

Original article

Quantitative, rather than qualitative, differences in CD69 upregulation in human blood eosinophils upon activation with selected stimuli

Background: The study aimed to investigate whether CD69 expression on granulocytes is subject to specific regulation by inflammatory mediators, and, if so, to identify these factors in relation to eosinophil activity markers such as the EG2 epitope and ECP release.

Methods: Peripheral blood leukocytes from healthy donors were used. The surface and intracellular distribution of CD69 was investigated with a whole-blood cell-membrane permeabilization technique, the FOG method, and flow cytometry. *In vitro* stimulation was performed with GM-CSF, IL-5, IL-5 plus eotaxin, LPS, and fMLP.

Results: A preformed intracellular pool of CD69 was demonstrated in both eosinophils and neutrophils, but not in monocytes. Almost no resting eosinophils, neutrophils, or monocytes expressed CD69 on the cell surface. However, *in vitro* stimulation with selected stimuli increased the proportion of CD69-positive eosinophils to various extents, with GM-CSF being the most and fMLP the least efficient stimulus. The neutrophils did not respond under these conditions. Increased expression of the EG2 epitope and initiation of degranulation preceded CD69 upregulation.

Conclusions: Eosinophils and neutrophils from healthy donors have a preformed intracellular pool of CD69, which is mobilized on the cell surface on eosinophils, but not on neutrophils, to various extents by selected stimuli. Monocytes, however, do not have a preformed intracellular pool of CD69. Our data indicate that a kinetic order exists among the EG2 expression, the degranulation process, and CD69 upregulation. Due to a quantitative, rather than a qualitative, upregulation of CD69 by stimuli associated with both allergic and bacterial inflammation, CD69 may be a potential activity marker of clinical value.

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Eosinophils are associated with a number of diseases, especially asthma, atopic dermatitis, and helminthic parasite infection (1). Histopathologic studies have shown an increased influx of eosinophils to the allergic site of inflammation with subsequent tissue damage, and it is now generally believed that eosinophilic accumulation is a characteristic feature of allergic inflammation (2, 3). Neutrophils, on the other hand, are recruited into sites of acute bacterial infection, but that does not exclude the possibility of neutrophilic contribution to the pathophysiology in allergic diseases (4–6).

No single granulocyte activity marker has been described that alone provides a useful tool for monitoring asthma or allergic inflammation. Increased number of eosinophils has been used as a

monitoring marker in the management of asthma, and it has been reported that the total number of peripheral blood eosinophils (PBE) correlates with disease severity (7, 8). However, not only the number of PBE but also their state of activation could reflect an ongoing allergic inflammatory process (9, 10). The expression of the EG2 epitope on intracellular eosinophil cationic protein (ECP) is one among several markers that have been used to estimate eosinophil activity both *in vitro* and *in vivo* (11–14). Another marker used to identify eosinophil activation, ECP is released during the allergic inflammatory process (15–17).

The antigen CD69 is a 60-kDa glycoprotein composed of two subunits of 27 and 33 kDa, respectively

(18). The ligand of CD69 is still to be identified, but a membrane-bound molecule is more likely than a soluble factor, since cross-linking of CD69 is required for optimal stimulation (19, 20). Surface expression of CD69 has been observed on various cells, such as activated T and B cells (18, 21, 22), natural killer (NK) cells (23), monocytes (24), neutrophils (25), and platelets (26). However, PBE from healthy subjects do not express a significant amount of CD69 on the cell surface (27). On the other hand, the main body of eosinophils recovered from bronchoalveolar lavage (BAL) in patients with eosinophilic pneumonia (28), as well as with asthma (27), express this antigen on the cell surface. Collectively, these data indicate that CD69 can be expressed on eosinophils recruited into inflammatory/infectious sites.

Given that the surface expression of CD69 on human eosinophils may vary in relation to the state of activation, our present study focused on the *in vitro* regulation of CD69 in peripheral blood eosinophils, neutrophils, and monocytes. The specific aim was to investigate whether CD69 expression is subject to specific regulation by inflammatory mediators, and, if so, to identify these factors in relation to eosinophil and neutrophil function.

Material and methods

Preparation of peripheral blood leukocytes

Peripheral blood from healthy, nonallergic blood donors (aged 18–65 years) was collected in test tubes containing citrate (Vacutainer, 5 ml, with 0.5 ml 0.129 M 9NC, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

The blood was hemolyzed in 150- μ l portions by dilution in 3 ml isotonic NH_4Cl -EDTA lysing solution (154 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.2), and incubated for 5 min at 15°C. The leukocyte suspensions were then centrifuged at 300 g for 6 min at 4°C and washed with 2 ml 0.15 M phosphate-buffered saline (PBS), pH 7.4, at 4°C, supplemented with 0.1 mM EDTA and 0.02% NaN_3 (PBS-EDTA), and kept on ice before further processing. This preparation is referred to as mixed leukocytes.

In some experiments, mononuclear cells were isolated from the peripheral blood of healthy, nonallergic blood donors by Ficoll-Hypaque centrifugation, as previously described (29).

Purified eosinophils were used in some experiments. Granulocytes were purified with Ficoll-Hypaque centrifugation, and eosinophils were then purified with anti-CD16 magnetic particles, as previously described (30).

In vitro activation of blood leukocytes

Mixed leukocytes were resuspended in 200 μ l HEPES (10 mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated calf serum (RPMI) in the presence of the following activators: phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co, St Louis, MO, USA) (10^{-7} M), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co) (5×10^{-7} M), lipopolysaccharide (LPS) (Sigma Chemical Co) (100 μ g/ml), recombinant human granulocyte/macrophage-colony stimulating factor (rhGM-CSF) (Nordic BioSite AB, Täby, Sweden) (10^{-9} –1.0 μ g/ml), recombinant human eotaxin (eotaxin) (Prepro Tech, Inc., Rocky Hill, NJ, USA) (200 ng/ml), or recombinant human interleukin-5 (rhIL-5) (Immunokontakt, Frankfurt, Germany) (100 ng/ml) (31).

Incubation times were as follows: GM-CSF, LPS, fMLP, and IL-5 120 min, IL-5 plus eotaxin 105 min + 15 min, and PMA 0–120 min. All incubations were performed at 37°C. Mixed leukocytes were incubated, in parallel, with medium alone at 4°C (nonstimulated cells) and at 37°C (control cells) at the respective time points. After the *in vitro* activation, the cells were washed once with 2 ml PBS-EDTA and centrifuged at 300 g for 5 min.

Cell membrane fixation and permeabilization (FOG method)

The peripheral blood leukocyte preparations were treated according to a cell membrane permeabilization technique, the FOG method (32). This procedure was done to obtain a clear differentiation between eosinophils and neutrophils in a scatter plot employing flow cytometry. Briefly, the mixed blood leukocytes were incubated for 10 min at 20–22°C in 200 ml phosphate-buffered 4% (w/v) paraformaldehyde (PFA) (Sigma Chemical Co) and then washed in 3 ml PBS-EDTA at 4°C (centrifugation at 400 g, 7 min). The fixed leukocytes were then permeabilized by incubation in 200 ml 0.74% *n*-octyl- β -D-glucopyranoside (OG) (Sigma Chemical Co) for 6 min at 20–22°C. The permeabilized cells were finally washed once in 3 ml PBS-EDTA at 4°C (centrifugation at 400 g, 7 min), resuspended in PBS-EDTA, and kept on ice.

Immunofluorescence staining of cell-surface and intracellular antigens

Cell-surface expression of CD69 and CD61 was detected with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD69 (clone: L78, Becton Dickinson) or CD61 (clone: Y2/51, DAKO A/S, Denmark). To obtain optimal binding, antibodies (20 μ l CD69 or 10 μ l CD61) to surface antigens were added to untreated mixed leukocytes (from 150 μ l of peripheral blood) in 100 μ l suspensions, before the FOG treatment.

For determination of the nonspecific binding, an isotype-matched IgG1 (10 μ l) (clone: DAK-GO1, DAKO A/S) control antibody was used in parallel.

Intracellular localized CD69 and ECP were detected by adding 20 μ l of FITC-conjugated monoclonal CD69 (Becton Dickinson) or EG2 (4 μ g/100 μ l) (Pharmacia & Upjohn, Uppsala, Sweden) antibody to FOG-treated mixed leukocytes (from 150 μ l of peripheral blood). Identification of monocytes was done by adding 10 μ l FITC-conjugated CD14 (clone: MY4-FITC, Beckman Coulter, Hialeah, FL, USA) to untreated mixed leukocytes in 100 μ l PBS-EDTA.

All incubations with monoclonal antibodies were performed for 30 min on ice, followed by two washes in 2 ml PBS-EDTA at 4°C (centrifugation at 300–400 g, 5 min). The different leukocyte preparations were finally suspended in 500 μ l PBS-EDTA before flow cytometry analysis.

Flow cytometry analysis

The different leukocyte preparations were analyzed in an EPICS XL flow cytometer (Beckman Coulter, Inc., Hialeah, FL, USA). Leukocyte subpopulations are distinguished by their different light-scattering properties; forward scatter (FS) reflects the cell size, and side scatter (SS) reflects the complexity/granularity. In the present study, three different leukocyte clusters were distinguished in untreated blood samples: lymphocytes, monocytes, and granulocytes (neutrophils plus eosinophils) (Fig. 1).

The FOG method, through fixation and permeabilization of mixed blood leukocytes, causes a decrease in light-scatter properties of all leukocyte populations, with the exception of the eosinophils. The alteration of light-scattering properties leads to the detection of three separated leukocyte populations designated lymphocytes, neutrophils (neutrophils plus monocytes), and eosinophils (33) (Fig. 1). The PBE were identified by their high scatter signals, surface expression of CD9, and expression of the EG2 epitope on intracellular ECP, as previously described (11, 33). Eosinophil and neutrophil analysis was based on minimums of 1000 and 10000 cells, respectively, within the defined gate. The monocytes in untreated leukocyte preparations were identified by their characteristic scatter properties and CD14 expression. A minimum of 2000 cells was analyzed within the monocyte cluster, which contained >90% CD14-positive cells.

The instrument was calibrated daily with standardized 10- μ m fluorospheres (Flow-Check, Beckman Coulter). Flow-set (Beckman Coulter), another fluorosphere with controlled fluorescence intensity, was used to standardize the mean fluorescence intensity (MFI) before each experiment. The proportions and absolute numbers of immunostained PBE and PBN were determined by the use of isotype-matched control

antibodies to define the cutoff for positively labeled cells. Quantification of the respective antigens was obtained by measuring the MFI units of the respective positively labeled cell population.

Analysis of ECP levels after *in vitro* activation

Mixed leukocyte populations were activated *in vitro* with different stimuli as described above. After *in vitro* activation, samples were centrifuged at 300 g for 5 min, and the cell-free supernatants were transferred to new tubes and stored at –70°C before ECP analysis, by CAP system immunoassay (CAP ECP FEIA Pharmacia). The cutoff was set to 2 μ g/ml.

Statistics

The results are expressed as medians (interquartile range) if not otherwise stated. Statistical evaluation was made by the nonparametric method (Wilcoxon paired test), and differences were considered statistically significant at $P < 0.05$.

Results

Surface expression of CD69 on eosinophils and neutrophils upon *in vitro* stimulation with GM-CSF

Mixed leukocytes from three healthy blood donors were incubated *in vitro* with different concentrations of GM-CSF (10^{-9} –1.0 μ g/ml) for 120 min at 37°C. Control cells were incubated in RPMI alone at 37°C. The proportions of CD69-positive control eosinophils and neutrophils were 18.6% (14.6–21.7%) and 7.1% (6.2–8.7%), respectively. Incubation with GM-CSF increased, in a dose-dependent manner, the proportion of CD69-positive eosinophils considerably. In the following experiments, a concentration of 0.2 μ g/ml GM-CSF was selected for further *in vitro* stimulation (results are expressed as mean [range] [$n=3$]) (9) (Fig. 2).

Intracellular expression and mobilization of CD69 in eosinophils and neutrophils

A low number of nonstimulated (4°C) eosinophils and neutrophils expressed CD69 on the cell surface; 4.8% (3.9–6.2%) and 1.1% (0.6–1.6%), respectively. Incubation with GM-CSF 2×10^{-7} g/ml for 120 min significantly ($P > 0.05$; $P > 0.05$) increased the surface expression of CD69 to 79.2% (65.8–86.0%) positive eosinophils and 4.6% (4.1–6.6%) positive neutrophils ($n=7$) (Fig. 3).

To investigate whether eosinophils and neutrophils possessed a preformed intracellular pool of CD69, we incubated permeabilized leukocytes with monoclonal antibodies against CD69. A majority of both non-stimulated (4°C) eosinophils (91.0% [85.7–96.3%]) and neutrophils (97.0% [96.8–99.2%]) possessed specific

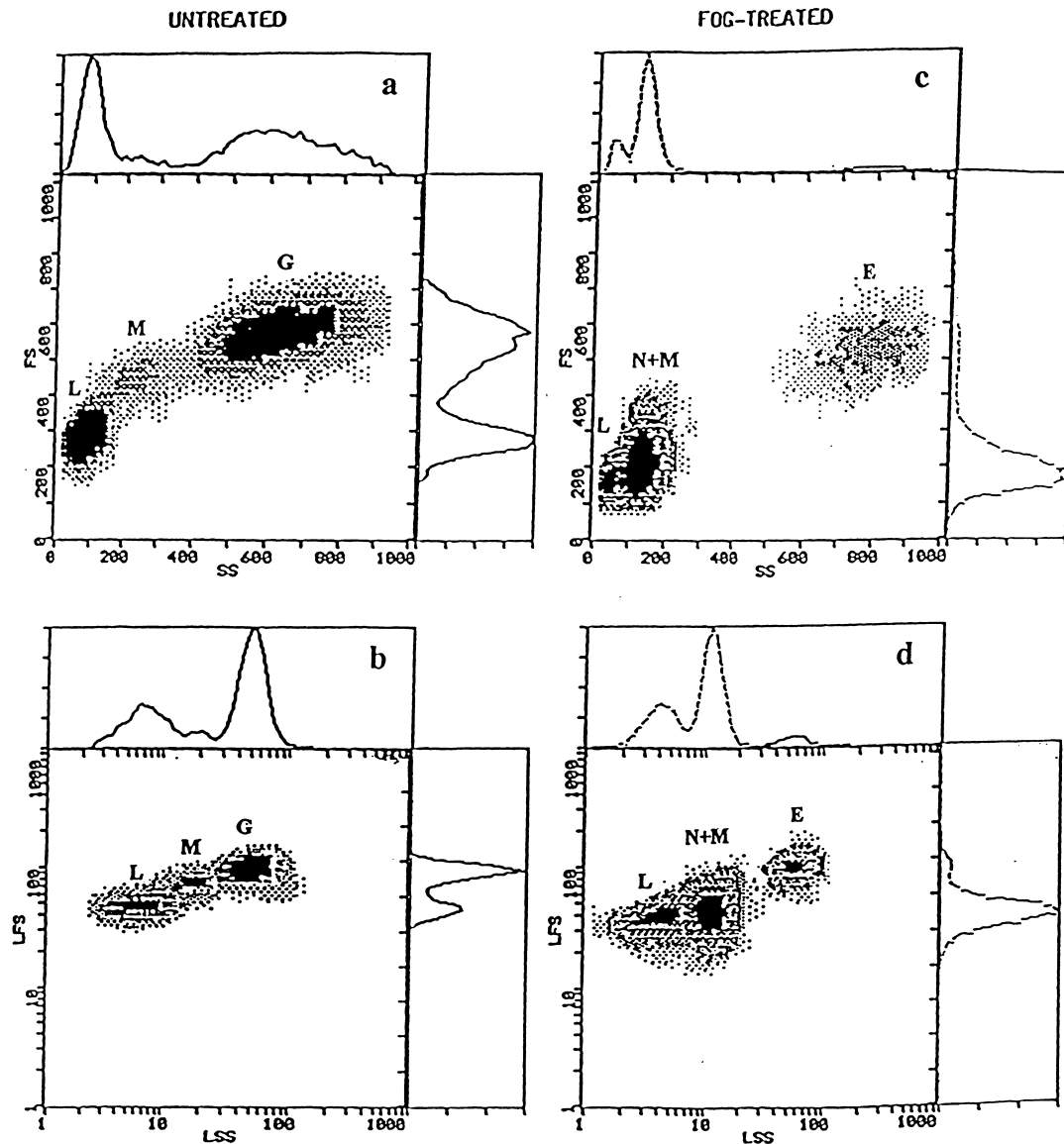


Figure 1. Effect of FOG treatment of scatter properties of distinguishable leukocyte subsets in unseparated peripheral blood. Leukocyte populations are indicated in two-parameter scatter-plot histograms by either linear (a, c) or logarithmic amplification (b, d). Histograms are from representative experiment. L: lymphocytes, M: monocytes, G: granulocytes, N: neutrophils, E: eosinophils, FS: forward scatter, SS: side scatter.

intracellular immunostaining for CD69 ($n=7$) (mean [minimum–maximum]). *In vitro* stimulation with GM-CSF (0.2 $\mu\text{g}/\text{ml}$ for 120 min) induced a significant ($P>0.05$) decrease in the proportion of eosinophil, but not neutrophil ($P=0.063$), expression of intracellular CD69; 64.1% (52.1–70.3%) and 96.7% (92.2–98.0%), respectively ($n=7$) (Fig. 3).

Surface expression of CD69 on eosinophils and neutrophils upon *in vitro* stimulation with GM-CSF, IL-5, IL-5 plus eotaxin, LPS, and fMLP

To investigate *in vitro* whether a spectrum of stimuli could mobilize the preformed intracellular pool of CD69, we incubated the leukocytes with RPMI, GM-CSF for 120 min, IL-5 alone for 75 or for 60 min with

additional time with eotaxin for 15 min, or with the bacteria-related products LPS and fMLP for 120 min. The results are summarized in Fig. 4. Only 3.5% (1.2–8.7%) and 0.6% (0.4–1.1%) of nonstimulated eosinophils and neutrophils, respectively, were positively stained for surface CD69 ($n=7$).

Incubation with GM-CSF resulted in a significant increase in the surface expression of CD69 on eosinophils ($P<0.005$) (86.0% [62.6–87.6%]), but not on neutrophils (4.4% [1.6–6.6%]). Incubation with IL-5 increased significantly ($P<0.05$) the proportion of CD69-positive eosinophils (49.2% [34.3–68.2%]) ($n=7$). No further significant increase in CD69-positive eosinophils (42.9% [40.76–59.4%]) was obtained when they were incubated with IL-5 in

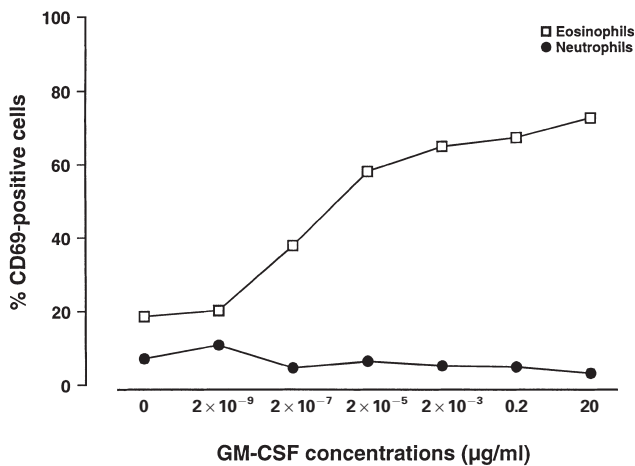


Figure 2. Distribution of surface-expressed CD69 on eosinophils and neutrophils after incubation with different concentrations of GM-CSF. Results are expressed as mean (range) ($n=3$).

combination with eotaxin. Hardly any neutrophils expressed CD69 on the cell surface, either before (0.2% [0.1–0.9%]), or after stimulation with IL-5 alone (1.9% [1.5–2.3%]) or in combination with eotaxin (2.2% [3.3–1.9%]) ($n=7$).

After stimulation with LPS for 120 min, we detected a significantly higher expression ($P<0.005$) of CD69 on 40.7% (27.9–49.7%) of eosinophils than on nonstimulated cells. Only 2.2% (1.6–4.5%) of neutrophils stained positive for CD69 after LPS stimulation ($n=7$). A similar pattern was noted after incubation with fMLP, which significantly ($P<0.05$) increased the CD69 expression on the eosinophils to 17.1% (5.5–24.7%), and to 4.3% (2.0–5.0%) on neutrophils ($n=7$) (Fig. 3).

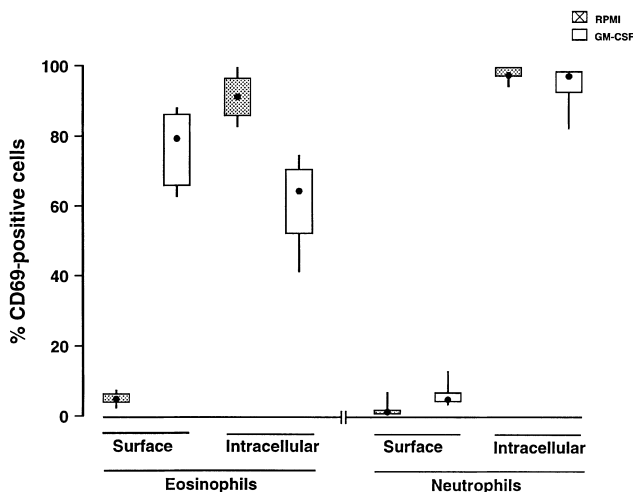


Figure 3. Distribution of intracellular and surface-expressed CD69 in eosinophils and neutrophils. Intracellular expression of CD69 in resting (open) eosinophils and neutrophils after stimulation with GM-CSF 2×10^{-7} g/ml for 120 min (filled). Surface expression of CD69 on resting (hatched) eosinophils and neutrophils after stimulation with GM-CSF 2×10^{-7} g/ml for 120 min (cross-hatched). Boxes enclose interquartiles with medians and ranges marked ($n=7$).

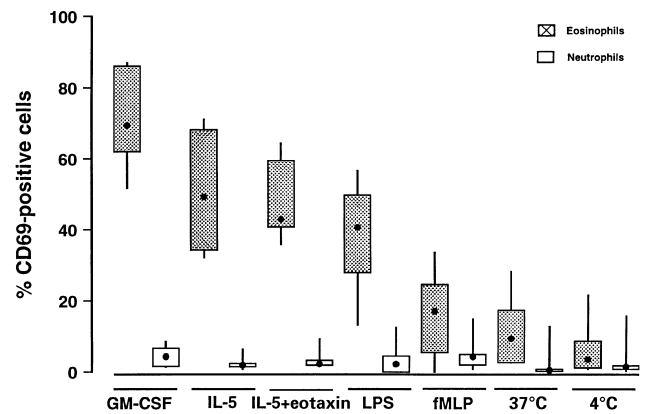


Figure 4. Surface expression of CD69 on eosinophils (filled) and neutrophils (open) after *in vitro* incubation with different stimuli. Boxes enclose interquartiles with medians and ranges marked ($n=7$).

To determine whether CD69 upregulation on eosinophils is due to a direct or a cascade effect, we incubated purified eosinophils with selected stimuli. With all stimuli except LPS, the results were similar to those when mixed leukocytes were used. When purified eosinophils were used, we detected a significantly lower expression ($P<0.05$) of CD69 after LPS stimulation.

Time course of eosinophil surface and intracellular expression of CD69 and EG2 epitope in relation to ECP release

Incubation with GM-CSF resulted in a time-related progressive increase of CD69 expression on eosinophils. At 60 min of incubation, the proportion of CD69-positive eosinophils was 23.1% (17.3–29.6%); at 120 min, it was 71.0% (62.0–86.0%). Incubation with RPMI alone at 37°C for different time periods (0, 5, 15, 30, 60, or 120 min) did not cause any increase in CD69 expression ($n=7$) (Fig. 5).

Eosinophils incubated in GM-CSF for different time periods showed a continuous increase in intracellular expression of the EG2 epitope; at 120 min, there was a significantly higher expression ($P<0.05$) (42.9% [29.4–49.2%] MFI) ($n=7$) than in nonstimulated eosinophils (24.7% [22.8–28.2%] MFI) ($n=7$) (Fig. 6).

ECP release was examined in the supernatant after stimulation with GM-CSF 2×10^{-7} g/ml ($n=7$) at different time points (0, 5, 15, 30, 60, and 120 min). Stimulation with PMA 10^{-7} M for 15 min was used as a positive control for degranulation ($n=7$). The amount of ECP released increased progressively to 57.8 (35.3–134.0) µg/ml after stimulation with GM-CSF for 120 min, while the level of ECP released after incubation with RPMI alone progressively increased to 29.9 (22.7–48.6) µg/ml (Fig. 7). Stimulation with PMA increased ECP levels in the supernatant gradually over time, and reached >24.0 µg/ml at 15 min (Fig. 7).

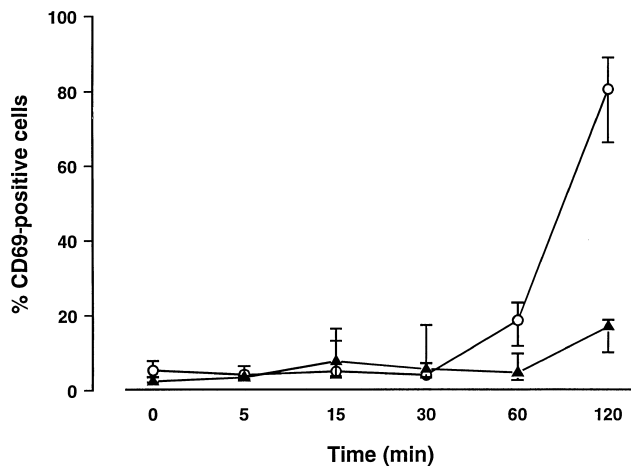


Figure 5. Expression of CD69 after *in vitro* incubation with RPMI (filled symbols) and GM-CSF (open symbols) at different time periods ($n=7$).

Surface and intracellular expression of CD69 in monocytes

To investigate whether nonstimulated monocytes also possess a preformed intracellular pool of CD69, permeabilized leukocytes from healthy blood donors were analyzed. Our results showed that 7.1% (5.08–7.31%) of the monocytes stained positive for intracellular CD69 ($n=7$). The corresponding value for surface staining was 0.5% (0.1–1.8%) (Fig. 8). Hardly any nonstimulated monocytes, (7.1% [5.1–7.3%]) expressed CD69 on the cell surface. Control cells, incubated with RPMI alone for 120 min, increased their CD69 expression to 28.9% (10.1–43.7%) (Fig. 8).

To investigate whether platelets, which are known to be adhesive (33, 34) and to coexpress CD69, could contribute to the overall CD69 expression on monocytes, we immunostained mixed leukocytes with the platelet-specific monoclonal antibody, anti-CD61 ($n=7$). We found that 18.0% (23.6–25.0%) of non-stimulated monocytes stained positive for CD61.

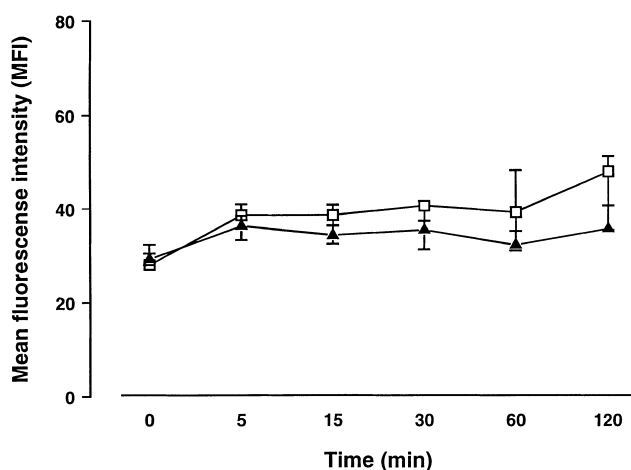


Figure 6. Expression of EG2 epitope on intracellular ECP in eosinophils after *in vitro* incubation with RPMI (filled symbols) and GM-CSF (open symbols) at different time periods ($n=7$).

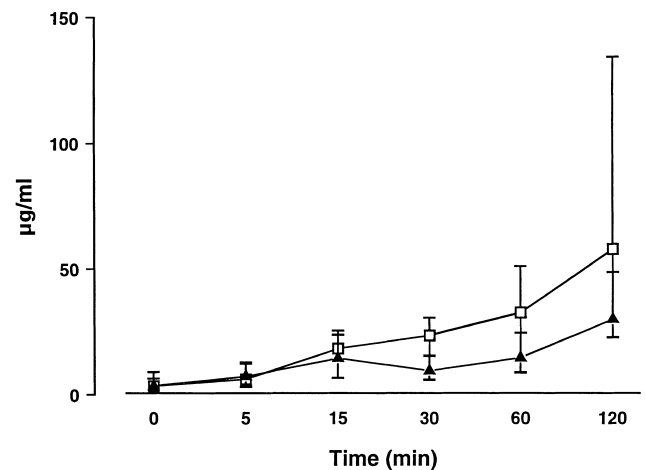


Figure 7. Level of released ECP after *in vitro* incubation of mixed leukocytes with RPMI (filled symbols) and GM-CSF (open symbols) at different time periods ($n=7$).

Incubation with GM-CSF 0.2 µg/ml for 120 min increased the percentage of CD61-positive monocytes significantly ($P<0.005$) to 30.5% (23.6–47.7%). Moreover, separation of mononuclear cells with Ficoll-Paque induced a significantly higher CD61 expression on stimulated monocytes ($P<0.001$) (43.1% [25.8–47.9%]) than on nonstimulated monocytes. Increased CD61 expression was parallel with increased CD69 expression (data not shown).

Discussion

In this study, the regulation of CD69 in human peripheral blood eosinophils and neutrophils from healthy individuals was investigated in relation to *in vitro* activation with GM-CSF, IL-5, IL-5 plus eotaxin, LPS, and fMLP. We were able to demonstrate a preformed intracellular pool of CD69 in both eosinophils and neutrophils. The intracellular pool of CD69

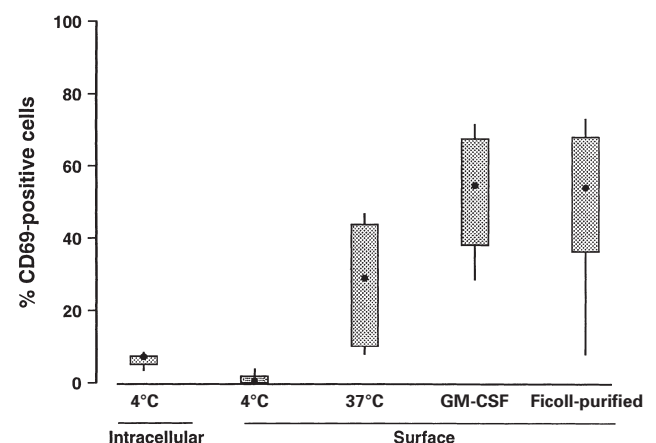


Figure 8. Distribution of intracellular and surface-expressed CD69 in monocytes. Boxes enclose interquartiles with medians and ranges marked ($n=7$).

was mobilized on the cell surface on eosinophils to different degrees by selected stimuli, with GM-CSF being the most efficient and fMLP the least efficient stimulus. In contrast, CD69 in neutrophils was not mobilized on the surface under these experimental conditions.

Previous reports have identified CD69 on different cell types, including activated T cells, B cells, NK cells, monocytes, neutrophils, and platelets (18, 21–25). In our present study, we demonstrated that almost all PBE and PBN have a preformed intracellular pool of CD69, and approximately 5% of resting PBE and 1% of resting PBN from healthy individuals express the antigen on the surface. These figures are in line with previously reported data (25, 34). However, it has been proposed that eosinophils recovered from BAL in patients with asthma (27) and eosinophilic pneumonia (28, 35) do express cell-surface CD69. Furthermore, stimuli such as GM-CSF, IL-3, IL-4, IL-5, IL-13, and IFN- γ can upregulate CD69 on eosinophils *in vitro* (27, 28, 34, 36, 37).

Eosinophils are derived from multipotent stem cells in the bone marrow in the presence of eosinophilopoietic cytokines such as IL-5 and GM-CSF. Besides promoting the terminal differentiation of the eosinophil precursors, these cytokines enhance different aspects of eosinophil function, such as chemotaxis, prolonged survival, and activation (38). When measuring the surface expression of CD69 on eosinophils and neutrophils after *in vitro* stimulation with cytokines such as GM-CSF and IL-5, we observed an increased expression of CD69 on eosinophils, but not on neutrophils. In another series of experiments, we detected a decrease in the intracellular pool of CD69 in PBE after stimulation with GM-CSF, whereas the preformed pool in neutrophils was not significantly changed. However, we noted a significantly increased number of surface CD69 on neutrophils, an effect which was less pronounced than on eosinophils. These results indicate that different mechanisms regulate the cell-surface mobilization of CD69 in eosinophils than in neutrophils.

To investigate whether additional stimuli, more related to infections, also may upregulate CD69, we used the bacteria-related products LPS and fMLP. Approximately 50% of the eosinophils responded with CD69 surface upregulation after LPS stimulation, and approximately 17% responded after fMLP stimulation. These results indicate that stimuli related to bacterial infection are less efficient than more eosinophil-specific stimuli, in the context of CD69 upregulation properties, and that the observed differences between these families of stimuli are more quantitative, than qualitative in nature. When we used LPS as stimulus, a significantly lower ($P < 0.01$) degree of CD69 expression was measured on purified eosinophils than on eosinophils in mixed leukocytes. This might be due to a cascade

effect whereby LPS activates the monocytes, which produce a factor or factors that promote CD69 upregulation. It is plausible that such a factor is GM-CSF, which is released upon LPS stimulation (39).

The neutrophils did not respond with CD69 upregulation under the experimental conditions used in the present study. The function of CD69 in neutrophils is not fully understood, and our results do not exclude the possibility that CD69 may be mobilized on the cell surface on neutrophils under different *in vitro* conditions.

In contrast to De Maria et al.'s report that CD69 is expressed on all purified monocytes (24), our results show that the main body of nonstimulated peripheral blood monocytes, without prior steps of purification, neither have a preformed intracellular pool nor express CD69 on the cell surface. The reason for this conflicting finding is unclear, but previous studies have shown that platelets, which are known to express CD69 (26), adhere to monocytes under different *ex vivo* conditions (40, 41). Our presented data demonstrate that purification with gradient centrifugation results in a higher number of CD61-positive monocytes than nonseparated monocytes, indicating that adherence of platelets to monocytes occurs during the separation process. Since platelets constitutively express CD69, *ex vivo* adherence of platelets to monocytes may be a plausible explanation of previous reports of CD69-positive monocytes.

In this study, we compared the expression of CD69 with other activity markers, the intracellular expression of the EG2 epitope on ECP, and the release of ECP. Stimulation with GM-CSF resulted in a pronounced upregulation of CD69 that peaked late during the actual incubation period. This kinetic pattern was in contrast to the EG2 and ECP pattern, which showed a more gradual and moderate increase, without any pronounced peak during late incubation. These results indicate that increased expression of the EG2 epitope, and the ECP release, precede the main upregulation of CD69, upon GM-CSF stimulation. The eventual impact of these findings is not clear, but may indicate that a kinetic order exists between degranulation and CD69 upregulation, and that the physiologic role of surface CD69 is primary after the initiation of the degranulation process. In line with this hypothesis is the notion that ligation of anti-CD69 induces apoptosis in eosinophils (42). One may hypothesize that a faulty upregulation of CD69 may play an important role in defect apoptosis, which may lead to prolonged survival of inflammatory cells in a state that permits degranulation. The consequence of this might be increased tissue damage, as observed in asthma and lung fibrosis.

In summary, our results show that both eosinophils and neutrophils have a preformed intracellular pool of CD69, and that hardly any eosinophils and neutrophils in the circulation of healthy individuals express CD69

on the cell surface. The intracellular pool can be mobilized on the surface of eosinophils, but not of neutrophils, by GM-CSF, IL-5, and LPS and, to a lesser extent, by the bacteria-related product fMLP. This indicates that a more quantitative than qualitative difference in CD69 upregulation abilities among stimuli exists. Moreover, our data indicate that a kinetic order exists between the degranulation process and CD69 upregulation, and that CD69 is an activity marker that appears later during activation than the increased expression of the EG2 epitope and the ECP release.

For further delineation of the role of CD69 on PBE and PBN in allergic disorders as compared to bacterial infections, analysis of CD69 expression on PBE and PBN from patients with inflammatory processes of different magnitude and nature would seem to be important.

Acknowledgments

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