

Original article

Cytokine production from sputum cells after allergenic challenge in IgE-mediated asthma

Background: Th2 cytokine production from airway cells is thought to govern the eosinophilic airways inflammation in allergic asthma. Induced sputum has become a widely used technique to assess airways inflammation.

Methods: By applying the technique of induced sputum to collect airways cells, we have assessed the spontaneous production of a set of cytokines, including interleukin-4, 6, 10, interferon- γ and tumour necrosis factor- α , 6 h after a bronchial allergenic challenge with *Dermatophagoides pteronyssinus* (Dpt) in 12 sensitized asthmatics and compared the results obtained after inhalation of saline as control. A group of eight healthy non-allergic subjects was enrolled to control for any non-specific effect of Dpt. Cytokines were measured by a dynamic immunoassay during a 24-h sputum cell culture.

Results: Allergen challenge in sensitized asthmatics caused an acute and a late bronchospasm together with a rise in sputum eosinophil counts. Afterwards allergen sputum cells from allergic asthmatics displayed a rise in their production of IL-4 ($P < 0.01$), IL-6 ($P < 0.05$) and IL-10 ($P < 0.05$) when compared to saline. By this time sputum generation of IL-4 in atopic asthmatics was greater than in healthy subjects ($P < 0.001$). Furthermore, in allergic asthmatics there was a strong correlation between the rise in interleukin-4 production from sputum cells and the rise in sputum eosinophils ($r = 0.87$, $P < 0.001$).

Conclusions: Sputum cell culture is a useful model to assess cytokine production in allergic asthmatics who show a marked up-regulation of Th2 cytokines following acute allergen exposure. The rise in sputum eosinophil count following allergen challenge strongly correlates with the rise in IL-4 generation from sputum cells.

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Key words: allergen; asthma; cell culture; cytokines; sputum.

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Accepted for publication 8 July 2002

Asthma is seen as an eosinophilic airways inflammatory disease featuring cytokine production corresponding to the Th2 pattern (1, 2). In the majority of cases asthma is associated with atopy and the exposure to a relevant allergen is considered to drive cytokine production towards the Th2 profile (3, 4). This concept has arisen from studies having used bronchoalveolar lavages (5) and bronchial biopsies (6) taken during bronchoscopy. However some studies have challenged the view according to which secretion of IFN- γ , a classical Th1 cytokine, is repressed in the airways of allergic asthmatics (7). By using a one-step dynamic and sensitive immunoassay we have recently found that whole blood of allergic asthmatics differentiated from that of non-allergic asthmatics and healthy subjects by an increased spontaneous production of IL-4 and IL-6 contrasting with a reduced production of IFN- γ (8).

In this study we have asked whether the cells recovered from induced sputum may represent a suitable model to look at cytokine production in the airways, and how

useful the model could be to pick up changes in cytokine production following an experimental exposure of sensitized asthmatics to *Dermatophagoides pteronyssinus* (Dpt), the most common aeroallergen in our area. Thus we have assessed IL-4, IL-6, IL-10, IFN- γ and TNF- α production from cultured sputum cells after exposure to inhaled allergen vs. saline in allergic asthmatics and healthy non-allergic subjects. We have also investigated how the airways cytokine production may relate to change in sputum cell counts as well as to change in lung function.

Material and methods

Subjects

We have studied 12 steroid-naïve allergic asthmatics sensitized to house dust mites and eight healthy controls without any evidence of IgE-mediated sensitization to common aeroallergens (see below) whose demographic and functional characteristics are given in Table 1.

Table 1. Subject characteristics

Age	Sex	Smoking	FEV ₁ (%)	PC20M (mg/ml)	Dpt skin prick test	Total IgE (Ku/l)	Dpt IgE (Ku/l)
Asthmatics							
32	F	No	90	0.09	Positive	330	1.57
19	F	No	120	16	Positive	1940	> 100
21	F	No	97	0.16	Positive	756	> 100
24	F	No	90	2	Positive	205	26
21	F	No	112	0.97	Positive	1641	4
24	F	Yes	86	0.08	Positive	1044	> 100
22	M	No	101	1.4	Positive	984	< 0.7
47	F	No	108	0.37	Positive	482	10
47	F	No	124	0.60	Positive	1020	54
46	M	No	113	16	Positive	39	1.94
23	M	No	94	0.99	Positive	162	30.6
43	F	No	94	0.64	Positive	ND	ND
Healthy subjects							
44	F	No	100	> 16	Negative	33	< 0.7
27	M	Yes	136	> 16	Negative	20	< 0.7
28	M	Yes	90	> 16	Negative	ND	< 0.7
24	M	No	93	> 16	Negative	ND	ND
29	F	No	97	> 16	Negative	26	< 0.7
25	M	No	142	> 16	Negative	5	< 0.7
36	M	No	119	> 16	Negative	18	< 0.7
47	M	No	89	> 16	Negative	11	< 0.7

ND, not done.

Asthma was diagnosed on the basis of a clinical history of recurrent wheeze, breathlessness, or cough, associated with bronchial hyperresponsiveness to methacholine, as indicated by a provocative concentration of methacholine ≤ 16 mg/ml (PC20M). Methacholine challenges were performed according to a slightly modified Cockcroft's method, as previously described (9). IgE-mediated allergy (10) was defined as a positive skin prick test reaction (≥ 3 mm) to common aeroallergens (house dust mites, cat and dog dander, grass, tree and weed pollens, moulds). All allergic asthmatics selected for this study were sensitized to house dust mites as reflected by a positive skin prick test and/or a RAST greater than 0.70 KU/l (class ≥ 2 , Pharmacia).

Study design

Allergic asthmatics attended the laboratory for three visits (V1-V3), whereas healthy subjects had only two visits (V1 and V3). At visit 1 the subjects inhaled an isotonic saline solution (NaCl 0.9%) for 5 min and their FEV₁ was monitored after 5, 15, 30 and 60 min and then hourly until the sixth hour. At the end of this period the subjects received 400 µg inhaled salbutamol and underwent a sputum induction. Only allergic asthmatics had visit number two 1 week later, during which they underwent an allergenic challenge with increasing doses of *Dermatophagoides pteronyssinus* (Dpt) extract (0.2, 0.8 and 3.2 IR (Index of Reactivity)) inhaled 15 min apart (Stallergen, France) in order to establish the dose of allergen causing a fall of at least 20% in FEV₁. The challenge was stopped when FEV₁ had fallen by at least 20% or when the final dose of 3.2 IR (cumulative dose 4.2 IR) had been inhaled. The cumulative PD20 Dpt was determined by linear interpolation. Two subjects failed to develop a fall of at least 20% even after the final dose of 3.2 IR. On visit 3, which took place 15 days after visit 2 and/or 21 days after visit 1, allergic asthmatics inhaled the cumulative dose of allergen determined at visit 2, or the dose of 4.2 IR for those two patients in whom FEV₁ failed to fall by at least 20% at visit 2. Then

FEV₁ was monitored for 6 h as indicated for visit 1 and a sputum induction was performed afterwards. At visit 3 healthy subjects inhaled the maximal Dpt dose of 4.2 IR. The protocol was approved by the local ethics committee and each subject gave his/her written informed consent.

Sputum induction and processing

After pre-medication of the subjects with a 400-µg inhaled salbutamol, sputum was induced by inhalation of an hypertonic saline (NaCl 4.5%) aerosol delivered by an ultrasonic nebulizer (Ultra-Neb 2000, De Vilbiss, Somerset, USA) with an output set at 1.5 ml/min. Each subject inhaled the aerosol for three consecutive periods of 5 min for a total time of 15 min. For safety reasons, the peak expiratory flow rates (PEFR) were monitored every 5 min and the induction stopped when PEFR fell under 250 l/min.

The whole sputum was collected in a plastic container, weighed, and homogenized by adding an equal weight of 7 mM dithiotreitol (DTT, Sigma, St Louis, MO, USA) for 30 min at room temperature. The sample was then further processed as previously described (11), and the differential performed on cytopins stained with Diff-Quick.

Cytokine assays

Cytokines were measured by a modified one-step culture immunoassay procedure, as previously described (12). Twenty-five µl of a sputum cell suspension containing 4×10^6 cells/ml or 25 µl of cytokine standards (IL-4, IL-6, IL-10, IFN- γ , TNF- α) were placed in sterile and pyrogen-free microwells coated with mAbs against the cytokines (Biosource Europe, Belgium) and containing 200 µl RPMI. The microwells were capped and incubated at 37°C for 24 h to allow the immunocapture of the produced cytokines. The caps were then removed and the wells washed intensively to remove unbound cytokines. Horseradish peroxidase (HRP) anti-cytokine conjugate mAb (Biosource Europe) was then added to the wells for a time (determined according to the cytokine studied) at room temperature with continuous shaking. After washing, 100 µl of chromogen solution (TMP) were added to each well, and the plates were incubated at room temperature with continuous shaking. A volume of 200 µl stop solution (H₂SO₄) was added to each well. Because the colour intensity determined by the absorbency at 450 nm is proportional to the cytokine concentration in the sample, the exact cytokine concentration in each sample was then calculated by interpolation from the standard curve.

Statistical analyses

The changes in FEV₁ after challenge were expressed as mean $\pm$ SEM and analysed by a *t*-test. Sputum cell counts and cytokines were expressed as median (range). The intra-group comparisons between saline and allergen were performed by a paired Wilcoxon-rank sum test. Inter-group comparisons were performed by a Mann-Whitney test. Correlations between parameters were sought by the Spearman coefficient of correlation. *P*-values < 0.05 were considered as statistically significant.

Results

Bronchospasm

When compared to saline, inhalation of allergen in allergic asthmatics resulted in an acute bronchoconstriction,

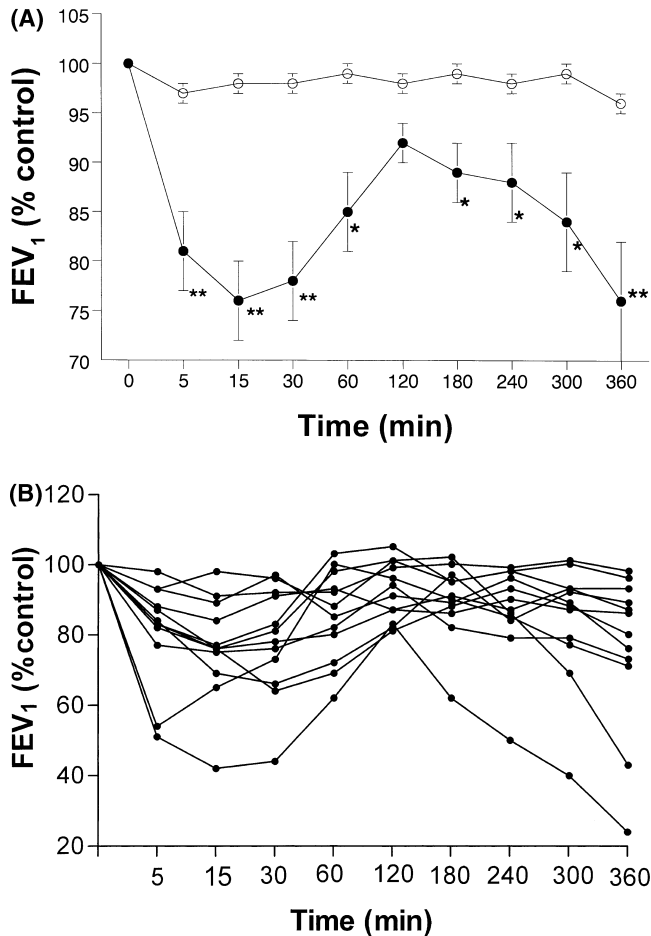


Figure 1. (A) Changes in FEV₁ following allergen (solid circles) vs. saline (open circles) challenge in allergic asthmatics. * $P < 0.05$ vs. placebo, ** $P < 0.01$ vs. placebo. (B) Individual changes in FEV₁ among allergic asthmatics following allergen challenge.

peaking at 15 min with a fall in FEV₁ reaching $24 \pm 5.4\%$ ($P < 0.01$). There was also a late bronchospasm peaking at 6 h with fall in FEV₁ of $21 \pm 7.5\%$ ($P < 0.01$) (Fig. 1A). Six out of the 12 asthmatics had a late bronchospasm characterized by a fall in FEV₁ greater than 20% from baseline at 6 h following allergen challenge (Fig. 1B). In healthy non-allergic subjects, no significant change in lung function occurred with either allergen or saline (always $< 3\%$ on average).

Sputum cell counts

The results are given in Table 2. One asthmatic patient had a pronounced late bronchospasm (fall in FEV₁ of 76%) that precluded sputum collection at 6 h post allergen. In the 11 remaining patients, the sputum eosinophil count measured at 6 h following allergen was greater than that found 6 h after saline inhalation ($P < 0.01$), whereas the converse was found for the macrophage and lymphocyte cell counts ($P = 0.06$ for

Table 2. Sputum cell counts

Cell type	Subject type	Saline	Dpt
Squamous (%)	HS	16 (5–51)	16 (3–30)
	AA	16 (4–44)	5 (1–43)*
Total non squamous $\times 10^6/\text{g}$	HS	0.65 (0.14–1.78)	0.68 (0.4–1.16)
	AA	0.56 (0.11–2.88)	0.85 (0.88–5.33)
Macrophages (%)	HS	39.8 (24.2–62.4)	57.2 (18.8–67.4)
	AA	53.7 (23.6–91)	27.2 (12.6–63.8)†
Lymphocytes (%)	HS	1 (0–2.2)	0.1 (0–4.2)
	AA	0.9 (0–7.6)	0.2 (0–2.4)†
Neutrophils (%)	HS	42.9 (3.8–57.4)	31.2 (6.6–67.6)
	AA	24.9 (0.6–73)	17.2 (1–66.2)
Eosinophils (%)	HS	1.6 (0–2.4)	0.4 (0.2–2.4)
	AA	6.2 (0–39)	26 (0–82.6)**‡
Epithelial cells (%)	HS	13.1 (0.4–52.8)	14.5 (0.4–33)
	AA	4.5 (0.2–28.2)	4.4 (1–72.2)

HS, healthy subjects; AA, atopic asthmatics.

Results are expressed as median (range) for eight healthy subjects and 11 atopic asthmatics.

* $P < 0.05$, ** $P < 0.01$ vs. V1 [saline].

† $P = 0.06$ vs. V1.

‡ $P < 0.05$ vs. healthy subjects.

Table 3. Cytokines generated by sputum cells

Cytokine	Subject type	Saline	Dpt
IL-4 (pg/ml 10^5 leuk)	HS	0 (0–9)	0 (0–2)
	AA	0 (0–51)	26 (0–406)**†
IL-6 (pg/ml 10^5 leuk)	HS	99 (12–807)	140 (0–773)
	AA	57 (5–690)	173 (21–962)*
IL-10 (pg/ml 10^5 leuk)	HS	20 (4–85)	11 (2–156)
	AA	7 (2–106)	37 (2–432)*
IFN- γ (U/ml 10^5 leuk)	HS	0 (0–0.1)	0 (0–0.1)
	AA	0.2 (0–6)	0.6 (0–21)‡
TNF- α (pg/ml 10^5 leuk)	HS	296 (56–671)	184 (18–446)
	AA	80 (13–490)	234 (41–852)

HS, healthy subjects; AA, atopic asthmatics.

Results are expressed as median (range) for eight healthy subjects and 11 atopic asthmatics.

* $P < 0.05$, ** $P < 0.01$ vs. V1 [saline].

† $P < 0.001$ vs. healthy subjects; ‡ $P = 0.07$ vs. healthy.

both). There was no significant difference with respect to neutrophil or epithelial cell counts. No significant change in any sputum cell counts occurred in healthy subjects after allergen vs. saline.

Cytokine production from sputum cells

The overall results are given in Table 3. In allergic asthmatics the production of IL-4 from sputum cells was increased after allergen challenge when compared to saline ($P < 0.01$). Furthermore the amounts of IL-4 generated from sputum cells after allergen in asthmatics were greater than those measured in healthy subjects ($P < 0.001$) at the corresponding visit (Fig. 2). In

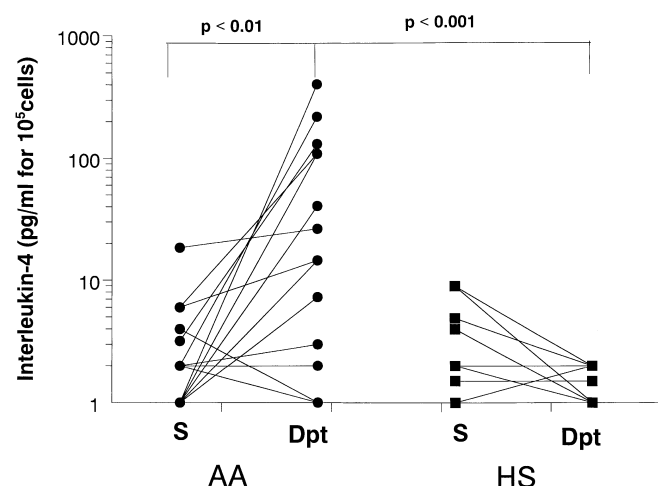


Figure 2. Interleukin-4 production from sputum cells cultured for 24 h in healthy subjects and allergic asthmatics 6 h after saline vs. allergen challenge.

asthmatics allergen challenge also resulted in an increased production of IL-6 ($P < 0.05$) and IL-10 ($P < 0.05$) when compared to saline. Inter-group comparisons however, failed to show any significant difference with respect to these two cytokines. There was no significant change in IFN- γ and TNF- α after allergen challenge in any of the groups tested. Inter-group comparisons after allergen showed that atopic asthmatics produced greater amounts of IFN- γ than healthy non-atopic subjects but the difference only approached statistical significance ($P = 0.07$). There was no significant difference between the groups with respect to TNF- α .

Correlations between cytokine production and the cellular profile in induced sputum and the late phase bronchospasm

In asthmatics the change in IL-4 strongly correlated with the change in sputum eosinophils ($r = 0.87$, $P < 0.001$) (Fig. 3). The changes in IL-10 and IFN- γ also followed those in sputum eosinophils ($r = 0.80$, $P < 0.01$ and $r = 0.72$, $P < 0.05$, respectively). None of the changes in cytokine generation significantly correlated with the intensity of the late phase reaction, albeit this approached the statistical significance for IFN- γ ($r = 0.59$, $P = 0.06$) and IL-10 ($r = 0.55$, $P = 0.08$), and IL-4 ($r = 0.53$, $P = 0.09$). Also the change in sputum eosinophils tended to correlate with the intensity of the late phase bronchospasm ($r = 0.54$, $P = 0.08$).

Discussion

In this study we have shown for the first time that experimental allergen exposure of sensitized asthmatics up-regulates the amount of IL-4, IL-6 and IL-10 spontaneously generated by sputum cells *ex vivo*. Furthermore

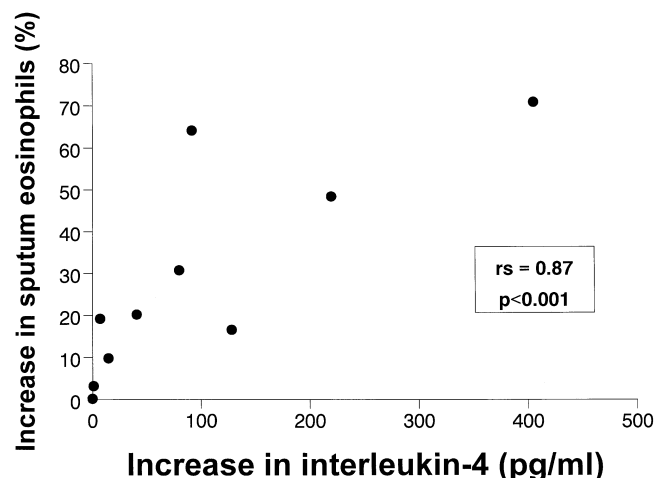


Figure 3. Correlation between the increase in interleukin-4 production from sputum cells collected 6 h after allergen vs. saline challenge and the change in sputum eosinophil count during the late bronchospasm in allergic asthmatics.

the sputum production of IL-4 appears to be strongly correlated with the extent of sputum eosinophilia.

Our results establish the usefulness of *ex vivo* sputum cell generation of cytokine as a suitable model to look at cytokine airways production. Importantly, because of the high sensitivity of our technique, the cytokines could be detected *ex vivo* without any additional stimulus in the culture medium. This differentiates our results from those of Liu et al. who had to add phytohaemagglutinin (PHA) in order to obtain detectable levels of IFN- γ in their sputum cell culture (13).

Interleukin-4 has been initially seen as a key cytokine in the regulation of IgE synthesis (14,15). However its potential role in several physiopathological events underlying asthma has widened over the years and IL-4 is currently believed to also play a role in acute allergic inflammation and airway remodelling (3,4). Recent clinical trials have shown some beneficial effects of a weekly treatment with inhaled IL-4 soluble receptor when tapering inhaled steroids in asthmatics, which indicates that this cytokine may indeed play a crucial role in the disease (16). Clearly our data show that production of IL-4 from airway cells sharply rises a few hours after contact with an allergen in sensitized subjects. Our observation confirms *in vivo* the ability of an allergen to release IL-4 from peripheral blood mononuclear cells of sensitized subjects *in vitro* (8). The source of IL-4 in our study is unclear, but recent data suggest that mast cells, together with T lymphocytes, are certainly valuable contenders in that respect (17–19). The strong correlation between sputum eosinophil influx post allergen and amounts of IL-4 generated by sputum cells lends support to a role for IL-4 in airways eosinophil recruitment (20). Alternatively it might also reflect that recruited eosinophils act as potent local producers of this cytokine (21).

Allergic asthmatics also displayed a rise in IL-6 and IL-10 production from sputum cells following allergen challenge. Since the two cytokines belong to the so-called Th2 cytokines, this observation strengthens the concept that allergen exposure promotes local Th2 cytokine secretion in sensitized subjects. Although IL-6 is considered as a pro-inflammatory cytokine that may contribute to airway lymphocyte activation (22), IL-10 is rather seen as an inhibitor of inflammation partly acting through an inhibition of lymphocyte proliferation and cytokine release (23). In the mouse model of asthma this cytokine was shown to reduce cellular recruitment into the airways after allergen exposure (24). In addition asthmatics were shown to have less IL-10 in their sputum fluid phase compared to healthy subjects (25). Therefore the rise in IL-10 production from sputum cells, which paralleled that in sputum eosinophils in our asthmatics, may appear somewhat surprising. However this might be seen as a protective mechanism attempting to control the acute inflammation caused by the allergen.

Clearly, the increased secretion of Th2 cytokines after allergen from airways cells of allergic asthmatics is not associated with a reduction in the production of IFN- γ , which actually also showed a rise after allergen. Under these circumstances the production of IFN- γ after allergen was even greater in asthmatics than in healthy subjects. Furthermore the change in IFN- γ followed the rise in sputum eosinophil counts. This indicates that the Th1 pathway may also be promoted in the airways of asthmatics after allergen exposure. The pattern of cytokine up-regulation following allergen in sensitized subjects might not be so selectively directed towards the Th2

pathway as previously thought. This is in keeping with a previous study demonstrating raised intracellular synthesis of IFN- γ in bronchoalveolar lavage lymphocytes from allergic asthmatics (7). Thus the low spontaneous secretion of IFN- γ from blood leukocytes of allergic asthmatics that we found in a previous study (8), is unlikely to result from natural allergen exposure, but rather seems to reflect a basic and perhaps genetic trend that characterizes allergic subjects. Thus the behaviour of cells from allergic asthmatics with respect to their IFN- γ production appears to be critically dependent on the body compartment selected and the stimuli applied.

Der p1, the major allergen of *Dermatophagoides pteronyssinus*, is endowed with protease activity (26). Thus we wondered whether this molecule might have an intrinsic property beyond its capacity to bind to specific IgE at the airways cell surfaces to promote their activation or degranulation. That is the reason why we designed the study so that we were able to compare sensitized vs. non-sensitized subjects with respect to their response to inhaled Dpt. When compared to saline, Dpt failed to change sputum cell counts or to modulate the production of cytokines in healthy non-allergic subjects. This suggests that a single inhalation of a high dose of Dpt is unable to elicit an airway inflammation when lacking specific IgE either in the serum or in the skin as detected by RAST or positive prick tests, respectively.

We conclude that sputum cells from allergic asthmatics collected 6 h after allergenic challenge showed increased release of a set of cytokines, including IL-4, IL-6, IL-10 and IFN- γ . Moreover the amounts of IL-4 generated strongly correlate with the extent of sputum eosinophilia.

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