

A microtiter assay for activation of eosinophils

Simultaneous monitoring of eosinophil adhesion and degranulation

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A simple microtiter assay for eosinophil activation is described. The assay used 1000–4000 eosinophils/microtiter well, and the design allows for a separate or simultaneous quantitative assessment of eosinophil adhesion to protein-coated microtiter wells and degranulation after stimulation with eosinophil-activating factors. The number of adherent eosinophils is quantified indirectly by expressing the amount of eosinophil cationic protein (ECP) extracted from the adherent fraction of cells in percentage of the total amount of ECP extracted from the cells added to the wells. Degranulation is quantified in the same way by expressing the amount of ECP or eosinophil protein X (EPX) released to the supernatant in percentage of the total amount of ECP or EPX. Known eosinophil-activating agents such as PMA, interleukin (IL)-3, IL-5, GM-CSF, and platelet-activating factor (PAF) all induced a time- and dose-dependent adhesion to albumin-coated wells, whereas L-PAF did not. Kinetic experiments showed that most adhesion occurred within the first 15–30 min, reaching a plateau around 60 min. After prolonged incubation, a decline in adhesion was detected. GM-CSF-induced adhesion was completely inhibited by incubation with monoclonal antibodies directed against the common β_2 -chain (CD18) of the LFA-1, Mac-1, p150,95 complexes. Monoclonal antibodies against CD11a, CD11b, CD11c, VLA-4 ALFA, ICAM-1, VCAM-1, Sialyl-Le^x, ELAM-1, and LECAM had no inhibitory effect. Simultaneous monitoring of adhesion and degranulation after stimulation of eosinophils in albumin-coated wells with either PMA or GM-CSF showed that adhesion always preceded degranulation. Replacing the albumin coating of the microtiter wells with IgG or secretory IgA augmented both the spontaneous and the PMA- or GM-CSF-stimulated responses. In conclusion, the assay allows dynamic evaluation of eosinophil activation and can be used to assess soluble eosinophil-activating factors as well as to study eosinophil activation by solid-phase-bound proteins.

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Abbreviations. ECP: eosinophil cationic protein; EPX: eosinophil protein X; ICAM: intracellular adhesion molecule; LFA-1: leukocyte function antigen 1; VLA-4: very late activation antigen; VCAM: vascular cell adhesion molecule; EDTA: ethylenediaminetetraacetic acid; IL-3: interleukin-3; IL-5: interleukin-5; GM-CSF: granulocyte/macrophage colony stimulating factor; PAF: platelet-activating factor; PMA: phorbol 12-myristate 13-acetate; DMEM: Dulbecco's modified Eagle's medium; HSA: human serum albumin; PBS: phosphate-buffered saline.

Activation of eosinophils is associated with upregulation of many physiologic and functional properties of the cells, and various methods have been used to monitor eosinophil activity or activation after *in vitro* stimulation. These methods include cytotoxic assays using antibody-coated schistosomula of *Schistosoma mansoni* as target organisms (1, 2), adhesion assays monitoring eosinophil adhesion

in vitro either to monolayers of cultivated endothelial cells or to serum/plasma-coated surfaces (3–6), chemotaxis assays (7, 8), and degranulation assays monitoring release of preformed granule proteins after incubation with serum-opsonized or immunoglobulin-coated particles (9–13). Moreover, methods that monitor *de novo* production and release of reactive oxygen metabolites generated in the respiratory burst (14, 15) or production of the lipid mediators leukotriene C₄ (LTC₄) and platelet-activating factor (PAF) have been used to follow eosinophil activation (16–18).

A high volume of blood is needed to obtain a sufficient number of eosinophils of a high purity if normal donors with low blood eosinophil counts are used. Thus, many studies have been performed using cells from donors with varying degrees of blood eosinophilia. However, eosinophils from donors with even mild eosinophilia may already be primed or activated *in vivo* (19) and may therefore not behave as normal resting eosinophils.

The regulation of eosinophils from resting circulating cells in the blood to fully activated degranulating eosinophils in the tissues probably depends on various consecutive priming and activating stimuli. The migration of eosinophils from the circulation into the tissues demands increased surface expression or activation of adhesion molecules such as the CD18/CD11 complexes and VLA-4, which are involved in adhesion of eosinophils to activated endothelial cells expressing the ligands ICAM-1 and VCAM-1 (3, 4, 6). Thus, adhesion may be regarded as one of the first functional events in the activation cascade, whereas degranulation and release of the cytotoxic cationic granule proteins probably represents one of the final functional events in this activation cascade.

This study aimed to develop a simple *in vitro* method enabling quantitative assessment of eosinophil activation by monitoring of eosinophil adhesion either alone or by simultaneous monitoring of eosinophil adhesion and degranulation. As an important priority, we wanted the assay to function with a low number of cells, making it possible to perform a high number of experiments on eosinophils obtained from normal blood donors with low blood eosinophil counts.

Material and methods

Blood samples and donors

Eosinophils from normal, nonatopic donors with blood eosinophil counts of less than $0.2 \times 10^9/l$ were used in the activation experiments. Blood samples were collected into vacutainers containing

ethylenediaminetetraacetic acid (EDTA) as anticoagulant (Exetainer, LABCO, UK).

Purification of eosinophils

Eosinophils were purified by the method described in detail by Hansel et al. (20). The method is based on Percoll gradient centrifugation (density 1.082 g/ml) (Percoll, Pharmacia, Uppsala, Sweden) to isolate granulocytes, lysis of red blood cells with 155 mM ammonium chloride (NH₄Cl), and, finally, immunomagnetic depletion of neutrophils by the magnetic cell separation system (MACS) using anti-CD16-coated MACS particles (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of eosinophil preparations was invariably $\geq 95\%$. Throughout the purification procedure, the cells were kept at 4°C in a Ca²⁺- and Mg²⁺-free medium.

Assay procedure

Coating of plates. Flat-bottom microtiter plates (Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C and for 4 h at 37°C with 1% human serum albumin (HSA) (Behringwerke Marburg, Germany) in 0.15 M NaCl, 250 µl/well. Before use, the wells were washed twice in incubation medium (Dulbecco's modified Eagle's medium [DMEM]) supplemented with 1% fetal calf serum (FCS) (Life Technologies, Paisley, UK). Where indicated in the text, the plates were coated with either IgG (Nordimmun, Novo Nordic, Bagsværd, Denmark) or secretory IgA (s-IgA) (Nordic Immunological Laboratories, Tilburg, The Netherlands) 1 µg/well in 50 µl NaCl, and then blocked with 1% HSA as described above.

Stimulation of cells. A volume of 50 µl of incubation medium/well or 50 µl of stimulating agent, diluted in incubation medium, was mixed with 50 µl cell suspension containing 1000–4000 purified eosinophils, and the plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The stimulating agents tested were PMA, PAF, L-PAF (Sigma, St Louis, MO, USA), recombinant human interleukin-3 (IL-3), IL-5, and GM-CSF (Genzyme, Boston, MA, USA). All stimulations were performed in duplicate wells.

Monitoring of adhesion alone. After incubation, the wells were emptied by inverting the plates and washed three times in 0.15 M NaCl (37°C). Adhering eosinophils were then extracted by addition of 200 µl extractions buffer (0.5% *N*-cetyl, *N,N,N*-trimethylammonium bromide [CTAB] in phosphate-buffered saline [PBS]). After two cycles

of freeze-thaw, the plates were centrifuged. The supernatants of the extracts were harvested and frozen at -20°C until assayed for the content of ECP measured by ELISA.

Simultaneous monitoring of adhesion and degranulation. After incubation, the supernatants and non-adhering eosinophils were transferred to tubes containing 1 vol. of ice-cold (PBS) with 5 mM EDTA. The adherent cells were then washed three times by gently flushing the wells with 200 μl of prewarmed saline (0.15 M NaCl, 37°C) and extracted in 200- μl extractions buffer, as described above. The three sets of wash solution were mixed/pooled with the corresponding tubes containing the nonadhering eosinophils and the supernatant from the incubation. After centrifugation (2000 g, 10 min at 4°C), the supernatants were recovered and frozen at -20°C until assayed for ECP and EPX.

Calculation of adhesion and degranulation

Adhesion and degranulation were calculated indirectly by expressing the amount of ECP extracted from the adhering cell fraction and/or the amount of ECP released to the supernatant in percentage of the total amount of ECP extracted from 50 μl of the cell suspension added to the wells. Total extraction of cells was performed by adding 1 vol. of 1.0% CTAB buffer to the cell suspension, followed by two cycles of freeze-thaw and centrifugation, as described above.

Measurement of ECP and EPX

ECP and EPX in supernatants and cell extracts were quantified by two sensitive ELISA methods described in detail elsewhere (21, 22). Briefly, both ELISAs are polyclonal, sandwich-type and use the biotin-avidin amplification system. The methods measure ECP and EPX in the ranges 15–1000 and 60–2000 pg/ml, respectively. Before measurement, the samples were diluted in PBS containing 0.1% Tween 20, 0.1% CTAB, 20 mM EDTA, and 0.2% HSA, pH 7.4.

Inhibition of adhesion

The inhibitory effect of mouse monoclonal antibodies (mAb) against various adhesion molecules and ligands, CD11a, CD11b, CD11c, CD18, VLA-4- α , ICAM-1, VCAM-1, Sialyl-Le^x, ELAM-1, and LECAM (CAMfolio, Becton Dickinson, Glostrup, Denmark), was tested by coincubation of antibodies with eosinophils and the activating agents.

Recovery experiments

Total recovery. After stimulation of eosinophils for various lengths of time with either medium alone, PMA, GM-CSF, serum-opsonized zymosan (Baker's yeast, Sigma, St Louis, MO, USA), or serum-opsonized Sephadex G-15 (Sephadex G-15, Pharmacia, Uppsala, Sweden), the total amount of ECP (i.e., ECP in the adhering cells, the nonadhering cells, and the supernatant) was extracted by adding 1 vol. of 1% CTAB in 0.15 M NaCl.

Differential recovery. After stimulation of eosinophils with medium, PMA, and GM-CSF for 1 h, the cells and supernatants were separated into adhering cells, nonadhering cells, and supernatants, as described above, and the ECP content in each fraction was determined.

Statistics

The Wilcoxon rank sum test and the Friedman test were used for paired observations, and the Mann-Whitney rank sum test and the Kruskal-Wallis test for unpaired observations. The coefficient of correlation was calculated as Spearman's rho. All calculations were done with SigmaStat software (Jandell Scientific, California, USA). *P* values of less than 0.05 were considered significant.

Results

Titration of cells

Fig. 1 shows the result of an experiment performed to find a suitable number of eosinophils/well. Each well received 100 μl cell suspension and 100 μl 1% CTAB buffer. Good precision and accuracy were achieved even with a low number of eosinophils/well. On the basis of these findings, the number of 1000–4000 eosinophils/well was chosen for all further experiments.

Eosinophil adhesion

The dose-response relationships and kinetics of eosinophil adhesion after stimulation with titrations of PMA, IL-3, IL-5, GM-CSF, PAF, and lyso-PAF are illustrated in Figs. 2 and 3. After incubation for 15 and 30 min, PMA, IL-3, IL-5, GM-CSF, and PAF all induced a dose-dependent eosinophil adhesion ($P \leq 0.005$), whereas lyso-PAF did not (Fig. 2). Comparison of the responses induced by the individual concentrations showed significant differences ($P = 0.03$) between the responses obtained by every second and third of the tested concentrations. After 1 h, the dose-response relationship was less

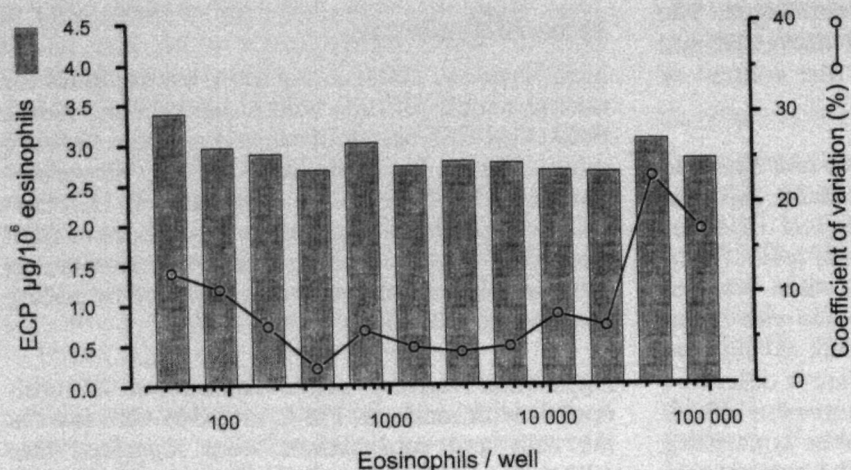


Fig. 1. Amount of ECP/ 10^6 eosinophils (bars) and coefficient of variation (solid line) calculated from amount of ECP extracted from 12 twofold dilutions of purified eosinophils. Final extraction volume was 200 μl . This experiment was performed in octuplicate with blood from donor with mild eosinophilia (eosinophil count: $0.55 \times 10^9/\text{l}$).

clear since most of the tested concentrations induced approximately the same level of adhesion (data not shown).

Kinetic studies showed that most adhesion occurred within the first 15–30 min reaching a significant peak or plateau ($P=0.03$) within 30 min (PMA) or 60 min (IL-3, IL-5, GM-CSF, and PAF) (Fig. 3). After prolonged incubation, a decline in the observed adhesion was seen. All the tested concentrations showed the same pattern (data not shown).

Inhibition of adhesion

To identify the molecule(s) responsible for the adhesion to the solid-phase-bound albumin, we investigated the inhibitory effect of monoclonal antibodies against various adhesion molecules and

ligands (Fig. 4). Anti-CD18 antibodies completely inhibited the GM-CSF-stimulated adhesion ($P=0.03$), whereas monoclonal antibodies against CD11a, CD11b, CD11c, VLA-4 α , ICAM-1, VCAM-1, Sialyl-Le^x, ELAM-1, and LECAM had no inhibitory effect.

Recovery experiments

After 120-min and 240-min incubations, a decline in adherence was observed (Figs. 3 and 6). We therefore tested whether the total amount of ECP was constant during incubation after stimulation. Cells were stimulated with either medium alone, PMA, GM-CSF, serum-opsonized Sephadex G-15, or serum-opsonized Zymosan. No decrease in recovery could be detected when the cells were stimulated with medium alone or serum-opsonized

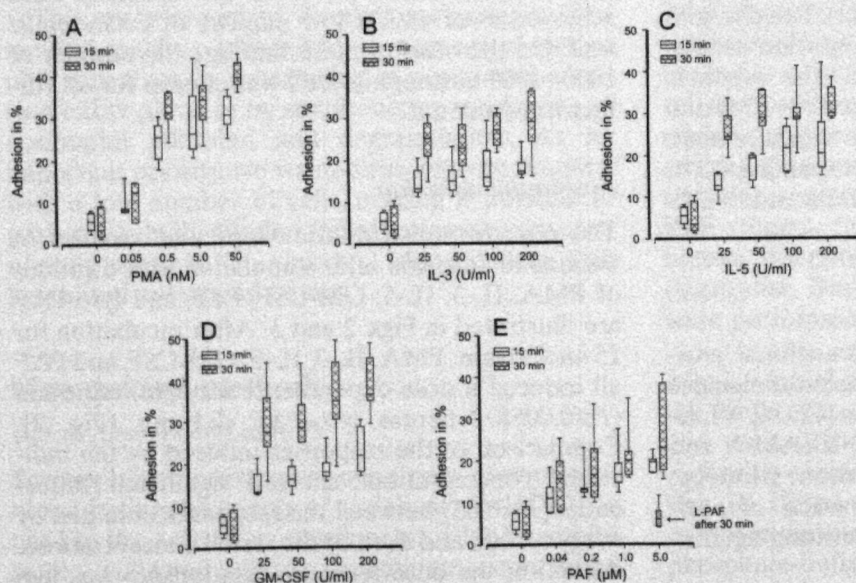


Fig. 2. Dose-response relationship of eosinophil adhesion after stimulation with A) PMA; B) IL-3; C) IL-5; D) GM-CSF; E) PAF for 15 min (open boxes) and 30 min (filled boxes). Insert in E indicates maximum lyso-PAF-stimulated adhesion after 30 min. Box plot indicates median, 25th and 75th percentiles, and range of six individual experiments.

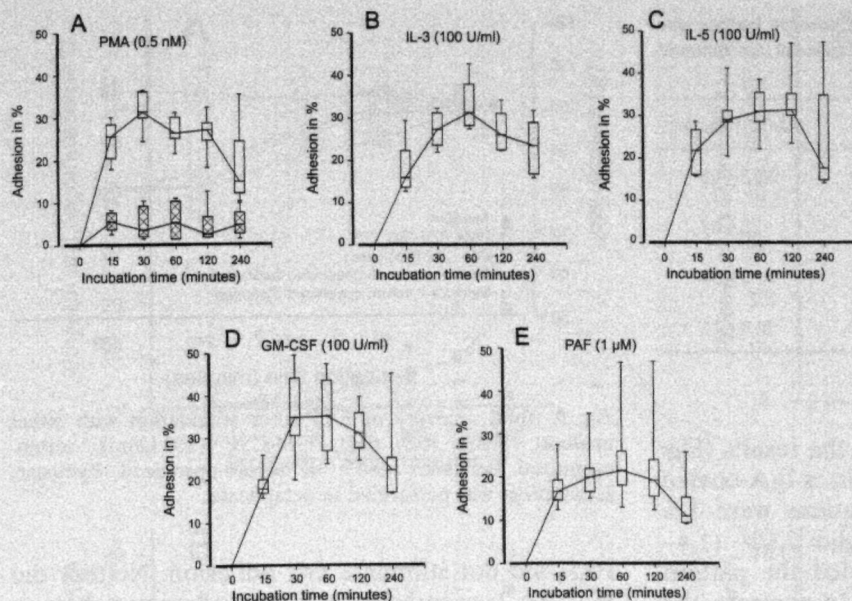


Fig. 3. Kinetics of eosinophil adhesion after stimulation with A) PMA, 50 nM; B) IL-3, 100 U/ml; C) IL-5, 100 U/ml; D) GM-CSF, 100 U/ml; E) PAF, 1 µM. Insert in A (filled boxes) indicates adhesion after stimulation with medium alone. Box plot indicates median, 25th and 75th percentiles, and range of six individual experiments.

Sephadex G-15 for up to 8 h. However, if cells were stimulated with serum-opsonized Zymosan, PMA, or GM-CSF, significant decreases in recoveries were detected after 2 h ($P \leq 0.03$). After continued incubation for 8 h only approximately 80% could be recovered ($P \leq 0.0002$) (Fig. 5). Furthermore, we investigated how much of the total amount of ECP could be recovered from the individual fractions, i.e., the adherent fraction of cells, the nonadherent fraction of cells, and the supernatant after stimulation and incubation for 1 h. Of the total amount of ECP, 10–15% was lost, probably during manipulation of the small volumes (Table 1). A negligible but significant percentage of ECP was released when cells were stimulated with PMA and GM-CSF compared to the amount released when cells were incubated in medium alone ($P < 0.0002$).

Simultaneous monitoring of adhesion and degranulation

Fig. 6A shows the results of simultaneous measurements of adhesion and degranulation after stimulation of cells with a single concentration of PMA (0.5 nM) and GM-CSF (100 U/ml). The adhesion response showed the same pattern as seen in the previous experiments and clearly preceded the release of ECP by approximately 2 h. Monitoring the adhesion with EPX as a marker protein instead of ECP showed the same pattern and order of magnitude within the first 1–2 h ($P > 0.7$) (Fig. 6A and B). There was a good correlation between the released percentage of ECP and EPX ($r_s = 0.96$, $P = 0.0$); however, the cells released almost double the percentage of EPX

compared to ECP ($P = 0.0$). This higher release of EPX was reflected in a steeper decrease in the amount of EPX recovered from the adhering cells compared to the recovery of ECP in the same samples when cells were stimulated with PMA ($P \leq 0.008$). The same trend, but not a statistically significant one ($P = 0.10$), was seen when cells were stimulated with GM-CSF (compare Fig. 6A and B).

In the same series of experiments, we also investigated whether coating of the wells with IgG and s-

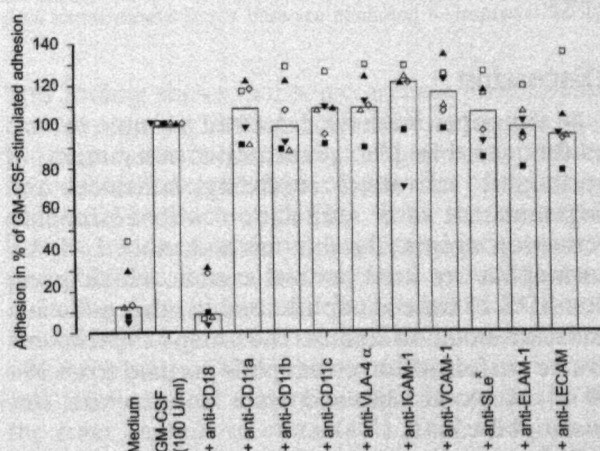


Fig. 4. Inhibition of GM-CSF (100 U/ml)-stimulated eosinophil adhesion by mAb against various adhesion molecules and ligands: CD18, CD11a, CD11b, CD11c, VLA-4- α , ICAM-1, VCAM-1, Sialyl-Le^x, ELAM-1, and LECAM. Bars indicate median adhesion of six individual experiments. GM-CSF-stimulated adhesion = 100%.

Table 1. Recovery of ECP (percentage of total ECP) in individual fractions after stimulation with medium, PMA, and GM-CSF for 1 h. Experiment was performed in octaplate

	Medium	PMA (0.5 nM)	GM-CSF (100 U/ml)
ECP in nonadhering fraction of cells	79.1 ± 9.9	33.4 ± 6.2	46.5 ± 5.6
ECP released to supernatant	0.5 ± 0.2	2.5 ± 1.2	2.5 ± 0.7
ECP in adhering fraction of cells	5.9 ± 1.7	52.0 ± 9.3	42.0 ± 5.2
Total recovery	85.5 ± 9.3	87.9 ± 8.9	91.0 ± 7.2

IgA instead of albumin influenced the results (Fig. 7). The spontaneous adhesions in s-IgA-coated wells after 15- and 30-min incubations were 1% (0.8–9.3%; median and range) and 2.1% (1.4–6.0%), respectively, which paralleled the pattern seen in the albumin-coated wells. In contrast, the spontaneous adhesions in IgG-coated wells after 15 min and 30 min were 34.4% (range 17.0–41.7%) and 46.5% (range 26.7–57.0%), respectively. The changed surface coatings of the wells also influenced the release pattern. The spontaneous release in IgG- and s-IgA-coated wells was increased compared to the release in albumin-coated wells ($P \leq 0.002$), particularly in IgG-coated wells, where the spontaneous releases of ECP and EPX were comparable and even higher than the GM-CSF-stimulated release in albumin-coated wells ($P \leq 0.003$). After stimulation with PMA and GM-CSF, the release of ECP and EPX was further increased, and, compared to the release kinetics in albumin-coated wells (compare Figs. 7A and B and 6A and B), the release curves were left-shifted by 1–4 h.

Discussion

In this paper, we have described a simple *in vitro* method enabling a quantitative assessment of eosinophil activation regarding adhesion and degranulation after stimulation with eosinophil-activating agents. By this method, only $1\text{--}4 \times 10^3$ eosinophils are used per test sample, which is less than 10% of the eosinophils used in other adhesion and degranulation studies. Thus, many experiments can be performed on eosinophils purified from 10–20 ml of blood obtained from donors with low eosinophil counts.

PMA and eosinophil-activating agents such as IL-3, IL-5, GM-CSF, and PAF, previously reported to activate eosinophils (5, 8, 12), all stimulated a time- and dose-dependent adhesion, within the first 15–30 min, reaching a plateau after approximately 1 h of stimulation. L-PAF, the inactive precursor of

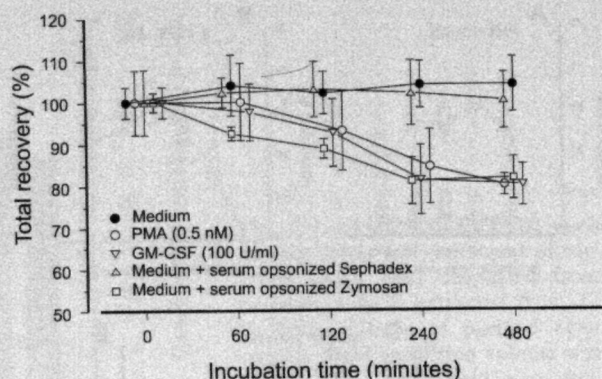


Fig. 5. Total recovery of ECP after stimulation with either medium, PMA (0.5 nM), GM-CSF (100 U/ml), serum-opsonized Sephadex G-15, or serum-opsonized Zymosan. Experiment was performed in octaplate.

PAF, did not stimulate any adhesion. Neither did IL-1, IL-2, or interferon-gamma (data not shown). Within the first hour, the dose response and kinetics of the cytokine- and PAF-stimulated adhesion closely paralleled what have been described by others studying eosinophil adhesion to either protein-coated surfaces (6, 23) or monolayers of endothelial or epithelial cells (5, 24).

A high interindividual donor variation was seen (compare Figs. 2, 3, and 6), indicating that the source of eosinophils is important. Eosinophils were occasionally activated either *in vivo* or during purification, as judged from a high spontaneous adhesion which could not be distinguished from the positive control (PMA 0.5 nM) (data not shown). The cells were kept at 4°C in a Ca^{2+} - and Mg^{2+} -free medium to avoid *in vitro* activation during purification. Moreover, we took care that donors had not been exercising or under physical stress before blood donation, as some experiments have indicated that this may cause some activation of eosinophils *in vivo*.

After continued incubation for more than 1 h, a time-dependent decline in adhesion was seen. This agrees with data reported by Walsh et al. (6), who counted eosinophils adhering to plasma-coated glass surfaces after stimulation with IL-3, IL-5, GM-CSF, and PAF, and described detachment of cells after incubation for 2 h. Horie & Kita (23), however, who quantitated eosinophil adhesion to albumin-coated microtiter wells by determination of EDN/EPX in extracts, found no such decline, despite a concomitant release of EDN. Down-regulation of CD18 on activated or low-density eosinophils has been reported (25) and could be responsible for the detachment of cells during prolonged incubation. However, by our method, which quantified the number of eosinophils by extraction and measurement of ECP in the extract,

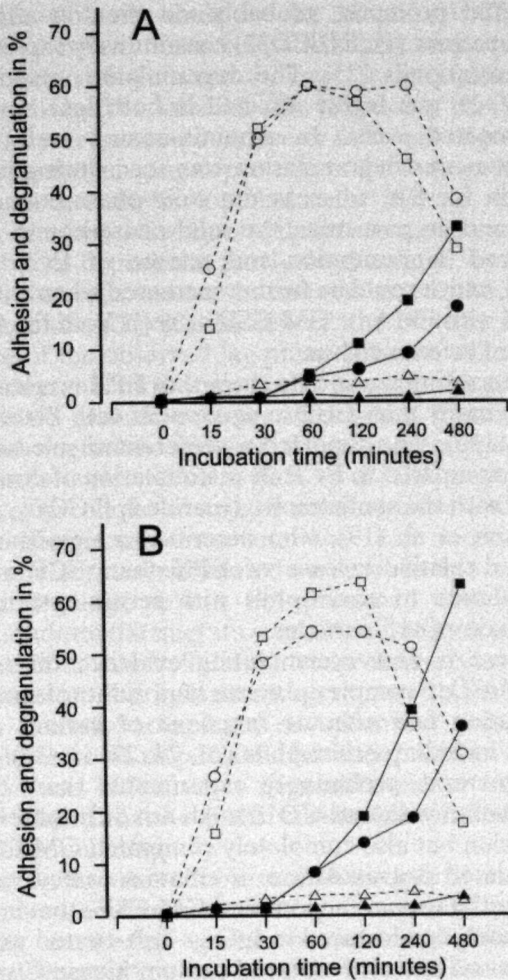


Fig. 6. Simultaneous monitoring of adhesion (open symbols, broken line) and degranulation (closed symbols, solid line) in albumin-coated wells after stimulation of cells with either medium (triangles), PMA (0.5 nM) (squares), or GM-CSF (100 U/ml) (circles). A) Monitoring adhesion and degranulation with ECP as marker protein. B) Monitoring of adhesion and degranulation with EPX as marker protein. Data points represent median values of four individual experiments. Error bars are excluded to improve clarity.

the decline in adhesion could be explained not only by detachment of cells but also by release of ECP from the adherent cells. Moreover, it has been reported that some granule protein may be lost to assay due to intracellular degradation after activation of eosinophils (26).

Therefore, we tested the total recovery of ECP during stimulation and incubation for 8 h and the recovery in the individual fractions of cells and supernatant after 1 h. A time-dependent decline in total recovery of ECP was seen when eosinophils were stimulated with PMA, GM-CSF, or serum-opsonized zymosan (phagocytizable particles), but not when stimulated with medium alone or serum-opsonized Sephadex (nonphagocytizable particles).

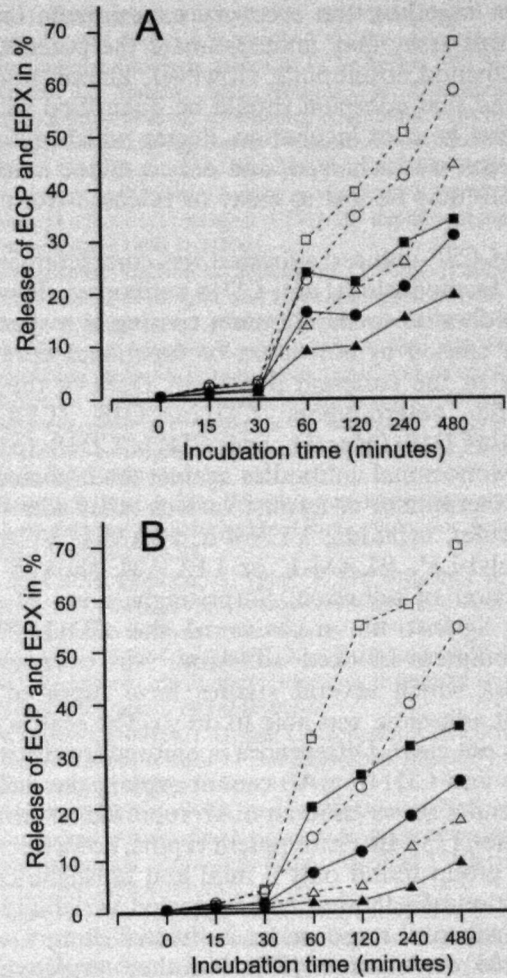


Fig. 7. Release of ECP (closed symbols, solid line) and EPX (open symbols, broken line) in IgG-coated wells (A) and s-IgA-coated wells (B) after stimulation with either medium (triangles), PMA (0.5 nM) (squares), or GM-CSF (100 U/ml) (circles). Data points represent median values of four individual experiments. Error bars are excluded to improve clarity.

This finding shows that some granule protein may be lost to assay during eosinophil activation and prolonged incubation, and could be explained by intracellular degradation of granule proteins, as suggested by Bach et al. (26). Thus, the measured decline in adhesion during prolonged incubation is probably due to a combination of several factors, including detachment of cells, release of ECP, and loss of ECP. A good recovery of ECP was achieved when the sum of ECP recovered in the individual cell fractions and supernatants was compared to the total amount of extractable ECP after 1 h of incubation. Despite a different distribution of ECP among the individual fractions according to whether the cells were incubated with stimulating agent or medium, the sum of recoveries was the same. Approximately 10–15% of the total ECP was lost during manipulation of the small volumes.

Taken together, the recovery experiments imply that this assay may underestimate the percentage of activated eosinophils. However, kinetic studies showed that adhesion should be quantified within the first hour of incubation, during which optimal adhesion was achieved, and only a minor amount of ECP may be lost to assay or released from the cells.

GM-CSF-induced adhesion was completely inhibited by monoclonal anti-CD18 antibodies, showing that adhesion to the albumin coating is a specific event caused by activation or functional upregulation of the common β -chain (CD18) of the β_2 -integrin heterodimers CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150, 95). Monoclonal antibodies against the α -chains of this heterodimer or against various other adhesion molecules, including VLA-4- α , ICAM-1, VCAM-1, Sialyl-Le^x, ELAM-1, or LECAM, showed no inhibition of adhesion. Surprisingly, none of the mAb against the α -chains of the CD11/CD18 heterodimers blocked adhesion. Not even anti-CD11b, which several studies have reported to inhibit adhesion, was able to do so. The reason for this is not clear. Differences in epitope specificities of the anti-CD11b mAb cannot explain the different results, as we used an mAb reported to inhibit adhesion (23). In a subsequent report, however, the same group found only a mild and nonsignificant inhibition of adhesion and suggested variability of the eosinophil response by individual donors as a possible explanation (27). Another explanation could be the experimental design. Horie & Kita (23) preincubated cells and antibodies for 10 min before stimulating the cells with cytokines, whereas we coincubated eosinophils, mAb, and GM-CSF during the entire incubation period. Elimination of the preincubation step had no effect on the inhibition when anti-CD18 mAb was used.

The low amount of ECP recovered in the supernatant after 1 h led us to monitor the kinetics of adhesion and degranulation simultaneously in albumin-coated wells. In the same series of experiments, we also followed the IgG- and s-IgA-induced degranulation. Serum-opsonized or immunoglobulin-coupled Sepharose beads are known to induce eosinophil degranulation (9, 12, 13, 28, 29). To avoid the potential loss of ECP, which may be adsorbed to the large surface exposed by the Sepharose beads, we coupled IgG and s-IgA directly to the microtiter wells. Monitoring adhesion and degranulation in albumin-coated wells clearly showed that activation for adhesion preceded degranulation, which started after 1–2 h.

The spontaneous adhesion in s-IgA-coated wells paralleled the spontaneous adhesion in albumin-coated wells, but, in IgG-coated wells, eosinophils

adhered promptly, probably via the low-affinity IgG receptor (Fc γ RII/CD32) constitutively expressed on eosinophils (25). The degranulation response, however, was highly affected in both IgG- and s-IgA-coated wells. In albumin-coated wells, no spontaneous degranulation was seen during incubation for 8 h, whereas the solid-phase-bound s-IgA and, in particular, the solid-phase-bound IgG induced degranulation and release of ECP and EPX, which could be further increased when stimulated with PMA or GM-CSF, as seen from the left-shifted release curves.

Interestingly, our data show that EPX is released more easily than ECP, in agreement with Peterson et al. (30), who reported a preferential release of EPX compared to ECP after stimulation of eosinophils with the synthetic triglyceride IpOCOC₉, and Carlson et al. (19), who described a significantly higher relative release of EPX than ECP after stimulation of eosinophils with serum-opsonized Sephadex G-15 particles.

There is now accumulating evidence that the CD11/CD18 complex plays an important role in the activation and effector functions of various cell types including eosinophils (23, 24, 27, 31–34). By our method, preliminary experiments show that incubation with anti-CD18 mAb not only inhibited adhesion but also completely eliminated GM-CSF-stimulated degranulation in albumin-coated wells and both the spontaneous and GM-CSF-stimulated degranulation induced in IgG-, s-IgA-coated wells, whereas direct activation of protein kinase C with PMA bypassed the inhibitory effect of anti-CD18 mAb (data not shown). These preliminary observations agree with other reports suggesting an important role for the CD11/CD18 complex in eosinophil degranulation, as degranulation was inhibited when eosinophil adhesion was prevented by either incubation with anti-CD18 mAb or mechanical stirring (27, 33, 35).

Many studies on β_2 -integrin-dependent eosinophil adhesion have been performed with serum/plasma-coated surfaces, monolayers of IL-1- and tumor-necrosis factor- α -activated endothelial cells, or monolayers of transfected cell lines expressing adhesion molecules or ligands (3–5, 36). We have chosen to use albumin-coated surfaces to obtain a system as simple as possible that avoids interference from serum/plasma proteins such as immunoglobulins, complement factors, plasma fibronectin, or cytokines produced by endothelial cells. It is not easy to explain why functional upregulated CD11/CD18 on activated eosinophils binds to an abundant protein such as albumin. Naturally, precautions should be taken when discussing the *in vivo* relevance of data generated *in vitro*, but one theoretic explanation could be that

plasma albumin binds to the functional CD11/CD18 complex, preventing circulating eosinophils, if activated above a certain level, from interacting with activated endothelial cells expressing ICAM-1. This interaction could induce or facilitate degranulation and damaging deposition of the toxic granule proteins on the endothelial cells. Instead, these cells are found in the circulation as an increased number of hypodense, partly degranulated, or EG-2-positive cells, as seen in diseases associated with eosinophilia (37, 38). Moreover, the biologic relevance of the observed lag-phase between adhesion and degranulation may be to prevent release of cytotoxic granule proteins during the transendothelial migration, allowing the cells to migrate to the inflammatory focus. Kita et al. (39) reported that extracellular matrix proteins, such as laminin and fibronectin, attenuate the activation and degranulation of stimulated eosinophils. In view of these findings, it can be speculated that after transendothelial migration additional stimuli in the tissue from extracellular matrix proteins, cytokines released at the inflammatory site, or interaction with immune complexes or opsonized parasites would then be responsible for either further activation and induction of degranulation, or inhibitory stimuli that keep the eosinophils primed but nondegranulating.

In conclusion, a simple *in vitro* method of investigation of eosinophil-activating factors and monitoring eosinophil activation regarding adhesion and degranulation has been developed. The assay employs less than 10% of the eosinophils used in other published adhesion/degranulation studies, allows a dynamic evaluation of eosinophil activation, and can be used to assay soluble eosinophil-activating factors as well as to study eosinophil activation by solid-phase-bound proteins.

Acknowledgments

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