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Lower airways inflammation in allergic rhinitis: a comparison with asthmatics and normal controls

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Clinical and Experimental Allergy

Summary

Background Allergic rhinitis (AR) and asthma represent a continuum of atopic disease. AR is believed to pre-dispose an individual to asthma. Compared with asthmatics and normal controls, the inflammatory response in the lower airways of rhinitis is not fully elucidated. To test the hypothesis that the inflammatory response in the airways of subjects with AR is at a level intermediate between that in normal controls and asthmatics, we have characterized bronchial inflammation and cytokine mRNA levels in non-asthmatic allergic rhinitis and compared it with subjects with allergic asthma and with normal controls.

Methods Endobronchial mucosal biopsies were obtained at bronchoscopy from 14 allergic rhinitis, 16 asthmatics and 21 normal controls. Biopsies were embedded into glycol methacrylate resin for immunohistochemical analysis of cellular inflammation and snap frozen for semi-quantitative PCR analysis of cytokine mRNA levels.

Results Airway inflammation in rhinitic subjects was characterized by an increase in submucosal eosinophils, mast cells and the mRNA expression of TNF- α , at an intermediate level between healthy and asthmatics. In addition, CD3⁺ and CD8⁺ lymphocytes in the epithelium, the endothelial expression of vascular adhesion molecule-1 and IL-1 β mRNA were higher in the allergic rhinitis compared with both normal controls and asthmatics, whereas growth-related oncogene α -mRNA was decreased in AR compared with both healthy and asthmatics. Airway inflammation in the asthmatic group was characterized by higher numbers of eosinophils and mast cells, together with an increase in TNF- α -mRNA compared with both healthy and rhinitis. IFN- γ mRNA was the highest in normal controls and lowest in the asthmatics.

Conclusions In individuals with AR the present data suggest an intermediate state of airway inflammation between that observed in normal individuals and subjects with clinical asthma. It is also indicated that IFN- γ production by CD8⁺ T lymphocytes could be protective against the development of airway hyperresponsiveness. Further work is needed to evaluate this hypothesis.

Keywords allergic rhinitis, asthma, bronchial mucosa, cytokine, inflammation

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Introduction

In the industrialized countries of the world, there is evidence of an increasing epidemic of asthma [1–4], the cause of which remains unclear. Individuals with pre-existing allergic rhinitis (AR) have an increased risk for the development of asthma [5, 6], particularly in the

presence of the following risk factors: a family history of asthma and atopy [7, 8]; skin test reactivity to common aeroallergens [9]; or bronchial hyperresponsiveness (BHR) [10]. Asymptomatic BHR is generally thought to be caused by airway inflammation [11, 12], and has been shown to precede the development of asthma in both adults and children [13, 14]. This asymptomatic inflammation has been measured indirectly by the measurement of exhaled nitric oxide [15, 16] and directly quantified by the

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examination of induced sputum [17] and bronchial biopsies [18], both of which identified increased numbers of mast cells and eosinophils in atopic non-asthmatic subjects outside the pollen season. Asymptomatic lower airways inflammation may thus represent one of the earliest stages in the development of asthma.

To improve the understanding of the relevance of atopy to the development of asthma, we have characterized the inflammation in the lower airways of non-asthmatic, asymptomatic subjects with AR. We tested the hypothesis 'The inflammatory response in the airways of subjects with AR is at a level intermediate between that observed in normal controls and asthmatics'. In the cellular processes involved, we hoped to be able to suggest a mechanism for the induction and maintenance of airway inflammation in such atopic individuals, ultimately leading to the development of asthma.

Materials and methods

Subjects

Three groups of subjects were recruited into the study: allergic rhinitics ($n = 14$), asthmatics on short-acting β_2 -agonists on demand ($n = 16$), and normal controls ($n = 21$). At inclusion, all subjects underwent skin prick testing to a standard panel of 10 aero-allergens, birch, timothy, mugwort, cat, horse, dog, *Dermatophagoides pteronyssinus* [house dust mite (HDM)], *Dermatophagoides farinae* (HDM), cladosporium (mould) and alternaria (mould). A positive result was recorded if the skin prick test (SPT) resulted in a weal reaction equal to or greater than the histamine control at 15 min. Forced expiratory volume in 1 s (FEV₁) was measured in all subjects using a computerized pneumotach spirometer (Vitalograph, Buckinghamshire, UK). In order to determine airway responsiveness, methacholine challenge with nebulized concentrations of methacholine ranging from 0.03 to 32 mg/mL was performed in the allergic rhinitics and the asthmatics. The provocative concentration of methacholine was calculated on the log-dose-response curve as the concentration of methacholine required to result in a decline in FEV₁ of 20% (PC₂₀) [19].

The inclusion criteria for subjects in the asthmatic group were a clinical history of asthma, classified according to GINA guidelines as intermittent to mild persistent [20]. All asthmatics had a positive SPT against at least one of the tested aero-allergens, FEV₁ > 80% of predicted and BHR with a PC₂₀ < 8 mg/mL. In the asthmatic group, 14 out of 16 subjects were tested positive for birch or timothy; 12 of these subjects also had a positive SPT against cat, horse, dog, mite or mould. The remaining two subjects had a positive SPT against cat, horse, dog, mite or mould.

Table 1. Characteristics of the subjects in each study group

Subject group	Healthy controls	Allergic rhinitis	Allergic asthma
Number in group	21	14	16
Mean age in years (range)	24 (21–29)	25 (22–34)	24 (18–32)
Gender (male : female)	12 : 9	7 : 7	8 : 8
FEV ₁ (% predicted)	98 ± 15	97 ± 15	99 ± 15
Geometrical mean of PC ₂₀ (mg/mL)	Not assessed	> 8.0	2.7 ± 1.8
Skin prick test to pollens	Negative	Positive	Positive

FEV₁, forced expiratory volume in 1 s.

The subjects with AR were recruited on the basis of a clinical history of seasonal AR requiring medical treatment only during the pollen season. They were classified as mild intermittent according to ARIA guidelines [21]. All subjects in the rhinitic group had a positive SPT against at least one of the tested aero-allergens, FEV₁ > 80% of predicted and no hyperresponsiveness (PC₂₀ > 8 mg/mL) as well as no history of asthma. In the rhinitic group, all subjects were tested positive for birch or timothy. In addition, four subjects also had a positive SPT against cat, horse, dog, mite or mould.

Normal controls had a negative history of atopy, negative SPTs to a standard panel of aero-allergens and FEV₁ > 80% predicted. The characteristics of the three groups are detailed in Table 1. Exclusion criteria in all groups included any history of smoking, airway infection within 6 weeks before or during the study, and current use of any medications (other than short-acting β_2 -agonists on demand in the asthmatic group). The study took place outside the pollen season. The local ethics committee approved the study and the subjects gave their written informed consent.

Bronchoscopy

Bronchoscopy was performed at the Department of Respiratory Medicine and Allergy, University Hospital, Umeå, Sweden. Subjects were premedicated with 1.0 mg of atropine given subcutaneously 30 min before the procedure. Topical local anaesthetic was achieved using lidocaine. All subjects tolerated the procedure well. A flexible video bronchoscope (Olympus BFIT200, Tokyo, Japan) was inserted through the mouth via a mouthpiece with the subjects in the supine position. Four to six endobronchial mucosal biopsies were taken for immunohistochemistry and PCR using fenestrated forceps (Olympus FB-21C, Tokyo, Japan). The biopsies were obtained either from the anterior aspect of the main carina and the subcarina of the second to fourth generation airways on the right side, or from the posterior aspect of the main carina and the corresponding subcarina on the left side.

Immunohistochemistry

The coded biopsy specimens were processed into glycol methacrylate (GMA) resin for immunohistochemistry as described previously [22]. Immunohistochemical staining was performed using the strept-avidin biotin-peroxidase technique and monoclonal antibodies directed against specific cell markers including mast cells (AA1), eosinophils (EG2), neutrophils (NOE), lymphocytes (CD3⁺, -4⁺, -8⁺, -25⁺), macrophages (CD68⁺) and adhesion molecules [E- and P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1)]. Positively stained nucleated cells were counted separately in the epithelium and in the submucosa excluding mucosal glands, blood vessels and muscle in two sections at least 10 µm apart. The area of the submucosa and the length of the epithelium counted were measured using computed-assisted image analysis. To be included in the study, the biopsies had to have a minimum area of 0.46 mm², as this has been previously shown to give representative data [23]. The results were expressed as cells/mm² and cells/mm, respectively. For each adhesion molecule, the number of blood vessels stained was presented as a percentage of the total number of vessels stained using the pan-endothelium marker EN4 in consecutive sections (2 µm apart). Analysis of the biopsies was performed with the observer blinded to the subject group.

Polymerase chain reaction

For semi-quantitative PCR, biopsies were analysed as described previously [24]. Samples were immediately frozen in liquid nitrogen and total RNA was subsequently extracted with Trizol and quantified by measuring optical density at 260 nm. RNA samples were reverse transcribed using avian myeloblastoma reverse transcriptase to produce cDNA. PCR amplification was performed with primer pairs specific for adenine phosphoribosyl transferase (APRT), IL-1β, IL-5, IL-8, TNF-α, IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF) and growth-related oncogene α (GRO-α). The conditions for each primer pair were established in advance and regulated using a Perkin Elmer thermal cycler (Perkin Elmer, Warrington, UK). After 30 cycles, the resulting gene transcripts were detected by a digoxigenin-ELISA method. The levels of cytokine transcripts were normalized to APRT and expressed as a percentage (level of cytokine products/level of APRT product × 100).

Statistics

Data were not normally distributed and are therefore presented as medians with 25th and 75th percentiles. Comparison of baseline immunohistochemistry indices

and PCR data between the three groups was performed using the Kruskal–Wallis ANOVA test. If a significant difference was observed between the three groups, the Mann–Whitney *U*-test was used for *post hoc* analyses. *P*-values < 0.05 were considered to be statistically significant.

Results

Immunohistochemistry

Analysable biopsies were obtained from 13 out of the 14 rhinitics who underwent bronchoscopy and from all the asthmatics (*n* = 16) and all the normal controls (*n* = 21). The biopsies had a mean submucosal area of 1.49, 1.36 and 1.38 mm², in the rhinitic, asthmatic and normal subjects, respectively, and an average epithelial length of 2.52, 3.18 and 3.38 mm.

Compared with normal controls, subjects with AR had elevated numbers of eosinophils in the bronchial submucosa (*P* = 0.022) (Fig. 1a), as well as increased numbers of mast cells (*P* = 0.022) (Fig. 1b), CD3⁺ lymphocytes (*P* = 0.029) (Fig. 1c) and CD8⁺ lymphocytes (*P* = 0.022) (Fig. 1d) in the epithelium. The increased numbers of epithelial lymphocytes in the allergic rhinitic subjects were also elevated when compared with the asthmatic group (Figs 1c and d). The increased cellular infiltration in the allergic rhinitics was paralleled by an increase in the endothelial expression of VCAM-1 compared with both normal controls (*P* = 0.003) and asthmatics (*P* = 0.001) (Fig. 2).

As expected, submucosal eosinophil numbers (*P* = 0.032) and epithelial mast cell numbers (*P* < 0.001) were increased in asthmatics (Figs 1a and b) compared with normal controls.

There were no differences between the three groups in the numbers of neutrophils, macrophages, CD4⁺ lymphocytes or in the endothelial expression of E- and P-selectin, and ICAM-1.

Polymerase chain reaction

Suitable biopsies for PCR were obtained from all the rhinitics (*n* = 14) and asthmatics (*n* = 16) and from 18 of the 21 normal controls.

Semi-quantitative PCR showed an increase in mRNA for IL-1β and TNF-α in the allergic rhinitics (*P* < 0.001 and *P* = 0.041) (Figs 3a and b), whereas mRNA for GRO-α and IFN-γ (*P* = 0.009 and 0.037) were decreased (Figs 3c and d), when compared with normal controls. Compared with the asthmatics, subjects with AR expressed a lower level of GRO-α mRNA (*P* = 0.002), while IFN-γ mRNA was higher (*P* = 0.007).

In the asthmatic subjects, mRNA levels for IL-1β and TNF-α were increased (*P* < 0.001 and *P* = 0.009), whereas

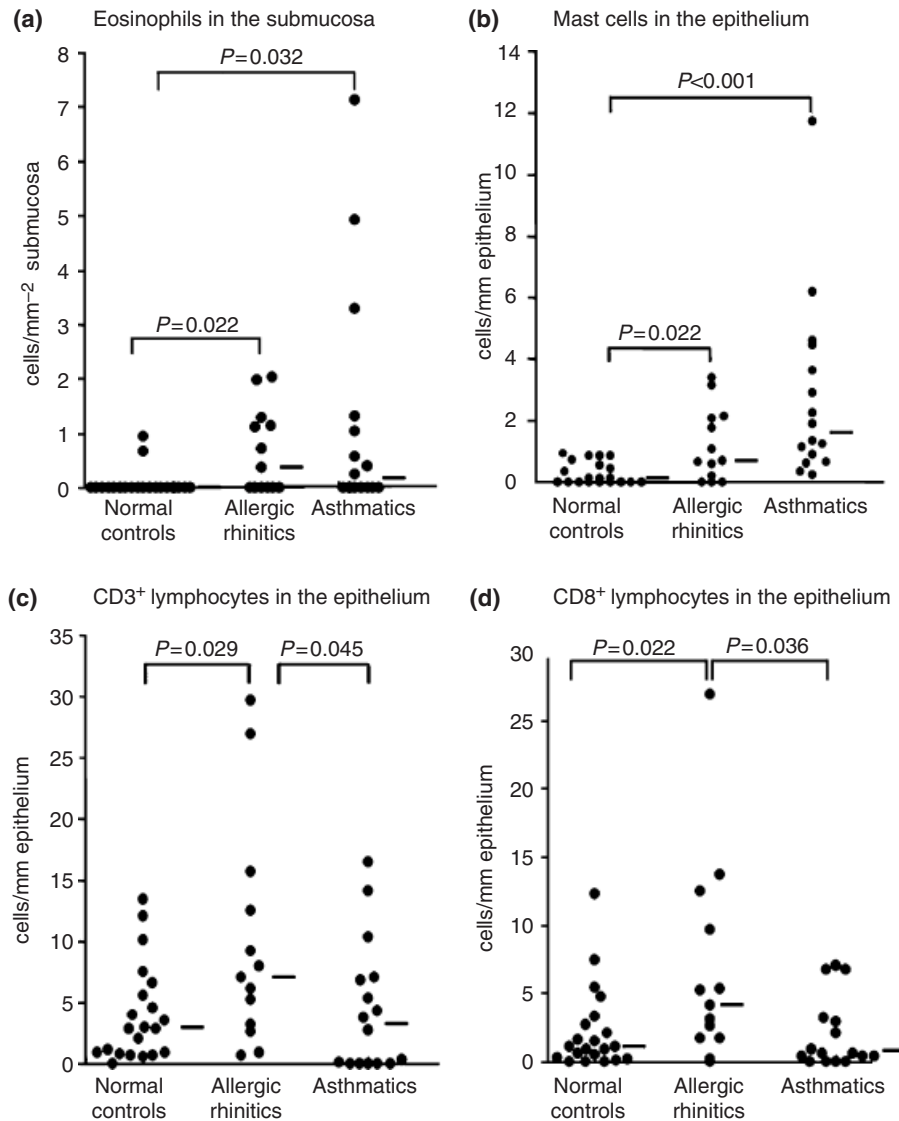


Fig. 1. Graphs showing the number of eosinophils in the bronchial submucosa (a), and mast cells (b), CD3⁺ lymphocytes (c) and CD8⁺ lymphocytes (d) in the bronchial epithelium of the normal, allergic rhinitic and asthmatic subjects. Significant P -values are shown.

IFN- γ mRNA was decreased ($P=0.002$), compared with the normal controls.

The mRNA levels for IL-5, IL-8 and GM-CSF did not differ between the three groups.

Discussion

The results from the present study demonstrate that allergic rhinitic subjects show evidence of inflammation in the lower airways when studied outside the pollen season. The numbers of mast cells and eosinophils were intermediate compared with the numbers in the lower airways of asthmatic and control subjects. However, the numbers of CD3⁺ and CD8⁺ lymphocytes and the endothelial adhesion molecule VCAM-1 were significantly higher in the allergic rhinitic subjects compared with both

allergic asthmatics and normal subjects. In allergic rhinitis, the inflammatory cellular response was paralleled by an increase in mRNA levels for IL-1 β and TNF- α and decreases in GRO- α and IFN- γ .

The eosinophil is considered to be a major effector cell of allergic disease as a result of its ability to produce inflammatory mediators such as leucotrienes, major basic protein and eosinophil peroxidase and cationic protein [25–28]. The presence of eosinophils in the lower airways of asthmatic, rhinitic and atopic non-asthmatic individuals has been described previously [18, 29, 30], with eosinophil numbers the highest in the asthmatics and intermediate in the atopic subjects compared with healthy controls [18, 29]. In this present study and in that of Braunstahl et al. [30], although eosinophil numbers were greater than in normal controls, similar numbers were

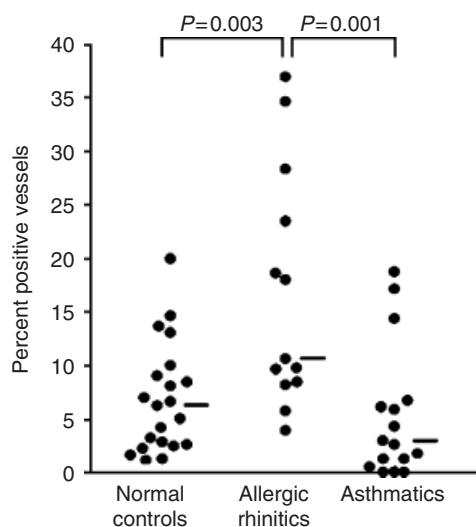


Fig. 2. Graph showing the endothelial expression of vascular adhesion molecule-1 in the normal, allergic rhinitic and asthmatic subjects. Significant *P*-values are shown.

observed in both asthmatic and allergic rhinitic airways, suggesting that the presence of eosinophils is related to atopy and this alone is not enough to cause a symptomatic airway disease.

In this current study, we did not study markers of eosinophil activation. However, Lam et al. [31] and Alvarez et al. [32] have examined this in bronchoalveolar lavage (BAL) and sputum, respectively, before and after antigen challenge in both asthmatics and allergic rhinitics. Before antigen challenge, neither observed any differences in leukotriene or eosinophil cationic protein levels. However, signs of eosinophil activation were seen following antigen provocation in both groups, with a greater response in asthmatics. In an electron microscopic study of bronchial biopsies from asthmatics and atopic individuals, Djukanovic et al. [18] observed degranulation of eosinophils in both groups. These observations, together with the study of Leckie et al. [33] in which treatment of asthmatics with an anti-IL-5 antibody reduced both blood and sputum eosinophilia but had no effect on airway hyperresponsiveness (AHR), raise some questions about the exact role of the eosinophil in its contribution to the development of symptomatic asthma.

The role of the mast cell in asthma is well established: they contribute to the activation and perpetuation of the inflammatory response [34–37]. In active disease, the mast cell is usually located within the bronchial epithelium, at the interface of the internal and external environment, where it can respond to allergens and other exogenous stimuli. In this study, we observed significantly more mast cells in the bronchial epithelium in the allergic rhinitics compared with the normal controls. Consistent with previous studies, no differences in sub-

mucosal mast cell numbers were observed between subjects with AR, allergic asthma and healthy controls [18, 29–31, 38].

In the allergic inflammatory response, activation of mast cells leads to the release of mediators contributing to the early phase as well as the late-phase of asthmatic inflammation [39, 40]. Lam et al. [31] reported raised, although not statistically significant, levels of histamine in BAL before antigen challenge in asthmatics compared with rhinitics. Tryptase was detectable in the sputum in seven out of 31 asthmatics at baseline but was completely undetectable in rhinitic subjects [32]. BAL levels of IL-4 and IL-5, two cytokines known to be released by mast cells [39], were not different between asthmatics and rhinitics [41]. Djukanovic et al. [18] observed ultrastructurally that mast cells in asthmatics exhibited signs of degranulation compared with those from atopic controls, which, despite showing some signs of degranulation, were generally intact. Taken together, these data suggest a difference in the activation status, rather than in numbers, of mast cells between asthmatic and rhinitic individuals and that this may contribute to the BHR observed in asthmatics.

T lymphocytes are also implicated in the development of asthma with T helper 2 (Th2) cell activation and production of cytokines, playing an important role in the development and maintenance of allergic disease and hyperresponsiveness [42, 43]. We did not observe any differences in lymphocyte numbers within the submucosa between the three groups. However, in the rhinitic group, a significant increase in epithelial CD8⁺ lymphocytes was detected compared with both normal controls and asthmatics. This would account for the increase in total number of T lymphocytes (CD3⁺) that was also evident. CD8⁺ T cells have dual functions – some are cytotoxic and others mediate T cell suppression. For example, depletion of CD8⁺ T cells leads to an enhanced Th2 response and BHR before sensitization in both rat and murine challenge models [44, 45]. It is thus possible that the increased numbers of CD8⁺ cells in the airways of the rhinitics may represent a protective phenotype, preventing the development of asthma.

The significance of the increase in endothelial expression of VCAM-1 in the allergic rhinitics compared with both the normal controls and asthmatics remains unclear. Zangrilli et al. [41] observed no difference in soluble VCAM-1 in BAL of asthmatics and rhinitics before antigen challenge. VCAM-1 is important in recruiting eosinophils and activated T lymphocytes from the circulation into the airways of asthmatic subjects [46]. However, in this study there was no correlation between endothelial expression of VCAM-1 and eosinophil or lymphocyte numbers. Other work has suggested that the extent of both VCAM-1 and ICAM-1 expression in asthmatics may be increased after allergen challenge [47]. Furthermore, in asthmatics, a significant association has been found between the

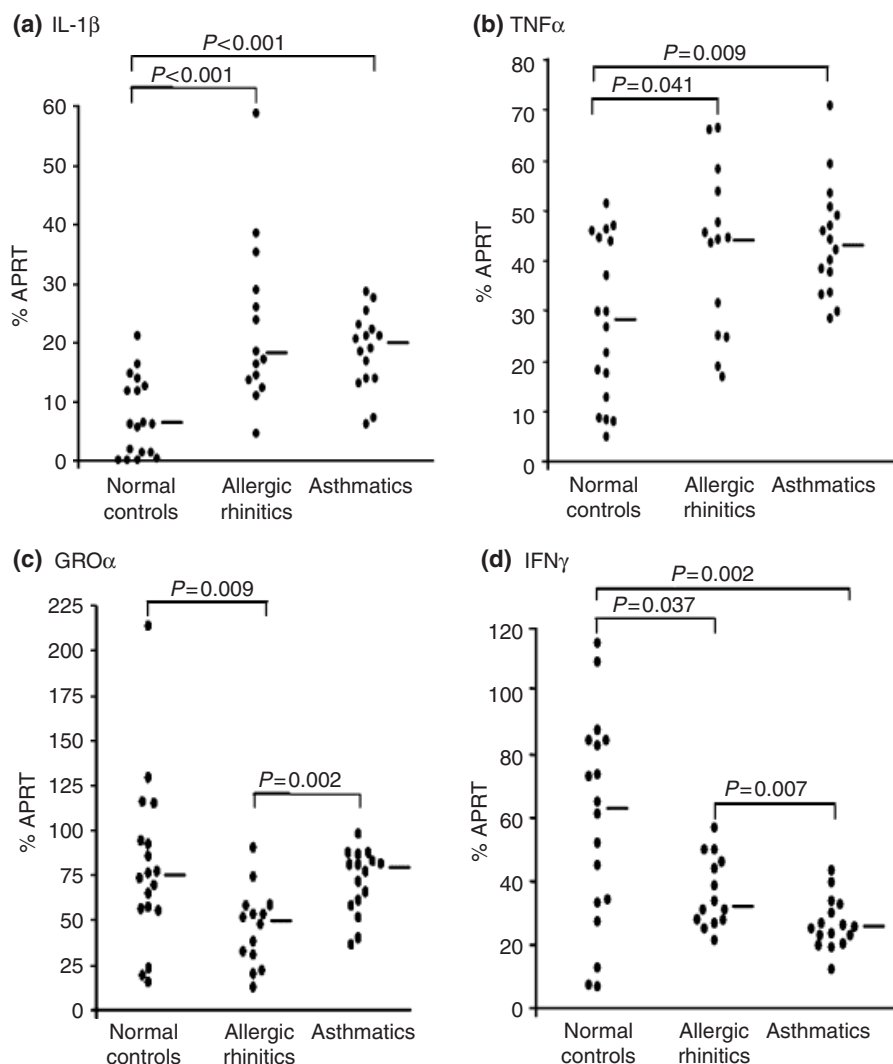


Fig. 3. Graphs showing the mRNA expression for IL-1 β (a), TNF- α (b), growth-related oncogene α (GRO- α) (c) and IFN- γ (d) in the normal, allergic rhinitic and asthmatic subjects. Significant P -values are shown.

expression of these adhesion molecules and the number of eosinophils [47]. The lack of correlation in allergic rhinitis may reflect the intermediate inflammatory status within the bronchial mucosa.

IL-1 β and TNF- α are both pro-inflammatory cytokines that can contribute to the initiation and perpetuation of the inflammatory response, including the activation of the endothelium with increased expression of adhesion molecules and subsequent inflammatory cell recruitment (reviewed in [48]). mRNA levels of both these cytokines were increased in the airways of both the rhinitics and asthmatics compared with normal controls, with no difference detected between the two atopic groups. Increased expression of these cytokines has previously been reported in asthma [49–53]; again, as with the eosinophil, our findings suggest that this may be a feature of atopy rather than asthma.

The relevance of the decreased mRNA levels for the neutrophil chemokine GRO- α , in the rhinitics compared with both the normal controls and asthmatics is unclear. No differences were observed in neutrophilic inflammation between the three subject groups. This therefore remains a matter for further investigation.

IFN- γ is considered an inhibitory cytokine, inhibiting allergic inflammation while promoting cell-mediated cytotoxic responses [48]. Our observation of a disease-related decrease in IFN- γ levels is thus not unexpected. There are no previous studies comparing mRNA levels of IFN- γ in bronchial biopsies from rhinitics with those of normal subjects or asthmatics. In studies of BAL cells from asthmatics that have been stimulated *ex vivo*, increases in IFN- γ are reported compared with normal controls [53, 54]. However, if BAL cells are not stimulated, no differences are observed [55]. When comparing IFN- γ

expression in lung tissue samples from subjects who died of asthma to those with asthma who died of other causes, or to biopsies from normal subjects, no differences were observed [56]. Ying *et al.* [57] has studied IFN- γ -mRNA expression in bronchial biopsies and observed a decrease in the number of IFN- γ -expressing cells in asthmatics compared with controls. Nonetheless, consistent with the murine model [58], one possibility is that IFN- γ production by CD8⁺ T lymphocytes protects against the development of AHR. The present data indicated a possible relationship between the mRNA levels for IFN- γ and PC₂₀ methacholine in the asthmatic and allergic rhinitic subjects, but this did not reach statistical significance ($\rho = 0.337$, $P = 0.07$). As mRNA expression does not necessarily lead to protein synthesis, a stronger relationship may be observed if this were examined.

In conclusion, we have demonstrated the presence of inflammatory changes in the lower airways of both asthmatic subjects and non-asthmatic subjects with AR. The allergic rhinitics had a pattern of mast cell and eosinophil inflammation similar to that in the asthmatics but they had increased numbers of epithelial CD8⁺ T cells and increased endothelial expression of VCAM-1. In addition, we found increased mRNA for IL- β and TNF- α in both asthmatic and rhinitic subjects compared with normal controls as well as a disease-related decrease in IFN- γ . Further work is required to characterize the functional properties of the infiltrating CD8⁺ T cells and to investigate the contribution of IFN- γ to protect against the development of asthma in subjects with AR.

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