by skin prick test (SPT) and/or Phadebas radio allergo sorbent test (RAST), 5-16 years old at enrolment. Exclusion criteria were: a) other sensitizations, including grass, trees, pet dander, Alternaria species or Aspergillus species; b) courses of specific immunotherapy with the same allergens in at least the 5 previous years; c) chronic or recurrent inflammation of oral mucosa; d) low compliance of the patients; e) systemic immunologic or metabolic disease; f) malignancies; g) major anatomic alterations of the upper airways; h) severe atopic diseases, i.e. noncontrolled asthma and severe atopic dermatitis; i) chronic systemic and nasal corticosteroid treatment and l) forced expiratory volume in 1 second (FEV_1) <70% on pharmacological treatment. All the patients were asked to continue their normal housecleaning activities. Avoidance measures were to remain unchanged throughout the study in order to maintain the same level of HDM exposure.

Ten sex- and age-matched healthy subjects, [8.5 (6, 12.5) yrs of age], with no history of upper or lower respiratory tract symptoms in at least 4 weeks before the evaluation, served as a control group. They had normal pulmonary function parameters, negative results to the standardized SPT and normal IgE serum levels (<100 KU/L). The Ethics Committee of Giannina Gaslini Institute approved the study and the Italian Ministry of Health was notified. All patients were informed in detail about the experimental procedure and provided written informed consent.

Experimental design

Allergic children were evaluated in three occasions, 2 to 4 days apart, while control children were studied in two days. On the first day, both allergic children and controls underwent SPT, serum total and allergen-specific IgE levels determination, pulmonary function evaluation and nasal brushing. On the second day, allergic children and controls underwent bronchial inhalation challenge with methacholine (MCh) while, on the third day, only allergic children were stimulated by nasal allergen challenge, followed by nasal brushing.

None of the children evaluated had changes in any 'respiratory or allergic' parameters during the two days which separate baseline from post challenge brushing.

Diagnosis of allergy

SPT

Sensitization to the most common classes of aeroallergens was evaluated as previously described [15]. The allergen panels tested included: *Dermatophagoides pteronyssinus, Dermatophagoides farinae* (5,000 PNU/ml), *Parietaria officinalis* (1,000 UP/ml), mix of Graminae, Compositae, Betulaceae, Oleaceae, mix of Aspergillus, Cladosporium, *Alternaria tenuis* (10,000 PNU/ml), cat or dog skin scale allergen extracts (1:20), (Bayropharm, Milan-Italy). The reactions were recorded within 15 minutes: a wheal diameter 3 mm larger than the negative control was considered as a positive reaction.

Total and allergen-specific IgE

Serum total IgE levels were determined by paper radio immunosorbent test (PRIST) kits (Pharmacia) [15]. Serum specific IgE against *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* were measured using RAST (Pharmacia, Sweden).

Lung function and MCh bronchial challenge

Lung function evaluation, MCh bronchial challenge and nasal allergen challenge were performed on separate days in atopic children and in controls. All children were able to perform forced expiratory manoeuvres. Forced vital capacity (FVC), FEV_1, forced expiratory flows at 25-75% of the vital capacity (FEF25-75%) were measured by spirometry (Med Graphics, Pulmonary Function System 1070 series 2, Med Graphics Corporation; St. Paul, MN, USA) [16]. On each occasion, three forced expiratory manoeuvres were obtained and the best values were retained. All children had baseline FEV_1 >80% than predicted. Aerosols were delivered by a SM-1 Rosenthal breath-activated dosimeter (SensorMedics) driven by compressed air (30 lb./in.^{2}) with 1-s actuations. Aerosol output at the mouth was 10 µl per actuation. Aerosols were inhaled during quiet tidal breathing in a sitting position. MCh solutions were prepared on each study day in 0.9% pyrogen-free saline. The challenge was started from a MCh dose of 0.02 mg. The dose was then doubled until FEV_1, measured within 1 minute after MCh inhalation, was below 80% of control value (inhalation of saline). The MCh dose causing a 20% decrease in FEV_1 (PD_{20}MCh) was calculated by interpolation of the dose-response curve [16].

Nasal allergen challenge

The procedure [8] was performed in atopic children when patients were asymptomatic. The subjects had to discontinue any drug at least 15 days before the challenge.

Briefly, to rule out non-specific hyperreactivity to the delivery system, challenges with the vehicle used for the allergen (diluent such as 10% glycerinated saline solution) were performed. A series of challenges with increased doses of allergen (2, 4, 8 BU/ml ALK-ABELLÓ) started at standard time intervals (usually 15 min) until nasal clinical hypersensitivity reaction was elicited (nasal obstruction, rhinorrhoea, nasal or ear itching, nasal sneezing). Allergen mixture (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* extract) solution were blown into the nose using a metered pump spray delivering 1 puff/volume (80µl) in one nostril, with the patient holding his or her breath in full inspiration to avoid bronchial provocation. After spraying, the patients were allowed to wipe their noses but not to blow them. Nasal brushings were performed at baseline and 30 min after clinical reaction.

Collection of nasal epithelial and inflammatory cells

Nasal brushings were collected at baseline (in atopic children and in controls) and in a separate day 30 minutes after nasal allergen challenge (in atopic children) [17]. Briefly, after washing the nasal cavity with saline, a channel cleaning brush designed for fiberoptic bronchoscopes (model BW-15SH, Olympus, Japan) was inserted in the nostril along the tip of the inferior turbinate and the adjacent median nasal wall under direct visualization, using a headlamp. A few backward-forward and rotatory movements collected nasal cells and the material adhering to the brush was removed by brisk agitation in 5 mL of sterile medium (RPMI 1640). The cell suspension was filtered and centrifuged at 500 x g for 5 min. The cell pellet was washed once and resuspended in Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}). The differential cell count was determined on cytocentrifuge preparations (Cytospin; Shandon Southern Products Ltd, Runcorn, UK): the air-dried slides were stained with Romanovsky stain (Diff-Quick, Merz & Dade AG, Dudingen, Switzerland) and evaluated by light microscopy (Carl Zeiss, Oberkochenk, Germany) [17]. The proportions of eosinophils and neutrophil 'contaminating' the epithelial cells were determined by counting 800 cells/sample and expressed as percentage of leukocytes recovered. The proportion of intact and degranulated eosinophils was also determined by light microscopy.

Evaluation of ICAM-1 and HLA-DR expression by nasal epithelial cells

Fifty µL of cell suspensions obtained by nasal brushing were placed in round-bottom microtiter 96 well plates (Costar Corp. Cambridge, Mass., USA) and stained for 30 minutes at 4°C with monoclonal antibodies to human ICAM-1 or HLA-DR (Sera Lab, Crawly Down, UK) fluorescein isothiocyanate-conjugates, as previously described [18]. A monoclonal antibody against human platelets (PTF 19) was used as a negative control preparation. After washing, the cells were fixed in PBS plus 1% paraformaldehyde (Eastman Kodak Co. Rochester, NY, USA). ICAM-1 and HLA-DR expression was analyzed by single colour immunofluorescence flow cytometry (FACS scan, Becton Dickinson Immunocytometry Systems, Mountain View, Ca, USA). The intensity of fluorescence was expressed as mean fluorescence channel (mfc) [18].

Statistical analysis

All data were expressed as median values plus lower and upper quartiles. PD_{20}MCh values were expressed as geometric mean. The Wilcoxon rank test was used when appropriate. Relationships between independent variables were assessed by the Spearman correlation test when appropriate. P values ≤ 0.05 were considered statistically significant.

Results

Lung function, bronchial hyperreactivity and nasal inflammatory indices at baseline

Evaluation of the pulmonary function parameters showed normal values (≥ 90% of predicted) in all allergic patients, with no differences with controls (not shown). In contrast, a detectable bronchial hyperresponsiveness to MCh was observed only in allergic children (312.17μg PD_{20}MCh) and in none of the control group (> 2400 μg PD_{20}MCh). At baseline, as compared to controls, a significant higher expression of ICAM-1 and HLA-DR ($p < 0.05$), (Figures 1A and 1B), associated with a higher proportion of eosinophils ($p=0.001$), but not of neutrophils ($p=0.169$) was observed (Figures 1C and 1D).

Changes in nasal inflammatory parameters after allergen challenge

Nasal allergen challenge was followed by lower respiratory tract symptoms, i.e. cough and/or wheezing, requiring the administration of inhaled salbutamol in 6 out of the 11 children evaluated.

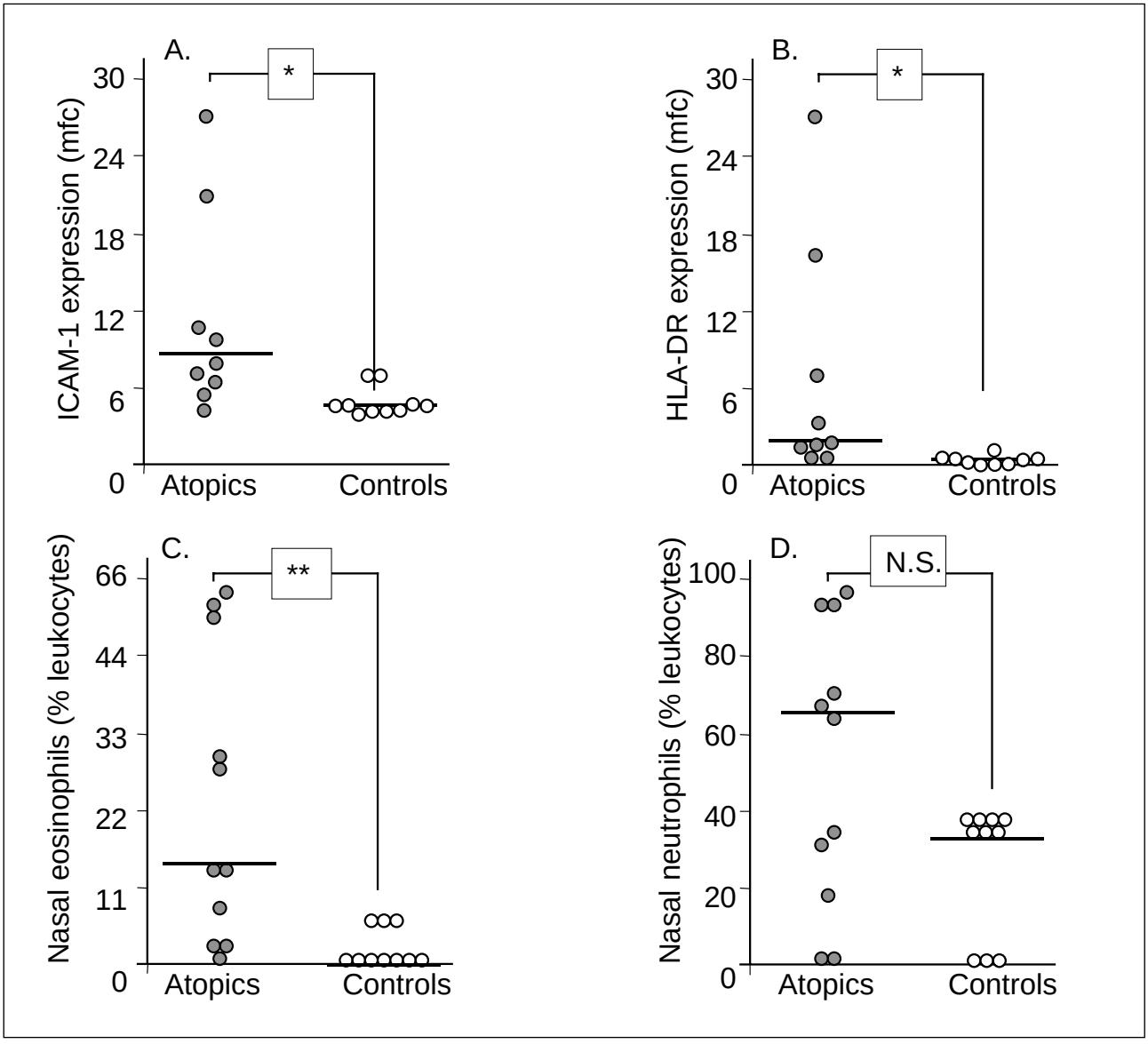


Figure 1. Nasal inflammation parameters at baseline in atopic children and in non atopic normal controls: A) Intercellular adhesion molecule-1 (ICAM-1) expression by nasal epithelial cells; B) HLA-DR expression by nasal epithelial cells; C) Percentage of eosinophils in nasal brushings; D) Percentage of neutrophils in nasal brushings. ICAM-1 and HLA-DR expression, measured as mean fluorescence challenge (mfc), and eosinophil and neutrophil proportions, expressed as percentage of the total nasal brushing leukocytes are shown on the ordinate, and the two study populations on the abscissa. The horizontal lines represent median values. * = $p<0.05$, ** = $p<0.01$. N.S.: Not statistically significant.

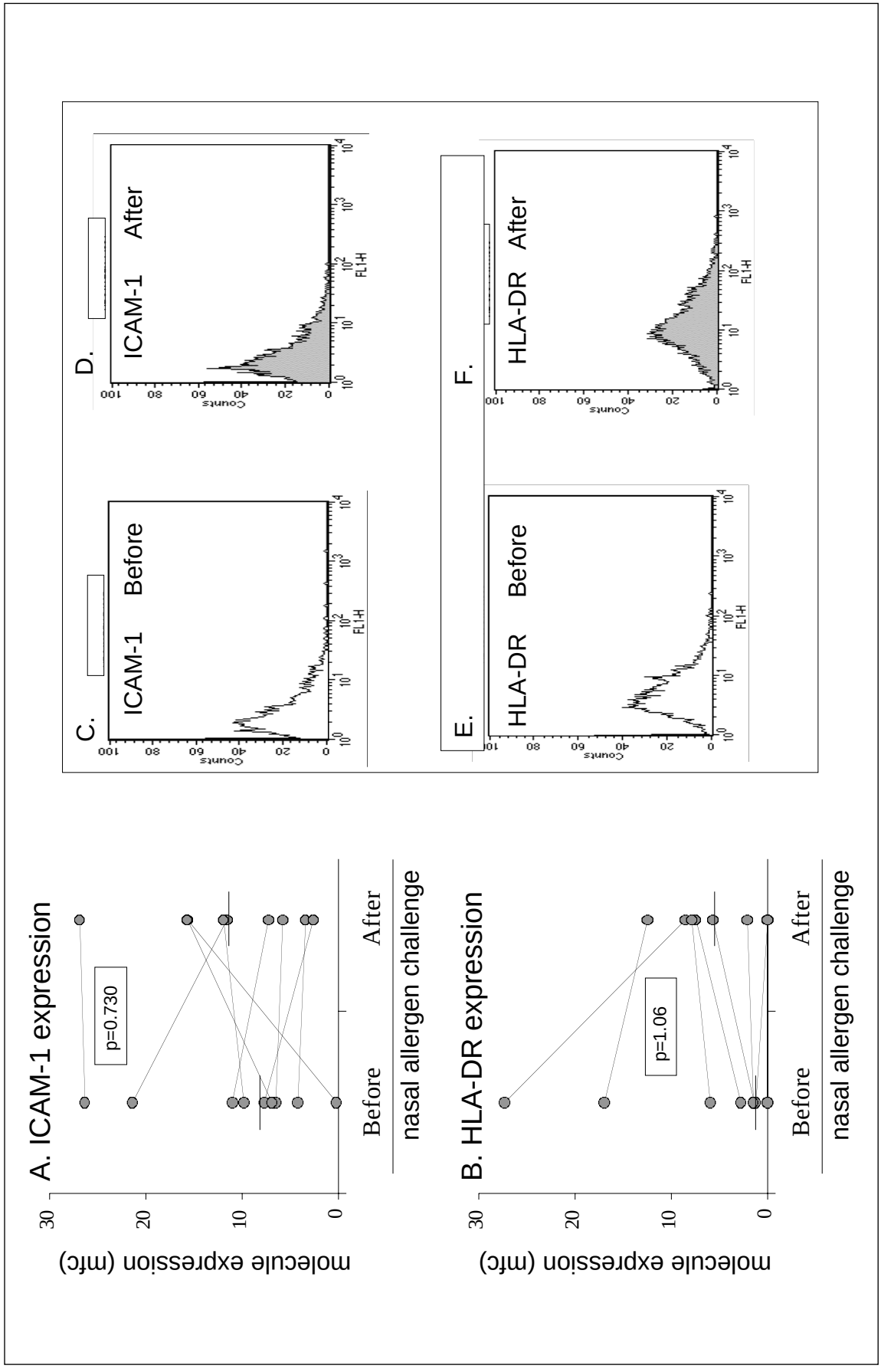


Figure 2. Expression of surface molecules, ICAM-1 and HLA-DR, by nasal epithelial cells of atopic children at baseline and after nasal allergen challenge. In (A) and (B), ICAM-1 and HLA-DR expression is indicated as mean fluorescence channel (mfc) on the ordinate, while the two study points are reported on the abscissa. The horizontal lines represent median values. In (C)-(F), flow cytometric histograms of ICAM (C), (D) and HLA-DR (E), (F) expression by nasal epithelial cells, before (C), (E) and after allergen challenge (D), (F), representative of one atopic individual.

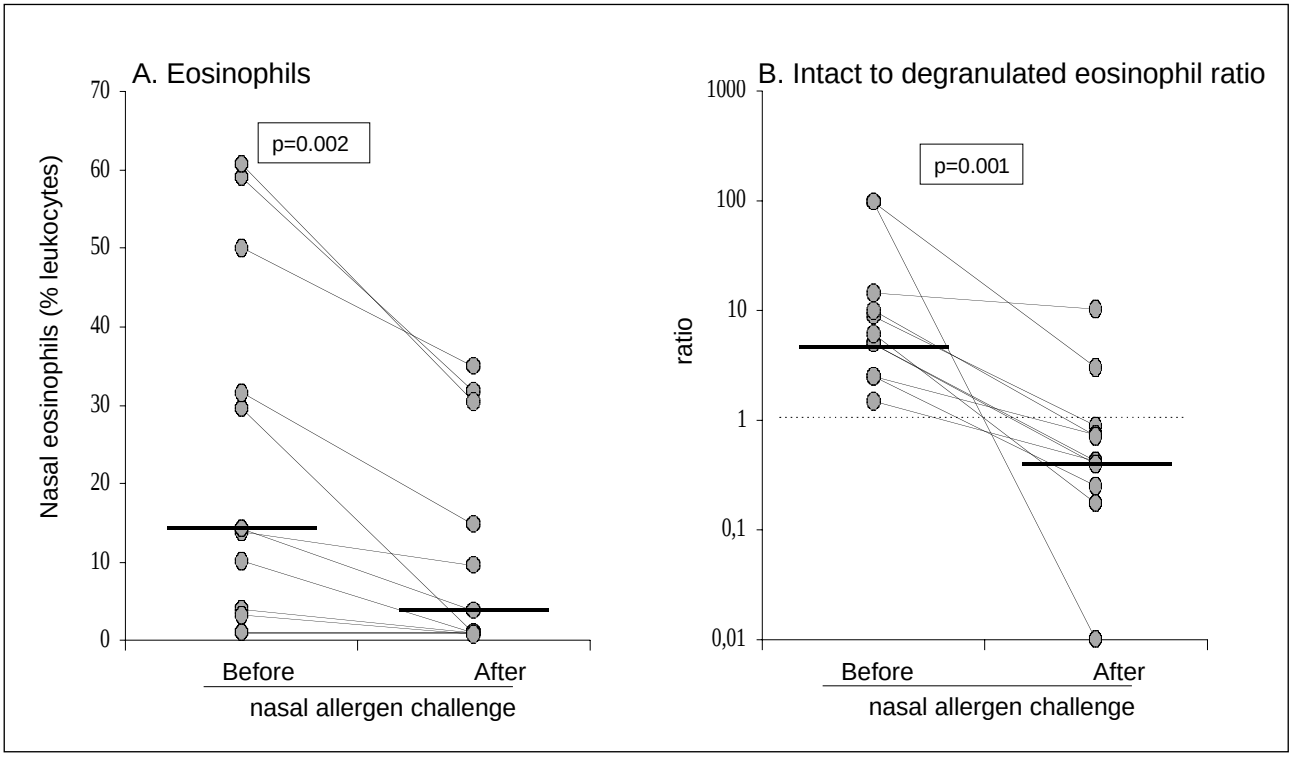
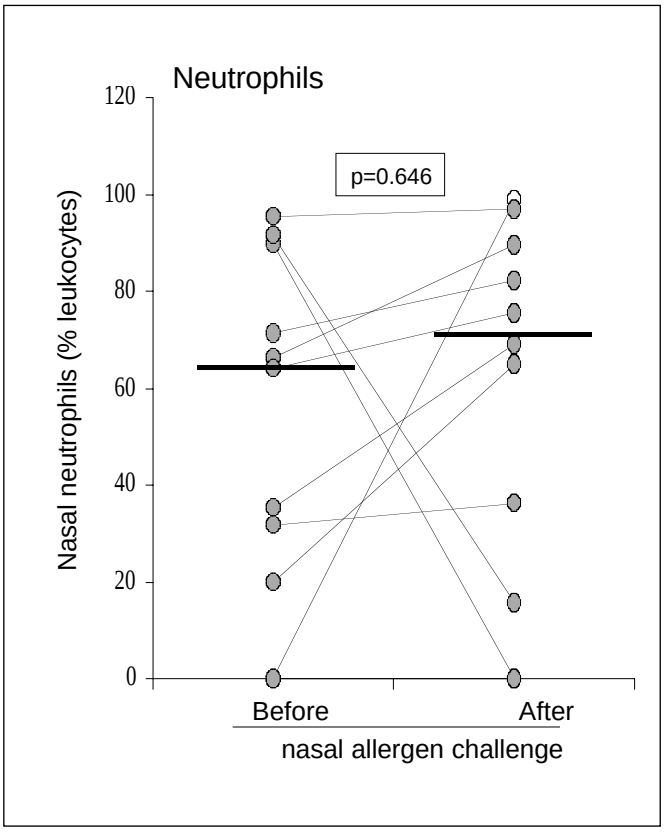


Figure 3. Percentage of intact eosinophils (A) in nasal brushings and 'intact to degranulated eosinophil' ratio (B) in atopic children at baseline and after nasal allergen challenge. Eosinophil proportions, expressed as percentage of the total nasal brushing leukocytes and the 'intact to degranulated eosinophil' ratio are shown on the ordinate, and the two study points on the abscissa. The horizontal lines represent median values.

Figure 4. Percentage of neutrophils in nasal brushings of atopic children at baseline and after nasal allergen challenge. Neutrophil proportions, expressed as percentage of the total nasal brushing leukocytes, are shown on the ordinate, and the two study points on the abscissa. The horizontal lines represent median values.

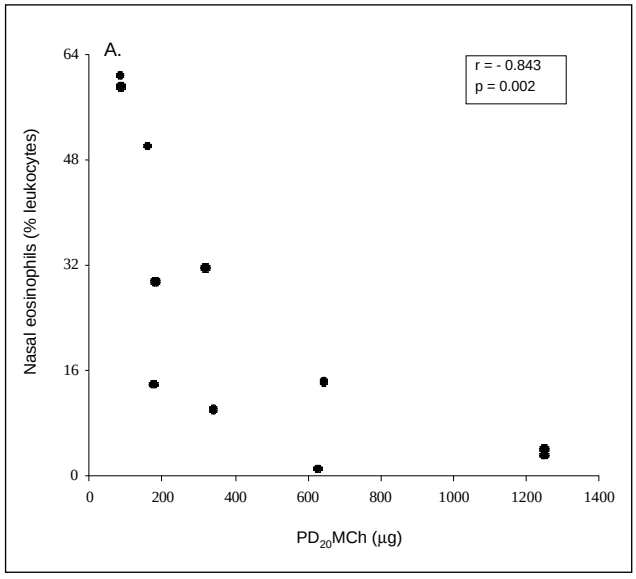


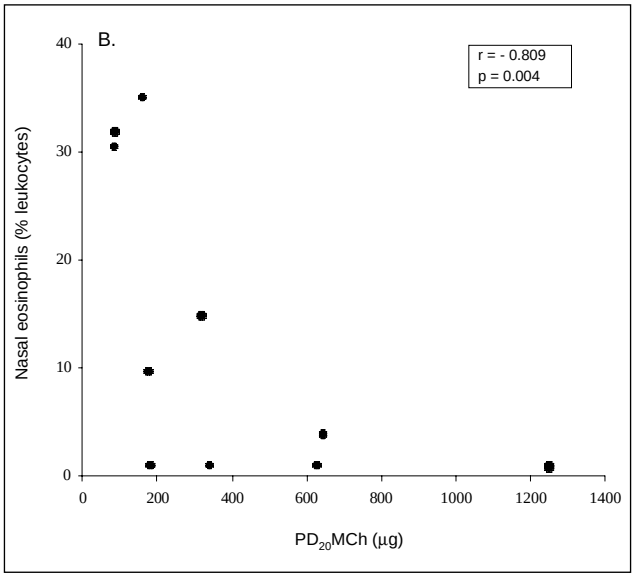
Nasal allergen challenge, performed in the allergic patients, did not induce significant changes in the intensity of the expression of ICAM-1 (p=0.73) or HLA-DR by epithelial cells (p=1.0) (Figures 2A, 2B, 2C and 2D). In contrast, allergen challenge was followed by a significant decrease in the proportion of eosinophils (p=0.002), (Figure 3A) associated to a significant decrease in the 'intact to degranulated eosinophil' ratio being 6.14 (3.74-56.69) before allergen challenge and 0.43 (0.33-1.94) after allergen challenge (p=0.0013) (Figure 3B); no change in neutrophilia was observed (Figure 4), (p=0.646).

Correlation between nasal inflammatory indices and pulmonary functions

At baseline, both in the allergic and in the control group, no correlations between ICAM-1 or HLA-DR expression and eosinophil or neutrophil proportions was detected (not shown). Similarly, no correlations were found between nasal inflammation indices (ICAM-1 or HLA-DR expression and inflammatory cell proportion) and pulmonary function parameters (FVC, FEV_1, and FEF25-75%) (not shown). Only in allergic children, a statistically significant correlation was observed between eosinophil proportion and bronchial reactivity to MCh, expressed as PD_{20}MCh (r = - 0.84, p = 0.002), (Figure 5A).

After nasal allergen challenge, no correlations were observed between nasal eosinophilia or neutrophilia and ICAM-1 or HLA-DR expression or between the allergen-induced changes in nasal inflammation indices (table 2) and at baseline pulmonary function parameters (not shown). In contrast, significant correlations were detected between bronchial reactivity to MCh, and nasal eosinophilia after allergen challenge (r = - 0.81, p = 0.004) or nasal allergen induced-changes in eosinophilia (r = - 0.75, p = 0.010), (Figure 5B and 5C).





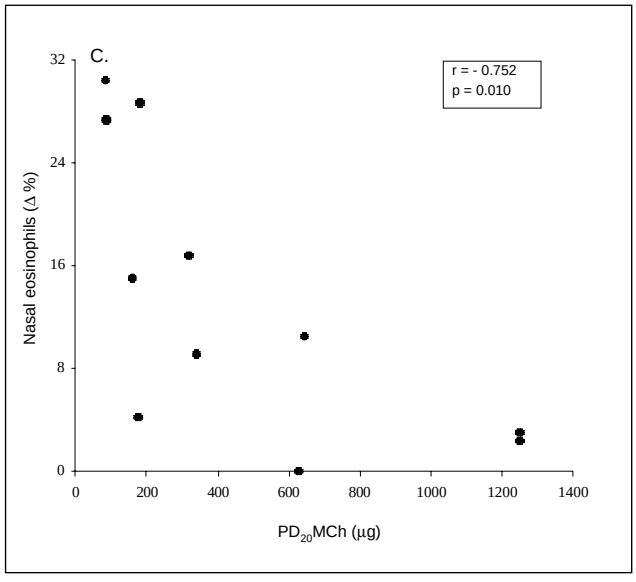


Figure 5. Correlation between bronchial reactivity to methacholine (MCh) and nasal brushing eosinophil proportion before (A) or after allergen challenge (B) or changes in eosinophil proportion induced by allergen challenge (C). Eosinophil proportion, or changes in eosinophil proportion, expressed as Δ eosinophils between baseline and allergen challenge-induced cell proportions, are shown on the ordinate, whereas bronchial reactivity to MCh, expressed as PD_{20} MCh, is shown on the abscissa.

Discussion

Evaluating children sensitized to HDM, with rhinoconjunctivitis and asthma, by nasal brushing we have shown an increase in nasal eosinophilia and in ICAM-1 expression by nasal epithelial cells and a correlation between eosinophilia and bronchial reactivity to MCh. After nasal allergen challenge, a significant decrease in nasal eosinophil percentage, associated to a significant decrease in the 'intact to degranulated eosinophils' ratio, was observed and correlations were still present between bronchial reactivity to MCh and nasal allergen challenge-induced changes in nasal eosinophilia.

The inflammatory process underlying allergic rhinitis is triggered and subsequently maintained by exposure to allergens with induction of allergen-specific IgE synthesis by B-cells [19,20]. Through the interaction with the high affinity receptor for IgE, FcεRI, these allergen-IgE complexes activate mast cells and basophils to release proinflammatory mediators, able to recruit eosinophils and basophils and to increase expression of adhesion molecules involved in leukocyte migration and activation by endothelial and epithelial cells [19,21]. In agreement with previous observations, we found increased proportions of eosinophils associated with an overexpression of ICAM-1 in the nasal epithelium of our patients with perennial rhinitis, as compared to controls [17, 22-24].

Our knowledge of the mechanisms involved in the pathogenesis of allergic rhinitis have greatly improved by nasal challenge studies, through the evaluation of cells and mediators released in the nasal fluid during the early- and late-phase allergic reaction [5]. Experimental studies have shown that after allergen challenge, the activation and degranulation of mast cells and basophils occurs [25] and results in the release of products thought to play an important role in the induction of the inflammatory events that characterize the late-phase reaction [5,7,9,10,26].

Evaluating the very early events following allergen challenge, we here have found a significant decrease in the total number of eosinophils infiltrating the nasal epithelium, associated with a relative decrease in the proportion of intact cells as compared to degranulated cells. These changes in eosinophil proportion were likely related to a reaction to allergen since, in preliminary experiments performed in a different study, no modification in the nasal eosinophilia after challenge with the inert diluent was detected [17]. In addition, also MCh challenge has been shown not to influence eosinophilia in the airways [27,28]. Although the mechanism characterizing eosinophil degranulation *in vivo* is poorly understood, this phenomenon may be, at least in part, related to a FcεRI-mediated activation and degranulation of these cells, as observed in the present paper. Indeed, eosinophil from allergic individuals overexpress the FcεRI on their membrane [13], a characteristic that in mast cells has been associated with a greater binding affinity to IgE molecules, and increased release of cell-derived products [21]. In addition, as also shown in the present report, a high proportion of degranulated eosinophils may be detected in the nasal mucosa in allergic rhinitis after artificial and natural allergen challenge [29]. Evaluating by transmission electron microscopy biopsy specimens from patients with allergic rhinitis, nasal polyposis, asthma and inflammatory bowel diseases, Erjefalt and co-authors found a greater eosinophil degranulation pattern in the first condition despite a similar number of tissue eosinophils [29]. The concept of an early degranulation of eosinophils following allergen exposure is also supported by the demonstration that, in latex allergy, elevation of the levels of the eosinophilic cationic protein (ECP) may be detected as early as 2 hours after nasal challenge, while a significant increase in eosinophil numbers is observed only 6 hours after challenge [30].

In addition to a FcεRI-mediated activation, allergen challenge may induce eosinophil activation through an IgE-independent pathway. Indeed it has been shown that, because of their enzymatic proteolytic activity, HDM allergens may directly activate, at least *in vitro*, airway epithelial cells to release cytokines and chemokines [31] and to alter the epithelial tight junctions, thereby increasing epithelial permeability [32].

ICAM-1, an adhesion molecule involved in the process of leukocyte migration, appears to be upregulated on nasal epithelial cells by mast cell-derived preformed products, such as histamine, as early as 1 hour after stimulation [14,33,34]. Increased expression of ICAM-1 has been shown in the nasal mucosa of adult allergic individuals [25], as early as 6 hours after nasal allergen provocation [8]. In accordance with other reports [33,34], we did not detect differences in adhesion molecule expression soon after allergen challenge. These conflicting results may be based on different patient population or on different methodological approaches to assess adhesion molecule expression.

In this study, we also found correlations between bronchial reactivity to MCh and eosinophil percentages in nasal brushing, 'at baseline' and after nasal allergen challenge. In asthmatic patients, the relationship between eosinophilic inflammation and airway hyperresponsiveness or airflow limitation is unclear [35,36]. Indeed, while lung function may correlate with airway eosinophil numbers [36], no clear evidence of a close relationship between bronchial hyperresponsiveness and inflammation has been shown [35]. In allergic individuals, however, bronchial responsiveness to MCh or to histamine significantly correlated with serum IgE levels and blood eosinophilia respectively [37] or with the decrease in circulating eosinophils, that follows allergen inhalation challenge [38]. A significant correlation between bronchial reactivity to MCh and the decrease in eosinophil proportions after nasal allergen challenge was detectable also in the present study.

Induction of neurogenic reflexes and the release of cytokines, chemokines, lipid mediators, neurotransmitters and neuropeptides are some of the mechanisms that may explain the links between the inflammatory processes characterizing allergic rhinitis and the occurrence or worsening of non-specific bronchial responsiveness or of airflow limitation in the lower airways [3-5, 19].

Since nasal allergen challenge induced lower respiratory tract symptoms, i.e. cough and/or wheezing, requiring the administration of inhaled salbutamol in an important proportion of the children evaluated, we could not study possible changes in bronchial responsiveness shortly after allergen challenge.

In *summary*, the results here presented further support the view that asthma and rhinitis can be considered as closely-related entities influenced by common pathogenetic processes, linked by similar physiologic characteristics, sustained and amplified by inter-connected mechanisms in atopic individuals.

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