Increased interleukin-13 mRNA expression in bronchoalveolar lavage cells of atopic patients with mild asthma after repeated low-dose allergen provocations

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Immune and inflammatory responses mediated by cytokines are essential in the pathophysiology of asthma. The aim of this study was to analyse the cytokine mRNA profiles in bronchoalveolar lavage (BAL) cells of patients with mild atopic asthma, before and after induction of a subclinical allergic airway inflammation. For this purpose, eight patients with mild atopic asthma received low-dose allergen inhalations equivalent to 10% of a provocational dose causing a 20% fall in forced expiratory flow in 1 sec (PD20) for 7 weekdays. BAL was performed before and after low-dose provocations in patients, and without provocation in five healthy controls. Alveolar macrophages (AM) were enriched by negative selection, using magnetic beads, to enable separate studies of the BAL cells. Using a semiquantitative RT-PCR technique, the mRNA expression of macrophage-derived cytokines interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, IL-13, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β was analysed. After low-dose provocations, we observed a significant increase in the expression of IL-13 mRNA (P = 0.01) in BAL cells enriched for AM of the asthmatic patients. The increased IL-13 mRNA positively correlated with the proportion of BAL fluid eosinophils (r = 0.7, P = 0.05). Moreover, a tendency was found towards an increased IL-1 and a reduced IL-6, IL-8, IFN-γ and TNF-α expression by the BAL cells. Comparing asthmatic patients before low-dose provocations and healthy controls, a significantly higher expression of IL-6 (P < 0.003), IL-10 (P < 0.005) and TGF-β (P < 0.003) and a significantly lower expression of IL-8 (P < 0.005) and TNF-α (P < 0.01) was detected in the patients. In summary, repeated low-dose allergen provocations of asthmatic patients results in a modified BAL cell cytokine mRNA profile with increased production of IL-13, that may be of importance for the development of a Th2-like immune response. A possible source of the increased IL-13 mRNA is AM, which may have a more active function in the allergic inflammation than previously thought.

Key words: asthma; low-dose provocation; alveolar macrophages; IL-13.

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways, influenced by genetic and environmental factors, and characterized by bronchial hyperreactivity, eosinophil infiltration, elevated serum IgE levels and excessive airway mucus production (1). Bronchial inflammation reflects a cell-mediated immune process, in which cytokine production by activated T cells may orchestrate the immune response. Thus, the pathology of asthma is thought to be mediated primarily by the type 2 cytokines IL-4, IL-5 and IL-13 (2,3).

In animal models, IL-13 has been recently implicated in allergic asthma, acute lung inflammatory injury and pulmonary granuloma formation (4–7). IL-13 shares receptor components and biological properties with IL-4 such as induction of IgE synthesis and downregulation of IL-12 and IFN-γ production, thereby favouring a type 2 cytokine response (8,9).

Repeated low-dose allergen exposure is a good method for studying the development of allergic inflammation in asthma. This model has been shown to provide a realistic
gradual worsening of the asthmatic response with airway eosinophilia and increased IL-5 in induced sputum, associated with airway hyperresponsiveness without development of marked acute bronchoconstriction (10–12). In a recent study, we described an increased responsiveness to methacholine and airway inflammation after repeated low-dose allergen provocations in a group of patients with mild asthma (13). In the present report, we used the same group of patients to analyse the mRNA cytokine expression in BAL cells enriched for alveolar macrophages (AM). We found a significant increase in the expression of IL-13 mRNA, and a trend towards a reduced expression of the pro-inflammatory cytokines IL-6 and IL-8 and of the Th1 associated cytokines IFN-γ and TNF-α. Our results are in line with the notion that a repeated exposure for low doses of allergen elicits a Th2 deviated immune response, and suggest the low-dose allergen provocation model to be appropriate for studies of early inflammatory events in allergic asthma.

Materials and methods

SUBJECTS

Eight patients with atopic asthma, seven females and one male, with a median age of 29 years (range 24–41 years) were included in the study. The patients had a history of mild allergic asthma to pollen and/or to animal dander with a positive skin prick test and positive radioallergoabsorbent test (RAST, Pharmacia, Uppsala, Sweden). All patients were in a stable phase of the disease with a median forced expiratory volume in 1 sec (FEV1) 93% of the predicted normal value (range 80–101%) and median total IgE values of 79 kU l⁻¹ (range 25–170 kU l⁻¹). [For individual data see Lensmar et al. (13).] No medication was taken except for occasional β₂-agonists. All patients were free from symptoms of airway infection for at least 4 weeks prior to the study. The study was performed outside the pollen season and patients allergic to animal dander did not have pets, and were asked to avoid animal contact during the study period. Five healthy subjects (two females) without symptoms or a history of allergy, with a median age of 25 years (range 23–27 years) were selected as controls. The local Ethics Committee approved the study, and all patients gave informed consent.

STUDY DESIGN

A first bronchoscopy with BAL was performed 2–3 weeks before starting the allergen provocation. Basal FEV₁ was measured before the first dose of allergen as well as before and 15 min after each allergen inhalation. Allergen doses of birch or grass pollen (Aquagen, ALK, Copenhagen, Denmark) were selected according to previously determined PD₉₀ (the allergen dose causing 20% fall in FEV₁). Inhalation doses of allergen corresponding to 10% of PD₉₀ were administrated in the mornings, and at the same time daily during 7 weekdays [see Lensmar et al. (13) for more details]. The same dose was given every day and varied from 8·4 SQ (arbitrary units related to the concentration of the allergen) to 243 SQ between the individuals. A second BAL was performed 1 day after completed allergen provocation. All bronchial provocations were performed by using a dosimeter controlled jet nebulized Spira Electro 2 (Respiratory Care Center, Hameenlinna, Finland). BAL was performed once in the Control group without any preceding provocation.

BAL AND AM PREPARATION

BAL was performed using a flexible fibre-optic bronchoscope (Olympus BF Type P20; Olympus Optical Co. Ltd, Tokyo, Japan) as previously described (14). Briefly, the patients were pre-medicated with Morphine–Scopolamine (Pharmacia and Upjohn, Uppsala, Sweden) and administered inhaled β₂-agonist 10 min before the bronchoscopy. The right middle lobe was instilled with five aliquots of 50 ml sterile buffered saline solution (PBS), and the fluid was gently aspirated after each aliquot, collected in a sterile siliconized bottle and kept on ice. The lavage fluid was filtered through a single layer of dacron net (type AP32, Millipore, Bedford, Ireland) and centrifuged at 400 g for 10 min at 4°C. The cell pellet was resuspended in RPMI 1640 (Sigma-Aldrich Co, St Louis, MO, U.S.A.), counted in a Bürker chamber and the viability assessed by cellular exclusion of trypan blue. Cytospin preparation was performed by centrifugation of 6 × 10⁶ cells at 500 g for 3 min (Cytospin 2, Shandon Southern Instruments, Runcorn, U.K.). One slide was stained in May–Grünwald Giemsa for evaluation of cell differentials, and another in Toluidine blue for assessment of metachromatic cell counts.

AM from BAL were enriched by negative selection using anti-CD2 coated magnetic beads (Dynalbeads, Dynal, Oslo, Norway) according to the manufacturer instructions, resulting in a depletion of T-lymphocytes and natural killer (NK) cells. The purity of this population was determined by May–Grünwald Giemsa and Toluidine blue stainings.

RNA EXTRACTION, cDNA SYNTHESIS AND RT-PCR

Total cellular RNA was extracted from 1–2 × 10⁶ enriched AM with RNAzoB (Biotec Labs, Houston, TX, U.S.A.) according to the manufacturer’s instructions. The RNA was quantitated by spectrophotometry at 260 nm and stored at −70°C. The integrity of the RNA sample was assessed by electrophoresis in a 1.4% agarose gel (BRL, Gaithersburg, MD, U.S.A.) with 0.2 μg ml⁻¹ ethidium bromide. RNA was denatured at 90°C for 5 min and chilled on ice. First strand cDNA synthesis was performed in a final volume of 20 μl containing: 10 μl of denatured RNA (1–5 μg), 4 μl buffer (5 ×, BRL), 1.5 μl dithiothretiol (100 mm, BRL), 2 μl dNTP (dATP, dCTP, dGTP and dITP, 5mm each, Pharmacia, Uppsala, Sweden), 0.5 μl RNasin (40U μl⁻¹, Promega, Madison, W), 1.0 μl random hexamer primers pd(N)₆ (1 μm, Pharmacia) and 1.0 μl reverse transcriptase from murine Moloney leukemia virus (200 U μl⁻¹, BRL).

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The samples were incubated at 40°C for 45 min and then heated for 5 min at 95°C.

The cDNA was amplified by PCR in a 20 μl reaction mixture containing 2 μl of the target cDNA, 0.25 mM primers, 2 mM MgCl₂, 0.2 μM dNTPs, 0.025 U μl⁻¹ Taq polymerase (Sigma) using a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, U.S.A.). An appropriate dilution of the cDNA samples and the number of cycles for each primer were chosen after preliminary experiments, ensuring that the samples remained within the exponential range of amplification (data not shown). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to check the quality of the cDNA preparation as well to normalize the cDNA concentrations. The expression of each cytokine was calculated in relation to G3PDH expression. The cytokines and primer sequences used are shown in Table 1.

PCR was conducted for 30–40 cycles under the following conditions: denaturation at 94°C, annealing at 62°C for cytokines (55°C for G3PDH), and extension at 72°C. IL-1, IL-6, IL-10, IL-12, IL-13 and TNF-α primers were synthesized on a DNA Synthesizer (Applied Biosystem, Foster City, CA, U.S.A.) while IL-8, IFN-γ, TGF-β and G3PDH primers were purchased from Clontech (Palo Alto, CA, U.S.A.). Phytohaemagglutinin stimulated mononuclear cells expressed all cytokines tested for, and thus served as a positive control to ascertain that each primer yielded a PCR product of the correct size (data not shown). The PCR products were visualized on a 1–6% agarose gel containing 0.2 μg ml⁻¹ ethidium bromide, and analysed using a DC120 Zoom Digital Camera connected to ID Image Analysis Software (Kodak, Eastman Kodak Co. Rochester, NY, U.S.A.). The relative mRNA levels were expressed as the ratio of the net intensity detected in a given cytokine band divided by that of its respective G3PDH band. The specificity of amplified PCR products for G3PDH and IL-13 was confirmed by Southern blot and hybridization with ³²P-labelled specific probes, according to standard protocols. Radioactive products were scanned and analysed in a Phosphoimager (Molecular Dynamics, U.S.A.).

STATISTICAL ANALYSIS

The Mann–Whitney U-test and the Wilcoxon matched pairs test were used for intergroup and intragroup comparisons, respectively. Correlations were calculated using Spearman rank correlation test. A P-value of less than 0.05 was considered statistically significant.

Results

PATIENTS AND BAL SAMPLES

After 7 weekdays of repeated low dose allergen provocations, an allergic inflammatory reaction, subclinical in six out of eight asthmatic subjects, was observed as determined by increased airway methacholine reactivity, increased BAL fluid total cell and eosinophil counts and increased serum eosinophilic cationic protein (ECP) levels. Detailed data are

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 s</td>
<td>GCCAATGACTCAGAGGAAGA</td>
<td>328</td>
</tr>
<tr>
<td>IL-1 as</td>
<td>TCTCAGGCACCTTCACGAC</td>
<td>315</td>
</tr>
<tr>
<td>IL-6 s</td>
<td>TGAACTCCTTCCTCACAAGC</td>
<td>289</td>
</tr>
<tr>
<td>IL-6 as</td>
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<td>352</td>
</tr>
<tr>
<td>IL-8 s</td>
<td>ATGACTTCCAAGCTGGCTGGCT</td>
<td>420</td>
</tr>
<tr>
<td>IL-8 as</td>
<td>TCTCAGCCCTCTTCAAAAACTTCTC</td>
<td>353</td>
</tr>
<tr>
<td>IL-10 s</td>
<td>ACCAAGACCCACATCAAGC</td>
<td>427</td>
</tr>
<tr>
<td>IL-10 as</td>
<td>GAGGTCAATAAGGTTTCTCAAG</td>
<td>363</td>
</tr>
<tr>
<td>IL-12p40 s</td>
<td>CAGCAGTGGTCTATCTCTTG</td>
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</tr>
<tr>
<td>IL-12p40 as</td>
<td>CCAGCAGGTTGAAACGTCCA</td>
<td>353</td>
</tr>
<tr>
<td>IL-13 s</td>
<td>TGTGACACCGTCATTTGCT</td>
<td>427</td>
</tr>
<tr>
<td>IL-13 as</td>
<td>ATGTAAGAGCAGGTCTTTCAC</td>
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<tr>
<td>IFN-γ s</td>
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<tr>
<td>IFN-γ as</td>
<td>CTTCCTTTTCGTTCTTTCGTGTTCGCTT</td>
<td>363</td>
</tr>
<tr>
<td>TNF-α s</td>
<td>TTAGCTCAGTAAAGCATGATC</td>
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</tr>
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<td>TTATCCTCACGTCTCCAGGCC</td>
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</tr>
<tr>
<td>TGF-β s</td>
<td>GCCCTGGACACCACTATTTGCT</td>
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<tr>
<td>G3PDH s</td>
<td>TGAAGGTCTGAGTCAACGGGATTTGCT</td>
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<td>CATGTTGGGCGCATGAGGTCCACACC</td>
<td>983</td>
</tr>
<tr>
<td>IL-13 probe</td>
<td>TCTGTTTCATCGGATGAT</td>
<td>983</td>
</tr>
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</table>

s: sense; as: antisense
presented in Lensmar et al. (13). To be able to perform other studies in parallel with the detection of cytokine mRNA following allergen provocation, BAL cells were enriched for AM, resulting in a purity of ≥97% macrophages, as assessed by May–Grünewald Giemsa and Toluidine blue stainings. Contaminating cells in the enriched AM fraction were (mean values) lymphocytes 1.5%, neutrophils 1.0%, eosinophils 0.5% and a small number of mast cells (2.3 in 10 visual fields, magnification ×16).

CYTOKINE mRNA EXPRESSION BEFORE AND AFTER LOW-DOSE ALLERGEN

A semiquantitative RT-PCR was performed to analyse cytokine mRNA expression, using mRNA of the housekeeping gene G3PDH to enable comparisons of each cytokine mRNA transcript expression in BAL samples before and after provocation [Fig. 1(a)]. The expression of IL-13 mRNA was found to be statistically significantly enhanced \((P = 0.01)\). IL-13 transcripts were detected only in two out of the eight asthmatics (2/8) before provocation and were found to be upregulated in all eight patients after provocation [Fig. 1(a and b)]. In three of the patients, the specificity for IL-13 on the amplified PCR material was investigated and confirmed by probing transferred products with an IL-13 probe complementary to sequences internal to the primers [Fig. 1(c)]. A tendency was observed to a reduced mRNA expression for IFN-\(\gamma\) (7/8), IL-6 (6/8), IL-8 (6/8) and TNF-\(\alpha\) (5/8) and to an increased expression of mRNA for IL-1 (5/8) after the allergen provocations (Fig. 2). Finally, IL-10, IL-12 and TGF-\(\beta\) mRNA were detected but without any distinct patterns before or after provocation.

The post-challenge increase in the expression of IL-13 showed a positive correlation with the increased BAL fluid proportion of eosinophils \((P = 0.05, r = 0.7)\), (Fig. 4) and with the serum ECP values \((P = 0.09, r = 0.7)\), although a significant \(P\)-value was obtained only with the former parameter. A significant positive correlation was also found between the post-challenge change in BAL cell concentration and proportion of neutrophils and the change in IL-8 mRNA expression \((P = 0.002, r = 0.9\) and \(P = 0.01, r = 0.8\), respectively).

CYTOKINE mRNA EXPRESSION IN PATIENTS VersUS HEALTHY CONTROLS

A significantly higher mRNA expression for IL-6 \((P = 0.003)\), IL-10 \((P = 0.005)\) and TGF-\(\beta\) \((P = 0.003)\) was found in patients (before allergen provocation) vs. controls [Fig. 3(a)], while IL-8 \((P = 0.003)\) and TNF-\(\alpha\) \((P = 0.008)\) mRNA transcripts were significantly lower in the former group [Fig 3(b)]. The same pattern of cytokine expression was observed when comparing the asthmatics after provocation with healthy controls, except for IL-13 that was significantly higher \((P = 0.005)\) expressed in asthmatics than in controls but only after low-dose allergen provocation. No significant differences were observed in the mRNA expression of IL-1, IL-12, IL-13 and IFN-\(\gamma\) cytokines between the two groups.

Discussion

Low-dose allergen provocation has proven to be a useful model for studying airway hyperresponsiveness as well as the inflammatory process underlying mild bronchial asthma (10–12). Recently, we demonstrated a subclinical inflammatory reaction after low-dose provocation in a group of mild atopic asthmatic patients (13). In the present study,
FIG. 2. PCR analysis of interferon (IFN)-γ, interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)-α and IL-1 mRNA in BAL cells enriched for alveolar macrophages (AM) obtained from mild asthmatic patients before and after low-dose allergen provocation. Data are presented as a ratio of cytokine/G3PDH mRNA net intensity obtained by Digital Image Analysis Software (Kodak, Eastman Kodak Co., Rochester, NY, U.S.A.). (b) Before and (a) after low-dose allergen provocation; 1-8: patient numbers.
FIG 3. PCR analysis of interleukin (IL)-6, IL-8, IL-10, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β mRNA in bronchoalveolar lavage (BAL) cells enriched for AM obtained from mild asthmatic patients and healthy control subjects. (a) Cytokines expressed at higher levels; and (b) lower level, in mild asthmatic patients compared with healthy controls. Data are presented as a ratio of cytokine/G3PDH mRNA net intensity obtained by Digital Image Analysis Software. (B) before low-dose allergen provocation (n=8); (C) controls (n = 5). *P < 0.01 and **P < 0.005.
The same group of patients was analysed for cytokine expression in BAL cells by using a semiquantitative RT-PCR technique. Following 7 weekdays of repeated low-dose allergen provocations, we found a significantly increased IL-13 mRNA expression accompanied by increased IL-1 but reduced IL-6, IL-8, IFN-γ and TNF-α mRNA expressions, in BAL cells enriched for AM.

The enhanced \textit{in vitro} production of immunoglobulin E (IgE) observed in asthmatic patients has been reported to be mediated by IL-13, and therefore IL-13 may play a central role in the pathogenesis of IgE-mediated allergic diseases (15,16). The upregulation of IL-13 transcripts in BAL cells of patients with mild asthma after low-dose allergen provocations, as reported here, is in good agreement with the recently demonstrated elevated expression of IL-13 mRNA in the bronchial mucosa of patients with asthma (17). IL-13 selectively induces vascular cell adhesion molecule (VCAM)-1 expression on endothelial cells, favouring a VCAM-1/very late antigen (VLA)-4 dependent accumulation of inflammatory cells at the site of the inflammatory reaction (18,19). Moreover, IL-13 enhances survival of eosinophils and may contribute to the pathological activities of these cells in asthma (20). In line with these reports, we found a positive correlation between the post-challenge increase in the proportion of eosinophils and the elevated IL-13 mRNA expression. Besides cell infiltration, IL-13 may also be important for airway obstruction (21), as shown by blocking or adding IL-13 in experimental animal models (4,5).

In particular T lymphocytes, but also mast cells, basophilic granulocytes and dendritic cells, are capable of producing IL-13 (22–24). On the other hand, human eosinophils are not considered as a source of IL-13 (25). In our study, the vast majority (>97 %) of BAL cells were AM, which only recently were recognized as a potential source of IL-13 (26). We found no correlation between the numbers of contaminating cells and the increased IL-13 mRNA expression, and suggest that AM may be the source of the elevated IL-13 mRNA expression following low-dose allergen provocations. To confirm such an IL-13 production by AM, one could apply \textit{in situ} hybridization techniques, something we were unable to perform in this study due to lack of material.

Many biological activities of IL-13 are shared with IL-4, probably due to the common IL4Rα chain for IL4R and IL-13R, required for signal transduction (4,8,27). IL-13 may affect T cell functions and Th2-cell differentiation indirectly through its downregulatory effects on the production of IL-12, IFN-γ and TNF-α (9,28). This observation is in agreement with the decreased expression of IFN-γ transcripts in our study. Although IFN-γ expression has previously been considered to be restricted to T and NK cells, unstimulated or IL-12-stimulated AM as well as AM from tuberculosis patients have been shown to produce and secrete IFN-γ (29,30). Also the downregulation of the pro-inflammatory cytokines IL-6 and TNF-α and the chemokine IL-8 after low-dose inhalations may be due to the inhibitory capacity of IL-13 on the monocyte/macrophage production of cytokines (6,8).

In the BAL cells of patients before allergen challenge vs. controls, there was a significantly higher expression of the Th2 associated cytokines IL-6 and IL-10, as well as of TGF-β that has been implicated in airway wall remodelling activity in asthma. In contrast, the cytokines IL-8 and TNF-α were expressed at lower levels in asthmatics than in controls. Our results are in line with previous reports showing an increased expression of IL-10 mRNA in BAL fluid from asthmatics, an inhibitory capacity of IL-10 on macrophage cytokine production and increased levels of TGF-β in BAL fluid from asthmatics (31–34).

In summary, the cytokine pattern in BAL cells from patients with mild atopic asthma after repeated low-dose allergen provocations is characterized by a significant IL-13 upregulation and an IFN-γ downregulation in addition to an inhibition of pro-inflammatory cytokine expression, i.e. in part a Th2 deviated immune response. Although the origin of IL-13 was not firmly established in this study, AM is a possible source as they made up >97 % of the investigated cells. This could indicate that AM play a more regulatory role in the pathogenesis of allergic asthma than previously believed.

**Acknowledgements**

We would like to thank Margitha Dahl and Gunnel de Forest for excellent technical assistance and Dr Jan Wahlström for fruitful discussions.

This work was supported by The Swedish Foundation for Health Care Sciences and Allergy Research, The Swedish Medical Research Council (71X-12621), The Swedish Heart Lung Foundation and The Karolinska Institute.

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