

Increased expression of surface activation markers on neutrophils following migration into the nasal lumen

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Summary

Background The sequence of events following the recruitment of a free-flowing neutrophil in the peripheral circulation, via adhesion, migration and release of mediators, to a neutrophil on the surface of the nasal epithelium is a co-ordinated process. Little is known about the state of neutrophil activation following this course of events.

Objectives To investigate the expression of surface activation markers on neutrophils, reflecting activation during their recruitment to the nose, and to see whether the inflammatory process during allergic rhinitis influences this process.

Method Nine healthy controls and 12 patients with grass pollen-induced intermittent allergic rhinitis were investigated during the peak of the pollen season. The expression of CD11b, CD66b and CD63 on the neutrophil cell surface, as a reflection of activation, was analysed using flow cytometry. Neutrophils were derived from peripheral blood and nasal lavage fluid. In addition, eosinophil cationic protein (ECP) and myeloperoxidase (MPO) as well as L-, P- and E-selectins in the nasal lavage fluid were analysed using RIA and ELISA, respectively.

Results A marked increase in the expression of all three CD markers on the neutrophil cell surface was noticed following migration from the bloodstream to the surface of the nasal mucosa. At the peak of the grass pollen season, the MPO levels increased, reflecting an increase in the total number of nasal fluid neutrophils. In parallel, the expression of CD11b was further augmented. The expression of the CD66b was reduced on neutrophils remaining in the circulation. In addition, the level of L-selectin was reduced on neutrophils derived from the blood during allergic inflammation.

Conclusion Neutrophils might become activated during their transfer from the blood to the surface of the nasal mucosa, but these changes may also be due to depletion of activated neutrophils in the blood via activated endothelial/epithelial adhesion and chemoattractant measures. The increased expression of surface activation markers during allergic rhinitis suggests roles for neutrophils in the inflammatory process.

Keywords allergic rhinitis, CD11b, CD63, CD66b, flow cytometry, nasal lavage, neutrophils, selectins
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Introduction

Neutrophils can be viewed as 'enzymatic storehouses' capable of destroying engulfed microorganisms and antigen–antibody complexes [1]. They originate in the bone marrow, circulate in the blood for a few hours and are signalled to sites of inflammation by a combination of specific adhesion molecules and chemotactic stimuli. In order to reach the airways, they have to adhere to the endothelium and migrate through the airway epithelium [2]. CD11b, CD 63 and CD66b are three membrane-bound glycoproteins which, by regulating the adhesive activity, are involved in neutrophil migration and activation. They are present intracellularly in the

membrane of different subsets of cytoplasmic granules, and mobilization of the granules causes an increased expression of them on the cell surface, thereby reflecting cellular activation [3–6]. The neutrophil enzymatic content is believed to play a role in the development of allergic rhinitis and we have previously demonstrated that neutrophils, on their way from the blood to the nasal epithelium, stimulate secretion from serous and seromucous glands via the release of elastase [7–9]. Although there is little doubt that the attraction of these cells to the nose is of importance in creating the symptoms of rhinitis, the information about neutrophil activation following recruitment is scarce. The present study was designed to investigate if neutrophils in the bloodstream and nasal lumen expressed differences in their sets of CD glycoproteins that reflected cellular activation. Furthermore, we examined if this expression was altered during the peak of the pollen season in patients with allergic rhinitis.

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Materials and methods

Subjects

The study included 12 patients with symptomatic grass pollen-induced allergic rhinitis and nine healthy donors as control. The median ages of the patients and the controls were 40 (range 20–69) and 43 (range 19–61) years, respectively. Three patients and four donors were women. The diagnosis of grass pollen-induced allergic rhinitis was based on a history of seasonal allergic rhinitis for at least 2 years, and positive skin prick tests (SPTs) against grass pollen. Patients were excluded if they had a history of perennial rhinitis, had positive SPTs tests against perennial allergens, had been given local or systemic glucocorticoid treatment during the 6 weeks before the start of the study, or were pregnant. The healthy donors were all symptom free, had no history of allergic rhinitis or any other atopic disease, and had negative SPTs against a panel of allergens, including grass pollen. Patients with aspirin-induced airway symptoms were excluded from the study along with subjects with a history of concurrent purulent nasal infection within 6 weeks before the study or nasal surgery during the last year. None of the participating patients suffered from asthma that required continuous medication.

This study was approved by the Local Ethics Committee of Lund University and informed written consent was obtained from all subjects. Subjects were recruited through advertisements and physician referrals.

Study design

The rhinitis patients were instructed to record daily symptoms from eyes, nose and lungs during the anticipated grass pollen season. A nasal lavage was performed during the peak of the season, as estimated by the pollen count. At the same time, 20 mL of blood was drawn and heparinized. The patients were instructed not to use any anti-allergic medication prior to the nasal lavage with the exception of ocular sodium cromoglycate and the occasional use of oral antihistamines. None of these drugs were allowed within 48 h of the lavage. Local and oral steroids were not allowed. The healthy donors were tested during the same week as the allergic rhinitis subjects.

SPT and pollen count

SPTs were performed with a standard panel of 10 common airborne allergens (ALK, Copenhagen, Denmark) including pollen (birch, timothy, artemisia), house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*), moulds (*Cladosporium* and *Alternaria*) and animal allergens (cat, dog and horse). Tests were performed on the volar side of the forearm with saline buffer as negative and histamine chloride (10 mg/mL) as positive controls. The weal reactions were measured after 20 min and designated as 4+, 3+, 2+, 1+ or 0 depending on the size in relation to histamine [10]. The rhinitis patients exhibited 3+ or 4+ reaction to timothy, but were negative to the other allergens. All healthy donors in the control group were negative to the 10 allergens when tested with the standard panel.

Pollen counts were performed with the aid of a pollen trap (Burkard Seven Day Recording Volumetric Spore Trap,

Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK), located on the roof (30 m above ground level) of the hospital building where the investigations were performed. The sucking rates were adjusted to 10 L/min, and the total number of pollen grains dispersed during a study week was calculated as the summation of the daily values (particles/m³ air) [11].

Symptom scores

All rhinitis patients recorded daily symptoms from eyes, nose and lungs: from the eyes itching, redness and tear flow, from the nose sneezing, nasal blockage and watery discharge, and from the lungs coughing, wheezing and trouble of breathing. Each subsymptom was graded from 0 to 3, where 0 represented no symptoms, 1 represented mild symptoms, 2 represented intermediate symptoms and 3 represented severe symptoms.

Nasal lavage and mediator analysis

Nasal lavage fluids were obtained using a 30 mL syringe and the 'nasal pool' technique [12]. After clearing excess mucus by forceful exsufflation, each nasal cavity was rinsed with 8 mL 0.9% NaCl solution, repeated five times. The suspension from both nasal cavities, ranging from 12 to 15 mL, was then centrifuged in 400g, with slow acceleration and unbraked deceleration, at room temperature for 10 min. The supernatant was removed and 1 mL PBS solution with 10% fetal calf serum and 1% Varidase[®] was added to the centrifuged cells.

Albumin, eosinophil cationic protein (ECP) and myeloperoxidase (MPO) were determined in the lavage fluid using competitive radioimmunoassays from Pharmacia & Upjohn Diagnostics AB (Uppsala, Sweden). Selectins were analysed in the nasal lavage fluid using ELISA kits from R&D Systems (Abingdon, UK).

Flow cytometry

Heparinized whole-blood and nasal lavage cell suspension (see above) were used for the cell analysis. The whole blood was put on ice and red blood cells were lysed using formic acid. Blood and lavage cells were incubated with a mixture of a phycoerythrin (PE)-conjugated monoclonal antibody against CD16 (Dako, Glostrup, Denmark) and a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against CD11b, CD63 or CD66b (Immunotech, Marseille, France) or, as a control, CD16 antibody and an isotype-matched FITC-conjugated irrelevant monoclonal antibody (Mab IgG1; Immunotech) at the same concentration as the specific antibodies.

Cells were analysed using a Coulter Epics XL-MCL flow cytometry system (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a 488 nm argon laser for excitation and filters detecting emission at 515–545 nm (for FITC) and 565–595 nm (for PE). Background autofluorescence was monitored for each sample by analysing cells labelled with dual-colour isotype-matched irrelevant mouse-Ig. Neutrophils were gated using their characteristics in side scatter and forward scatter, and their identity was further confirmed by their characteristic CD16 signal. The mean fluorescence

intensity (MFI) in the FITC channel was determined for the CD11b, CD63 and CD66b antibodies, respectively, and the control antibody. The MFI for the specific antibodies was subtracted from the MFI for the control antibody, representing background. Each analysis was ended at a score of 3000 cells.

Statistics

Results are expressed as mean $\pm$ SEM in the text, whereas the median is shown in the figures. Statistical comparisons were made by Student's *t*-test, Bonferroni corrections were performed when appropriate and *P*-values less than 0.05 were considered to be statistically significant.

Results

The expression of CD11b, CD66b and CD63 on the neutrophil cell surface was analysed in nasal lavage fluid and blood from nine healthy donors. The expression of all three glycoproteins was more pronounced in neutrophils derived from the nasal lavage fluid than from the blood; CD11b: 6.9 ± 1.6 vs. 2.6 ± 0.4 MFI_{mean} ($P < 0.05$), CD66b: 12.8 ± 2.5 vs. 1.6 ± 0.2 MFI_{mean} ($P < 0.05$); and CD63: 2.0 ± 0.8 vs. $0. \pm 0.01$ MFI_{mean} ($P < 0.05$) (Fig. 1).

The rhinitis patients were evaluated and sampled during the peak of the season as estimated by the pollen count (Fig. 2). Throughout the season, all patients recorded their daily symptoms from the eyes, nose and lungs, and all exhibited severe symptoms of allergic rhinoconjunctivitis at the time of the sampling (Fig. 2).

At the peak of the season, the rhinitis patients expressed higher levels of ECP, MPO and albumin in nasal lavage fluid than the healthy subjects; ECP_{mean}: 15.0 ± 4.3 vs. 2.9 ± 0.8 $\mu\text{g/L}$ ($P < 0.05$); MPO_{mean}: 197.7 ± 52.9 vs. 52.1 ± 19.5 $\mu\text{g/L}$ ($P < 0.05$); and albumin_{mean}: 15.2 ± 3.4 vs. 3.8 ± 1.0 mg/L ($P < 0.05$), (Fig. 3). The expression of CD11b on neutrophils derived from the nasal lavage fluid was higher among the rhinitis patients at the peak of the season than among the healthy donors; CD11b: 10.4 ± 10.4 vs. 6.9 ± 1.6 MFI_{mean} ($P < 0.05$). No such differences were noted for CD66b

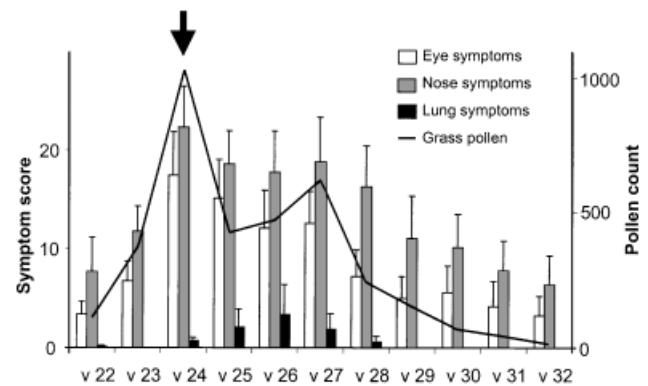


Fig. 2. Symptom score and pollen count obtained during the grass pollen season. Daily symptom scores for eyes, nose and lungs obtained from patients with grass pollen-induced intermittent allergic rhinitis. The arrow represents the week chosen for nasal lavages and concomitant blood sampling. Symptom scores are presented as weekly summations $\pm$ SEM, $n = 12$.

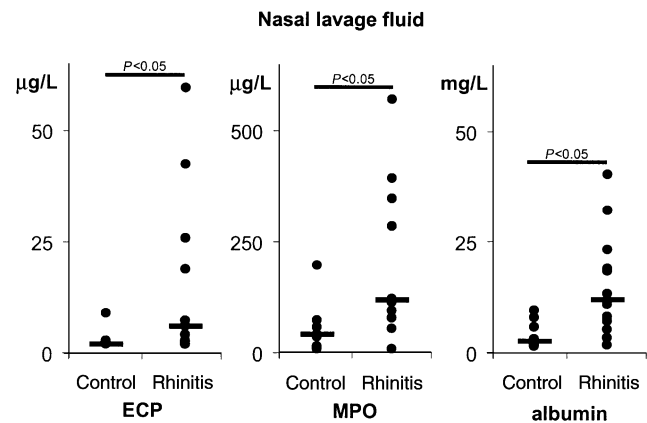


Fig. 3. Levels of eosinophil cationic protein (ECP), myeloperoxidase (MPO) and albumin in nasal lavage fluid obtained from patients with grass pollen-induced intermittent allergic rhinitis at the peak of the pollen season. Horizontal bars represent the median values (ECP_{median} and MPO_{median}), $n = 9-12$.

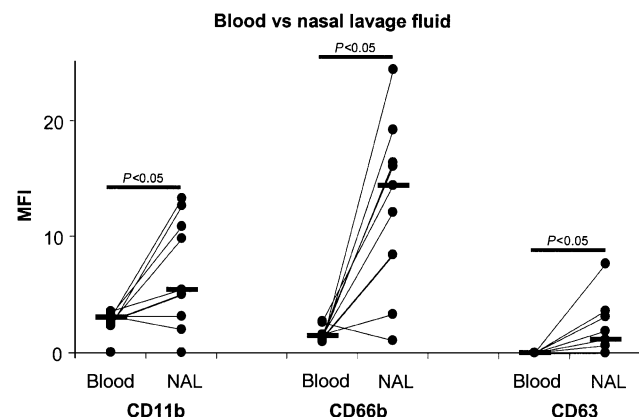


Fig. 1. Expression of CD11b, CD66b and CD63 on the cell surface of neutrophils derived from peripheral blood and nasal lavage fluid in healthy control subjects. Data are expressed as mean fluorescence index (MFI), horizontal bars represent the median values (MFI_{median}), $n = 9$.

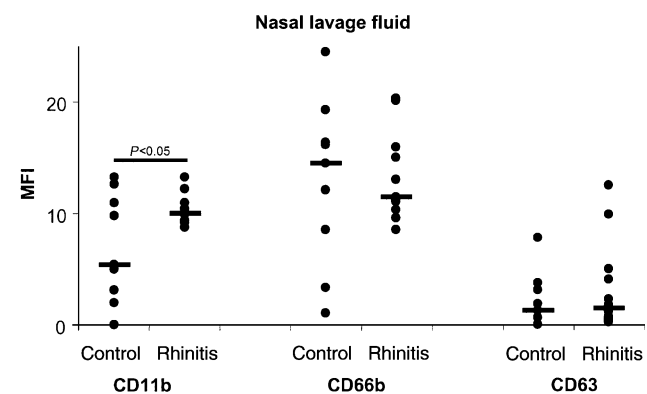


Fig. 4. Expression of CD11b, CD66b and CD63 on the cell surface of neutrophils derived from nasal lavage fluid in healthy control subjects and in patients with grass pollen-induced intermittent allergic rhinitis at the peak of the pollen season. Data are expressed as MFI, horizontal bars represent the median values (MFI_{median}), $n = 9-12$.

(13.3 ± 1.2 vs. 12.8 ± 2.5 MFI_{mean}) and CD63 (3.2 ± 1.2 , vs. 2.0 ± 0.8 MFI_{mean}), (Fig. 4). In contrast, the expressions of CD11b on neutrophils in blood were significantly lower in

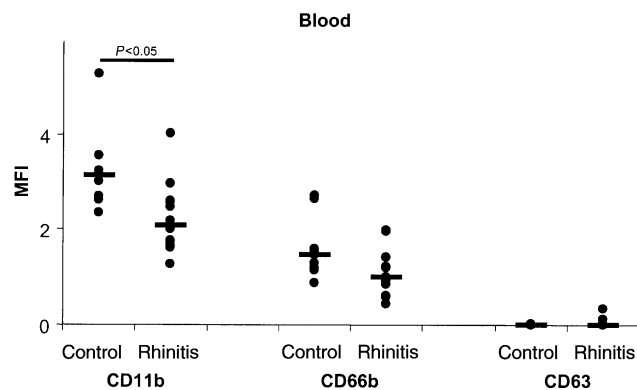


Fig. 5. Expression of CD11b, CD66b and CD63 on the cell surface of neutrophils derived from the peripheral blood of healthy control subjects and from patients with grass pollen-induced intermittent allergic rhinitis at the peak of the pollen season. Data are expressed as MFI. Horizontal bars represent the median values (MFI_{median}), $n = 9-12$.

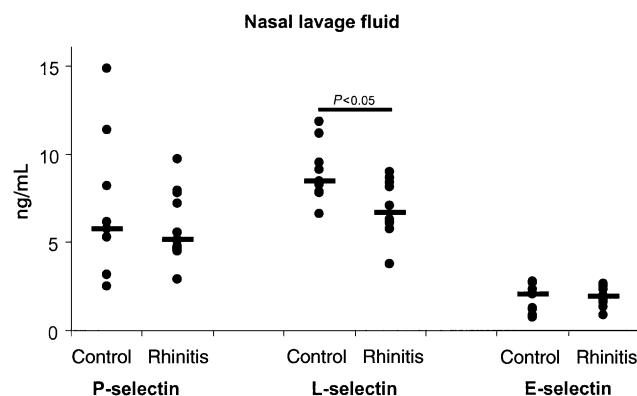


Fig. 6. Expression of P-, L- and E-selectins in nasal lavage fluid from healthy control subjects and from patients with grass pollen-induced intermittent allergic rhinitis at the peak of the pollen season. Data are expressed as ng/mL. Horizontal bars represent the median values, $n = 9-12$.

rhinitis patients than among the healthy donors, CD11b: 2.2 ± 0.2 vs. 3.2 ± 0.3 MFI_{mean} ($P < 0.05$), whereas CD66b did not change significantly; 1.1 ± 0.1 vs. 1.6 ± 0.2 MFI_{mean} ($p = NS$). The expression of CD63 was close to zero both among patients and donors (Fig. 5).

Selectins were measured in the nasal lavage fluid. The levels of L-selectin were markedly lower among the rhinitis patients compared to the healthy donors; 6.68 ± 0.47 vs. 8.43 ± 0.56 ng/mL ($P = 0.01$). No such differences were found for P- and E-selectins, 5.14 ± 0.56 vs. 5.72 ± 1.32 ng/mL and 1.94 ± 0.15 vs. 2.03 ± 0.28 ng/mL (P- and E-selectins, respectively) (Fig. 6).

Discussion

The current study demonstrates that there is a marked increase in the expression of the glycoproteins CD11b, CD63 and CD66b on the cell surface of neutrophils following their migration from the bloodstream to the surface of the nasal

mucosa during healthy conditions. At the peak of the grass pollen season, the MPO levels increased, reflecting an increase of the total number of nasal fluid neutrophils. In parallel, the expression of CD11b was further augmented. In contrast, during the same period the expression of the CD11b is reduced on the surface of circulating neutrophils. In nasal lavage fluid, the levels of L-selectin, another adhesion molecule associated with neutrophil recruitment, are also reduced at the peak of the season.

Normally, a considerable number of neutrophils migrate through the venules of the nasal mucosa. From the lamina propria they continue through the epithelium and become a part of the nasal secretion. The migratory procedure begins with the 'capture' of circulating neutrophils by endothelial cells, followed by rolling along the vessel wall. Then, if an appropriate stimulus is at hand, the rolling leucocytes firmly adhere to endothelial cells thus positioned for passing between the endothelial cells into the tissue parenchyma [13-15]. Numerous regulatory molecules are involved at different stages of the migration process. Selectins are one such group of regulators present in different forms on both the neutrophil and on the endothelial cell. These glycoproteins are up-regulated following neutrophil activation and play a pivotal role in the 'capture' and rolling of the neutrophils [15, 16]. Two other important glycoproteins involved in the migratory process are CD11b and CD66b. Both CD11b, as a part of the CD11b/CD18 complex, and CD66b are expressed on the neutrophil surface following activation and both are implicated in the change of the neutrophil adhesive properties necessary for migration. CD63 is often used as a marker of azurophilic granule release, even though its actions are less well known. In analogy with CD11b and CD66b, it is believed to be involved in the adhesion of neutrophils following activation [3, 4]. Accordingly, the increase in the expression of CD11b, CD66b and CD63 presently reported might reflect neutrophil activation as part of the migratory process. These glycoproteins are during normal, unstimulated, conditions located within the neutrophil granules and they are expressed as a consequence of different granules fusing with the plasma membrane, resulting in the release of granule contents and a simultaneous up-regulation of CD antigens on the cell surface. From their position on the cell surface, the CD antigens are free to interact with their close environment. It is therefore conceivable that the activated neutrophil might also be involved in other physiological and pathophysiological processes related to the nose. We have previously demonstrated that neutrophils release elastase during the migratory process, resulting in nasal hypersecretion [7-9]. The constant flow of neutrophils into the nose has also been suggested to be a reflection of a 'physiological inflammation' in the nasal mucosa, implying some sort of activation, probably induced by continuous exposure to micro-organisms and inhaled particles [17, 18].

Neutrophils from allergic patients respond to allergen stimulation with the release of cytotoxic enzymes like MPO [19]. Moreover, neutrophils release pro-inflammatory cytokines [20] that may induce influx and degranulation of eosinophils [21-23]. MPO is often used as a marker of neutrophils and ECP, a granule protein normally connected with eosinophils, as a marker for eosinophils [24]. Neuro-

phils have recently also been shown to contain and release small amounts of ECP [25, 26]. In the present study, patients with grass pollen-induced intermittent rhinitis were investigated at the peak of the grass pollen season during a period with severe symptoms from both nose and eyes. They all displayed increased levels of MPO and ECP in the nasal lavage fluid, reflecting the fact that the amount of leucocytes that had migrated from the blood to the nose had increased dramatically. At the same time as the amount of neutrophils had increased the expression of CD11b on their surface was further augmented, indicating an increased activation at the peak of the season. In addition, neutrophils with low levels of CD11b can be observed in nasal lavage fluid from control subjects, but not in lavage fluid from the allergic patients. This might represent an increased state of activation of neutrophils in allergic rhinitis. At the same time, the low levels seen during control conditions might argue against a direct role for CD11b and CD66b in the migration process. The change in CD expression seen might also be due to depletion of activated neutrophils in the blood via activated endothelial/epithelial adhesion and chemoattractant measures or perhaps, although less likely, present in the mucosa after 'usual' entry through non-activated endothelial cells into the tissue.

The reduced expressions of glycoproteins on neutrophils isolated from the blood found during the present investigation are in line with our previous findings [27] and might indicate that all neutrophils with the ability to express CD11b already had been recruited to the nose, making up for the increased amount of cells found in the lavage fluid. It is also conceivable that these reductions might reflect some form of negative feedback mechanism at the peak of the season in conjunction with the presentation of severe symptoms. The simultaneous finding of reduced L-selectin levels in the nasal lavage fluid might be a reflection of the same process. Even though most soluble adhesion molecules in serum are reported to be up-regulated during periods of respiratory manifestations of allergic symptoms [28, 29], some, like L-selectin, are reported to be down-regulated during the early stage of the season [30]. L-selectins are expressed on the neutrophil surface and initiate neutrophil binding to endothelial cells, whereas P- and E-selectins are mainly associated with epithelial cells [31].

Neutrophils derived from sputum have been reported to express higher levels of CD11b and lower levels of L-selectin compared with peripheral blood neutrophils, and similar results have been reported for eosinophils. However, no difference was found between sputum and blood levels, respectively, when data obtained from healthy subjects were compared with results obtained on patients with atopic asthma (mild through severe) [32]. This rendered the conclusion that the altered expression of these adhesion molecules on sputum granulocytes compared with blood-derived granulocytes is not related to exposure to inflammatory mediators specific for asthma, but rather due to factors associated with the migration [32]. However, the presently reported augmentation of CD11b at the peak of the allergy season supports the idea of a direct involvement of neutrophils and neutrophil-related products in the allergic reaction.

The expression of CD63, found on neutrophils in the lavage fluid, was not affected by the pollen season. CD63 is present

in the membrane of azurophil granules and its mobilization to the cell surface correlates well with the release of azurophil granule contents. The mobilization of azurophil granules generally requires much stronger stimuli than the mobilization of granules containing CD11b and CD66 [33, 34]. This may explain why CD63 was not found on the neutrophils in the blood and why their relative expression level was low relative to CD11b and CD66.

Although current concepts of allergic rhinitis pathogenesis focus on the role of CD4⁺ T cells and eosinophilic inflammation, there is evidence that neutrophilic inflammation may be of importance [35]. The present results show that the expression of surface activation markers in neutrophils both in blood and nasal lavage fluid are altered in patients with intermittent allergic rhinitis at the peak of the pollen season. Further research is needed to find out if these changes are caused specifically by allergen exposure *in vivo* or if it is a process generally associated with airway inflammation. Thus, there is a call for pre-, peak- and post-seasonal comparisons as well as investigations of neutrophil activation during viral, bacterial and COPD processes.

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