

Influx of Neutrophils into the Airway Lumen at 4 h after Segmental Allergen Challenge in Asthma

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Key Words

Asthma allergic · Segmental allergen challenge ·
Bronchoalveolar lavage · Neutrophilic granulocytes ·
Interleukin-8 · Chemotaxis

Abstract

Background: Segmental allergen challenge is a powerful tool to study inflammatory reactions in asthmatic airways. There is little information on the early events at 5 min and 4 h after allergen challenge with respect to the cell influx and the chemokine interleukin-8 (IL-8). **Methods:** Seven mild to moderate allergic asthmatics (AA group), 5 allergic nonasthmatics (ANA group) and 5 nonallergic controls underwent segmental allergen challenge, with allergen doses based upon skin reactivity. Bronchoalveolar lavage (BAL) samples were obtained before, 5 min and 4 h postchallenge, and were analyzed for cell numbers and differential counts, eosinophil and neutrophil chemotactic activity, and levels of IL-8. **Results:** At 5 min postchallenge, no changes were observed compared to baseline. At 4 h postchallenge, an increase was found in the number of neutrophils and the levels of IL-8, which was dependent on the dose of allergen in the AA and ANA group. At the same allergen dose, the increases in neutrophils and

levels of IL-8 were calculated to be 91 and 67 times higher, respectively, in AA than in ANA. Levels of IL-8 correlated with the number of neutrophils and with the in vitro neutrophil chemotactic activities in BAL fluid. **Conclusions:** Neutrophil chemotactic activity is increased in BAL fluid at 4 h after segmental allergen challenge. We suggest that apart from IgE-mediated mast cell degranulation, additional local factors in the airways determine the degree of IL-8 increase and neutrophil influx.

Introduction

Allergen challenge followed by bronchoalveolar lavage (BAL) is a powerful tool to study pathophysiological reactions in the airways of patients with allergic asthma. After inhalation of an allergen two phases of bronchoobstruction can be distinguished [1]. Within minutes an early bronchoobstructive reaction occurs, which is believed to result mainly from smooth muscle contraction as a consequence of mediator release from mast cells upon crosslinking of receptor-bound specific IgE by allergen [2]. Four to six hours after allergen exposure a second obstructive reaction frequently occurs. It may last for up to 24 h and is accompanied by an increase in nonspecific bronchial hyperrespon-

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siveness [3]. During this late phase response, an increase in inflammatory reactions of the bronchial (sub)mucosa can be observed [4, 5].

A number of studies on local reactions in the airways after segmental allergen challenge or whole lung challenge have focused on time points 24–96 h after challenge, when the late inflammatory reaction has developed completely. The bronchoalveolar eosinophilia described during this late inflammatory reaction is a prominent finding and considered to be characteristic of asthma [6]. The role of the neutrophil in the allergen-induced inflammatory process is less obvious. After exposure of asthmatic subjects to either common allergens or occupational sensitizers, several investigators have observed increased numbers of neutrophils in BAL fluid or bronchial biopsies, and they have suggested that neutrophils play a role in the pathophysiology of the allergen-dependent reactions [7–11]. In other studies the occurrence of neutrophils was observed in asthmatics as well as in nonasthmatics and controls, and it was therefore attributed to nonspecific factors, like endotoxin contamination of the allergen solution or the endoscopic challenge procedure per se [7, 9, 11]. The chemokine interleukin-8 (IL-8) that is a chemoattractant for neutrophils has been demonstrated to be increased in BAL fluid both in stable asthmatics [12] and after allergen challenge [13].

The neutrophil has the potential of causing mucosal damage by producing cytokines, proteases, oxygen metabolites and lipid-derived mediators, such as prostaglandins and leukotriene B₄ [14]. As mucosal inflammation and bronchial hyperresponsiveness are characteristic features of chronic asthma, it is important to obtain information on the occurrence of neutrophils in the allergen-induced inflammatory responses. Recently, murine studies have reported that neutrophils are especially recruited to the lung early after allergen challenge, whereas eosinophil recruitment is maximal at later time points [15]. There are only few studies in allergic asthmatics (AA) and allergic nonasthmatics (ANA) investigating time points before 6 h postchallenge.

The aim of the present study was to investigate both the profile of cell recruitment and the presence of granulocyte chemotactic activity in BAL fluid at 4 h after allergen challenge. The 4 hour time point was chosen, because in allergic subjects challenged with common aeroallergens, an increase in bronchial hyperreactivity has been identified from 3 h after whole lung challenge, and it marks the onset of the clinical late asthmatic reaction [16]. This suggests the occurrence of relevant inflammatory events in the airways at 3 to 4 h after allergen challenge. To examine whether chemoattractive activity is present in BAL fluid after allergen challenge, we performed chemotaxis assays with puri-

fied neutrophils and eosinophils. In addition, we measured the levels of IL-8, one of the main chemoattractants for neutrophils.

Methods

Subjects

Seventeen volunteers (7 mild to moderate AA, 5 ANA and 5 non-allergic healthy controls) were selected for the study (table 1).

All allergic subjects had a positive skin prick test for house dust mite allergens and/or grass pollen. Asthma was defined according to the American Thoracic Society (ATS) criteria and included a history of recurrent episodes of wheezing, chest tightness and dyspnea and a normal lung function between asthmatic attacks [17]. None had experienced an exacerbation of asthma during at least 2 months before the investigation. None of the subjects used anti-inflammatory medication, in particular they had not used corticosteroids for at least 3 months prior to the study. Inhaled bronchodilators were withheld at least 8 h before all investigations. The 5 ANA subjects had no past or present history of wheezing, chest tightness, or recurrent episodes of reversible airway obstruction. Two had a history of seasonal or perennial rhinitis and the other 3 allergic subjects were presently asymptomatic.

The nonallergic subjects had no positive wheal-and-flare response in the skin prick tests with a panel of common inhalant allergens. They had no history of asthma nor did they have signs of seasonal or perennial rhinitis.

None of the allergic and nonallergic subjects had experienced recent airway infection. The study was approved by the Internal Review Board of the Academic Medical Center, Amsterdam, and it was performed after written informed consent had been obtained.

Study Design

At an initial visit, all subjects completed a respiratory and allergy questionnaire, and a full medical history, physical examination and skin prick tests. A blood sample was taken to determine white blood cell count and specific and total IgE. Baseline spirometry and a histamine provocation test were performed. Within 4 weeks, subjects returned to the clinic for allergen challenge. A second baseline spirometry was done and intracutaneous skin dose-response series with house dust mite or grass pollen were performed to determine the allergen dose for the allergen provocation procedure.

Determination of Allergen Dose. Skin tests were performed to determine the concentration of the allergen producing a 10-mm wheal response, 15 min after intracutaneous injection [18]. The AA group and ANA group underwent an allergen challenge with 1 ml of this allergen concentration brought to a final volume of 5 ml with saline, for each of the lung segments. The nonallergic subjects (control group) were challenged with the highest dose of allergen applied in the allergic group.

Fiberoptic Bronchoscopy and Segmental Allergen Challenge. In all subjects a baseline BAL was performed of the lingula and two postchallenge BALs (at 5 min and 4 h) of the right middle lobe segments. Bronchoscopy was performed as described previously [19]. Briefly, after premedication with atropine and codeine and after local anesthesia with lidocaine, a flexible fiberoptic video-bronchoscope (Olympus p200) was wedged into a subsegment of the lingula. Seven

Table 1. Subject characteristics

	Subject No.	Age years	Sex	FEV ₁ % predicted	PC ₂₀ hist ^a mg/ml	Skin threshold ^b ng/ml
AA group	1	26	F	113	0.8	0.1
	2	35	M	92	4.0	0.1
	3	21	M	88	5.7	0.1
	4	30	M	120	16.0	1.0
	5	28	M	105	2.5	6.0
	6	20	M	91	7.2	100
	7	22	F	109	9.0	100
ANA group	1	20	M	90	11.0	60
	2	20	F	102	14.2	60
	3	31	M	115	>32	60
	4	19	F	104	19.0	600
	5	23	F	97	15.0	6,000
Control group	1	22	F	103	>32	>6,000
	2 ^c	43	F	121	6.2	>6,000
	3	39	F	114	>32	>6,000
	4	26	F	121	32	>6,000
	5	39	M	100	>32	>6,000

PC₂₀hist = Concentration of histamine that caused a fall in FEV₁ of 20% of the baseline value.

^a AA v. ANA group: $p < 0.02$; AA v. control group: $p < 0.01$.

^b Skin test threshold: concentration of allergen producing a 10-mm wheal response.

^c No selective BAL obtained at 4 h after challenge.

successive 20-ml aliquots of prewarmed NaCl (154 mM) were instilled and each aspirated immediately with low suction. After the control lavage, allergen diluted to a final volume of 5 ml was administered in both the lateral and the medial segment of the right middle lobe, and the bronchoscope was retracted above the carina for 5 min. Next a lavage was performed of one of the challenged segments and the bronchoscope was withdrawn. Four hours after challenge, the bronchoscopy was repeated and the remaining segment of the right middle lobe was lavaged. Transcutaneous oxygen saturation was monitored throughout the bronchoscopy.

One hour after each bronchoscopy measurements of forced expiratory volume in 1 s (FEV₁) were performed. Subjects were observed for at least 3 h after the last bronchoscopy.

Lung Function Tests

FEV₁ and inspiratory vital capacity (IVC) were measured with a dry rolling-seal spirometer (SensorMedics BV, Bithoven, The Netherlands) according to standardized guidelines [20]. Bronchial reactivity to histamine was determined by a 2-min tidal-breathing method [21]. The histamine threshold (PC₂₀hist) was defined as the interpolated concentration of histamine that caused a fall in FEV₁ of 20% of the baseline value.

Lavage Fluid and Cell Processing

The first three aliquots were combined and named pool 1. The aliquots four to seven were combined and named pool 2. The pools were immediately centrifuged at 500 *g* and 4°C. The cell-free supernatant was stored at -80°C. Pool 2 was divided in two equal portions. To one of the portions ethylenediaminetetraacetic acid (EDTA;

Merck, Darmstadt, Germany) was added (final concentration 2.8 mM). All investigations were performed in pool 1 and pool 2.

Cells were resuspended in phosphate-buffered saline (140 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 0.5% (wt/vol) bovine serum albumin (Boseral DEM, Organon Teknika b.v., Boxtel, The Netherlands; PBA). The number of erythrocytes was counted with Dacie suspension [1.2% (wt/vol) trisodium citrate · 2 H₂O + 0.4% formaldehyde 37%] in a Fuchs-Rosenthal counting chamber. All pools contained less than 1×10^5 erythrocytes/ml, indicating no significant bleeding during bronchoscopy. Total cell number was determined by counting manually in a Bürker counting chamber. Cells were cytocentrifuged at 500 rpm for 2 min in a Shandon Cytocentrifuge (model cytopsin 2) and stained with Romanovsky (Diff-Quik) and Jenner-Giemsa. For differential cell counts, a total of 1,000 cells were enumerated with the investigator being blinded for subject identity and lavage time point. Epithelial cells, macrophages, lymphocytes, neutrophils and eosinophils were identified.

Chemotaxis Assays

Neutrophil and eosinophil migration was measured by a modification of the method according to Boyden with a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, Md.) [23]. Briefly, BAL fluid or incubation buffer was added to the lower compartments. Two filters were placed between the lower and the upper compartments. The lower filter had a pore width of 0.45 µm (Millipore, Bedford, Mass., type HA) and the upper filter (cellulose nitrate) a pore width of 3 µm (thickness 150 µm; Sartorius, Göttingen, Germany; type SM 113). Before use, the filters were soaked in incubation buffer. Normal human eosinophils and neutrophils were obtained by Percoll-Hy-

paque isolation. The purity of both the isolated eosinophils and neutrophils was greater than 95%. Eosinophils were primed with IL-5 (100 pM) or buffer (control) for 30 min at 37°C and washed with incubation buffer. Subsequently, the cells were placed in the upper compartments (25 µl of 2×10^6 /ml) of the Boyden chambers. The chambers were then incubated for 90 min at 37°C. The upper filters were removed, fixed in butanol/ethanol (20/80%, v/v) for 10 min and stained with Weigert solution [composition: 1% (v/v) hematoxylin in ethanol mixed with 70 mM FeCl₃ solution at a 1:1 ratio]. The filters were dehydrated with ethanol, made transparent with xylene and fixed upside down. The chemotactic responses of the cells were quantified with an image analyzer (Quantimet 600S, Leica RXM, Leica Cambridge) using Quantimet 600 Control Software (QUIC, version 2.02) and a custom-made software program written in Qwin. An automated microscope was used to step through the filters at a given number of levels in the Z direction with a certain Z level interval. Cells were counted at each of these levels. The mean migrated distance of all cells present in the filter (chemotactic index) was used as a readout for chemotaxis. Optimal concentrations of C5a and IL-8 served as positive controls. All data were corrected for the migration induced by the incubation buffer.

Enzyme-Linked Immunosorbent Assay for IL-8

The levels of IL-8 in BAL fluid were measured by enzyme-linked immunosorbent assay (ELISA) [12]. The lower limit of detection of this ELISA was approximately 5 pg/ml. Intra- and interassay coefficients of variation were less than 10%. Response curves of BAL fluid were parallel to that of the calibrator in the measuring range from 5 to 200 pg/ml. The levels of IL-8 were not affected by freezing and thawing procedures.

Measurement of Lipopolysaccharide Content

Lipopolysaccharide (LPS) was measured by a commercially available chromogenic limulus amoebocyte lysate assay (Endosafe, Charleston, S.C.). The linear standard curve of this assay, showing a relation between the absorbances and the LPS concentrations for a dilution series of a standard endotoxin, ranged from 1.25 to 10 pg/ml (1 ng/ml = 12 endotoxin units/ml). Samples containing less than 2.1 pg/ml are within United States Pharmacopeia (USP) standards for endotoxin concentrations and are considered safe and nonpyrogenic for intravenous injection [24].

Statistical Analysis

When appropriate, logarithmic transformation was performed to obtain normally distributed values. The Student t test was used for paired analysis. For group comparisons we used one-way analysis of variance (ANOVA). Linear regression analysis and Pearson's test were used for variables of normally distributed values; a two-tailed p value of less than 0.05 was considered significant. Analyses were performed with the SPSS/PC+ statistical package (version 6.1, Chicago, III).

Results

All subjects completed the procedure successfully. From one control subject it was not possible to obtain a selective BAL sample 4 h after challenge. The procedure was well-tolerated by all subjects and no major decrease in lung-

function (FEV₁) occurred in any subject. Two subjects in the AA group received salbutamol before bronchoscopy, because of wheezing during local anesthesia. Results in these subjects did not differ from the other subjects in the AA group. The volume of BAL fluid recovered was similar in the three groups (% recovery, mean $\pm$ SE: pool 1, 49.8 $\pm$ 8.2%; pool 2, 77.4 $\pm$ 8.8%). Separate analysis of pool 1 and pool 2 showed essentially the same results. Therefore, the combined data of pool 1 + pool 2 are shown.

Skin Test Threshold, Allergen Dose and Levels of LPS

The doses of allergen that produced a 10-mm wheal response in the skin test were lower in the AA group than in the ANA group. Therefore, total doses of allergen delivered with the segmental challenge were lower in the AA group than in the ANA group (median and range): AA group, 1.0 ng (0.1–100) and ANA group, 60 ng (60–6,000) (fig. 1). The levels of LPS delivered with the allergen solution were below 2.1 pg/ml in all subjects (within USP standards for endotoxin concentrations, and therefore considered safe and nonpyrogenic). The highest concentration of LPS delivered was 0.25 pg/ml (in 1 subject from the ANA group and in the control subjects).

Total and Differential BAL Fluid Cell Counts

At baseline, no significant differences were observed in total and differential BAL fluid cell counts between the three groups (table 2). At 5 min postchallenge, no changes were observed in total and differential BAL fluid cell counts when compared to baseline in any of the groups (table 2).

At 4 h postchallenge the number of neutrophils was increased in all three groups (AA group: $p < 0.0025$; ANA group: $p < 0.04$; control group: $p < 0.02$). No significant differences in increase were observed among the three groups (ANOVA: $p > 0.05$). In the AA and ANA group the increase in number of neutrophils correlated significantly with the dose of allergen delivered with the challenge procedure (AA group: $r = 0.87$, $p = 0.011$; ANA group: $r = 0.95$, $p = 0.005$; fig. 1).

The slopes of the regression lines in figure 1 did not differ significantly (slope $\pm$ SE of ¹⁰log-transformed data: AA group: 0.750 ± 0.191 , ANA group: 0.845 ± 0.114 ; $p > 0.05$). Parallel line analysis with common slope showed a significant difference between the intercepts ($p < 0.001$). This indicates an increased susceptibility for allergen-dependent influx of neutrophils in the AA group compared to the ANA group. At the same allergen dose, the calculated increase in neutrophils in the AA group was 91 times (95% confidence interval: 22.4–369) higher than in the ANA group.

Table 2. Cells ($\times 10^3/\text{ml}$) in BAL fluid before, (0) and at 5 min and 4 h postchallenge

Cells	AA group (n = 7)			ANA group (n = 5)			Control group (n = 5)		
	0	5 min	4 h	0	5 min	4 h	0	5 min	4 h
Mac	107 (60–221)	105 (60–190)	89 (54–202)	123 (94–154)	97 (78–192)	106 (71–116)	96 (21–385)	112 (39–323)	67 (55–418)
Lymphocytes	3.0 (0.05–9.8)	3.2 (1.1–6.0)	6.2 (0.18–18.4)	3.6 (1.0–12.3)	2.0 (1.2–15.1)	2.2 (0.65–3.41)	3.6 (0.56–5.4)	4.9 (1.9–5.7)	6.7 (1.3–21.2)
Eosinophils	0.82 (0–1.9)	0.58 (0–2.7)	1.4 (0–8.6)	0.18 (0–0.99)	0.19 (0–1.1)	0.14 (0–0.62)	0.09 (0–2.2)	0.28 (0.06–0.95)	0.27 (0–2.2)
Neutrophils	1.1 (0.14–1.9)	0.9 (0.44–1.6)	24.1 ^a (0.27–478)	0.99 (0.55–1.7)	1.4 (0.25–4.1)	5.2 ^b (1.9–111)	1.2 (0.34–2.7)	3.0 (0.06–11.3)	7.7 ^c (3.4–11.2)
Epithelial cells	2.3 (0.56–36)	2.5 (0.16–8.9)	0.40 (0.17–2.9)	4.6 (1.1–5.8)	5.4 (2.6–7.8)	0.65 (0–7.3)	1.6 (0.65–9.5)	3.0 (1.5–4.5)	0.44 (0–2.8)
Total	120 (62–223)	121 (68–195)	217 (89–574)	131 (102–165)	125 (83–206)	118 (76–227)	104 (24–392)	129 (42–345)	78 (64–455)

Mac = Alveolar macrophages. Median and range are shown. ^a $p < 0.0025$; ^b $p < 0.04$; ^c $p < 0.02$; all compared to baseline.

No significant differences were observed in the total number of cells, number of lymphocytes, or in the number of eosinophils recovered 4 h after challenge when compared with baseline in any of the three groups. For all cell types essentially the same results were obtained when percentage of total cells were analyzed.

Neutrophil and Eosinophil Chemotactic Activity from BAL Fluid before and at 4 h Postchallenge

In the allergic individuals a significant increase was observed in neutrophil chemotactic activity from the BAL fluid at 4 h postchallenge (tested with normal blood neutrophils) when compared to baseline samples [mean neutrophil migration, geometric mean and SE, AA + ANA group: before: $4.8 \mu\text{m}$ (3.4); at 4 h: $12.3 \mu\text{m}$ (2.2); $p < 0.02$]. Eosinophil chemotactic activity was detected in the unconcentrated BAL fluid at 4 h postchallenge in 2 subjects from the AA group only (data not shown).

Interleukin-8

At baseline no significant differences were observed in levels of IL-8 among the three groups. At 5 min after allergen challenge there was no significant increase in the levels of IL-8 in the BAL fluid compared to baseline in any of the three groups. At 4 h postchallenge levels of IL-8 were increased in all three groups (AA group: $p < 0.02$; ANA group: $p < 0.04$; control group: $p = 0.05$). No significant differences were observed in increases in the three groups (ANOVA: $p > 0.05$). In the AA and ANA group the increase in levels of IL-8 correlated significantly with the dose of al-

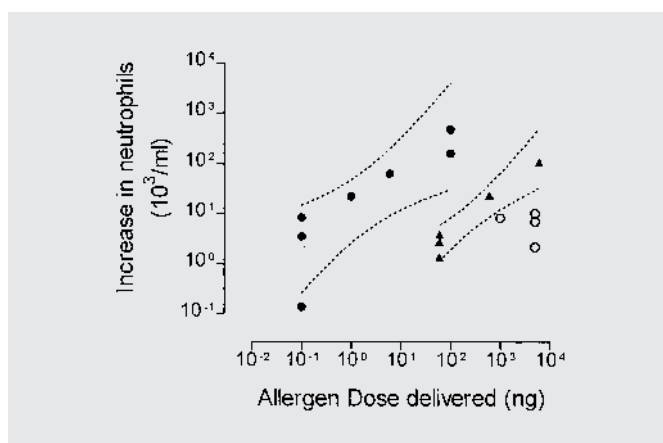


Fig. 1. Increase in total number of BAL neutrophils in the three groups at 4 h after allergen challenge. ● = AA group; ▲ = ANA group; ○ = nonallergic subjects (control group). At 4 h postchallenge the number of neutrophils was significantly increased in all groups, but no significant differences were observed among the three groups (ANOVA: $p > 0.05$). In the AA and ANA group the increase in the number of neutrophils correlated significantly with the dose of allergen delivered with the challenge procedure (AA group: $r = 0.87$, $p = 0.011$; ANA group: $r = 0.95$, $p = 0.005$). Dotted lines represent 95% confidence interval of the regression line.

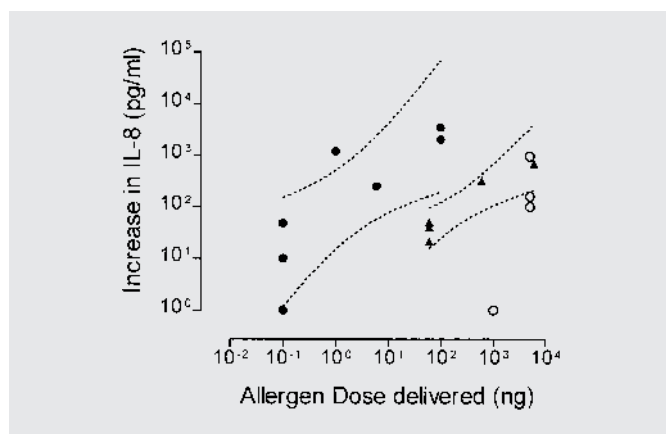


Fig. 2. Increase in levels of IL-8 in BAL fluid in the three groups at 4 h after allergen challenge. ● = AA group; ▲ = ANA group; ○ = non-allergic subjects (control group). At 4 h postchallenge levels of IL-8 were increased in all three groups, but no significant differences were observed among the three groups (ANOVA: $p > 0.05$). In the AA and ANA group the increase in the levels of IL-8 significantly correlated with the dose of allergen delivered (AA group: $r = 0.84$, $p = 0.018$; ANA group: $r = 0.96$; $p = 0.012$). Dotted lines represent 95% confidence interval of the regression line.

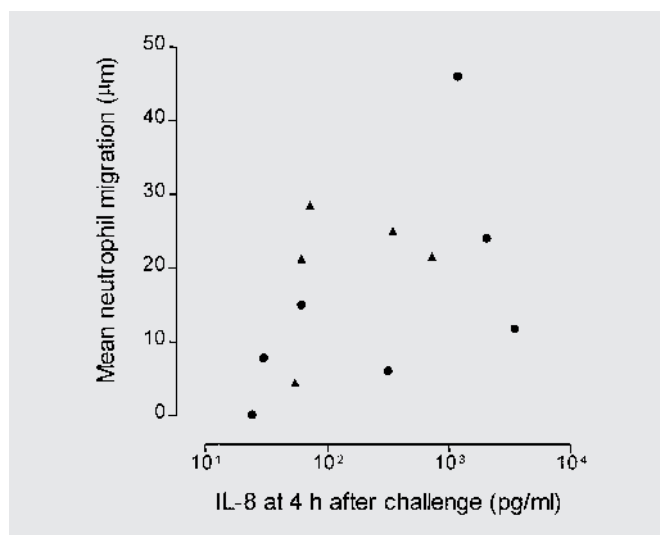


Fig. 4. Relationship between levels of IL-8 in BAL fluid and the mean migration of normal neutrophils towards BAL fluids in the chemotaxis assay. ● = AA group; ▲ = ANA group (AA group: $r = 0.76$, $p < 0.05$; AA + ANA group: $r = 0.63$, $p < 0.03$).

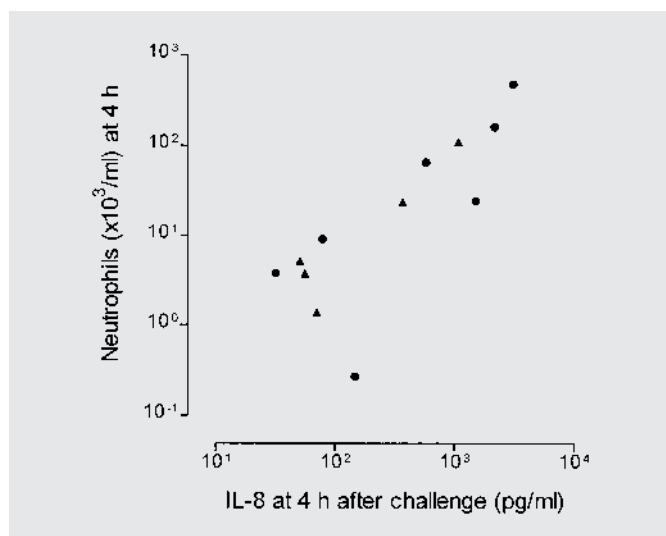


Fig. 3. Relationship between number of neutrophils and levels of IL-8 in BAL fluid. In the allergic subjects the levels of IL-8 at 4 h after allergen challenge correlated with the number of neutrophils at that time point. ● = AA group; ▲ = ANA group (AA group: $r = 0.77$, $p < 0.05$; ANA group: $r = 0.93$, $p < 0.025$; AA + ANA group: $r = 0.81$, $p < 0.002$).

lergen delivered (AA group: $r = 0.84$, $p = 0.018$; ANA group: $r = 0.96$, $p = 0.012$; (fig. 2). The slopes of the regression lines did not differ significantly (slope $\pm$ SE of $^{10}\log$ transformed data: AA group: 0.798 ± 0.231 , ANA group: 0.690 ± 0.124 ; ($p > 0.05$). Parallel line analysis with a common slope showed a significant difference between the intercepts ($p < 0.019$). At the same allergen dose, the calculated increase in IL-8 was in the AA group 67 times (95% confidence interval: 12.5–361) higher than in the ANA group.

In the allergic subjects the levels of IL-8 at 4 h after allergen challenge correlated with the number of neutrophils at that time point (AA group: $r = 0.77$, $p < 0.05$; ANA group: $r = 0.93$, $p < 0.025$; AA + ANA group: $r = 0.81$, $p < 0.002$; fig. 3). Linear regression analysis of the relation between IL-8 and neutrophils did neither show a difference in slope nor in intercept between the AA group and the ANA group, indicating no difference between the two groups in the ratio of IL-8 to neutrophil influx.

At 4 h postchallenge the levels of IL-8 in BAL fluid from the allergic subjects correlated with the mean migration of normal neutrophils towards BAL fluids in the chemotaxis assay (AA group: $r = 0.76$, $p < 0.05$; AA + ANA group: $r = 0.63$, $p < 0.03$; fig. 4).

Discussion

Our study shows that at 4 h after segmental allergen challenge, there is an increase in the number of neutrophils, levels of IL-8 and neutrophil chemotactic activity in BAL fluid from AA, ANA and healthy controls. In the AA and ANA groups a significant relationship was found between the dose of allergen delivered and the increase in the numbers of neutrophils and levels of IL-8. The two allergic subject groups showed the same dose-dependent increase in both parameters (equal slopes of the regression lines). However, at the same allergen dose, the increase in numbers of neutrophils and levels of IL-8 was a factor 91 and 67 higher, respectively, in the AA group than in the ANA group. At 4 h postchallenge, levels of IL-8 correlated significantly with the numbers of neutrophils with similar IL-8 to neutrophil influx ratios for the two groups. Furthermore, *in vitro*, the increased neutrophil chemotactic activity in BAL fluid obtained at 4 h postchallenge correlated significantly with levels of the neutrophil chemoattractant IL-8 in these BAL fluid samples.

Generally, the eosinophil is considered to be the most important inflammatory cell in asthma, but neutrophils have also been implicated in the pathophysiological mechanisms [7, 25]. Increased levels of IL-8 have been described in BAL fluid of stable asthmatics [12, 26]. Several studies have found increased numbers of neutrophils in the airways of allergic subjects after allergen challenge. Some of these studies have suggested that this increase might be nonspecific or procedure-dependent [13, 27]. Others have shown that the increase might be allergen-dose-dependent [11]. Few studies analyzed neutrophil influx at 4 h postchallenge, a time point around which in murine studies especially neutrophils are recruited to the lung [15]. Teran and coworkers [13] investigated BAL fluid of asthmatics at 4 h postchallenge, and also observed an increase in the number of neutrophils and levels of IL-8, which did not differ significantly from the increase in the segments challenged with saline. Therefore, the increase was considered to be procedure-dependent. In our study, in which we used low doses of allergen, we took the allergen dose into account in the evaluation of the results, and observed an allergen dose dependency of the increase in numbers of neutrophils and levels of IL-8 in both allergic groups. This indicates that the observed phenomenon is not induced by the challenge procedure *per se*. Studies including saline challenge as a control should substantiate this aspect further.

In our study, allergen doses delivered with the segmental challenge were based upon skin reactivity for the allergen. The doses given were aimed at obtaining the same response

in all allergic subjects. This design was based on a study from Metzger et al. [28], demonstrating a relationship between the allergen dose required for a positive skin test and the dose required to produce a visible immediate response in the airways. If in our study the effects in the airways at 4 h postchallenge had been simply related to the early skin test reactions, in all subjects a similar influx of neutrophils was expected with only random variation. However, in both allergic groups an allergen dose dependency was observed. The transformation of similar early skin reactions to dose-related differences in inflammatory changes in the airways at 4 h may be caused by the inability to predict immediate allergic reactions in the lung from the skin test. For example, differences in mast cell reactivity in airways and skin may underlie the observed dose dependency. This, however, is unlikely since measurements of tryptase at 5 min postchallenge revealed no relationship with the allergen dose (unpubl. results). Furthermore, the inflammatory response in nonallergic controls is not expected to be caused by mast cell activation. Therefore, we propose that apart from IgE-mediated mast cell activation and subsequent allergic inflammatory events additional reactions result in the observed dose dependency. Direct triggering of epithelial cells, macrophages and/or monocytes by their receptors or indirect triggering via antibody (IgG/IgE) or by products of complement activation with the subsequent release of active cytokines (IL-1, IL-8, TNF- α) might play a role in such a pathway.

In the AA group both the increase in levels of IL-8 and the number of neutrophils occurred at lower allergen doses than in the ANA group. This difference may result from an increased number, reactivity and/or the accessibility of target cells in asthmatic airways. Epithelial cells, alveolar macrophages and other types of cells obtained from asthmatics have been shown to release more easily inflammatory mediators *in vitro*, including IL-8, than cells of nonasthmatics [29]. Furthermore, the denudation of the bronchial epithelium and the increased permeability of the respiratory membrane in asthmatics may result in an increased accessibility of target cells in the bronchial mucosa [30]. This may cause a release of chemotactic mediators, like IL-8, and subsequent recruitment of neutrophils at lower allergen doses.

No difference was found in the relationship between increase in IL-8 and neutrophil influx between the two allergic groups. This suggests that the difference between the AA and ANA groups originates from an enhanced release of neutrophil chemoattractants rather than from an increased response of neutrophils to chemoattractants.

We are not aware of data comparing AA and ANA for inflammatory reactions early after allergen challenge which are not related to IgE-mediated mast cell degranulation. However, an increased susceptibility of AA for induction of IgE-dependent reactions has been reported [31]. Our study suggests that the dose of allergen delivered in a segmental challenge may be a critical determinant of the inflammatory reactions, which should be taken into account in the analysis. Dupuis and colleagues [11] and more recently Jarjour and coworkers [32] have considered this for IgE-dependent inflammatory reactions.

One of the cytokines that probably plays a role in the recruitment of inflammatory cells to the lung is IL-8. We and others previously reported increased levels of IL-8 in BAL fluid from clinically stable patients with asthma as compared to healthy controls [12, 26]. Here, the number of patients included does not permit a definitive conclusion as to whether basal IL-8 levels are also increased in the lower airways of ANA. In the present study we found, at 4 h postchallenge, an increased neutrophil chemotactic activity and increased levels of IL-8 in BAL fluid from all subjects. Levels of IL-8 correlated significantly with the number of neutrophils and with the neutrophil chemotactic activity at 4 h postchallenge. This points to a role of IL-8 in the recruitment of neutrophils to the lung. The present study does not provide an answer as to the cellular source of IL-8.

We observed eosinophil chemotactic activity in unconcentrated BAL fluid from 2 AA subjects. Teran et al. [33]

showed an increase in immunologically reactive RANTES in BAL fluid at 4 h after challenge, which reached up to 263 pg/ml in concentrated BAL fluid. They showed that RANTES purified from BAL fluid was chemotactic for eosinophils starting at RANTES levels of about 0.5 ng/ml. Probably any eosinophil chemotactic activity in unconcentrated BAL fluid obtained at this early time point after challenge is below the detection limit in most patients.

We suggest that apart from IgE-mediated mast cell degranulation, additional local factors in the airways determine the increase in neutrophils and levels of IL-8 at 4 h after allergen challenge. Although allergen dose and method of administration are nonphysiological in segmental allergen challenge, the greater susceptibility of AA to an allergen-induced influx of neutrophils may indicate that neutrophilic inflammation contributes to the pathophysiology of asthma under natural allergen exposure.

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