

## Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction

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**ABSTRACT:** We wanted to determine whether changes in bronchial hyperresponsiveness (BHR) following allergen challenge show a time relationship with inflammatory events in the airways of allergic asthmatic subjects.

Lavage was performed and endobronchial biopsies were taken via the fiberoptic bronchoscope, before, and 3 and 24 h after, allergen challenge, on separate occasions, in nine dual asthmatic responders.

The numbers of activated eosinophils, identified by immunohistochemistry, using the monoclonal anti-eosinophil cationic protein antibody, EG2, were significantly increased both at 3 h and at 24 h in the submucosa and bronchial lavage. A significant negative correlation was found between the number of EG2<sup>+</sup> cells in the submucosa and in the bronchial lavage 24 h after the allergen challenge ( $r=-0.70$ ). At 24 h, the amount of eosinophil cationic protein (ECP) was increased in the bronchial lavage. A significant correlation was observed between the amount of ECP at 3 h and the log provocative dose of house dust mite producing a 20% fall in forced expiratory volume in one second ( $PD_{20}$  HDM) ( $r=0.63$ ).

The results suggest a recruitment of activated eosinophils to the submucosa and, further, to the epithelial lining, followed by degranulation. This process has already started 3 h after allergen challenge, and lasts for at least 24 h, which may result in mucosal damage and subsequent allergen-induced increase in BHR, before and after the late asthmatic reaction.

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Bronchial hyperresponsiveness (BHR) increases upon natural exposure to airborne allergens or occupational asthma inducers [1, 2]. An enhanced BHR has been observed following the late asthmatic response (LAR) induced by allergen provocation in sensitized human subjects [3-5].

More recently, it has been shown that the development of a LAR is also accompanied by an early increase in BHR 3 h after allergen inhalation, but response may differ depending on the stimulus used to measure BHR [6, 7]. These observations suggest that the tissue damage, which is supposed to cause the increase in BHR may, already have occurred before the LAR is clinically evident. Several factors may be responsible for the tissue damage. Important candidate mediators are inflammatory cells and their products in the bronchial submucosa [8]. In asthmatics, eosinophils are present in increased numbers in blood, and found in bronchoalveolar lavage fluid (BAL) [9, 10]. Major basic protein (MBP) has been found in increased concentrations in sputum of asthmatic patients [11]. Histological studies have shown that eosinophils often reside in the bronchial submucosa of

asthmatics, and are increased in the epithelium and submucosa, compared to normal subjects and atopic non-asthmatics [12-14].

After bronchial challenge with an allergen in patients in whom a LAR develops, there is an increase in the number of eosinophils in the BAL fluid during the LAR, but not 2 h after the early asthmatic reaction [15]. At the present time, it is unclear how findings in the lavage fluid relate to rapidly changing inflammatory events in the bronchial wall.

The aim of this study was to investigate whether changes in BHR following allergen challenge show a time relationship with inflammatory events in the airways.

The numbers and activation state of eosinophils in the submucosa of endobronchial biopsies, in bronchial lavage (BL) and BAL, and eosinophil cationic protein (ECP) in the lavage, were measured before, and 3 and 24 h, after allergen challenge, in symptomatic atopic asthmatic subjects with a documented dual asthmatic response.

Immunohistochemical staining of fresh frozen sections and cytopins with a monoclonal antibody (EG2) specific for ECP was employed, to identify activated eosinophils

[16]. To obtain functional correlates for the cells studied, their numbers in the BL, BAL, and submucosa were related to the amount of ECP in the BL and the clinical response on allergen challenge.

## Methods

### Subjects

Nine asthmatic subjects (3 women, 6 men) participated in the study. The clinical characteristics of the patients are given in table 1. All asthmatic subjects were selected on the basis of positive intracutaneous skin tests to common inhalant allergens, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* (Diephuis, Groningen, The Netherlands), and increased specific immunoglobulin E (IgE) (normal <0.35 peripheral resistance units (PRU)·ml<sup>-1</sup>) for house dust mite (HDM) allergen (Phadezym radio-allergosorbent test (RAST), Pharmacia, Uppsala, Sweden) and increased bronchial responsiveness to inhaled histamine (normal >32 mg·ml<sup>-1</sup>) [17]. Forced expiratory volume in one second (FEV<sub>1</sub>) had to be above 65% of the predicted value.

The patients had experienced no acute asthmatic attacks or respiratory tract infection for at least 2 months. None of the patients received oral corticosteroids or high dose inhaled corticosteroids (no more than 600 µg inhaled budesonide daily). All medication was withheld before the study days: inhaled corticosteroids two weeks, cromoglycate one week, and β-adrenergic drugs 24 h before the study.

None of the participating subjects were smokers or used medication that could interfere with the outcome. All subjects gave written informed consent, and the study was approved by the Medical Ethics Committee of the University Hospital Groningen. All subjects were able to stop their maintenance treatment before the study days.

In all subjects two HDM challenges and three bronchoscopy procedures were performed, according to the protocol. All patients developed a dual asthmatic response after allergen challenge.

### House dust mite provocation

Allergen solutions were prepared from stock solutions of *Dermatophagoides pteronyssinus*, diluted in phosphate-buffered saline (PBS) with 0.03% human serum albumin (HSA) and with 0.5% phenol (Diephuis Laboratories, The Netherlands), to produce a range of increasing fivefold concentrations of 80 to 10,000 Noon-equivalents (Ne)·ml<sup>-1</sup>. The solutions were administered using the breath-actuated dosimeter APS (Jaeger GmbH, West Germany), containing 3 ml solution, which allows air at 20 pounds per square inch (psi) into the nebulizer for 0.4 s. The output was 9.04±0.43 µl per 0.4 s of nebulization. Subjects inhaled the aerosolized allergen solutions in 10 inhalations, from end-tidal volume to full inspiratory capacity, through a mouthpiece whilst wearing a noseclip. Allergen inhalation was performed at 15 min intervals, each subject starting with the lowest dose. The dose of HDM causing a fall of more than 20% of the post-PBS value in FEV<sub>1</sub> was considered as a threshold value. The provocation dose, required to reduce FEV<sub>1</sub> by 20% of the post-PBS baseline value (PD<sub>20</sub>) was derived by linear interpolation.

### Lung function

Pulmonary function testing consisted of inspiratory slow vital capacity (VC) and FEV<sub>1</sub>, using a water-sealed spirometer.

The patients used a Microspirometer (Micro Medical Ltd, Rochester, UK) for FEV<sub>1</sub> measurements every hour (three blows at 2 min intervals, the highest of the three

Table 1. - Clinical data of the asthmatic patients

Pt no.	Sex	Age yrs	PC <sub>20</sub> mg·ml <sup>-1</sup>	IgE kU·l <sup>-1</sup>	EOS cells·µl <sup>-1</sup>	VC l	FEV <sub>1</sub> l·s <sup>-1</sup>	FEV <sub>1</sub> % pred	EAR % FEV <sub>1</sub>	LAR % FEV <sub>1</sub>	Med
1	M	21	1.3	1470	297	5.50	4.10	82	39	34	Bud, C, T
2	F	22	0.4	1012	616	4.95	2.95	75	26	22	N, T
3	M	21	4.4	440	167	4.35	3.57	97	26	30	T
4	F	23	0.6	735	320	4.25	3.60	100	30	34	Bud, T
5	M	20	9.8	2440	407	6.15	4.50	95	39	29	Bud, T
6	M	34	4.9	162	319	5.15	4.30	96	40	24	Bud, T
7	M	23	17.8	661	431	6.10	5.90	104	29	23	S
8	M	28	0.4	534	410	5.50	4.00	91	30	22	Bud, S
9	F	22	1.4	266	220	4.35	3.70	108	43	20	C, T
Mean		24	4.6	856	354	5.14	4.07	94	34	26	
SEM		1.5	2.0	238	44	0.24	0.28	3.5	2.2	1.8	

PC<sub>20</sub>: provocative concentration of histamine producing a 20% fall in FEV<sub>1</sub>; EOS: blood eosinophils; EAR and LAR: early and late asthmatic reactions, representing the % decrease in FEV<sub>1</sub>; IgE: total immunoglobulin E in serum; VC: vital capacity; FEV<sub>1</sub>: forced expiratory volume in one second; % pred: initial FEV<sub>1</sub>/predicted FEV<sub>1</sub>×100; Med: regular medication; C: cromoglycate; N: nedocromil; T: terbutaline; S: salbutamol; Bud: budesonide.

values being recorded), during the evening hours on the day of the challenge. The following indices were selected to describe the FEV<sub>1</sub> time-response curves after administration of HDM, compared with the FEV<sub>1</sub> after the control solution: 1) maximal decrease in FEV<sub>1</sub> from the post-PBS baseline value during 9 h; and 2) the Microspirometer measurements, used as supplementary data in the analysis of the LAR.

#### *Bronchoscopy procedure*

The procedure was performed, as described by the American Thoracic Society [18]. All bronchoscopies were performed by the same bronchoscopist (RA).

Vital capacity VC and FEV<sub>1</sub> were measured before the procedure. Local anaesthesia was administered before introducing a flexible bronchoscope, type Olympus B1-IT10. Later, extra local anaesthesia was administered, if necessary. Supplementary oxygen was available. At first the bronchoscope was introduced in the lateral segment of the middle lobe. Lavage was performed, using 5 separate aliquots of 20 ml sterile, warm 0.9% NaCl, instilled into the middle lobe segment.

The fluid was gently aspirated directly after each aliquot and collected in plastic tubes. The tubes were placed in ice and immediately brought to the laboratory for analysis.

The bronchoscope was then passed into the right lower lobe, and four to five biopsies were taken from the subcarinae using a standard, fenestrated, cup forceps. The exact locations from which the biopsies had been taken during the first and second procedure, were carefully noted and efforts were made during the second and third occasion to obtain the biopsies from the same carina but at the opposite side, in order to avoid the exact sites.

Each specimen was assigned an alphanumeric code, and the clinical and physiological data were not revealed to the pathologist interpreting the specimens.

Immediately after the procedure, all patients inhaled 0.5 mg terbutaline, and 1,200 µg budesonide, and remained for at least 2 h in the department for clinical observation, while measuring VC and FEV<sub>1</sub>.

#### *Processing of lavage fluid*

In the laboratory, the lavage fluid was pooled in two fractions: tubes one and two, defined as bronchial lavage (BL), and tubes three to five, defined as bronchoalveolar lavage (BAL).

Both fractions were centrifuged separately (10 min, 4°C, 400×g), and the supernatants stored at -80°C. The cells were washed twice with RPMI 1640 medium, with 25 mmol hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer, and L-glutamine (Gibco, Paisley, Scotland).

The cell fraction was suspended in 1 ml RPMI+1% bovine serum albumin. The cells were counted using a Coulter counter, and May-Grünwald-Giemsa-stained slides

were used for differential counting, as described previously [15].

From each fraction, cytopspins were made, using a Shandon cytospin-2 centrifuge (Shandon Inc, Pittsburgh, USA), with a 100 µl volume of the cell suspension. Slides were air dried, wrapped in aluminium foil, and stored at -80°C until use.

#### *ECP measurements*

ECP measurements in the BL supernatants (not in BAL supernatants) were performed using a double antibody radio-immunoassay (Pharmacia Diagnostics AB, Uppsala Sweden), as previously described by PETERSON *et al.* [19]. The assay is based on the competition of radiolabelled ECP for the binding sites of a specific monoclonal antibody. Calibration is realized against pure ECP. The detection limit is <2 µg·l<sup>-1</sup>. The procedure was performed following the instructions of the manufacturers.

#### *Processing of biopsy specimens*

Within 20 min of bronchoscopy, each biopsy specimen was removed from isotonic saline, and individually embedded in Tissuetek II OCT Compound (Miles, Naperville, Illinois, USA), and then frozen by immersing in -80°C isopentane. Samples were stored at -80°C until use.

Frozen sections (4 µm) were cut on a Bright LOTF/AS cryostat (Huntingdon, Cambridge, UK), dried for 30 min under a hairdryer (room temperature), and stored at -20°C until use.

#### *Immunoperoxidase staining*

Lavage cytopspins and biopsy tissue sections were immunostained with the EG2 monoclonal antibody (Sanbio, Uden, The Netherlands). This antibody can identify activated eosinophils in which a portion of the ECP molecule is cleaved to reveal the epitope to which the antibody is directed [16]. The immunoperoxidase staining procedure was performed as described previously by TIMENS and POPPEMA [20]. In short, slides were dried immediately from the freezer under a hairdryer, and fixed in acetone for 10 min at room temperature. After fixation, and after each following step, the slides were washed in phosphate-buffered saline (PBS), pH 7.4, for 5 min. The slides were incubated with 50 µl of the EG2 solution (1:50) for 60 min. In all cases, control sections were incubated with PBS or irrelevant antibodies (not reactive with haemopoietic antigens).

As a second step, the slides were incubated for 30 min with a 1:300 biotinylated rabbit anti-mouse F(ab')<sub>2</sub> fragments solution (Dakopatts, Glostrup, Denmark), supplemented with 1% human AB serum, followed by incubation with peroxidase-conjugated streptavidin (Dakopatts, Glostrup, Denmark), 1:300, supplemented with 1% human AB serum for 30 min.

3-amino-9-ethylcarbazole (AEC) (Aldrich Chemical Co., Milwaukee, Wisconsin, USA), together with  $H_2O_2$ , was used as histochemical reagent for the demonstration of peroxidase reactivity, resulting in a reddish-brown reaction product. The slides were counterstained with fresh haematoxylin and mounted with glycerol-gelatin (Merck, Darmstadt, FRG) and a coverslip.

### Quantification

In each cytospin, the number of positive cells was determined, counting a total of 300 cells. Only intact and clearly identifiable cells were counted. In many of the biopsies, the epithelial layer was not well-preserved, and as a result, no counts were made at the luminal side of the epithelial basement membrane. With an eyepiece graticule, the number of positively stained cells was counted in 10 areas of  $100 \times 100 \mu m^2$  ( $10,000 \mu m^2$ ) in the submucosa adjacent to the basement membrane. All of the counting was done by one person (MS), at a magnification of  $8 \times 25$ .

In order to account for the variation in numbers of EG2 positive cells in different parts of the biopsy, and to make sure that the average count was a reliable mean, counting was accomplished as follows: at least three sections were counted, which were at least  $50 \mu m$  apart in the biopsy. In each section 3-4 areas were chosen at random along the basement membrane. The reliability of this counting method was tested by observing the change of the mean after each individual area count [21].

In the bronchial biopsies, the mean did not change significantly after 10 counts. Individual means were calculated as the average of 10 area counts.

### Study design

The study was performed from January to May. VC, FEV<sub>1</sub> and PC<sub>20</sub> histamine (table 1), were determined one week prior to the study and these were regarded as baseline values.

Subjects attended the laboratory on four occasions. On the first occasion, a steady-state bronchoscopy was performed. Subjects attended the hospital at 8:30 A.M. and, after completing a questionnaire, undergoing physical examination and establishing that the VC and FEV<sub>1</sub> were within baseline values, a fiberoptic bronchoscopy procedure was performed. Immediately after the procedure the subjects received bronchodilating therapy, inhaled corticosteroids, and started with their maintenance treatment until the next procedure, as described.

Three weeks later, the subjects attended the hospital in the morning at 8:00 A.M. Allergen challenge with HDM was started at 8:30 A.M., provided that the VC and FEV<sub>1</sub> were within baseline values, and after the same precautions as described for the first occasion. Spirometry was performed as described. Exactly 3 h after the last dose of allergen, the second bronchoscopy procedure was performed, when the VC and FEV<sub>1</sub> had returned to baseline values. Immediately after this procedure the subjects received medication as described earlier.

At least four weeks later, subjects attended the hospital at 8:00 A.M., and after the same precautions, allergen challenge with HDM was started at 8:30 A.M. At 5:00 P.M. the subjects went home, having received instructions on how to use a Microspirometer (Micro Medical Ltd, Rochester, UK) for FEV<sub>1</sub> measurements every hour (three blows at 2 min intervals, the higher of the three values being recorded), until the subject went to bed. They also received careful instructions about what to do in case of chest complaints.

Twenty four hours after the last dose of allergen, the third bronchoscopy procedure was performed, when the VC and FEV<sub>1</sub> had returned to baseline values, and after the same precautions as described above.

### Data analysis

Total serum IgE, PC<sub>20</sub> histamine and PD<sub>20</sub> HDM values were logarithmically transformed for statistical analyses. Differences were considered to be significant if  $p < 0.05$ . The mean submucosal cell counts from the biopsy sections, and cell counts from the lavage, before and after allergen challenge, were compared with Wilcoxon's matched-pairs, signed-ranks test. Correlation coefficients were calculated by least-squares linear-regression analysis.

Subjects were considered to have a LAR if their maximal decrease in FEV<sub>1</sub>, measured from 4 h after the onset of the EAR, was 20% or more, and/or if a decrease of more than 20% was shown by spirometry values at home.

## Results

We studied nine atopic asthmatics, whose clinical characteristics are shown in table 1. All subjects studied tolerated the bronchoscopy procedure well. In none did the procedure have to be terminated, and none complained of distressing dyspnoea. None of the subjects had clinical adverse effects during and after the procedure.

### Immunohistology of endobronchial biopsies

In all patients, biopsies were taken from the subcarinae of the right lower lobe after the lavage, which was performed in the right middle lobe, on all three occasions.

Counting of cells stained immunohistochemically for activated (EG2<sup>+</sup>) eosinophils was possible in the submucosa in eight patients before the allergen challenge, and in all patients 3 h and 24 h after the challenge. Reliable counting in the epithelium was not possible, as a consequence of epithelial damage, especially after the allergen challenges. In the mucosa, the activated eosinophils often demonstrated a pronounced concentration of the cells immediately under the epithelial basement membrane but were also diffusely distributed throughout the tissue. In particular, 3 h after the allergen challenge, a shift of the activated eosinophils was seen to the basement membrane and the epithelial layer (fig. 1).

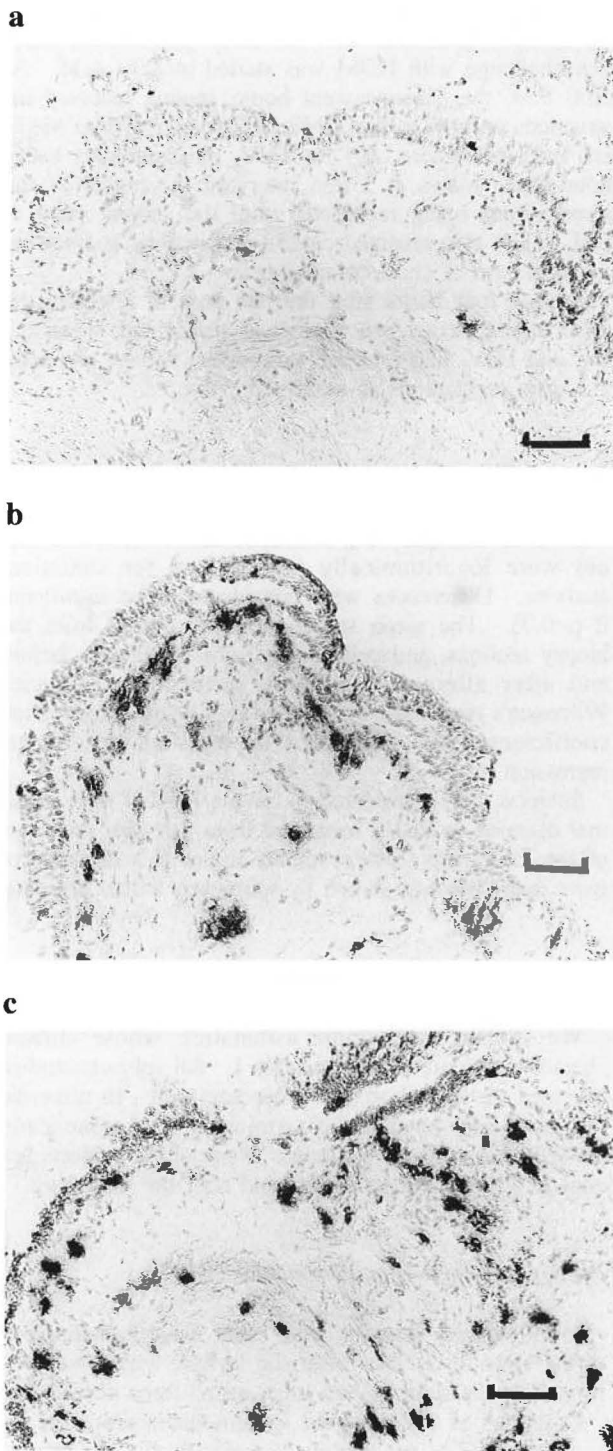


Fig. 1. - a) Cryostat section of a biopsy specimen from a patient before house dust mite (HDM) challenge, incubated with the anti-EG2 monoclonal antibody and stained with the indirect immunoperoxidase method. b) A similarly stained section from the same subject 3 h after HDM challenge. The mucosa reveals surface epithelium, basement membrane and lamina propria, with an abundant distribution of EG2 positive cells throughout the whole section. Moreover, most of the positive staining cells are located close to the basement membrane, attached to the membrane and in the epithelium. c) A similarly stained section, again in the same patient, 24 h after challenge. There is still an extensive distribution of EG2 positive cells, however, most of them are now located in the surface epithelium. Original magnification for all three parts is 140 $\times$  (bar=0.1 mm).

In the immunohistology preparations a significant increase in the number of EG2<sup>+</sup> cells·0.01 mm<sup>-2</sup> is demonstrated 3 and 24 h after the challenge, compared with the prechallenge data, from median 1.45 to 2.63 and 2.31 ( $p < 0.05$ ), respectively, (fig. 2). A significant negative correlation is found between the number of EG2<sup>+</sup> cells·0.01 mm<sup>-2</sup> in the submucosa and in the BL, 24 h after the allergen challenge ( $r = -0.70$ ,  $p < 0.05$ ) (fig. 3).

No clear correlations could be observed between the numbers of/or changes in EG2<sup>+</sup> cells·0.01 mm<sup>-2</sup> in the submucosa and clinical parameters, such as the PD<sub>20</sub> HDM, PC<sub>20</sub> histamine and % decrease in FEV<sub>1</sub> of the LAR.

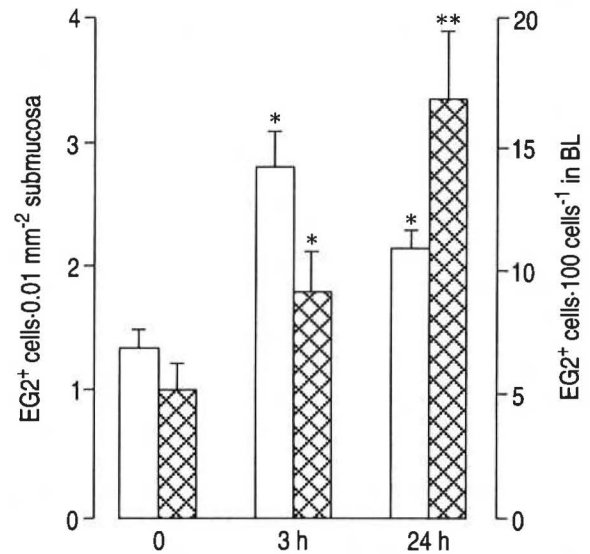


Fig. 2. - EG2 positive cells·0.01 mm<sup>2</sup> in the submucosa (open bars), and EG2 positive cells·100 cells<sup>-1</sup> in the bronchial lavage (BL) (hatched bars), before and 3 and 24 h after house dust mite (HDM) challenge. Mean $\pm$ SEM. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  compared with prechallenge values.

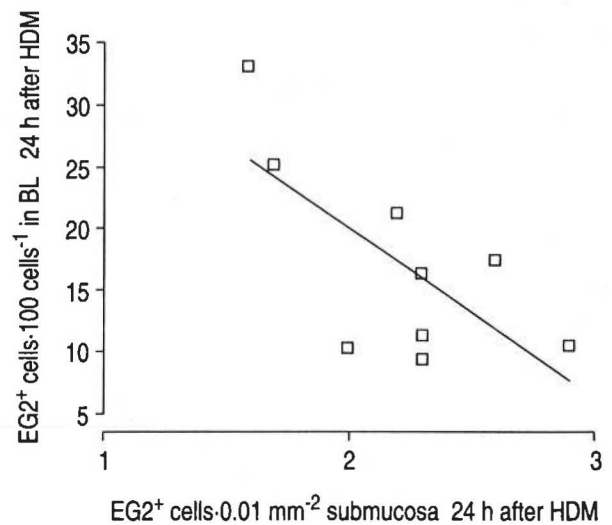


Fig. 3. - Correlation between the number of EG2 positive cells·0.01 mm<sup>2</sup> in the submucosa and in the bronchial lavage 24 h after the house dust mite (HDM) challenge.  $r = -0.70$ ,  $p < 0.05$ .

*BL and BAL, 3 and 24 h after challenge*

There was no significant difference in viability, recovery, total cell number and cell count per ml in the BL, 3 and 24 h after the HDM challenge, when compared with the steady-state lavage (table 2). A significant increase in the percentage of eosinophils from a mean of  $5.8 \pm 1.2$  (SEM) to  $9.7 \pm 1.9$  ( $p < 0.05$ ) at 3 h was observed in the BL. Twenty four hours after the challenge, this increase in percentage eosinophils was even more pronounced, to  $17.2 \pm 3.6$  ( $p = 0.01$ ), compared with the steady-state. At 3 h after challenge, 7 of the 9 subjects had a greater percentage of eosinophils, and at 24 h 8/9 subjects. In contrast to the findings in the BL, no significant changes in the percentage of eosinophils could be observed in the BAL, both at 3 h and at 24 h after challenge.

Table 2. - Total fluid recovery and harvested numbers of cells, before, and 3 h and 24 h after HDM challenge

	Fluid recovery ml	Cells $\times 10^5 \cdot \text{ml}^{-1}$
<b>Bronchial lavage</b>		
Before	$17.0 \pm 2.0$	$1.1 \pm 0.2$
3 h	$15.4 \pm 1.5$	$0.9 \pm 0.2$
24 h	$19.1 \pm 1.2$	$1.1 \pm 0.2$
<b>Bronchoalveolar lavage</b>		
Before	$40.4 \pm 4.2$	$1.9 \pm 0.2$
3 h	$40.7 \pm 3.0$	$2.0 \pm 0.3$
24 h	$43.2 \pm 2.9$	$3.5 \pm 0.6$

Data are presented as mean  $\pm$  SEM. HDM: house dust mite.

The number of activated (EG2) eosinophils in the BL increased significantly 3 and 24 h after the challenge from  $5.0 \pm 1.1$  to  $9.0 \pm 1.7$  ( $p < 0.05$ ) and to  $16.9 \pm 2.7$  ( $p = 0.007$ ), respectively, (fig. 2). In the BAL, however, only a trend in increase in the EG2<sup>+</sup> cells could be observed from  $2.7 \pm 0.9$  to  $5.3 \pm 1.5$  and to  $12.6 \pm 3.8$ .

A correlation was found in the BL between eosinophils and EG2<sup>+</sup> cells ( $r = 0.82$ ,  $p < 0.01$ ) 24 h after the HDM challenge. No significant correlations were seen between the number of EG2<sup>+</sup> cells in the BL and BAL and clinical parameters.

*ECP measurements in the BL*

The amount of ECP increased from  $2.24 \pm 0.95$  to  $3.93 \pm 0.91 \mu\text{g} \cdot \text{l}^{-1}$  at 3 h, this increase, however, being non-significant. At 24 h, the amount of ECP had increased to  $10.22 \pm 3.13 \mu\text{g} \cdot \text{l}^{-1}$ , ( $p < 0.01$ ). The difference between 3 and 24 h was also significant ( $p < 0.05$ ). No correlation could be observed between the increase in the amount of ECP and the changes in the number of EG2<sup>+</sup> cells in the biopsies, the number of EG2<sup>+</sup> cells in the BL and the percentage of eosinophils in the BL. A significant correlation was observed between the log PD<sub>20</sub> HDM and the amount of ECP 3 h after challenge ( $r = 0.63$ ,  $p < 0.05$ ).

**Discussion**

The results of this study not only demonstrate a significant increase in activated eosinophils in the submucosa and in the BL, 3 and 24 h after allergen challenge, but also indicate a shift of the activated eosinophils from the submucosa through the basement membrane to the epithelial lining. These data, in combination with the increase in ECP in the BL, suggest that the activated eosinophils mainly degranulate at the level of the epithelial lining. As far as eosinophils are concerned, it also appears that lavage samples reflect the events taking place in the submucosa.

In an earlier study, we were able to demonstrate a significant increase in allergen-induced BHR 3 and 24 h after challenge, and a dissimilarity in response when using methacholine or adenosine 5' monophosphate (AMP) [7]. These findings suggested that several mechanisms are responsible for the observed increase in BHR. It is important to realize that the FEV<sub>1</sub> had returned to pre-challenge values, and that the subjects were without complaints between the early and late reaction. In the present study, we were able to investigate submucosal biopsy and lavage findings at the mentioned time points in allergic asthmatic subjects.

Fractional processing of separate bronchial and bronchoalveolar samples was chosen, since asthma is merely a disease of the bronchial airways, and differences between bronchial and alveolar samples have been demonstrated [22].

To our knowledge, this is the first study comparing biopsy and lavage findings, using separate fractions, at three different time points, before and after HDM challenge, in the same patients. In our earlier study [15], performing BAL during the late asthmatic reaction and immediately after the early asthmatic reaction, no eosinophilia was present 2 h after the EAR in patients who developed a LAR and those who did not. In the present study, an increase in activated eosinophils both in the biopsies and BL (but not in the BAL) is seen exactly 3 h after allergen challenge.

Comparing the results, migration of eosinophils from the bronchial submucosa to the bronchial lumen in a relatively short period after the EAR is suggested. This interpretation is supported by recent observations, performing BAL before and 4 h after HDM challenge [23]. However, our data do not entirely support the findings by Rossi *et al.* [23] concerning the 24 h BAL. Instead of a decrease in eosinophils in patients who developed a LAR and an increase in patients who did not, we found a significant increase in the percentage of eosinophils in the BL in the patients developing a LAR, and no changes in the BAL. Our BL observations are in accordance with the lavage (BL and BAL) data of METZGER and co-workers [24, 25], in which the number of eosinophils was still significantly increased at 24 h, and after local allergen challenge even at 48 h. The differences observed may be a consequence of separating lavage recovery into BL and BAL, the first being more sensitive and representative of changes in the bronchial wall.

The correlation between EG2<sup>+</sup> eosinophils in the submucosa and in the epithelium, demonstrated by DJUKANOVIC *et al.* [13], and the increased number of EG2<sup>+</sup> eosinophils 3 h after challenge in the biopsies and bronchial lavages in our study, suggest not only that both sides of the bronchial wall represent a continuum of eosinophil recruitment, but also that their activity is more important than just the number of eosinophils. The findings reflect a dynamic process involving inflammatory cell movement, especially activated eosinophils, and change in activity. As part of this process, eosinophils secrete their granule products and presumably a range of newly-formed mediators, including leukotriene C<sub>4</sub> (LTC<sub>4</sub>), platelet activating factor (PAF), and 15-hydroxyeicosatetraenoic acid (15-HETE) (26, 27), resulting in epithelial damage.

Using a cumulative clinical scoring system and eosinophil enumeration, BOUSQUET *et al.* [12] demonstrated a correlation between the severity of asthma and the numbers of eosinophils in peripheral blood, lavage fluid and epithelium. Our finding of a correlation between the amount of ECP and the PD<sub>20</sub> HDM is of interest. It provides evidence that the amount of products released by the eosinophil relates to disease severity. It has been demonstrated that the instillation of small amounts of ECP produces epithelial cell damage and patchy denudation of the epithelial cell layer, both in the trachea and in the bronchi [28], and from platelet-activating factor, it has been reported to produce a long-lasting bronchial hyperresponsiveness when given by inhalation to normal people [29]. Shedding and desquamation of the epithelium in the airways of asthmatics have been consistent findings.

LATTINEN *et al.* [30] showed extensive epithelial damage in bronchial biopsies, and JEFFERY *et al.* [31] have shown that the extent of surface epithelium loss correlates well with the degree of methacholine responsiveness.

Another finding in bronchial biopsies taken from asthmatics is a thickening of the lamina reticularis below the basement membrane. There is evidence that myofibroblasts may be responsible for the subepithelial fibrosis [32]. Furthermore, eosinophils can secrete a fibroblast growth factor, and ECP stimulates the synthesis of hyaluron and proteoglycan in human fibroblasts [33, 34].

The fibrosis may cause a decline in pulmonary function, and the presence of contractile myofibroblasts could contribute to a persistent hyperresponsiveness. Eosinophils could, thus, be involved in the genesis of these changes. The changes occur where activated eosinophils and the deposition of ECP was demonstrated in our study, and other investigations [12–14].

In conclusion, we have established that activated eosinophils are already infiltrating the airways at 3 h after HDM challenge, and are especially evident in the submucosa, but also seen in the lavage. Elevated ECP levels in BL fluid are correlated with the amount of house dust mite allergen given. Although there is no direct evidence that these activated eosinophils are, by themselves, responsible for the allergen-induced increase in BHR 3 and 24 h after allergen challenge, this and other studies support the concept that their activity may result in epithelial damage, leading to an increase in BHR.

These findings, which are broadly consistent with other biopsy and BAL studies in humans, also suggest that activated eosinophils play a crucial role in pathological processes in the human airway following allergen inhalation. The findings imply that allergen avoidance, and treatment focusing on reduction of eosinophil recruitment and activation, may be of benefit in allergic asthmatic patients.

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## References

- O'Byrne PM, Dolovich J, Hargreave FE. – Late asthmatic responses. *Am Rev Respir Dis* 1987; 136: 740–751.
- Cockcroft DW. – Mechanism of perennial allergic asthma. *Lancet* 1983; i: 253–256.
- Postma DS, Koëter GH, de Vries K. – Clinical expression of airway hyperreactivity in adults. *Clin Rev Allergy* 1989; 7: 321–343.
- Cartier A, Thomson NC, Frith PA, Roberts R, Hargreave FE. – Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and changes in airway caliber. *J Allergy Clin Immunol* 1982; 70: 170–177.
- Gökemeyer JDM. – Hyperreactiviteit van de luchtwegen. Thesis. Wolters-Noordhoff. Groningen, 1976.
- Durham SR, Craddock CF, Cookson WO, Benson MK. – Increases in airway responsiveness to histamine precede allergen-induced late asthmatic responses. *J Allergy Clin Immunol* 1988; 82: 764–770.
- Aalbers R, Kauffman HF, Koëter GH, *et al.* – Dissimilarity in methacholine and adenosine 5'-monophosphate responsiveness 3 and 24 hours after allergen challenge. *Am Rev Respir Dis* 1991; 144: 352–357.
- Djukanovic R, Roche WR, Wilson JW, *et al.* – Mucosal inflammation. *Am Rev Respir Dis* 1990; 142: 434–457.
- Horn BR, Robin ED, Theodore J, Van Kessel A. – Total eosinophil counts in the management of bronchial asthma. *N Engl J Med* 1975; 292: 1152–1155.
- Wardlaw AJ, Dunette S, Gleich GJ, Collins JV, Kay AB. – Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137: 62–69.
- Frigas E, Loegering DA, Solley GO, Farrow GM, Gleich GJ. – Elevated levels of eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clin Proc* 1981; 56: 345–353.
- Bousquet J, Chané P, Lacoste JY, *et al.* – Eosinophilic inflammation in asthma. *N Engl J Med* 1990; 323: 1033–1039.
- Djukanovic R, Wilson JW, Britten KM, *et al.* – Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 1990; 142: 863–871.
- Bradley BL, Azzawi M, Jacobson M, *et al.* – Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial specimens from atopic subjects with asthma: Comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to

- bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88: 661-674.
15. De Monchy JGR, Kauffman HF, Venge P, *et al.* - Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am Rev Respir Dis* 1985; 131: 373-376.
16. Tai PC, Spry CJF, Peterson C, Venge P, Olson I. - Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein. *Nature* 1984; 309: 182-184.
17. de Vries K, Goei JT, Booy-Noord H, Orie NGM. - Changes during 24 hours in the lung function and histamine hyperreactivity of the bronchial tree in asthmatic and bronchitic patients. *Int Arch Allergy Appl Immunol* 1962; 20: 93-101.
18. Summary and recommendations of a workshop on the investigative use of fiberoptic bronchoscopy and bronchoalveolar lavage in asthmatics. *Am Rev Respir Dis* 1985; 132: 180-182.
19. Peterson CGB, Enander I, Nystrand J, *et al.* - Radioimmunoassay of human eosinophil cationic protein (ECP) by an improved method. Establishment of normal levels in serum and turnover *in vivo*. *Clin Exp Allergy* 1991; 21: 561-567.
20. Timens W, Poppema S. - Lymphocyte compartments in human spleen: an immunohistologic study of normal spleens and non-involved spleens in Hodgkin's disease. *Am J Pathol* 1985; 120: 443-454.
21. Weibel ER. - *In: Stereological Methods*. Vol. 1. chapter 3. Sampling of tissue. London, Academic Press, 1979; pp. 63-100.
22. Rennard SI, Ghafouri MO, Thopson AB, Linder J, *et al.* - Fractional processing of sequential bronchoalveolar lavage to separate bronchial and alveolar samples. *Am Rev Respir Dis* 1990; 141: 208-217.
23. Rossi GA, Crimi E, Lantero S, *et al.* - Late-phase asthmatic reaction to inhaled allergen is associated with early recruitment of eosinophils in the airways. *Am Rev Respir Dis* 1991; 144: 379-383.
24. Metzger WJ, Richerson HB, Worden K, Monick H, Hunninghake GW. - Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. *Chest* 1986; 89: 477-483.
25. Metzger WJ, Zavala D, Richerson HB, *et al.* - Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. *Am Rev Respir Dis* 1987; 135: 433-440.
26. Wardlaw AJ, Kurihara K, Moqbel R, Walsh GM, Kay AB. - Eosinophils in allergic and non-allergic asthma. *In: Kay AB, ed. Clinical Immunology and Allergy: the Allergic Basis of Asthma*. London, Balliere Tindall, 1988; pp. 15-36.
27. Shaw RJ, Walsh GM, Cromwell O, *et al.* - Activated eosinophils generate SRS-A leukotrienes following IgG-dependent stimulation. *Nature* 1985; 316: 150-152.
28. Dahl R, Venge P, Fredens K. - The eosinophil. *In: Barnes PJ, Rodger I, Thomson N, eds. Asthma: basic mechanisms and clinical management*. London, Academic Press, 1988; pp. 115-130.
29. Cuss FM, Dixon CM, Barnes PJ. - Effect of platelet activating factor on pulmonary function and bronchial responsiveness in man. *Lancet* 1986; 2: 189-192.
30. Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T. - Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 1985; 131: 599-606.
31. Jeffery PK, Wardlaw AJ, Melson FC, Collins JV, Kay AB. - Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 1989; 140: 1745-1753.
32. Brewster CEP, Howarth PH, Djukanovic R, Wilson J, Holgate ST. - Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3: 507-511.
33. Pincus SH, Ramesh KS, Wyler DJ. - Eosinophils stimulate fibroblast DNA synthesis. *Blood* 1987; 70: 572-574.
34. Sarnstrand B, Westergren-Thorsson G, Hernas J, *et al.* - Eosinophil cationic protein and transforming growth-factor-A stimulates synthesis of hyaluran and proteoglycan in human fibroblast cultures. *Proc Intern Colloquium on Pulmonary Fibrosis*, 1988.