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REGULAR ARTICLE

Nitric oxide, IL-6 and IL-13 are increased in the exhaled breath condensates of children with allergic rhinitis

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ABSTRACT

Aim: To evaluate nitric oxide and interleukin (IL)-6, IL-8 and IL-13 in the exhaled breath of children with allergic rhinitis (AR), before and after intranasal allergen exposure.

Methods: A total of 49 children with AR – comprising 20 who also had episodic asthma (AR+A) and 29 without asthma (AR) – were compared with 34 healthy controls. Nitric oxide concentrations in exhaled air (eNO) and IL-6, IL-8 and IL-13 in exhaled breath condensates (EBC) were measured in winter, outside the natural allergen exposure season, before and after an intranasal allergen challenge.

Results: The mean concentrations of eNO, IL-6 and IL-13 were significantly higher in the two AR groups. The concentration of IL-8 was below the assay detection limit in all EBC samples. The intranasal allergen challenge increased IL-13/EBC levels in both AR groups, but did not influence mean concentrations of eNO, IL-6 or IL-8. No challenge-related changes in IL-13/EBC were observed in the allergen-exposed controls or placebo-exposed children.

Conclusion: Despite local application, the intranasal allergen challenge increased IL-13/EBC concentration in the AR children. As EBC reflects the status of lower airway segments, our observation may support the 'united airways' hypothesis, suggesting a functional link between the upper and lower airways.

INTRODUCTION

Allergic rhinitis (AR) was initially defined as a local process that exclusively affected the nasal mucosa. However, it is currently considered as a manifestation of an inflammatory reaction that involves the entire respiratory tract. This idea reflects the concept known as the 'united airways' hypothesis, which suggests a functional link between the upper and lower airways (1,2). This concept may be further supported by the relatively high incidence of patients with both AR and asthma. According to the 'united airways' hypothesis, both AR and asthma, although previously identified as distinct disorders, are now thought to be a common manifestation of an inflammatory response in the respiratory system (3).

The presence of an inflammatory reaction in the respiratory system may be detected and/ or monitored by the assessment of various proinflammatory cytokines and phospholipid derivatives, namely prostaglandins, thromboxanes and leucotrienes (4–6). These are mainly produced and released by mucosa-infiltrating leucocytes – Th lymphocytes, eosinophils, basophils and mucosal mast cells – and may be detected in sputum samples or through bronchoalveolar lavage (BAL). Scientists have recently developed a new research tool, which enables the use of liquefied exhaled air in biochemical and immunological studies (7). Exhaled air contains trace, but still detectable, amounts of various nonvolatile mediators of inflammation. It has been proven that the levels of these factors in exhaled air and exhaled breath condensate (EBC) correlate with the intensity of the inflammatory reaction, which affects the respiratory tract (8).

Due to the relatively low concentrations of these biologically active factors, it is necessary to use ultrasensitive essays to analyse EBC. The EBC process provides similar data to bronchoalveolar lavage (9,10), but unlike BAL, it is a noninvasive, risk-free procedure that can easily be

Key notes

- This study compared selected proinflammatory/allergic markers in 49 children with allergic rhinitis (AR) and 34 controls before, and after, an intranasal challenge.
- Nitric oxide and interleukins 6 and 13 were higher in AR subjects, and interleukin-13 in exhaled breath condensates increased further in response to the intranasal allergen challenge, but nitric oxide and interleukin-6 did not.
- The results may suggest a functional link between the upper and lower airways.

applied, even to very young children (11). This means that analysing EBC is a novel approach for research and diagnosis in respiratory system diseases. Several authors have focused on the relevance of the various biologically active allergic rhinitis and/or asthma factors involved in EBC, based on the 'united airways' concept (12,13). We previously reported how useful EBC was in locating a specific marker of allergic rhinitis in children (13). That paper reported that we found that cysteinyl leucotrienes (cysLT) are highly specific and sensitive AR indicators, especially in combination with decreased peak nasal inspiratory flow (PNIF) following the allergen provocation. The aim of this study, which continues our research into the use of EBC, was to analyse the concentrations of selected proinflammatory/allergic markers: nitric oxide and interleukin (IL)-6, IL-8 and IL-13, in the exhaled breath of children with allergic rhinitis. We then compared those results with the healthy controls, before and after an intranasal allergen challenge.

PATIENTS AND METHODS

Patients

The study groups have previously been described and their clinical characteristics are also shown in Table 1 (13). In brief, 83 children (mean age 11.9 ± 4.8 years) were recruited and the allergic rhinitis patients (n = 49) were further divided into two subgroups: 29 children who only had AR and 20 children with allergic rhinitis and episodic asthma (AR+A). Asthma recognition was based on the child's history of clinical symptoms and was classified as episodic, according to the updated 2009 recommendations of the Global Initiative for Asthma, Strategy for Asthma Diagnosis and Prevention (available from http://www.ginasthma.org). The children all suffered from seasonal allergic rhinitis were only monosensitised to grass pollen and were diagnosed according to indications of the Allergic Rhinitis and its Impact on Asthma (14). The control group (n = 34) comprised healthy individuals, who attended routine healthcare visits at the outpatient clinic of the Department of Paediatrics, Pneumonology and Allergology at Warsaw Medical University.

Based on the inclusion and exclusion criteria, all children were in a stable condition and without signs of infection, as verified by routine clinical assessment and peripheral blood screening, which determined total leucocyte counts and their subpopulations. The study protocol allowed selected medication, including second-generation histamine receptor type 1 antagonists, intranasal or inhaled glycocorticosteroids and inhaled β2-mimetics, upon request. However, patients did not receive oral histamine receptor antagonists on the assessment day and for a week before, and corticosteroids were stopped at least 2 weeks before the assessment. All the children and their parents gave written, informed consent to participate in the study. The experiment protocol was formally approved by the local bioethics committee (approval no. KB/93/2008).

Table 1 Characteristics of the two study groups of allergic rhinitis children, compared with the healthy controls

Parameter\patient group	AR (n = 29)	AR+A (n = 20)	Healthy control (n = 34)
Age (years ±SD)	12.0 ± 4.3	10.9 ± 3.9	12.4 ± 4.9
Sex distribution (Female/Male)	12/17	4/16	19/15
PEF_{SDS} (L/min)	0.5 ± 0.2	0.6 ± 0.1	0.3 ± 0.2
$FEV1_{SDS}$ (L)	−0.3 ± 0.2	−0.8 ± 0.6	−1.1 ± 0.5
IgE serum level – total (kU/L)	**199.9 ± 38.2***	**269.8 ± 54.7***	33.2 ± 9.8
IgE – timothy specific (kU/L)	**26.0 ± 10.1***	**20.3 ± 8.4***	0.1 ± 0.2
Medication used before the study	HRA, CS	HRA, CS, B2M	None
Patient allocation for allergen/ placebo challenge	15/14	10/10	17/17
Mean symptom score (±SD) after allergen/placebo challenge	**4.2 ± 0.7***/ 0.9 ± 0.7	**3.6 ± 0.5***/ 1.0 ± 0.8	0.8 ± 0.8/ 1.0 ± 0.7

AR = Allergic rhinitis; AR+A = Allergic rhinitis with asthma; SDS = Standard deviation score (*Z*-score; value after age-related correction); HRA = Histamine receptor antagonists; CS = Corticosteroids; B2M = β2-mimetics 12.
Mean values ± SD.
*statistically significant, when compared to control group.
The differences were considered as significant at $p < 0.05$.

Study procedure

The nitric oxide concentrations in exhaled air (eNO) were measured using a Sievers NO 280 device (GE Analytical Instruments, Boulder, CO, USA) and the results expressed as ppb (particles per billion) units. The EBC collection was performed using an EcoScreen condenser (Jäger, Hoechberg, Germany). Both, the eNO measurement and EBC collection, were performed before, and after, a double-blind, placebo-controlled intranasal allergen challenge. This was carried out in the winter, to rule out natural seasonal allergen exposure.

According to study protocol, the children from the three groups – allergic rhinitis only (AR), allergic rhinitis and asthma (AR+A) and healthy controls – were randomly divided into two arms, the allergen challenge and placebo, and subjected to the study procedure. After the children had adapted to the ambient conditions for 15–20 minutes, their baseline eNO measurement was taken and they underwent the 15-minute EBC collection procedure. Afterwards, based on the results of the randomisation, they received either 100 μL of the 5000 BU/mL grass pollen suspension (Allergopharma, Reinbek, Germany) or the same volume of placebo solution, containing just the diluent used in the allergen suspension, in each nasal cavity. The clinical symptoms were evaluated using a scoring system (15). After 20 minutes, the postchallenge eNO measurement was taken and the next 15-minute EBC collection was carried

out. Samples of breath condensate, of approximately 600–800 μL each, were immediately deep frozen and stored at −70°C, until needed for further analysis.

EBC assessment

The cytokine concentrations in EBC were estimated in duplicates, using Human IL-6, IL-8 and IL-13 Ultrasensitive ELISA kits, respectively, according to the detailed protocols provided by the manufacturer (Invitrogen, Camarillo, CA, USA). The absorbance of the analysed samples was measured using a Microplate Reader 550 device (BIO-RAD, Hercules, CA, USA). The cytokine concentrations in EBC were calculated based on the respective standard calibration curve and expressed in pg/mL. The assays sensitivity, corresponding to the lowest point of the standard calibration curve, was 0.1 pg/mL for all tested cytokines. To exclude possible contamination by saliva, ten randomly selected EBC samples from each group were additionally screened for the presence of amylase (16). However, this was not detected in any of the tested samples.

Statistical evaluation

The concentrations of exhaled nitric oxide and cytokines in EBC were compared between the study groups using the Mann–Whitney *U*-test. The changes in concentration of analysed factors, due to allergen or placebo application, were assessed using the Wilcoxon matched-pair test. The differences were considered as statistically significant at $p < 0.05$.

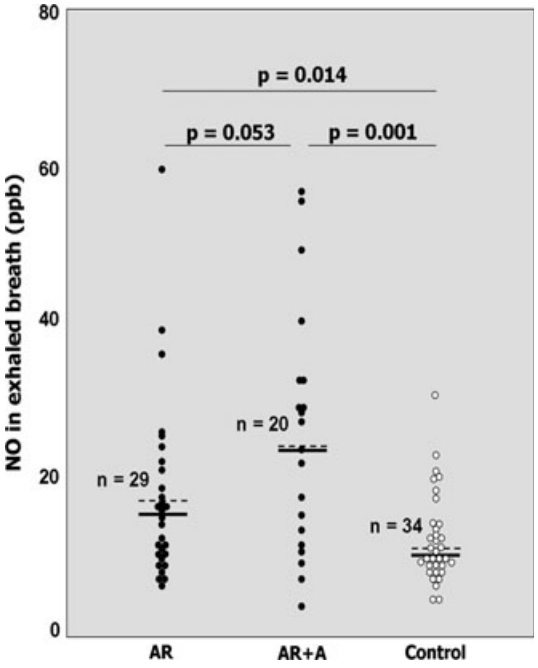

Figure 1 The concentration of nitric oxide (NO) in exhaled nitric oxide, expressed in particles per billion (ppb). Each dot represents one individual from respective group: patients with only allergic rhinitis (AR), allergic rhinitis with asthma (AR+A) and healthy controls. The mean values of NO concentration in each group were shown as dashed lines, whereas median values were indicated as solid lines.

RESULTS

Exhaled nitric oxide

The mean baseline exhaled nitric oxide (eNO) concentrations were measured in winter outside the season for natural allergen exposure. Both patient groups (AR and AR+A) showed statistically significantly higher levels than the healthy controls (18.1 ± 11.3 ppb and 24.8 ± 15.2 ppb vs 12.8 ± 5.3 ppb, respectively). The eNO levels did not differ statistically between the AR and AR+A groups (Fig. 1).

The intranasal allergen challenge did not influence the eNO concentrations measured before, and 20 minutes after allergen exposure, in any of the study groups. Similarly, no influence on eNO levels was observed after application of the placebo. Thus, in both the allergen and placebo arms, the calculated eNO changes (ΔeNO) did not differ between the study groups.

EBC cytokines

The mean baseline concentrations of IL-6 and IL-13 in EBC showed similar results to the eNO results, with significantly higher mean values in the AR and AR+A groups than the healthy controls. The IL-6/EBC levels were 0.23 ±0.10 pg/mL and 0.27 ± 0.11 pg/mL vs. 0.02 ± 0.05 pg/mL, and the IL-13/EBC levels were 0.41 ± 0.21 pg/mL and 0.49 ± 0.25 pg/mL vs. 0.10 ± 0.15 pg/mL, respectively. No statistically significant differences were observed between the AR and AR+A groups, regarding mean concentrations of both IL-6/EBC and IL-13/EBC (Fig. 2A,B). The levels of IL-8 in all analysed EBC samples were below the assay detection limit.

Intranasal allergen challenge

The intranasal allergen exposure resulted in a statistically significant increase in symptom scores in both patient groups (AR and AR+A). However, these changes were not observed in the allergen-challenged healthy individuals or in any of the children who received the placebo (Table 1).

The intranasal allergen provocation did not influence mean baseline levels of eNO, IL-6/EBC or IL-8/EBC, in any of the study groups. In contrast, the allergen challenge was associated with an increase in mean IL-13/EBC concentrations in both patient groups (AR and AR+A), but not in the healthy controls. No significant changes in IL-13/EBC levels were found in response to the intranasal application of the placebo solution in any of the study groups (Fig. 3).

DISCUSSION

Although it is nonspecific, the increased concentration of nitric oxide in exhaled air is currently considered as a sensitive marker of airways inflammation (17). Therefore, in addition to the well-established use of eNO in algorithm of

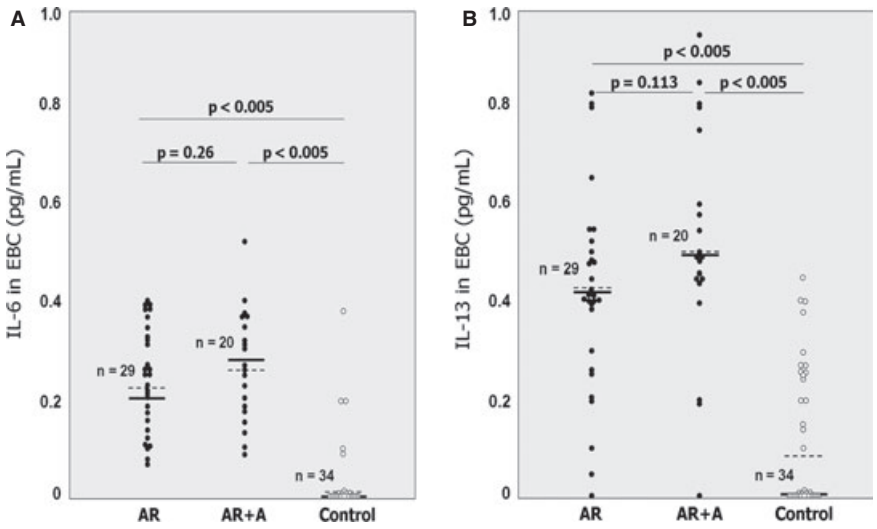

Figure 2 The concentrations of IL-6 (graph A) and IL-13 (graph B) in exhaled breath condensates, expressed in pg/mL. Each dot corresponds to one patient from the respective group: allergic rhinitis (AR), allergic rhinitis with asthma (AR+A) and healthy controls. Mean values of cytokine concentration in each group are indicated by dashed lines, and the median values are shown as solid lines.

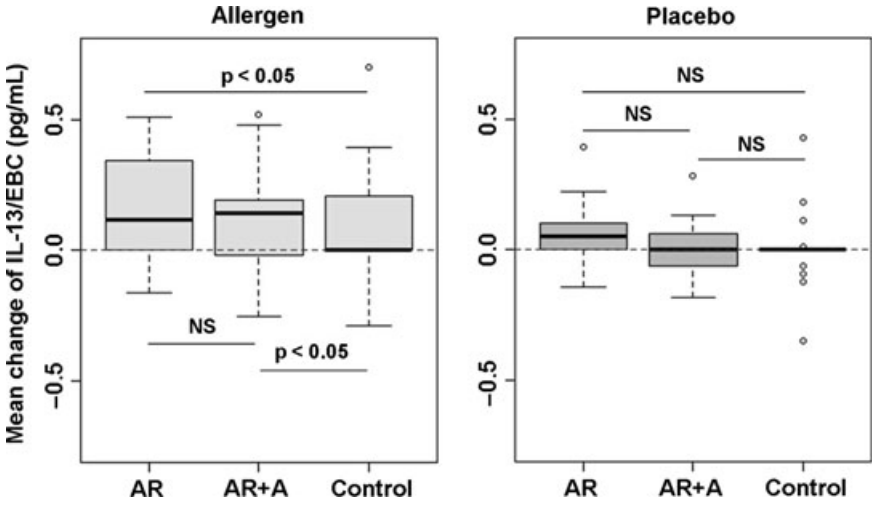

Figure 3 The mean IL-13/EBC changes in respect to challenge mode. The solid line in each box represents the median value, while the whisker ends correspond to the 9th and 91st percentiles, respectively. AR = allergic rhinitis, AR+A = allergic rhinitis with asthma and NS = nonsignificant.

asthma diagnostics and monitoring, this parameter may also be used to control other chronic diseases affecting the respiratory system (17,18). In common with other reports (17,19), our study also found that allergic rhinitis and asthma were associated with high concentrations of nitric oxide in exhaled air, even when the children were not exposed to natural allergens during winter. However, although eNO assessment is a nonspecific marker of inflammation, it failed to discriminate between allergic rhinitis individuals with and without asthma. Another observation that needs to be clarified is that the allergen challenge had no visible influence on the eNO levels in both of the allergic rhinitis groups. It is possible that this could be due to the short assessment time, which was insufficient to upregulate the expression of inducible nitric oxide synthase (iNOS). However, a similar lack of eNO changes after the intranasal allergen challenge was observed in another study, even after two, six and 24 hours (19). As suggested in that study, a single local application of the allergen may be inadequate to augment the inflammatory response measured by the eNO assessment. This issue clearly requires further research.

The usefulness of breath condensates in research focused on allergic inflammation has also been confirmed by studies that have assessed selected proinflammatory cytokines. For example, IL-6 was recognised as a marker of allergic asthma

(12,20). In our study, this cytokine was also elevated in the EBC samples of children with allergic rhinitis (and asthma), but not in the healthy controls. The allergen challenge did not influence its concentration in the EBC. A possible explanation of this result may be similar to the explanation regarding exhaled nitric oxide (19).

IL-8 was initially described as a key chemotactic factor for neutrophils and primed eosinophils. Although this chemokine is thought to reflect the inflammatory response to infection (21), it has been reported that the allergen challenge may also result in an increase in IL-8 in the nasal lavage of allergic rhinitis individuals (22). However, in our study, the IL-8 levels in all tested samples remained below the detection limit, probably due to very low cytokine concentration in EBC.

IL-13 displays similar activity to IL-4, stimulating the expression of proallergic Th2 cytokines, increasing IgE production and activating mast cells and eosinophils (23). Therefore, IL-13 is currently recognised as a good indicator of allergic inflammation and asthma (24,25). It has been shown that increased eosinophilia with local inflammation in grass pollen and dust mite allergy or submucosal fibrosis and asthma exacerbation correlates with high IL-13 levels in peripheral blood, sputum or mucosal biopsies, respectively (25,26). Interestingly, the other group did not detect considerable amounts of IL-13 in similar material (17). This discrepancy might possibly result from the use of less sensitive assays for cytokine detection in the mentioned studies.

In our experiment, we observed that all the patients who suffered from allergic rhinitis, but not the healthy controls, displayed significantly increased IL-13/EBC levels, which were easily detectable in winter, outside the natural allergen exposure season. However, if we consider allergic rhinitis as a local disease, this observation may be, at least to some extent, perplexing, especially because the contents of EBC reflect the condition of distal segments of the respiratory system. The influence of the intranasal allergen challenge on IL-13/EBC in both the allergic rhinitis groups, with and without asthma, also raises the same ambiguity. Interestingly, although the local allergen application did not stimulate any changes in the IL-6/EBC or IL-8/EBC levels during the same observation period, it did result in changes in the IL-13 levels. The rationale for this difference is not clear. *In vitro* experiments have proved that allergen/IgE cross-linking leads to fast upregulation of IL-13 mRNA in mast cells (27). The significant increase in IL-13 gene expression was observed within 20–30 minutes of stimulation (28). Furthermore, as IL-13 is constitutively expressed by mast cells (28), we can presume that some quantities of IL-13 may be stored and released from these cells in the course of their activation by the allergen challenge. It may be possible that, because we used an ultrasensitive assay, we were able to detect the release of preformed IL-13 from the mast cells. Finally, one could expect to see a measureable difference in eNO, IL-6/EBC or IL-13/EBC between the allergic rhinitis groups with and without asthma. However, this did not happen in our study. While this is difficult to understand, based on former concepts, all of these findings could be easily explained by the 'united airway' hypothesis (1–3). According to that, similar results in patient groups may be due to relatively mild, but comparable, intensities of inflammatory reactions in the respiratory tracts of those patients. This assumption may be supported by the observation that, in approximately 78% of asthmatic individuals, the onset of clinically overt asthma was preceded by symptoms of allergic rhinitis for several years (29). However, it still needs to be clarified whether the mentioned similarities between both patient groups resulted from well-controlled disease in the children already diagnosed with asthma or were due to the presence of subclinical asthma in individuals with allergic rhinitis, who were initially diagnosed as not having asthma (30). In conclusion, although our data are in agreement with the concept of the 'united airway', some aspects still require further research.

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