

Regulation of the release of eosinophil cationic protein by eosinophil adhesion

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Summary

Background Varying release of eosinophil granule proteins depending on the stimulus and environmental factors has previously been reported.

Objective To investigate the degranulation from adherent eosinophils by using mixed granulocytes.

Methods Granulocytes isolated by Percoll gradient centrifugation were incubated on plates coated with plasma and tissue fibronectin, fibrinogen or human serum albumin (HSA) and stimulated with Mn^{2+} , phorbol-myristate-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (f-MLP) and combinations thereof, respectively. The release of eosinophil cationic protein (ECP) was measured by radioimmunoassay.

Results Unstimulated eosinophils incubated in wells coated with plasma and tissue fibronectin, fibrinogen or HSA did not release any ECP. Furthermore, Mn^{2+} (5 mmol/L) did not induce release of ECP despite the fact that adhesion of eosinophils to these four proteins was induced. PMA stimulated a dose-dependent release of ECP. Contemporaneous stimulation of eosinophils with PMA and Mn^{2+} induced a dramatically increased release of ECP regardless of which protein the eosinophils were adhering to. A small but significant release of ECP was found when eosinophils incubated on plates coated with fibrinogen and HSA were stimulated by f-MLP. Contemporaneous stimulation of eosinophils with f-MLP and Mn^{2+} did not induce any synergistic effect on the release of ECP. On the contrary, Mn^{2+} inhibited the release of ECP induced by f-MLP from eosinophils. Serum-opsonized Sephadex particles stimulated a potent increase of the release of ECP up to 12%–14% in the presence of plasma fibronectin and, in particular, fibrinogen. The kinetics of eosinophil adhesion and degranulation showed that the cellular adhesion preceded the degranulation response and that the degranulation patterns depend on the stimuli and environment.

Conclusion The present study indicated that cellular adhesion plays an important role in the regulation of eosinophil degranulation, but that adhesion and degranulation can be induced separately.

Keywords: adhesion, $\beta 1$ integrins, $\beta 2$ integrins, degranulation, eosinophil, f-MLP, PMA, very late activation antigen

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Introduction

The eosinophil granulocyte is a potent inflammatory cell that is considered to play an important role in allergic

inflammation [1]. Deposition of eosinophil granule proteins and concomitant tissue damage have been documented, i.e. increased concentrations of eosinophil cationic protein (ECP) in samples of serum, sputum and bronchoalveolar fluid of patients with bronchial asthma [2]. Although both the release of ECP from eosinophils and, to a lesser extent, the release of myeloperoxidase (MPO) from neutrophils was increased in bronchial asthma, the number of eosinophils

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were significantly increased, whereas the number of neutrophils was normal [3]. In addition, the function of these two types of granulocytes are regulated by different mechanism [4], for instance, the functions of these granulocytes can be specifically regulated by the adhesion molecules VLA-4 and CD18, of which the former receptor is only expressed on eosinophils and the latter expressed on both cells.

Previous investigations have shown that cellular adhesion affects eosinophil degranulation. Eosinophil degranulation induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) or platelet-activating factor (PAF) was inhibited in stirred suspension of eosinophils and almost completely inhibited by anti-CD18 [5]. Integrin receptors are the most important adhesion molecules in leucocytes. It has been reported that adherence of neutrophils via $\beta 2$ integrins acts as a costimulus with cytokines for induction of the respiratory burst [6]. Taken together, it is clear that integrins can act as true signalling receptors in a variety of cell types [7]. On the other hand, the substance to which the cells adhere via $\beta 2$ and $\beta 1$ integrins might also affect the degranulation of cells. Previous studies have demonstrated that extracellular matrix proteins attenuated activation and degranulation of stimulated eosinophils [8].

From the results described above it is evident that the degranulation process can be affected by adhesion. The aim of the present study was to establish and compare the effect of contemporaneous stimulation of release of ECP by soluble and particulate stimuli during eosinophil adhesion to tissue and plasma fibronectin, fibrinogen and albumin. Granulocytes were isolated from the peripheral blood of normal, nonallergic subjects and degranulation was induced by phorbol-myristate-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (f-MLP) and serum-opsonized Sephadex G-15 particles, and eosinophil release of ECP was examined. The stimuli were chosen to represent different mechanisms of stimulation; PMA activating intracellular protein kinase C (PKC), f-MLP, which binds to its cell surface receptor and activates via a G-protein-dependent pathway, and serum-opsonized Sephadex particles, which mainly activates via binding of C3b to the complement receptor 3 (CR3-CD11b/CD18). Manganese ions were used to study the effect of specific integrin activation [9–11]. Furthermore, the eosinophil release response was compared with eosinophil adhesion response.

Materials and Methods

Material

Human plasma fibronectin, human tissue fibronectin, fibrinogen and human serum albumin (HSA) were purchased from Sigma Chemical Co (St Louis, Mo, USA). HSA was 96–99% albumin and the remainder was mostly globulins.

The monoclonal antibody (mAb) used in this study was anti-CD9-FITC (DAKO, A/S, Glostrup, Denmark). Sephadex G-15 was obtained from Pharmacia Biotech (Uppsala, Sweden). Propidium iodide (PI) was purchased from Orpegen Pharma (Heidelberg, Germany).

Preparation of protein-coated wells

Ninety-six well, flat-bottom, nontissue culture-treated, high protein binding plates (Greiner Labortechnik, Germany) were coated with plasma and tissue fibronectin, HSA and fibrinogen, 100 μ L, diluted in coating buffer (20 mmol $\text{NaH}_2\text{PO}_4/\text{L}$, 150 mmol NaCl/L , pH 8.0) at concentrations of 25 $\mu\text{g}/\text{mL}$ (fibrinogen) or 50 $\mu\text{g}/\text{mL}$ (the other three proteins) overnight at 37 °C, in a 5% CO_2 incubator. The wells were washed twice with 200 μ L of the assay buffer containing, NaCl (140 mmol/L), KCl (5 mmol/L), MgCl_2 (1 mmol/L), CaCl_2 (1 mmol/L), glucose (5.6 mmol/L), HSA (30 mg/L) and HEPES (10 mmol/L), with a pH of 7.4, and with 1% HSA (w/v). A further washing was carried out with 200 μ L of the same buffer with and without Mn^{2+} (5 mmol/L) just before use.

Isolation of granulocytes and eosinophils

Granulocytes were isolated from peripheral blood of non-allergic, normal volunteers by Percoll gradient centrifugation as previously described [12]. The purity of the granulocytes was >99% and 6 ± 3 (SD) % were eosinophils. The purified granulocytes were washed twice with the assay buffer plus 1% (w/v) HSA and subsequently suspended in the same buffer. The cell concentration was adjusted to $1 \times 10^9/\text{L}$.

For some of the experiments, isolation of eosinophils was performed according to the procedure described by Hansel *et al.* [13]. In short, granulocytes were isolated according to the procedure described above. The granulocytes were then incubated for 1 hour at 4 °C with magnetic beads coated with anti-CD16 (Miltenyi Biotech, Bergish Gladbach, Germany). Eosinophils and neutrophils were thereafter separated on a steel matrix column. The neutrophils with anti-CD16 beads attached stayed in the column, whereas eosinophils were eluted from the column. The purity of the isolated eosinophils was $\geq 99\%$.

Degranulation of eosinophils during adhesion to proteins

In brief, purified granulocytes or eosinophils were incubated with PMA (1×10^{-9} $\mu\text{mol}/\text{L}$), f-MLP (1×10^{-7} $\mu\text{mol}/\text{L}$) and Mn^{2+} (5 mmol/L) in protein-coated wells. The suspensions of granulocytes or purified eosinophils, 100 μ L at 1×10^9 cells/L or at the concentration of eosinophils, which

was equal to that in the normal population of granulocytes, in the assay buffer plus 1% HSA were added in triplicate to the wells and incubated for varying periods of time up to 90 min, in a humidified incubator at 37 °C and 5% CO₂. The supernatants were aspirated and centrifuged at 600g for 10 min at room temperature. The supernatants were then collected carefully and mixed with an equal volume of N-cetyl-N,N,N-trimethyl-ammonium bromide (CTAB)-solution (0.5% (w/v) CTAB, 0.15 mol NaCl/L). The ECP concentration of the supernatants were analysed by a double antibody competition radioimmunoassay as described earlier (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) [14]. Total intracellular ECP contents extracted from granulocytes solubilized with CTAB detergent solution were measured in parallel. Results from the degranulation experiments were expressed in percentage of the total amount (100%) of ECP in the respective population of eosinophils.

In the PMA stimulation experiments, granulocytes were pre-incubated with PMA for 15 min in a water bath at 37 °C before the cells were added to protein-coated wells. In the f-MLP and Mn²⁺ stimulation experiments, granulocyte suspensions with f-MLP were added to the protein-coated wells immediately.

Serum-opsonized Sephadex G-15 particles were prepared as described previously [3]. One volume of washed serum-opsonized Sephadex particles was mixed with two volumes of granulocytes with or without Mn²⁺ (5 mmol/L). The final cell concentration was 1 × 10⁹ cells/L and the final concentration of serum-opsonized Sephadex particles was 27.8 g/L. One hundred µL samples of this mixture of cells and particles were added to the protein-coated wells and incubated for 30, 60 and 90 min. In some experiments cell suspensions, with or without Mn²⁺, were added to coated wells and kept on ice for 15 min before the particles were added. The incubation was performed for 30 min at 37 °C in a 5% CO₂ atmosphere. The final concentrations of cells, particles and Mn²⁺ were the same as mentioned above.

Adhesion assay

Eosinophil adhesion was determined by measuring the ECP content of adherent cells by radioimmunoassay (RIA) (Pharmacia & Upjohn Diagnostics AB) as previously described [12]. Briefly, after the specified time of incubation, the supernatants were collected and stored for the degranulation assay. The wells were gently washed three times with warm (room temperature) assay buffer to remove nonadherent cells. The adherent cells were solubilized by the addition of 100 µL CTAB detergent solution. The ECP content in the extracts was measured by RIA and the relative number of adherent eosinophils (%) was calculated from a standard curve made of the total amount of ECP extracted from the cells initially added, as described above.

Effect of anti-CD18 and anti-CD49d on eosinophil adhesion and release of ECP

The inhibitory effect of mouse monoclonal antibodies against CD18 (clone L 130, Becton Dickinson, San Jose, CA, USA) and CD49d (clone HP2/1, Immunotech, Marseilles, France) was tested by co-incubation of granulocytes (1 × 10⁹/L) stimulated by PMA (1 × 10⁻⁹ mol/L) and Mn²⁺ (5 mmol/L) with the antibodies, at the final concentration of 10 µg/mL, in plasma fibronectin- and fibrinogen-coated wells. After 90 min of incubation samples for the determination of eosinophil release of ECP and eosinophil adhesion were obtained and processed as described above.

Measurement of cell death by propidium iodide and trypan blue uptake

Granulocytes (1 × 10⁹ cells/L in the assay buffer plus 1% HSA) with different stimuli (Mn²⁺ particles, PMA + Mn²⁺ or f-MLP + Mn²⁺) or without stimuli were added into wells coated with plasma fibronectin and fibrinogen in quadruplicate. After 90 min of incubation in an incubator at 37 °C and 5% CO₂, the plates was put on ice and 10 µL of optimally-titrated mAb CD9 and 7 µL propidium iodide (0.83 mg/L) were added to each well and incubated for 30 min and then the unadherent cells were transferred to tubes. After two final washings of the wells with the assay buffer, the samples were centrifuged and the supernatants removed. The adherent cells were detached by adding 100 µL EDTA solution (10 mmol/L, pH 7.4) to each well and incubated for 20 min at room temperature. After collecting these cells, they were resuspended in the assay buffer at a concentration of 1 × 10⁹ cells/L and analysed in an EPICS XL flow cytometer (Coulter Corporation, Miami, FL, USA). Eosinophils were separated from the neutrophils by the use of FITC-labelled anti-CD9 [15]. The relative amount of eosinophils stained with PI was measured.

To the wells with cells stimulated by serum-opsonized Sephadex G-15 particles, 500 µL EDTA solution was added to the tubes with cells and particles and incubated for 20 min at room temperature. The supernatant was collected by shaking the tube and then leaving it for 1–2 min. Owing to this treatment the particles sedimented, whereas the cells remained in the supernatant. The particles were washed twice more with EDTA-solution to detach the cells from the particles. The relative number of eosinophils stained with PI was measured as above.

For the trypan blue exclusion experiments, 50 µL granulocytes suspension with Mn²⁺, PMA + Mn²⁺ or f-MLP + Mn²⁺ were added to plasma fibronectin- and fibrinogen-coated wells and incubated for 90 min at 37 °C with 5% CO₂ in a humidified atmosphere. The supernatants were carefully aspirated. Fifty µL trypan blue solution was added to each

well followed by incubation for 5 min at room temperature. The trypan blue solution was gently aspirated and 100 μ L of PBS added to each well. The relative number of cells stained with trypan blue were evaluated in a blind fashion with an inverted microscope.

Statistical analysis

Data were analysed using the paired student's *t*-test by the use of CSS Statistica (Statsoft Inc, Tulsa, OK).

Results

Establishment of the optimal concentration of PMA and manganese ions

To examine eosinophil degranulation during adhesion to surfaces coated with extracellular matrix proteins, the optimal concentrations of PMA and Mn^{2+} were established. Cells were added to protein-coated wells and incubated for 1 h after pre-incubation for 15 min at 37 °C in a water bath. PMA induced a dose-dependent release of ECP regardless of whether eosinophils adhered to plasma fibronectin or fibrinogen. At the concentration of 1×10^{-8} mol/L PMA, a significant release of ECP from eosinophils adhering to both plasma fibronectin and fibrinogen was observed (Fig. 1a,b, $P < 0.005$, respectively). The release of ECP induced by PMA plus Mn^{2+} reached a plateau at the concentrations of 1×10^{-9} mol/L PMA and 5 mmol/L Mn^{2+} in presence of plasma fibronectin and fibrinogen ($P < 0.05$, respectively). No significant release of ECP was detected when eosinophil adhesion to plasma fibronectin or fibrinogen was induced by Mn^{2+} at the concentrations from 0.5 to 15 mmol/L.

Time course of the effect of PMA and Mn^{2+} on the degranulation of eosinophils

According to above experiments, the concentrations of 1×10^{-9} mol/L PMA and 5 mmol/L Mn^{2+} were chosen to investigate the time-dependent release of ECP when eosinophils adhered to plasma and tissue fibronectin, fibrinogen and HSA, respectively. As shown in Fig. 2a–d, unstimulated eosinophils did not release any ECP, regardless of whether the cells were incubated in wells coated with fibronectin, fibrinogen or HSA. In addition, Mn^{2+} did not induce any release of ECP in the presence of these four proteins either. PMA stimulated a weak but significant release of ECP from eosinophils adhering to HSA and to fibrinogen ($P < 0.01$ at the incubation time of 90 min, Fig. 2c,d) compared with unstimulated eosinophils, but not from eosinophils adhering to plasma and tissue fibronectin. Furthermore, there was no difference in the levels of

ECP release from eosinophils adhering to HSA and fibrinogen compared with those adhering to fibronectin, plasma and tissue form, respectively. PMA in combination with Mn^{2+} potentially enhanced the release of ECP from eosinophils adhering to the four proteins ($P < 0.05$ – 0.005) compared with PMA alone. This release of ECP was time dependent and the response appeared earlier than that induced by PMA alone, though no significant difference in the levels of release of ECP were found in response to the four different proteins.

Time course of the effect of f-MLP and Mn^{2+} on degranulation of eosinophils

To further assess the mechanism of release of ECP influenced by adhesion, we investigated the effect of f-MLP on the release of ECP when eosinophils were incubated in wells coated with plasma and tissue fibronectin, fibrinogen and HSA, respectively. The concentration of 1×10^{-7} mol/L of f-MLP was found to be optimal (results not shown). As shown in Fig. 3(a,b) f-MLP stimulated a weak and insignificant release of ECP from eosinophils in the presence of plasma and tissue fibronectin. On the other hand, a time-dependent small, but significantly increased, release of ECP was found ($P < 0.05$, at the incubation time of 75 min, Fig. 3c,d, respectively) when eosinophils in the presence of fibrinogen and HSA were stimulated by f-MLP as compared with unstimulated eosinophils. Fibrinogen and HSA supported a higher release of ECP induced by f-MLP at the incubation time of 75 min than plasma and tissue fibronectin ($P < 0.05$, respectively). F-MLP in combination with Mn^{2+} had no synergistic effect on the release of ECP in the presence of the four proteins. On the contrary, this combination inhibited the release of ECP to the background level ($P < 0.05$, for fibrinogen and HSA).

Time course of the effect of serum-opsonized Sephadex particles and Mn^{2+} on degranulation of eosinophils

Serum-opsonized Sephadex particles have previously been used to induce granulocyte degranulation in cell suspensions. The effect of washed serum-opsonized Sephadex particles on the degranulation of eosinophils in the wells coated with plasma fibronectin and fibrinogen was tested. As shown in Fig. 4(a,b), a potent increase of the release of ECP up to 12–14% in response to the washed serum-opsonized Sephadex particles was found in the presence of plasma fibronectin and fibrinogen. At the incubation time of 30–60 min, fibrinogen supported a higher level of release of ECP than plasma fibronectin ($P < 0.005$ – 0.05). The combination of washed serum-opsonized Sephadex particles and Mn^{2+} decreased the release of ECP in the presence of plasma fibronectin ($P < 0.05$) and fibrinogen ($P < 0.001$) at

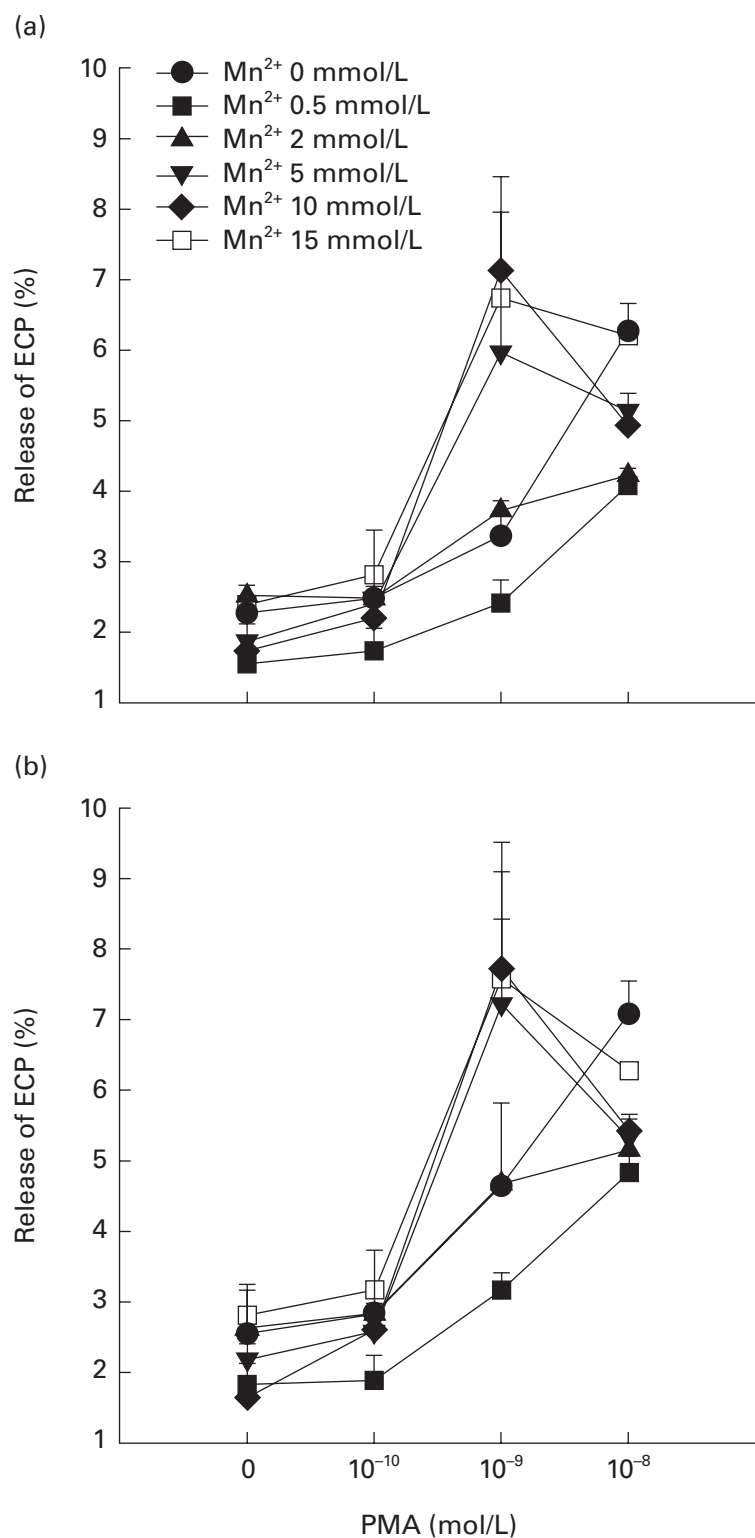


Fig. 1. Dose response of eosinophil release of ECP induced by PMA and Mn^{2+} . The granulocytes with different stimuli were added into the wells coated by plasma fibronectin (a) and fibrinogen (b) and incubated for 1 hour at 37 °C. Data represent the mean $\pm$ SEM of four experiments.

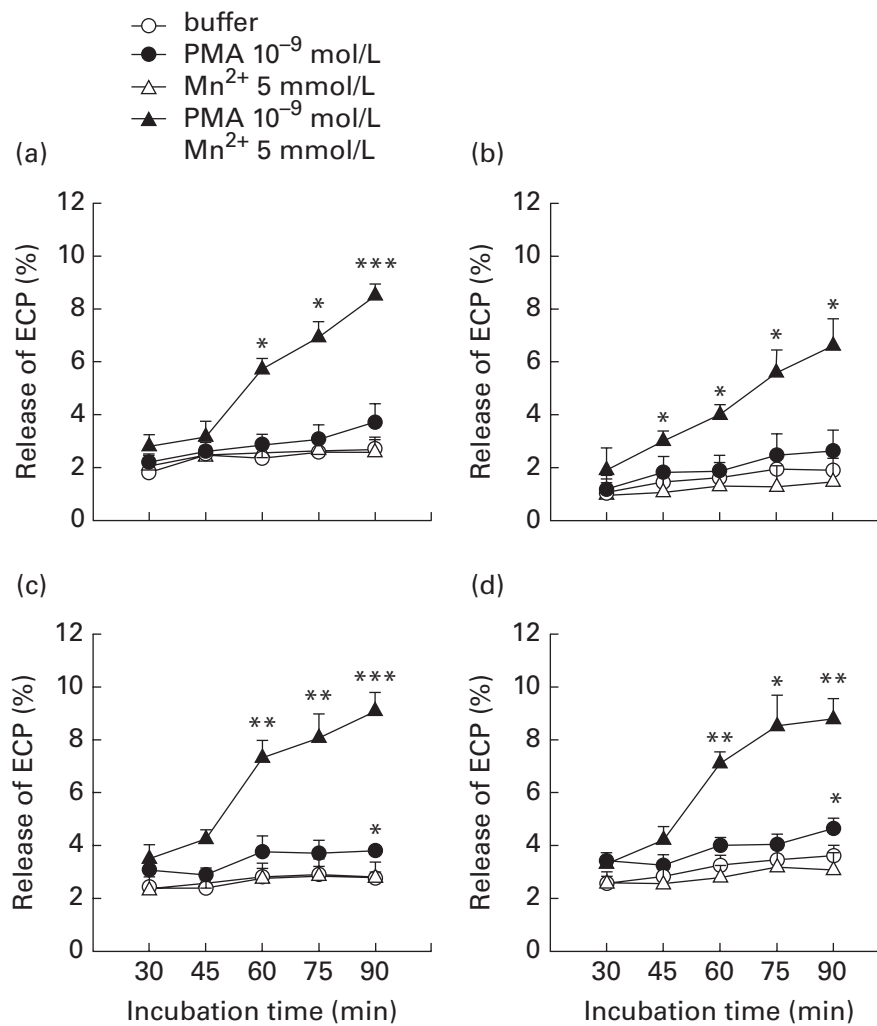


Fig. 2. The time course of the release of ECP induced by PMA, and PMA in combination with Mn^{2+} , during eosinophil adhesion to plasma fibronectin (a) and tissue fibronectin (b), fibrinogen (c) and HSA (d). Results are the mean $\pm$ SEM of four to five experiments. Significantly different results compared with that obtained in buffer only are represented by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

the incubation time of 30 min, when compared with the release of ECP induced by particles alone. At incubation times above 30 min the release of ECP showed the same levels with particles plus Mn^{2+} and with particles alone. There was no difference in the levels of the release of ECP from eosinophils adhering to plasma fibronectin and fibrinogen induced by the particles plus Mn^{2+} .

Release of ECP from isolated eosinophils

The release of ECP from isolated eosinophils was compared with that of eosinophils in the normal granulocyte population, used in the experiments described above, in parallel experiments using cells from the same donor (Table 1). Both after 15 and 90 min incubation, purified eosinophils stimulated by

serum-opsonized particles in the presence of Mn^{2+} showed a greater release of ECP ($P < 0.05$) than eosinophils in the normal population of granulocytes in the presence of fibronectin or fibrinogen. Isolated eosinophils stimulated by f-MLP in the presence of fibronectin showed a slightly higher release of ECP than eosinophils in the normal population of granulocytes. All the other stimuli, however, induced the same degree of ECP release of isolated eosinophils and eosinophils in the normal population of granulocytes.

The relationship between adhesion and degranulation of eosinophils

Figure 5a,b and Fig. 6a,b shows the results of simultaneous measurements of adhesion and degranulation after

Table 1a Comparison of the release of ECP from isolated eosinophils and eosinophils in the granulocyte population. Cells incubated in wells coated with plasma fibronectin

Incubation time	Release of ECP (%)			
	Eosinophils in granulocyte population	Isolated eosinophils	Eosinophils in granulocyte population	Isolated eosinophils
	15 min	15 min	90 min	90 min
<i>Stimuli</i>				
PMA	0.98 ± 0.50	1.76 ± 0.44	4.99 ± 2.78	3.32 ± 0.28
f-MLP	0.70 ± 0.35	1.81 ± 0.29*	6.59 ± 2.54	5.14 ± 0.58
Particles	2.58 ± 0.47	5.91 ± 2.13	8.79 ± 0.53	8.67 ± 0.92
PMA + Mn ²⁺	0.89 ± 0.45	1.30 ± 0.26	5.91 ± 0.89	7.49 ± 0.58
f-MLP + Mn ²⁺	0.74 ± 0.37	1.57 ± 0.16	5.61 ± 0.98	4.68 ± 0.59
Particles + Mn ²⁺	1.56 ± 0.06	4.34 ± 0.33*	9.19 ± 1.04	11.94 ± 1.43*

Table 1b Comparison of the release of ECP from isolated eosinophils and eosinophils in the granulocyte population. Cells incubated in wells coated with fibrinogen

Incubation time	Release of ECP (%)			
	Eosinophils in granulocyte population	Isolated eosinophils	Eosinophils in granulocyte population	Isolated eosinophils
	15 min	15 min	90 min	90 min
<i>Stimuli</i>				
PMA	0.97 ± 0.50	2.12 ± 0.02	5.23 ± 2.25	6.06 ± 0.93
f-MLP	1.12 ± 0.59	2.40 ± 0.46	5.81 ± 2.1	9.32 ± 0.97
Particles	2.56 ± 0.47	5.44 ± 1.78	8.40 ± 0.46	10.40 ± 1.42
PMA + Mn ²⁺	0.90 ± 0.45	2.00 ± 0.45	8.03 ± 0.53	8.14 ± 0.73
f-MLP + Mn ²⁺	0.72 ± 0.36	2.38 ± 0.32	4.47 ± 2.19	5.55 ± 0.29
Particles + Mn ²⁺	1.65 ± 0.13	4.54 ± 0.63*	9.48 ± 1.65	13.14 ± 1.99*

Release of ECP from isolated eosinophils and eosinophils in the granulocyte population was measured in parallel experiments using cells from the same donor. The number of isolated eosinophils was adjusted to the same number as that present in the granulocyte population. The results represent mean ± SEM of three experiments. Statistically significant differences between isolated eosinophils and eosinophils in the granulocyte population are indicated, * $P < 0.05$.

stimulation of cells by different stimuli. Mn²⁺ enhanced the adhesion of eosinophils to fibrinogen and particularly to tissue fibronectin (Fig. 5a,b), but no significant release of ECP was determined up to 90 min of incubation. However, PMA in combination with Mn²⁺ stimulated an increment of both adhesion and release of ECP regardless of the type of protein coating, where adhesion clearly preceded the release. The same pattern was seen when eosinophils adhered to HSA and plasma fibronectin induced by Mn²⁺ or PMA plus Mn²⁺ (data not shown). An insignificant stimulation of the adhesion of eosinophils to both

plasma fibronectin and fibrinogen was induced by f-MLP, meanwhile a small but significantly higher release of ECP was found in the presence of fibrinogen than in the presence of plasma fibronectin (Fig. 6a,b). Moreover, f-MLP plus Mn²⁺ enhanced the adhesion of eosinophils to both plasma fibronectin and fibrinogen, but not the release of ECP. Figure 4(a,b) show a background adhesion to plasma fibronectin and fibrinogen of unstimulated eosinophils of less than 10%. After stimulation of cells by serum-opsonized Sephadex particles, with or without Mn²⁺, almost no eosinophils adhered to these two proteins. During these

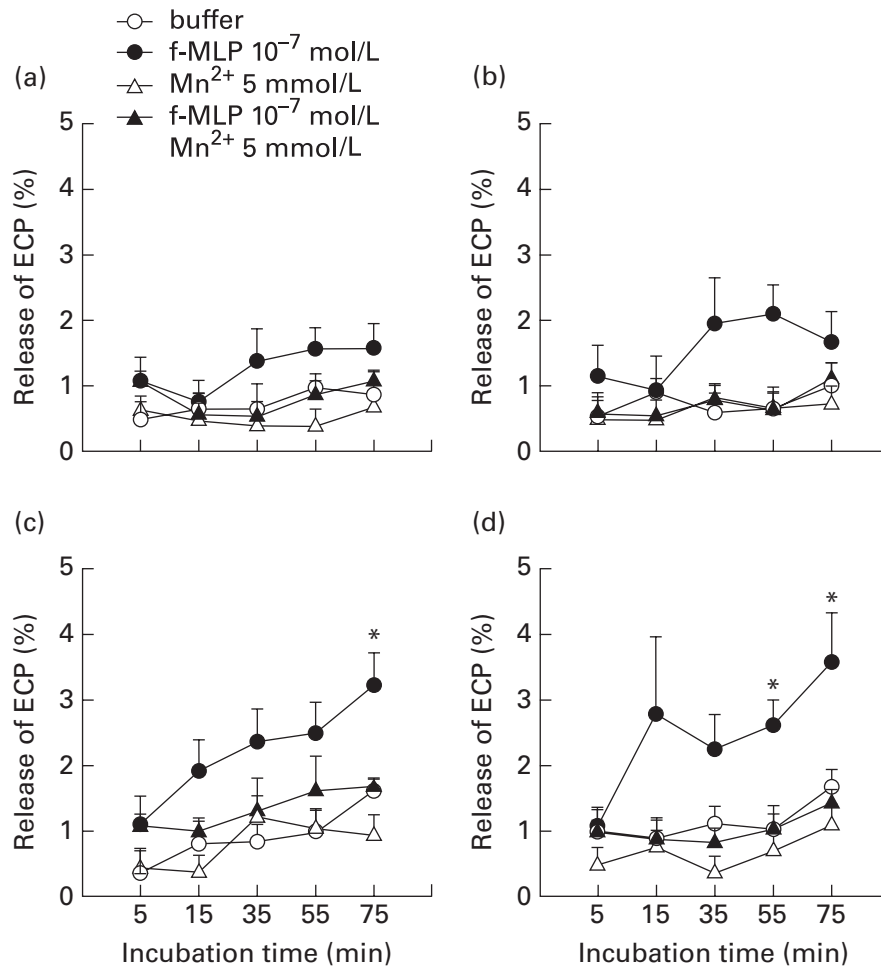


Fig. 3. The time course of the release of ECP induced by f-MLP and f-MLP in combination with Mn^{2+} during eosinophil adhesion to plasma fibronectin (a) and tissue fibronectin (b), fibrinogen (c) and HSA (d). Data represent the mean $\pm$ SEM of four experiments. Significantly different results compared with that obtained in buffer only are represented by * ($P < 0.05$).

Table 2. The relationship between eosinophil activation and cell death

PI-positive eosinophils (%)					Trypan blue-positive granulocytes (%)			
Buffer	Particles	Mn^{2+}	PMA + Mn^{2+}	f-MLP + Mn^{2+}	Buffer	Mn^{2+}	PMA + Mn^{2+}	f-MLP + Mn^{2+}
<i>Fibronectin</i>								
2.1 ± 0.4	$32.6 \pm 2.9^{**}$	$11.8 \pm 0.8^*$	$28.5 \pm 2.8^{**}$	$12.3 \pm 2.6^*$	4.0 ± 2.0	5.9 ± 0.9	$13.4 \pm 2.4^{**}$	6.4 ± 0.8
<i>Fibrinogen</i>								
2.7 ± 0.7	$34.7 \pm 4.2^{**}$	$15.6 \pm 3.4^*$	$31.6 \pm 8.8^{**}$	$15.9 \pm 3.1^*$	4.2 ± 1.5	8.0 ± 0.8	$19.2 \pm 6.2^*$	7.5 ± 1.2

The results shown are the means $\pm$ SEM of six experiments. The concentration of Mn^{2+} was 5 mmol/L, of PMA 1×10^{-9} mol/L, and of f-MLP 1×10^{-7} mol/L. The relative number of PI-positive eosinophils was measured by the use of flow cytometry and eosinophils were identified as CD9 positive granulocytes. Statistically significant differences between stimulated cells and cells in buffer are indicated by * $P < 0.05$ and ** $P < 0.01$.

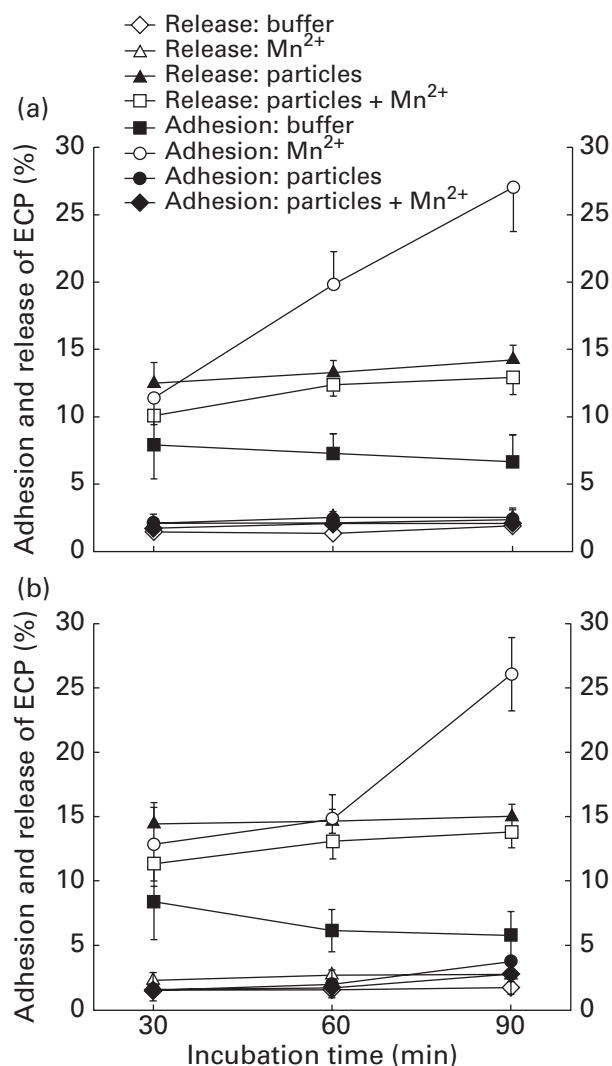


Fig. 4. The time course of the effect of serum-opsonized Sephadex G-15 particles combined with or without Mn^{2+} and Mn^{2+} alone on the release of ECP and eosinophil adhesion in the presence of plasma fibronectin and fibrinogen. Data represent the mean $\pm$ SEM of six experiments.

conditions a significant level of release of ECP was induced, as mentioned before.

The effect of anti-CD18 and anti-CD49d on eosinophil adhesion and release of ECP

The presence of anti-CD18, but not of anti-CD49d, significantly inhibited the release of ECP induced by PMA and Mn^{2+} from granulocytes adhering to fibronectin and fibrinogen (Fig. 7a). Comparison of the effect of anti-CD18 and CD49d on adhesion showed that both CD18 and CD49d inhibited eosinophil adhesion to fibronectin (Fig. 7b)

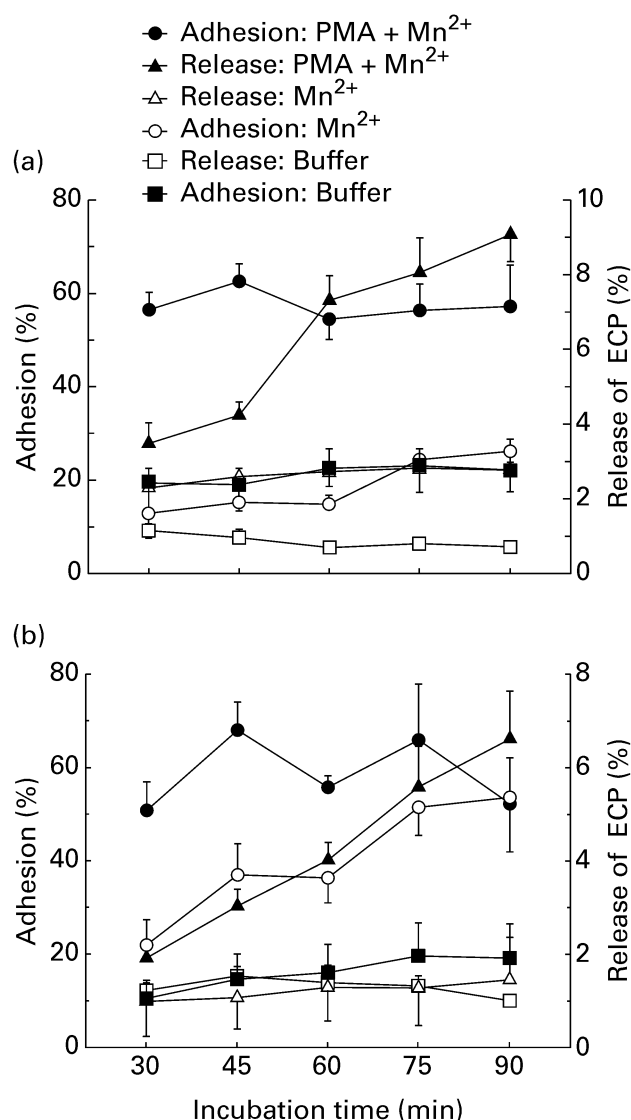


Fig. 5. The relationship between adhesion and degranulation of eosinophils induced by PMA in combination with Mn^{2+} or Mn^{2+} alone from granulocytes adhering to fibrinogen and tissue fibronectin. Data represent the mean $\pm$ SEM of four to six experiments.

whereas only CD18 inhibited the adhesion of eosinophils to fibrinogen.

Relationship between degranulation and cell death in eosinophils

The connection between the release of ECP and cell death was investigated. Granulocytes were incubated in the plasma fibronectin- and fibrinogen-coated wells for 90 min. The cells were then stained with propidium iodide or trypan blue indicating dead cells. The results are summarized in Table 2. Overall, the relative amounts of dead eosinophils were

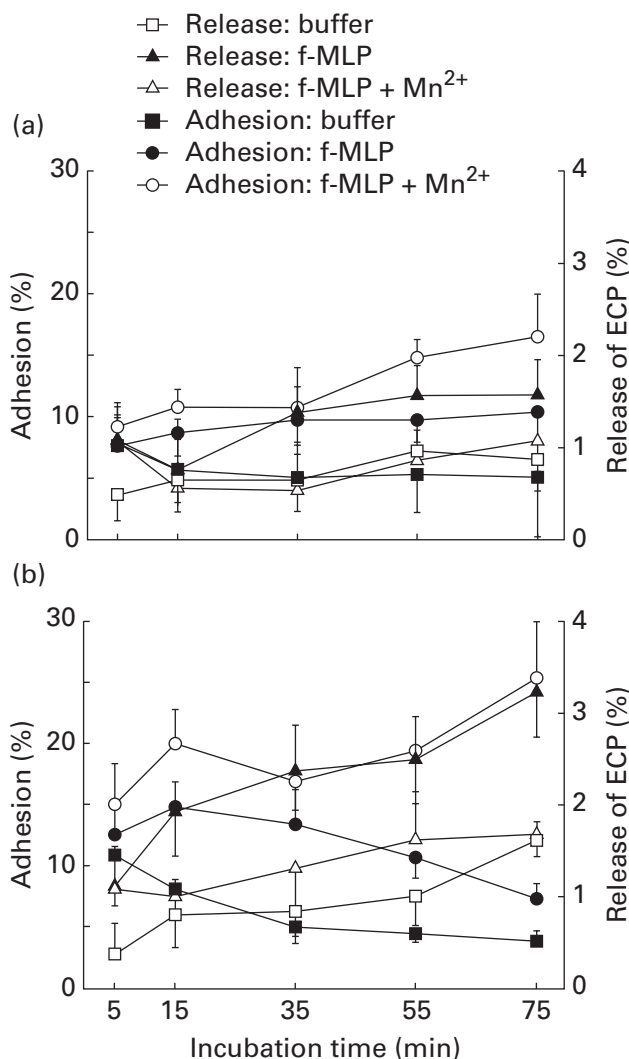


Fig. 6. The relationship of degranulation and adhesion of eosinophils induced by f-MLP or f-MLP plus Mn^{2+} during incubation of granulocytes in wells coated with plasma fibronectin and fibrinogen. Data represent the mean $\pm$ SEM of four experiments.

significantly increased in response to serum-opsonized Sephadex particles and PMA in combination with Mn^{2+} , compared with unstimulated cells. Mn^{2+} alone and f-MLP plus Mn^{2+} stimulated a small but significant cell death of eosinophils compared with unstimulated cells. However, no difference in the levels of dead cells was found in response to plasma fibronectin and fibrinogen alone.

The trypan blue exclusion experiments, even though they indicated a lower proportion of dead cells induced by PMA plus Mn^{2+} , f-MLP plus Mn^{2+} or Mn^{2+} alone, than in the PI experiments, showed a similar pattern of the cell death. In the trypan blue experiments it is, however, impossible to discriminate between eosinophils and neutrophils.

Discussion

The members of the integrin family of adhesion receptors have been reported to possess the intrinsic capability to trigger activation of selective eosinophil functions [16]. Our findings agree with the knowledge obtained from previous studies showing that adhesion molecules are not simply involved in cell–cell or cell–matrix interaction but may also modulate selective cell functions [7]. Manganese ions (0.5–15 mmol/L), which are known to activate integrins, did not induce any ECP release regardless of which protein the cells adhered to. PMA (1×10^{-9} mol/L) on the other hand stimulated a weak and late release of ECP from adherent eosinophils. The release of ECP induced by f-MLP from eosinophils in the presence of these four proteins demonstrated a similar pattern as that of eosinophils stimulated by PMA. A synergistic effect of PMA plus Mn^{2+} on the release of ECP from eosinophils during adhesion to these four proteins was found, whereas an inhibitory effect on the release of ECP induced by f-MLP plus Mn^{2+} was shown in the presence of the same proteins. The serum-opsonized Sephadex particles stimulated a potent release of ECP from eosinophils in the presence of plasma fibronectin, and particularly, of fibrinogen. Contemporaneous stimulation of eosinophils with these particles and Mn^{2+} initially induced a decrease of the release of ECP compared with that induced by particles alone. A release of 4–15% of the total eosinophil content of ECP is under the present experimental conditions equal to 30–100 μ g/l, which are physiologically measurable concentrations. The actual concentrations reached *in vivo* are however, strictly dependent on the number of accumulated eosinophils.

In the present study Mn^{2+} was used to separately activate the integrin receptors on the eosinophils and thereby induce adhesion. Induction of adhesion via $\beta 1$ and $\beta 2$ integrins by Mn^{2+} did not induce any release of ECP. These findings clearly demonstrate that cellular adhesion and degranulation can be separately regulated.

PMA is assumed to exert its action by a direct activation of protein kinase C [17]. Activation by PMA was found to induce a dose-dependent eosinophil degranulation independent of whether the eosinophils were adhering to fibronectin, fibrinogen or albumin. These results suggest that PMA removes the influence of extracellular matrix proteins on eosinophil degranulation, which has been demonstrated to attenuate activation and degranulation of eosinophils stimulated by PAF [5]. Our findings are also consistent with previous a report showing that PMA removes the environmental restrictions on the eosinophil respiratory burst [18]. The amplifying effect of Mn^{2+} -induced adhesion on PMA-stimulated release of ECP suggests that the signal transduction induced by integrin activation by Mn^{2+} and the activation of PKC by PMA synergistically promote

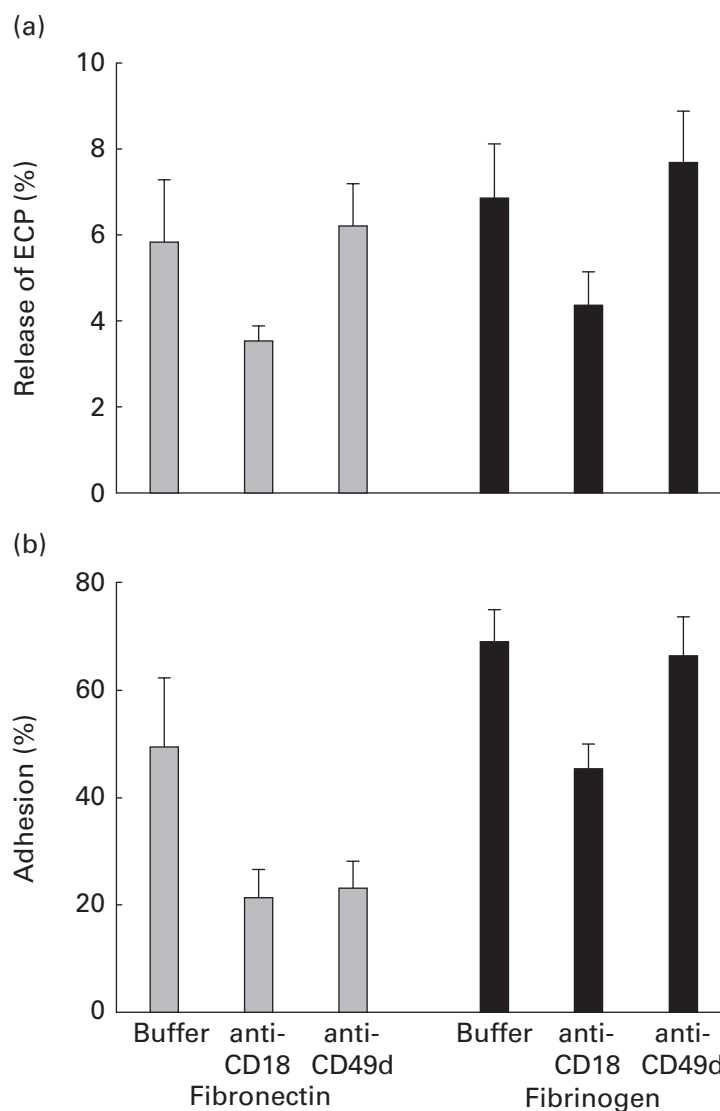


Fig. 7. The effect of anti-CD18 and anti-CD49d on eosinophil adhesion and degranulation from granulocytes adhering to plasma fibronectin and fibrinogen induced by PMA plus Mn^{2+} . The results represent mean $\pm$ SEM of four independent experiments. Significantly different results between results obtained in the presence and absence of antibodies, respectively, are represented by $*(P=0.05)$.

the degranulation of eosinophils. The signal transduction induced by integrin activation is not fully clarified but has been shown to involve phosphorylation of the $\beta 2$ subunit and in a later step also activation of PKC [19–21]. Phorbol esters are also reported to induce phosphorylation of the $\beta 2$ subunit of integrins [21]. Further studies using antibodies to the common $\beta 2$ chain and to the $\alpha 4$ chain of VLA-4 demonstrated that the release induced by PMA and Mn^{2+} in combination was dependent on $\beta 2$ integrins. This has previously been demonstrated in the case of eosinophils adherent to albumin stimulated by PMA [22]. Eosinophil adhesion to fibronectin was as expected inhibited by anti-CD49d [23] but also by anti-CD18. The latter effect could possibly be explained by the newly discovered $\alpha d\beta 2$ integrin [24], which was lately described to function as an alternative ligand for VCAM-1, and which in analogy with VLA-4 might also function as a ligand to fibronectin [25].

In the present study, f-MLP stimulated a low but significant release of ECP from eosinophils incubated in wells coated with fibrinogen and HSA but not from those in wells coated with fibronectin of either form. In a previous study it was demonstrated that chemoattractants can differentially regulate the avidity of $\beta 1$ and $\beta 2$ integrins in eosinophils. F-MLP induced a transient activation and then rapid deactivation of the avidity of VLA-4 but a prolonged activation of Mac-1 avidity [26]. Since previous investigations have demonstrated that eosinophils adhere to fibronectin via $\beta 1$ and $\beta 4$ integrins, to fibrinogen via $\beta 1$ and $\beta 2$ integrins and to HSA via $\beta 2$ integrins [23,26–30], the difference in f-MLP-induced release could thus be explained by a differential effect on $\beta 1$ and $\beta 2$ integrins. Another previous study also demonstrated that adhesion to laminin and fibronectin inhibited eosinophil degranulation induced by PAF [8]. Our results also showed that an increased adhesion to fibronectin

and fibrinogen induced by Mn^{2+} suppressed f-MLP-induced eosinophil degranulation.

Serum-opsonized Sephadex particles mainly activate eosinophils via the interaction between particle-bound C3bi and the $\beta 2$ integrin, CD11b/CD18, and $Fc\gamma RII$ (CD32), on the eosinophils [31–33]. Although the concentration of Sephadex particles in the present experiments was only one fourth of that used in previous experiments, in the absence of proteins, similar levels of release of ECP were obtained. These results indicate that the association between eosinophils and fibronectin, and fibrinogen, respectively, affected the cells even though they did not adhere firmly. The release of ECP was significantly higher in the presence of fibrinogen than of fibronectin, which indicates that priming via $\beta 2$ integrins is stronger than through $\beta 1$ integrins. The fact that activation by Mn^{2+} initially inhibited the release of ECP in the presence of fibronectin could be caused by the attenuation of release induced by adhesion to $\beta 1$ integrins described in a previous publication [8].

The C3b-coated Sephadex particles almost completely inhibited the adhesion to fibrinogen and fibronectin, irrespective of whether Mn^{2+} was present or not, indicating a competition between the activation pathways of particle bound C3b and surface bound fibrinogen/fibronectin. The competition between the activation of $\beta 2$ integrins via C3b and the activation of $\beta 2$ integrins by fibrinogen, and the activation of $\beta 1$ integrins by fibronectin, respectively, was equally potent. One of the mechanisms behind the result of this competition could be sterical hindrance in such a way that the particles prevent the association between eosinophils and fibrinogen/fibronectin. Another mechanism could be that C3b particles are a stronger and more rapid stimulus than fibrinogen/fibronectin. It is thus evident that adhesion via $\beta 1$ and $\beta 2$ integrins could regulate C3b-induced release of ECP from eosinophils.

Release induced by serum-opsonized Sephadex particles and PMA in combination with Mn^{2+} was shown to be associated with a considerable degree of cell death. This is in agreement with a previous publication showing that eosinophil degranulation stimulated by protein-coated beads was accompanied by cell death [34]. The degree of cell death measured in the present investigation was, however, not directly correlated to the degree of release. Significantly higher levels of release of ECP was induced by the particles than by PMA plus Mn^{2+} , whereas the amounts of dead cells were similar, thus indicating that the extent of cell death is dependent on the stimulus. Furthermore, stimulation of eosinophil adhesion to plasma fibronectin and fibrinogen by Mn^{2+} also increased the degree of cell death, but to a lower extent, whereas no considerable release of ECP was measured. The question whether cell death was associated with release due to apoptosis or necrosis is at present under investigation.

The results of the present investigation were obtained using the normally occurring granulocyte population containing approximately 5% eosinophils and 95% neutrophils. Comparison of the results obtained with isolated eosinophils and eosinophils in the granulocyte population, exposed to the same stimuli, in general demonstrated the same pattern of responses. The slightly but significantly higher release of ECP from isolated eosinophils induced by serum-opsonized particles could either indicate that neutrophils interfere with the function of eosinophils or that the eosinophils have been activated by the isolation procedure.

In conclusion this study indicated that cellular adhesion plays an important role in the regulation of eosinophil degranulation, but that adhesion and degranulation can be induced separately. Eosinophil adhesion preceded the release of ECP and there was no correlation between the levels of adhesion and release. A complex relationship between adhesion and C3b-induced degranulation was shown. A reversible association between eosinophils and fibrinogen/fibronectin-stimulated eosinophil C3b-particle induced degranulation, whereas a strong competition between $\beta 1$ and $\beta 2$ integrin-induced adhesion and C3b particle-induced degranulation was shown. On the other hand $\beta 1$ and $\beta 2$ integrin activation potentiated the release induced by PMA.

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References

- 1 Arm JP, Lee TH. The pathobiology of bronchial asthma. *Adv Immunol* 1992; 51:323–82.
- 2 Koller DY, Herouy Y, Götz M, Hagel E, Urbanek R, Eichler I. Clinical value of monitoring eosinophil activity in asthma. *Arch Dis Child* 1995; 73:413–7.
- 3 Carlson M, Håkansson L, Peterson C, Stålenheim G, Venge P. Secretion of granule proteins from eosinophils and neutrophils is increased in asthma. *J Allergy Clin Immunol* 1991; 87:27–33.
- 4 Sedgwick JB, Vrtis RF, Gourley MF, Busse WW. Stimulus-dependent differences in superoxide anion generation by normal human eosinophils and neutrophils. *J Allergy Clin Immunol* 1988; 81:876–83.
- 5 Horie S, Kita H. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. *J Immunol* 1994; 152:5457–67.
- 6 Nathan C, Srima S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright SD. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J Cell Biol* 1989; 109:1341–9.

- 7 Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992; 69 (1):11–25.
- 8 Kita H, Horie S, Gleich GJ. Extracellular matrix proteins attenuate activation and degranulation of stimulated eosinophils. *J Immunol* 1996; 156:1174–81.
- 9 Werfel SJ, Yednock TA, Matsumoto K, Sterbinsky SA, Schleimer RP, Bochner BS. Functional regulation of beta 1 integrins on human eosinophils by divalent cations and cytokines. *Am J Respir Cell Mol Biol* 1996; 14:44–52.
- 10 Bohnsack JF, Zhou XN. Divalent cation substitution reveals CD18- and very late antigen-dependent pathways that mediate human neutrophil adherence to fibronectin. *J Immunol* 1992; 149:1340–7.
- 11 Altieri DC. Occupancy of CD11b/CD18 (Mac-1) divalent ion binding site (s) induces leukocyte adhesion. *J Immunol* 1991; 147:1891–8.
- 12 Håkansson L, Nielsen LS, Teder P. Measurement of neutrophil and eosinophil adhesion to E-selectin, VCAM-1, and ICAM-1 by the use of transfected fibroblast cell lines. *J Immunol Methods* 1994; 176:53–66.
- 13 Hansel TT, De Vries IJM, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991; 145:105–10.
- 14 Peterson CG, Enander I, Nystrand J, Anderson AS, Nilsson L, Venge P. 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; 21:561–7.
- 15 Saito H, Yamada K, Breard J, Yoshie O, Mathe G. A monoclonal antibody reactive with human eosinophils. *Blood* 1986; 67:50–5.
- 16 Laudanna C, Melotti P, Bonizzato C, Piacentini G, Boner A, Serra MC, Berton G. Ligation of members of the beta 1 or the beta 2 subfamilies of integrins by antibodies triggers eosinophil respiratory burst and spreading. *Immunology* 1993; 80:273–80.
- 17 Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 1984; 308 (5961): 693–8.
- 18 Dri P, Cramer R, Spessotto P, Romano M, Patriarca P. Eosinophil activation on biologic surfaces. Production of O₂ in response to physiologic soluble stimuli is differentially modulated by extracellular matrix components and endothelial cells. *J Immunol* 1991; 147:613–20.
- 19 Wei J, Shaw LM, Mercurio AM. Integrin signaling in leukocytes: lessons from the alpha6beta1 integrin. *J Leukoc Biol* 1997; 61:397–407.
- 20 Ruoslahti E. Integrin signaling and matrix assembly. *Tumour Biol* 1996; 17:117–24.
- 21 Smyth SS, Joneckis CC, Parise LV. Regulation of vascular integrins. *Blood* 1993; 81:2827–43.
- 22 Egesten A, Gullberg U, Olsson I, Richter J. Phorbol ester-induced degranulation in adherent human eosinophil granulocytes is dependent on CD11/CD18 leukocyte integrins. *J Leukoc Biol* 1993; 53:287–93.
- 23 Anwar AR, Walsh GM, Cromwell O, Kay AB, Wardlaw AJ. Adhesion to fibronectin primes eosinophils via alpha 4 beta 1 (VLA-4). *Immunology* 1994; 82:222–8.
- 24 Grayson MH, van der Vieren M, Sterbinsky SA, Gallatin M, Hoffman PA, Staunton DE, Bochner BS. α d β 2 integrin is expressed on human eosinophils and functions as an alternative ligand for vascular cell adhesion molecule 1 (VCAM-1). *J Exp Med* 1998; 188:2187–91.
- 25 Matsumoto K, Sterbinsky SA, Bickel CA, Zhou DF, Kovach NL, Bochner BS. Regulation of alpha 4 integrin-mediated adhesion of human eosinophils to fibronectin and vascular cell adhesion molecule-1. *J Allergy Clin Immunol* 1997; 99:648–56.
- 26 Weber C, Katayama J, Springer TA. Differential regulation of beta 1 and beta 2 integrin avidity by chemoattractants in eosinophils. *Proc Natl Acad Sci U S A* 1996; 93:10939–44.
- 27 Diamond MS, Garcia Aguilar J, Bickford JK, Corbi AL, Springer TA. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J Cell Biol* 1993; 120:1031–43.
- 28 Davis GE. The Mac-1 and p150, 95 beta 2 integrins bind denatured proteins to mediate leukocyte cell–substrate adhesion. *Exp Cell Res* 1992; 200:242–52.
- 29 Walsh GM, Symon FA, Lazarovits AL, Wardlaw AJ. Integrin alpha 4 beta 7 mediates human eosinophil interaction with MadCAM-1, VCAM-1 and fibronectin. *Immunology* 1996; 89:112–9.
- 30 Altieri DC, Agbanyo FR, Plescia J, Ginsberg MH, Edgington TS, Plow EF. A unique recognition site mediates the interaction of fibrinogen with the leukocyte integrin Mac-1 (CD11b/CD18). *J Biol Chem* 1990; 265:12119–22.
- 31 Winqvist I, Olofsson T, Olsson I. Mechanisms for eosinophil degranulation; release of the eosinophil cationic protein. *Immunology* 1984; 51:1–8.
- 32 Albelda SM, Buck CA. Integrins and other cell adhesion molecules. *FASEB J* 1990; 4:2868–80.
- 33 Egesten A, Blom M, Calafat J, Janssen H, Knol EF. Eosinophil granulocyte interaction with serum-opsonized particles: binding and degranulation are enhanced by tumor necrosis factor alpha. *Int Arch Allergy Immunol* 1998; 115:121–8.
- 34 Weiler CR, Kita H, Hukee M, Gleich GJ. Eosinophil viability during immunoglobulin-induced degranulation. *J Leukoc Biol* 1996; 60 (4):493–501.