

The degree of natural allergen exposure modifies eosinophil activity markers in the circulation of patients with mild asthma

Fernvik E, Grönneberg R, Lundahl J, Hed J, Andersson O, Johansson SGO, Halldén G. The degree of natural allergen exposure modifies eosinophil activity markers in the circulation of patients with mild asthma. Allergy 1996; 51: 697–705. © Munksgaard 1996.

We have previously found that CD9, CD11b, and intracellular ECP (EG2) may be used as activity markers for eosinophils *in vitro*. The main object of the present study was to determine whether these markers can reflect eosinophil activation *in vivo* in relation to allergen exposure. For this purpose, six patients with a history of allergic rhinitis and occasional asthma symptoms during the pollen season participated. Blood donors served as controls. Peripheral blood eosinophils were analyzed according to the FOG method and flow cytometry, before and during one birch pollen season with high pollen load (HPL) and one with low pollen load (LPL). The CD9 expression on peripheral eosinophils from the patients was significantly increased both before ($P < 0.05$) and during ($P < 0.01$) HPL season, and CD11b expression solely during HPL season ($P = 0.01$) as compared to controls. The intracellular expression of the EG2 epitope was increased before ($P < 0.01$) and during ($P < 0.05$) HPL season, and increased significantly ($P < 0.05$) during season as compared to before. No changes were observed before and during LPL season. The proportion of eosinophils was increased both before ($P < 0.05$) and during ($P < 0.001$) the HPL season as compared to controls. The markers CD9, EG2, and, to a lesser extent, CD11b seem to detect activated eosinophils in the circulation, whereas EG2 may also reflect increased antigen exposure during season.

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Key words: ; CD9; CD11b; EG2; flow cytometry; FOG method; intracellular ECP; mild asthma.

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Accepted for publication 26 April 1996

Eosinophils are believed to be major effector cells in asthma. The number of peripheral blood eosinophils has often been used as a monitoring tool in the management of asthma, a correlation with the severity of the disease being reported (1, 2). It has been reported that an increased number of eosinophils in blood is also a risk factor for deterioration and exacerbation and can predict a favorable

response to corticosteroids (2). However, a normal eosinophil count in peripheral blood by no means excludes inflammatory activity (3). It has also been reported that patients with asthma have enhanced eosinophil numbers in sputum (4), bronchoalveolar lavage (5, 6), and airway biopsy samples (7, 8), and these eosinophils are believed to be in an activated state (9). The eosinophil contains several biologically active products with tissue-damaging properties, among which eosinophil cationic protein (ECP) is one of the most potent and important. Immunohistochemical studies have described increased staining with the monoclonal antibody EG2 to ECP in activated eosinophils (10, 11). However, these results are controversial since other investigators have been able to detect all eosinophils with EG2 irrespective of state of activation (12). It has also been reported that asthmatics have activated eosin-

Abbreviations: BHR, bronchial hyperreactivity; ECP, eosinophil cationic protein; EDTA, ethylene diaminetetraacetic acid; EG2, monoclonal antibody to ECP; FEV₁, forced expiratory volume in 1 s; FITC, fluorescein isothiocyanate; FOG, fixation and membrane permeabilization with OG; HPL, high pollen load; LPL, low pollen load; MFI, mean fluorescence intensity; OG, *n*-octyl-beta-D-glucopyranoside; PBS-EDTA, phosphate-buffered saline with EDTA; PD₂₀, provocative dose causing 20% reduction in FEV₁; PEF, peak expiratory flow; RAST, radioallergosorbent test; SPT, skin prick test.

ophils in the circulation, and that these cells exhibit a hypodense phenotype which *in vitro* is associated with increased metabolic activity and mediator release. However, in many ways, changes in membrane receptor phenotype are more clear-cut than quantitative markers such as density in terms of activation state (13). With the FOG method and flow cytometry, we have previously detected activated peripheral blood eosinophils in patients with asthma in active phase by means of increased expression of the EG2 epitope on intracellular ECP (14–16).

Recently, in *in vitro* studies, we have shown that CD9 may be a potential activity marker on peripheral blood eosinophils (17). The surface antigen CD9, also known as p24, was first identified by Kersey et al. using a monoclonal antibody directed against an acute lymphoblastic leukemia cell line (18). CD9, a member of the transmembrane 4 superfamily (19), is now known to be expressed on a variety of cells; e.g., eosinophils, platelets, basophils, stimulated T cells, early B cells, and endothelial cells. The biologic function of CD9 is not clearly understood, but CD9 has been reported to be involved in the regulation of the Ca^{2+} flux (20), and platelet and B-cell aggregation; to have protein kinase activity (21); and to require interaction with $\text{Fc}_\gamma\text{RII}$ (CD32) (22). Taken together, these observations suggest that CD9 plays a role in the initial phase of cell activation.

The cell-surface receptor CD11b, a member of the β_2 -integrin family, is a well-characterized adhesion molecule. It is expressed by all granulocytes and is considered a marker of eosinophil activity. CD11b is the α -chain which functions together with its β -chain CD18 in the receptor complex CD11b/CD18, often referred to as CR3. CD11b/CD18 functions both as a receptor for complement factor C3bi and as a general adhesion protein on monocytes and granulocytes. In resting state, eosinophils express only a minor part of their CD11b/CD18 content on their surfaces. However, upon *in vitro* stimulation, CD11b is rapidly mobilized from an intracellular pool and expressed in larger quantities on the cell surface (23). The expression of CD11b has been shown to be increased on circulating granulocytes in patients with allergen-induced asthma (24).

There is still no single biologic marker which can be used in the clinic to monitor the degree of inflammation in asthma. It would be preferable to have a standard method to follow markers for primed eosinophils and markers to measure the effect of allergen exposure.

Since the eosinophil is believed to be an important effector cell in allergic inflammation, the aim of the present study was to determine whether a

combination of the activity markers CD9, CD11b, and the EG2 epitope on intracellular ECP could reflect the degree of eosinophil priming *in vivo* in relation to allergen exposure during two birch pollen seasons in patients with mild asthma.

Material and methods

Study design

Bronchial responsiveness to inhaled histamine was assessed in all subjects before inclusion in the trial and estimated before and during the birch pollen season of 1994. Venous blood was drawn from each subject on four occasions, before and during each birch pollen season in 1993 and in 1994. At least 2 weeks had elapsed from when pollen counts started to increase before histamine challenge was performed and blood samples were taken. The pre-season blood samples were taken up to 2 months earlier in 1994 than 1993.

Blood was drawn and analyzed from healthy nonallergic blood donors, who served as controls, at the same time points as the patients, outside and during each pollen season; altogether, 51 controls for the analysis of the EG2 epitope on intracellular ECP, 45 controls for CD9, and 44 controls for CD11b. The same blood donors did not participate in both 1993 and 1994. Before participation in this study, all blood donors filled in a health declaration form in which they denied having allergic symptoms and any known history of allergy/atopy.

We used an old control material in which the percentage of eosinophils was calculated in peripheral blood from 35 healthy blood donors to compare with the percentage of eosinophils obtained in this study.

Subject characterization

Four men and two women, aged 28–52 years (mean age 36 years), participated in the study. They had a positive history of allergic rhinitis and occasional asthma symptoms, and positive skin prick test (SPT) and RAST (Pharmacia CAP System, Uppsala, Sweden) to tree and grass pollen and cat dander. Three subjects were also SPT and/or RAST positive to other pets, but none of the subjects had pets of their own. None of the patients were RAST positive to house-dust mite. Soluprick (10 HEP) and histamine dihydrochloride (10 mg/ml) (ALK, Copenhagen, Denmark) and sodium chloride with glycerol (diluent) were used for SPT on the volar aspect of the forearm. Three of the subjects were never smokers (nos. 3, 4 and 6), two were ex-smokers (nos. 1 and 2), and one subject (no. 5) was a current smoker with constant tobacco consumption (four cigarettes/

Table 1. Patient characteristics and laboratory parameters

	Subject	Age	Sex	Birch SPT/RAST	Alder SPT/RAST	Hazel SPT/RAST	Timothy SPT/RAST	Cat SPT/RAST	IgE kU/l
△	AH	28	M	4/4	nd/4	nd/3	3/1	4/3	91
○	AL	34	M	3/5	nd/5	nd/5	4/3	5/4	240
▽	AJ	30	F	4/4	nd/3	nd/3	3/4	2/2	250
◇	UH	38	M	3/5	nd/4	nd/4	3/2	5/3	200
★	MM	52	F	5/3	nd/3	nd/3	3/3	3/3	90
□	KG	33	M	4/5	nd/5	nd/4	3/4	4/4	400

SPT = skin prick test; RAST = radioallergosorbent test; nd = not done.

Analyzed out of season 1994.

Table 2. Baseline FEV₁, bronchial reactivity, and medication

	Subject	FEV ₁ % pred.	Histamine (end-point dose [μg])		1993 Pre-HPL	Medication		1994 Pre-LPL	LPL
			Pre-LPL	LPL		HPL			
△	AH	89	470	641	Inh. β ₂ ^c	Inh. β ₂ ^c Lorat.	Inh. β ₂ ^c	Inh. β ₂ ^c	Inh. β ₂ ^c Lorat.
○	AL	94	724	231	Inh. β ₂ ^c	Inh. β ₂ ^c Lorat. Bud.	Inh. β ₂ ^c	Inh. β ₂ ^c	Inh. β ₂ ^c
▽	AJ	90	2195	1552	Inh. β ₂ ^c	Inh. β ₂ ^c Lorat. Bud.	Inh. β ₂ ^c	Inh. β ₂ ^c	Inh. β ₂ ^c DSCG ^a Cetirizine
◇	UH	81	1351	>3520	Inh. β ₂ ^c Lorat.	Inh. β ₂ ^c Lorat. Bud.	Inh. β ₂ ^c	Inh. β ₂ ^c	Inh. β ₂ ^c Lorat. Bud.
★	MM	90	181	82	inh. β ₂ ^c	Inh. β ₂ ^c Terfen. Bud.	inh. β ₂ ^c Bud.	inh. β ₂ ^c	Inh. β ₂ ^c DSCG ^b Bud.
□	KG	89	294	204	Inh. β ₂ ^c	Inh. β ₂ ^c Terfen. BDP	Inh. β ₂ ^c	Inh. β ₂ ^c	Inh. β ₂ ^c

BDP = beclomethasone dipropionate 400 μg/d; Bud. = budesonide 400 μg/d; Cetirizine 10 mg × 1; DSCG = disodium cromoglycate; Inh. β₂ = inhaled β₂-agonist; Lorat. = loratidine 10 mg × 1; Terfen. = terfenadine 120 mg × 1.

^a = nasal spray; ^b = ocular drops; ^c = when necessary.

evening) during the whole study. The test subjects underwent outside pollen season SPT and RAST tests (Table 1). Histamine bronchial challenge was performed before and during the low pollen load (LPL) season, and the subjects were on the medication shown in Table 2. The subjects were included after giving informed consent, and they were given both oral and written information. The investigation was approved by the ethics committee of Huddinge University Hospital at Karolinska Institute, Stockholm, and was in accordance with the Helsinki Declaration II.

Bronchial responsiveness to histamine

Bronchial histamine provocations were made with a Spira Electro 2 nebulizer system (Hämeenlinna, Työkeskus, Tavastehus, Finland). The aerosol delivery time of the nebulizer was adjustable to 0.5 s with an onset of nebulization after 100 ml. The volume

of each inhalation was approximately 500 ml at a flow rate of 0.5 l/min. After baseline measurements of FEV₁, the subjects inhaled increasing doses (nine dose steps) of histamine. There were two, four, and eight inhalations of each of 1, 8, and 64 mg/ml buffered histamine diphosphate; i.e., increasing from an initial dose of 13.75 μg to a maximum dose of 3520 μg. FEV₁ was measured 3 min after each dose of histamine. The test was stopped when FEV₁ had decreased by 20% or more from the baseline value. The logarithmic histamine dose was plotted against FEV₁ to obtain the PD₂₀-value (the dose of histamine causing 20% reduction in FEV₁).

Birch pollen count

The birch pollen season in Stockholm in 1993 was more severe than usual with a maximum of 6103 pollen grains/m³. The total birch pollen count in Stockholm during this high pollen load (HPL)

season was 21 473 grains/m³. In 1994, the birch pollen season in Stockholm showed an LPL with a maximum of 53 pollen grains/m³ and a total birch pollen count of 457 grains/m³ (Laboratory of Palynology, Swedish Museum of Natural History, Stockholm).

Preparation of blood leukocytes

Leukocytes were prepared from peripheral blood. The blood was collected in tubes containing an appropriate concentration of EDTA (Vacutainer, 5 ml, with 50 µl of 21% EDTA) (Terumo, Leuven, Belgium) by the Vacutainer® technique. The blood was hemolyzed in 150-µl portions by dilution with 3 ml 4°C isotonic NH₄Cl-EDTA lysing solution (154 mM NH₄Cl, 1.5 mM KHCO₃, 0.1 mM EDTA, pH 7.2). After 5-min incubation at 15°C, the cells were centrifuged at 300 g for 6 min at 4°C. The leukocyte pellets were washed in 2 ml 4°C 0.15 M phosphate-buffered saline (PBS), pH 7.4, supplemented with 0.1 mM EDTA and 0.02% Na-azid (PBS-EDTA), and kept on ice until used.

Cell membrane fixation and permeabilization (FOG method)

The leukocytes were fixed and permeabilized by the FOG method (25). This was done in order to enable detection of eosinophils and to analyze intracellular antigens (14, 15). Stabilization of the cell membrane was performed by an incubation of the leukocytes in 200 µl 4% paraformaldehyde for 12 min at 20°C. The cells were then washed once in 3 ml 4°C PBS-EDTA by centrifugation at 400 g for 8 min at 4°C.

Cell membrane permeabilization was achieved by exposure of the cells to 200 µl 0.74% *n*-octyl-beta-D-glucopyranoside (OG) (Sigma Chemical, St Louis, MO, USA) dissolved in 0.15 M PBS, for 6 min at 20°C. The permeabilized leukocytes were then washed in 4°C PBS-EDTA by centrifugation at 400 g for 8 min at 4°C.

To calculate the percentage of eosinophils in the cell preparations, cells were resuspended in 0.5 ml PBS-EDTA for further analysis in the flow cytometer.

To obtain optimal binding of monoclonal antibodies to the cell-surface antigens and to avoid intracellular binding, antibodies to the cell surface were added to leukocytes before FOG treatment. This membrane permeabilization method has earlier been shown to have a minimal effect on cell-surface-bound monoclonal antibodies (25, 26).

Immunofluorescence staining of cell-surface and intracellular antigens

The cell antigens were detected by using fluorescein isothiocyanate (FITC)-conjugated monoclonal

antibodies against CD9 (10 µg/ml) (DAKO A/S, Denmark) and phycoerythrin (PE)-conjugated monoclonal antibodies against CD11b (CR3) (5 µg/ml) (DAKO A/S, Denmark), and the FITC-conjugated monoclonal antibody, EG2 (40 µg/ml) (Pharmacia AB, Diagnostics, Uppsala, Sweden), were used to detect intracellular expression of the EG2 epitope on ECP. FITC-conjugated mouse IgG1 and PE-conjugated IgG2a (DAKO A/S, Denmark and Becton Dickinson, Mountain View, CA, USA, respectively), used at comparable final concentrations, served as background controls to determine nonspecific binding to the leukocytes.

For analysis of surface expression of the CD9 and CD11b antigens, 10 µl (CD9) and 5 µl (CD11b) of the respective monoclonal antibody were added to untreated leukocytes resuspended in 100 µl PBS-EDTA before FOG treatment. The intracellular expression of ECP was detected by adding 20 µl of the EG2 antibodies to already FOG-treated, permeabilized leukocytes. The suspensions were incubated on ice for 30 min and then washed twice in 4°C PBS-EDTA.

To ensure the stability of the method, intra-assay variation was performed on peripheral blood from healthy blood donors. The intra-assay variations for the different markers were 5.3% (intracellular ECP, EG2 epitope), 2.1% (CD9), and 9.5% (CD11b), respectively.

In a previous comparative study (15) of routine differential counting with a Coulter STKS (five-part differential count) (Department of Clinical Chemistry, Karolinska Hospital) and the FOG method, the proportion of peripheral blood eosinophils from healthy blood donors (*n* = 35) was determined. This resulted in a median value of 3.1 (2.5–4.0)% eosinophils.

Analysis by flow cytometry

The different preparations of leukocytes were finally analyzed in an EPICS Profile II (Coulter, Inc., Hialeah, FL, USA) flow cytometer. In the flow cytometer, the cells were distinguished by their different light-scattering properties; forward scatter reflects the cell size, and side scatter reflects the cell complexity (granularity). In the present study, the leukocyte subpopulations were detected as separate clusters, and a minimum of 1000 cells were analyzed within the gate. The instrument was daily calibrated with standardized 10-µm fluorospheres, Immuno Check (Coulter Inc., Hialeah, FL, USA); if needed, the laser beam was adjusted. Another fluorosphere, Standard Brite (Coulter Inc., Hialeah, FL, USA), with controlled fluorescence intensity, was run before each experiment to obtain a standardization of the mean fluorescence intensity (MFI). After the

blood leukocytes had been treated according to the FOG method, an alteration of the leukocyte distribution was achieved. All leukocytes, except the eosinophils, decreased their light-scattering properties, and three leukocyte clusters could be distinguished, containing lymphocytes, neutrophils/monocytes, and eosinophils (14, 15).

The expression of antigens on and in leukocytes was presented as MFI of the percentage of positive cells.

Statistical analysis

The results are presented as median (interquartile). Statistical significance was evaluated by a non-parametric method (Mann-Whitney U-test), and differences were considered statistically significant at $P < 0.05$.

Results

Intracellular expression of the EG2 epitope on ECP in peripheral blood eosinophils

The intracellular expression of ECP in eosinophils was measured on four different occasions; before and during a pollen season with HPL in 1993, and before and during a season with LPL in 1994.

During the pollen season in 1993, all six patients showed significantly increased ($P < 0.05$) binding of the monoclonal antibody EG2 to intracellular ECP (40.3 [36.2–44.7]) MFI, as compared to before season (33.8 [32.4–36.6]). These values were significantly higher, both before ($P < 0.01$) and during

($P < 0.05$) the pollen season, when compared to controls (27.5 [24.9–30.9] MFI, $n = 16$ [before] and 26.1 [24.7–32.0], $n = 7$ [during]).

During the pollen season in 1994, the results were different; only one patient increased in intracellular ECP expression as compared to before the season. Median value for the six patients was 23.9 (18.3–30.3) MFI before, as compared to 22.8 (18.2–27.0) during the season. There were no significant differences at these time points when compared to controls (23.6 [21.4–32.0] MFI, $n = 18$ [before] and 18.8 [16.4–20.0], $n = 10$ [during]) (Fig. 1).

Expression of CD9 on peripheral blood eosinophils

We also measured the surface expression of CD9 on eosinophils before and during the two pollen seasons. During the HPL season in 1993, there was no significant change in CD9 expression as compared to before the season on eosinophils from patients (127.9 [112.2–136.7] MFI before compared to 130.9 [127.0–133.4] during). The CD9 expression was, however, significantly higher in patients when compared to healthy controls both before ($P < 0.05$) and during ($P < 0.01$) the pollen season (112.7 [103.0–119.6] MFI, $n = 20$ [before] and 107.6 [99.6–111.3], $n = 9$ [during]).

During the LPL season, CD9 expression did not change in the patient group compared to before the season (122.4 [112.8–139.6] MFI before compared to 114.8 [112.0–154.0] during). When CD9 expression on eosinophils from patients was compared to controls, we found no significant difference either

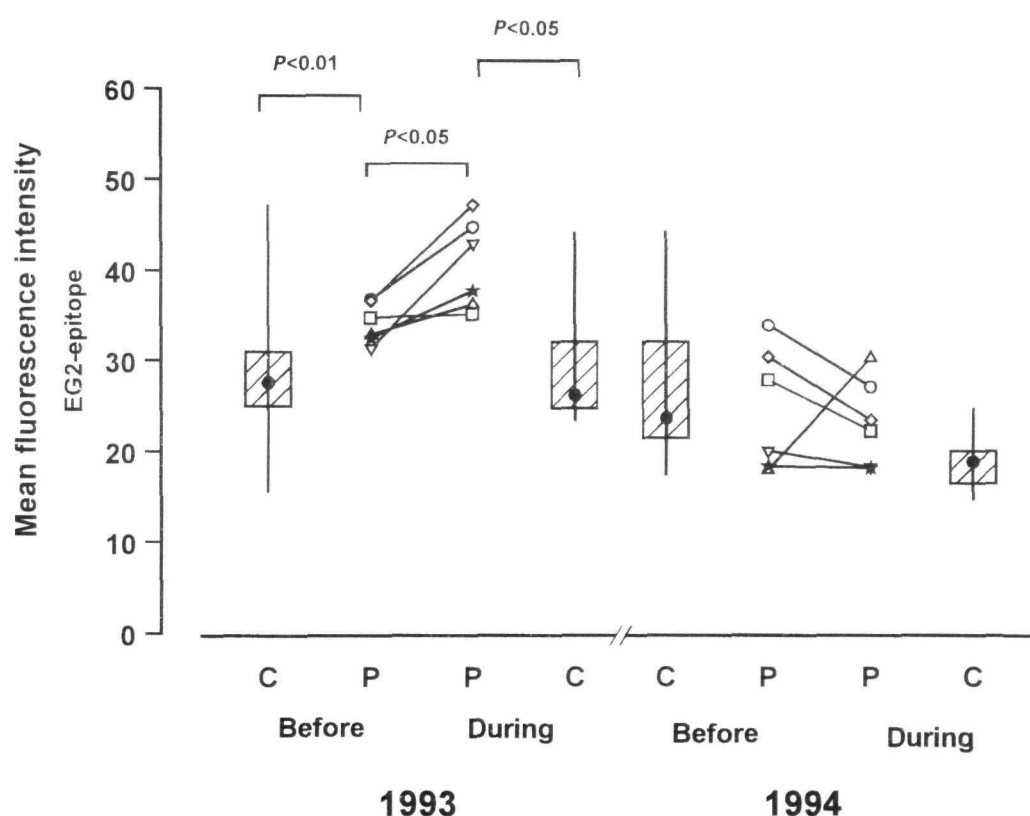


Fig. 1. Expression of EG2 epitope on intracellular ECP in peripheral blood eosinophils from patients (P) before and during high pollen load season in 1993, and low pollen load season in 1994, as compared to controls (C). Boxes enclose interquartiles with medians and ranges marked. EG2 binding was measured as mean fluorescence intensity of positive population.

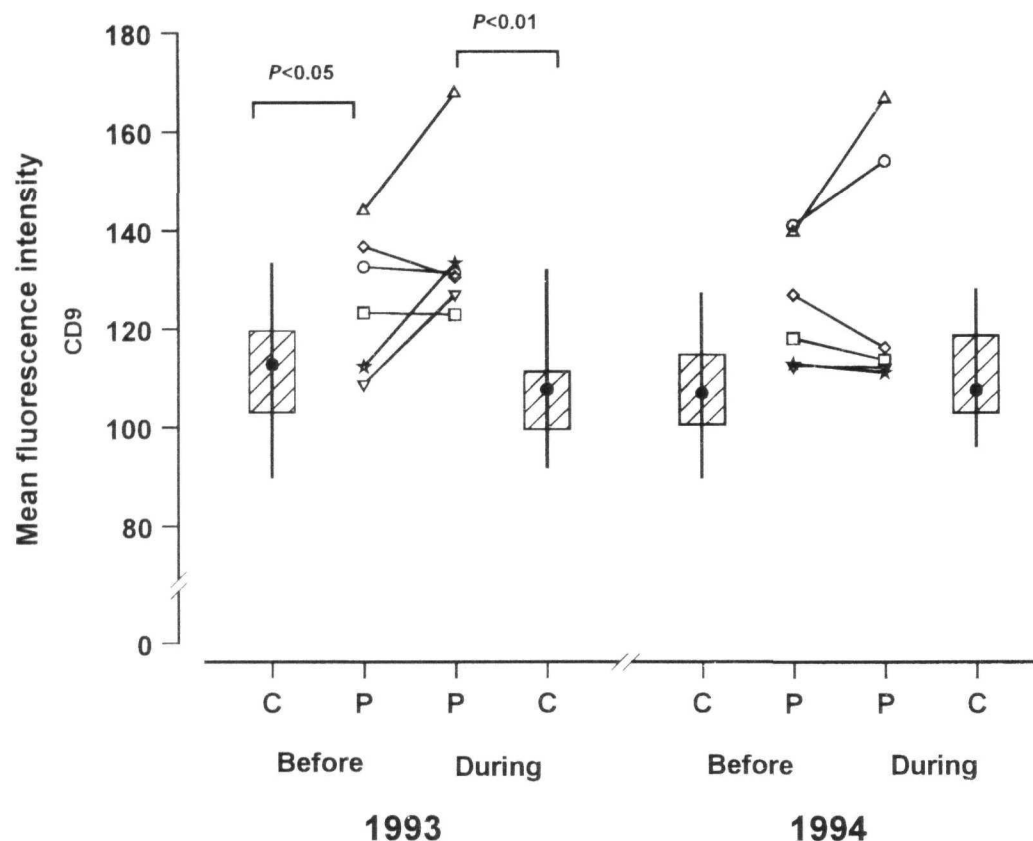


Fig. 2. Expression of CD9 on peripheral blood eosinophils from patients (P) before and during high pollen load season in 1993, and low pollen load season in 1994, as compared to controls (C). Boxes enclose interquartiles with medians and ranges marked. CD9 binding was measured as mean fluorescence intensity of positive population.

before ($P=0.06$) (106.9 [100.5–114.7] MFI, $n=6$) or during ($P=0.07$) (107.4 [102.9–118.5], $n=10$) the pollen season (Fig. 2).

Expression of CD11b on peripheral blood eosinophils

The surface expression of CD11b on eosinophils was measured before and during the HPL season. There was no significant change in CD11b expression on eosinophils from patients as compared to before the season (10.2 [9.3–14.6] MFI, compared to 12.9 [9.9–15.6]). However, there was a significantly increased ($P=0.01$) CD11b expression in the patient group as compared to the control group during the pollen season (7.2 [5.1–8.7] MFI, $n=6$), but no difference before the season (9.9 [8.2–10.4], $n=19$).

Measurements of the CD11b expression in 1994 (LPL) gave the following results: 7.4 (7.2–8.3) MFI before the season as compared to 9.0 (7.4–11.3) during the season. There was no significant increase when these data were compared to controls (7.3 [6.7–9.3] MFI, $n=9$ [before] and 11.4 [7.9–13.3], $n=10$ [during]) (Fig. 3).

Percentage of eosinophils in peripheral blood

The percentage of eosinophils in blood from the patients was determined by flow cytometry after treating the cells by the FOG method. The propor-

tion of eosinophils did not significantly change at any time point. However, when compared to our control group, the percentage of eosinophils was significantly higher both before ($P<0.05$) and during ($P<0.001$) the HPL season (Fig. 4).

Discussion

To monitor the degree of inflammation in asthma and to measure the effects of allergen exposure, it is preferable to have a standard method which can be used in the clinic. There is still no single biologic marker that is specific and feasible for these purposes. Instead of searching for one single marker, we suggest a combination of markers that reflect different biologic states in the activation sequence and recruitment of leukocytes in the circulation.

Since we want a simple method to monitor allergic inflammation, we present data in this study that are based on analysis of leukocytes in peripheral blood. However, we are aware that eosinophil activity in peripheral blood might not fully reflect the inflammatory process in the tissues, where the allergen challenge is exerted.

The eosinophil granulocyte is regarded as one of the effector cells that characterize inflammation in asthma (6, 9, 27). If eosinophil activity markers in the blood circulation do reflect the degree of local inflammation, one logical and simple way to monitor bronchial inflammation would be to study peripheral

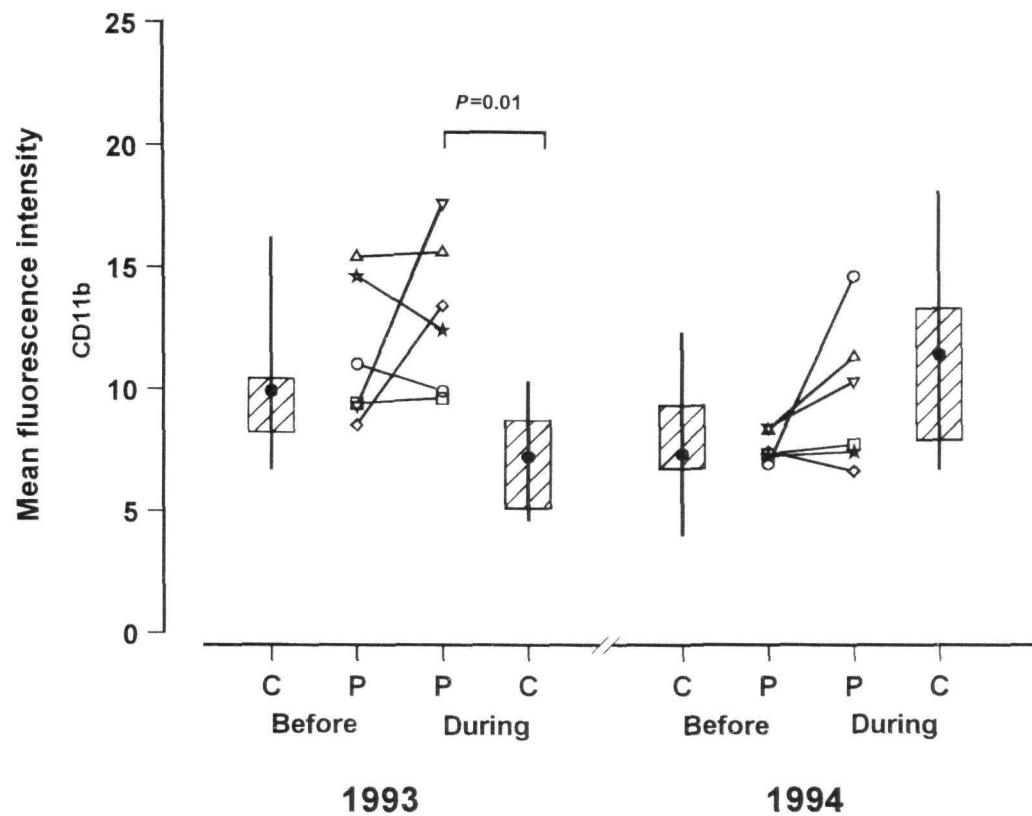


Fig. 3. Expression of CD11b on peripheral blood eosinophils from patients (P) before and during high pollen load season in 1993, and low pollen load season in 1994, as compared to controls (C). Boxes enclose interquartiles with medians and ranges marked. CD11b binding was measured as mean fluorescence intensity of positive population.

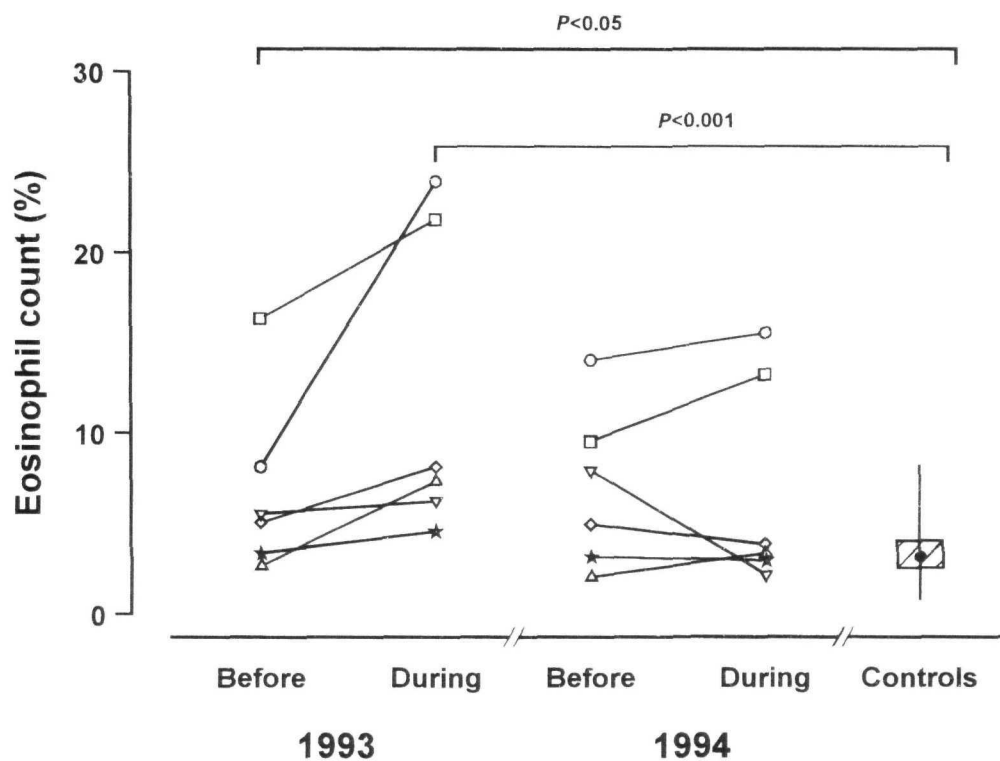


Fig. 4. Percentage of eosinophils in peripheral blood in patients measured by FOG method before and during high pollen load season in 1993, and low pollen load season in 1994. Box represents control group based on standard control material. Box encloses interquartiles with median and ranges marked.

blood eosinophils. It has been suggested that increased number of peripheral blood eosinophils reflects the severity of the asthmatic condition (9, 28). However, there is a wide variation in individual peripheral blood eosinophil counts. Although a relationship exists between eosinophil numbers and asthma severity on a population basis, it is unlikely that eosinophil count on an individual basis can be

related to disease activity. A raised peripheral blood eosinophil count indicates active asthma, but a normal blood eosinophil count by no means excludes it (3). In this study, we found that the proportion of eosinophils did not significantly change on any occasion; however, the patients significantly differed from the control group both before ($P < 0.05$) and during ($P < 0.001$) the HPL season.

In the present study, we have focused on three different eosinophil activity markers, the expression of the EG2 epitope on intracellular ECP, CD9, and CD11b, which reflect different activation stages, as previously shown in *in vitro* experiments (14, 15, 17, 23).

The results from this study demonstrate a significantly increased expression of the EG2 epitope on intracellular ECP in eosinophils from the circulation in asthmatic patients, as compared to healthy controls, both before and during a birch HPL season. We also detected a significantly higher expression of the EG2 epitope on intracellular ECP in the patients during the HPL season compared to themselves outside the season. The increased expression before the HPL season could be explained by early exposure to alder and hazel pollen, which was already detected in the air at the time of sampling, although, at this time point, the patients noticed only very limited clinical symptoms. Hence, the increased expression of intracellular ECP could be interpreted as an early activation of the peripheral blood eosinophils. The results indicate a further activation of peripheral blood eosinophils in patients with pollen-related asthma upon allergen exposure. During the LPL season, we did not notice any significant changes in intracellular ECP expression in our patient group.

Concerning the cell surface marker CD9, we detected an increased expression in the patient group before and during the HPL season, as compared to controls, but no further increase in CD9 expression was observed during the season. These results also reflect eosinophil activity both before and during the pollen season, but CD9 did not increase upon birch pollen exposure to the same extent as measured with intracellular ECP. As with intracellular ECP, we did not notice any significant changes in our patient group during the LPL season.

Concerning the expression of CD11b on eosinophils from the patient group, we detected a significant increase in receptor expression only on one occasion, when compared to healthy controls; namely, during the HPL season. This indicates that CD11b upregulation on eosinophils occurs mainly during high antigen load and that CD11b might not be a suitable early activity marker.

The method used in this study permits flow cytometric analysis of surface and intracellular antigens in eosinophils in unseparated whole blood. The method reduces *in vitro* manipulation and does not decrease already surface-bound monoclonal antibodies (25, 26, 29). To standardize the test and to minimize cell modulation, e.g., secretion of ECP, our samples were all processed within 1 h after the sample was taken.

Although the number of patients was limited, our data indicate that the markers EG2, CD9, and, to a lesser extent, CD11b reflect an activated state of peripheral blood eosinophils, an effect which may make them suitable to reflect events such as allergen exposure. A combination of the three markers could be a useful tool to improve the characterization of the inflammatory status in asthma with respect to allergen exposure.

Acknowledgments

We thank Lena Wehlin and Katarina Gustavsson of the Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska Hospital, and Marianne Claesson, Kerstin Örnefalk, and Randi Johansson Department of Medicine, Division of Respiratory and Allergic Diseases, Huddinge University Hospital, for excellent technical assistance. This study was supported by grants from the Swedish Heart-Lung Foundation, the Swedish Asthma and Allergy Association, the Swedish Society of Medicine, Consul Th. C. Bergh's Foundation, the Swedish Council for Work Life Research, the Swedish Medical Research Council (16X-105), Karolinska Institute, and the Swedish Allergy Research Foundation (SAS).

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