

Eosinophil cationic protein is stored in, but not produced by, peripheral blood neutrophils

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Summary

Background Eosinophil cationic protein (ECP) is an eosinophil-derived protein, which has been shown to be present in circulating neutrophils.

Objective To establish whether ECP is produced or internalized by peripheral blood neutrophils.

Methods This was done using microscopy, flow cytometry, fractionation of cells and RT-PCR techniques.

Results No ECP mRNA was detected after extensive cell purification to eliminate all traces of contaminating eosinophils. Examination of immunostained neutrophils by light, confocal, electron microscopy together with cell fraction experiments, established that ECP is present intracellularly and is mostly associated to cell granules. Uptake studies by flow cytometry and by using both cold and radiolabelled ECP showed that it is internalized by neutrophils and stored in some proportion in their primary granules. Upon stimulation with serum-treated Sephadex particles, the internalized ECP was partially released from cells.

Conclusion ECP is not produced but can be internalized by circulating neutrophils, which take it from the environment and partially store it in their primary granules.

Keywords confocal microscopy, electron microscopy, eosinophils, flow cytometry, fractionation, mRNA,

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Introduction

Eosinophil cationic protein (ECP) is a highly cationic protein found in the secretory granules of human eosinophils [1]. The protein is a member of the pyrimidine-specific ribonuclease family [2]. Six other members of the family are expressed in various tissues in humans, and their genes are located on chromosome 14 [3, 4]. One of the major biological properties of ECP is its cytotoxic capacity, which has been investigated in a number of studies. The protein is cytotoxic to viruses, bacteria, parasites and the cell line K562 (A. Trulsson et al. unpublished data) [5–7]. Moreover, ECP affects the proteoglycan metabolism of fibroblasts [8]. ECP is secreted from eosinophils upon cell activation by immunoglobulin-mediated mechanisms [9] or by particles coated with complement proteins such as C3b [10]. ECP can be detected in a number of biological fluids by means of sensitive immunoassays [11], and it has become an extensively used marker of eosinophil activity in patients with diseases such as asthma and other inflammatory disorders [12].

The clinical usefulness of the measurement of cellular markers is partly dependent on their cellular specificity. In the case of ECP, this specificity has been questioned in recent

publications, since ECP has been reported to be present in neutrophils and basophils [13, 14]. A still unsolved matter is whether the ECP association to neutrophils represents an artefact or the neutrophil has the actual capacity to synthesize ECP. The aim of the present work was to address these questions using a number of different techniques. We show that ECP is indeed present in peripheral blood neutrophils in small amounts, but also that neutrophils do not seem to have the capacity to produce this protein. Instead, neutrophils are able to take up and store ECP in their secretory granules and subsequently release it upon cell stimulation.

Materials and methods

Light and confocal microscopy

Samples were treated as previously described [15]. Peripheral blood was drawn in Venoject EDTA vials (0.05 mL, 0.47 M EDTA; Terumo, Leuven, Belgium) and divided into aliquots of 100 µL each. Two mL of PermeaFix™ (OPF; Ortho Diagnostic Systems Inc., Raritan, NJ, USA), diluted 1:1 in distilled water, were added to each tube. The suspension was mixed by gently pipetting it in and out. This procedure was repeated every 10 min during the fixation period of 40 min. The suspension was centrifuged for 4 min at 400 g, at 20 °C, and the supernatant was discarded. The pellet was resuspended with phosphate-buffered saline (PBS) containing 1.5% bovine serum albumin (BSA) and

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5% fetal calf serum and repeatedly mixed, as described above. After 10 min, the cell suspension was centrifuged again, obtaining a pellet of leucocytes. During this treatment with the protein-rich buffer, most of the red cells were lysed. The leucocytes were resuspended in PBS containing 0.2% BSA.

Immunocytochemistry Cells were centrifuged onto polylysine-coated microscope slides (Histolab, Gothenburg, Sweden) for 4 min at 80 *g* in a Shandon cytocentrifuge (Cytospin, Shandon, Astmore, UK). The slides were rinsed rapidly with PBS-0.2% BSA and immediately incubated with the primary anti-ECP antibody EG2 (2 µg/mL). Incubations were performed for 30 min in a humid chamber, at room temperature, and terminated by washing with PBS-0.2% BSA. The antigen-antibody complex was visualized by using a commercial APAAP kit and fast red substrate (K 670, Dako, Glostrup, Denmark). The primary antibody was omitted in negative controls. After counterstaining with Mayer's haematoxylin (Merck, Darmstadt, Germany) for 6 min, coverslips were mounted using fluorescence mounting medium (Dako) and examined in a Leica DRMB microscope. Fuji 200 film was used for the colour prints.

Confocal microscopy Cell samples, processed as a suspension, were incubated with 6 µg/mL FITC-labelled anti-ECP antibody (EG2) in PBS-0.2% BSA (pH 7.4). The incubation of cells with the irrelevant antibody anti- α -fetoprotein served as a control. After 30 min at room temperature, the samples were washed twice with PBS. A drop of 20 µL of each cell sample was applied onto a polylysine-coated coverslip and mounted as described above. Confocal microscopy was performed using a confocal laser-scanning microscope originally designed and built in EMBL (Heidelberg, Germany) and later modified in the Laboratory of Biophysics, University of Turku, Finland. The system was implemented around a Zeiss Axiovert 10 inverted microscope. A 488-nm line of an argon-ion laser was used for excitation, and the FITC fluorescence emission was filtered with a 530 ± 15 nm band-pass filter and detected with a single photon counting avalanche photodiode detector (SPAD) module of the type SPCM-AQ-131 (PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). Optical sections were acquired at 1.0 µm intervals with a Zeiss Neofluar 100×1.3 NA oil immersion objective.

Immunoelectron microscopy

Nine mL blood were collected in 4.5 mL EDTA tubes (Vacutainer system, Becton Dickinson, Meylan, France). Leucocytes were separated from red cells by sedimentation on Ficoll Hypaque (Amersham Biosciences AB, Uppsala, Sweden), by applying 1 mL blood on 2 mL Ficoll. After 30 min at room temperature, red blood cells sedimented to the bottom of the tube. The supernatant (leucocyte-containing plasma) was carefully collected and diluted with PBS up to 10 mL. Subsequently the cells were centrifuged at 180 *g* for 5 min. The cells were washed twice with PBS and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in cacodylate buffer for 2 h, on ice. After washing with Ringers buffer and subsequent dehydration in ethanol (50%, 75%, 90%, 95% and twice absolute ethanol, respectively), the cells were embedded in Lowicryl (KM4) (Agar Aids). Polymerization was performed by UV light (360 nm), at -20°C . Samples were sectioned to a thickness of 50 nm with an Ultratome V (LKB,

Bromma, Sweden) provided with a diamond knife (Diatome, Bienne, Switzerland). The sections were mounted on Formavar-coated golden grids.

The sections were first incubated in blocking buffer (1% BSA in PBS) to avoid non-specific binding and then with primary antibody MAb 611 (a gift from Dr C. Peterson, Pharmacia Diagnostics AB) at 1 µg/mL for 1 h, at room temperature. This MAb has been shown not to cross-react with EPX/EDN in ELISA assays. After rinsing with PBS-0.2% BSA, goat anti-mouse IgG antibody conjugated to 10 nm gold particles (British BioCell International, Cardiff, UK) was added and incubated for 30 min. Control samples were incubated with secondary antibody only, or alternatively the primary antibody was replaced by a non-relevant anti-human murine IgG (Dako Pats, Glostrup, Denmark). The samples were analysed in a Hitachi 100 electron microscope.

Preparation of cells for fractionation and RT-PCR

Blood was drawn from healthy individuals and eosinophils were isolated by negative magnetic immuno-selection, as described by [16] and [17], with minor modifications [18]. Mononuclear cells were separated from red cells and granulocytes by centrifugation on 67% Percoll (Amersham Biosciences AB, Uppsala, Sweden). Red cells in the pellet were then lysed twice with water. Granulocytes were washed twice with PBS containing 2% newborn calf serum (NBS). For eosinophil purification, the granulocyte mixture was incubated for 1 h with magnetic beads coated with anti CD16 antibody (Milenyi Biotech, Bergisch-Gladbach, Germany) in the proportion of 1.5 µL of magnetic beads and 3 µL buffer per 1×10^6 granulocytes, at 4°C . For neutrophil isolation, the granulocytes, at a final concentration of $2.5 \times 10^6/\text{mL}$, were incubated for 30 min with anti-CD9 antibody (Immunotech, Marseilles, France), at a final concentration of 2.5 µg/mL, at 4°C . The excess of antibodies were washed off and the granulocytes were incubated with magnetic beads coated with anti-IgG antibody (Milenyi Biotech) for 1 h, at 4°C , in the proportion of 20 µL bead suspension and 80 µL buffer per 10^7 cells. In the case of neutrophil isolation, the excess of beads was washed off after incubation. Subsequently, the cells were resuspended and loaded on a separation column, which was placed in a strong magnetic field. In case of eosinophil isolation the mixture of granulocytes was directly applied to the column. Non-retained cells were eluted with PBS-2% NBS. Cells were counted, centrifuged on cytospin slides and stained with May Grünwald-Giemsa. The purity of eosinophil or neutrophil was on average $>98\%$. Purified cells were used immediately.

To prepare mRNA from neutrophils for RT-PCR, we aimed to obtain neutrophils as close as possible to 100% purity. For this purpose, 40 mL of blood was drawn from an individual with low blood eosinophil counts. Neutrophils were then purified by the MACS system as described above but the procedure was repeated twice more using 4 µg/mL CD9 antibodies each time. Each time, the amount of remaining eosinophils was determined by counting 20 000 cells on Cytospin slides (one eosinophil in 20 000 cells represents a 0.005% contamination). After the first and second rounds of purification, contaminating eosinophils were 0.5% and 0.07%, respectively. After a final third round of purification, magnetically non-retained cells were eluted with two successive portions of PBS-2% NBS that were separately collected. The contaminating eosinophils in the

first collection tube were 0.02%, and less than 0.005% in the second tube.

Human fibroblasts (cell line 1523), used for the study of the sensitivity of detection of ECP mRNA described below, were cultured to confluence in Eagle's MEM (Gibco, Gaithersburg, MD, USA) containing 10% fetal calf serum. Eosinophils were mixed with fibroblasts in the final cell number proportions of 0.02, 0.07, 0.50 and 5.3%, with the total cell number being 1.5×10^6 cells.

U937 cells, from which RNA was prepared for RT-PCR-positive controls, were cultured in RPMI medium (Gibco) containing 10% fetal calf serum.

RT-PCR

RNA was extracted with Trizol (Life Technologies, Gaithersburg, MD, USA). The integrity of the RNA was examined by 1% agarose gel electrophoresis and staining with ethidium bromide, which showed intact ribosomal 18S and 28S RNAs. RT reactions were performed with 0.5 µg total RNA (from an estimated amount of 1.5×10^6 cells), 0.5 µg oligo-dT and RT (Life Technologies), in a total volume of 20 µL. Similar reactions using 0.1 µg total RNA and half the amount of the reagents were subsequently made. The RT mixtures were incubated for 1.5 h at 37 °C. A reaction with RNA and oligo-dT, but without RT, was performed as a control. The cDNA from the RT reactions was precipitated in 1/10 volume of 3 M sodium acetate and 2.5 volume of 99% ethanol, subsequently washed with 70% ethanol and finally resuspended in 10 mL distilled water. The cDNA obtained from 0.5 µg total RNA was used for PCR amplifications employing ECP-specific primers and, as normalization controls, β -actin-specific primers. Additionally, cDNA obtained from 0.1 µg was used for PCR amplifications using ECP, EPX/EDN and β -actin-specific primers. The sequences of the ECP mRNA specific primers were 5'-gccacagctcagagactgggaaca-3' (bp 302–321 and 597–602, spanning two exons) and 5'-acaggagcttagatgtgg-3' (bp 1046–1028), based on GenBank no. X16545. The exon spanning 5' primer abolishes the need for DNase treatment of the RNA before use in the RT-reaction. The EPX/EDN specific primers (located in two exons) were 5'-tctcacaggagctacagcgcg-3' (bp 326–346) and 5'-gaatcatc taagctcctgtatcag-3' (bp 1065–1088) based on GenBank no. X16546. The sequences of the β -actin specific primers, located in two exons, were 5'-tgacggggtcaccacactgtgccatcta-3 (bp 2133–2162) and 5'-ctagaagcatttgcggtggacgatggagg-3' (bp 3000–2971) based on GenBank no. M10277. PCR reactions consisted of 30 cycles of 30 s at 96 °C, 30 s at 53 °C and 1 min at 74 °C. Followed by an extension period of 5 min at 74 °C. The PCR products were visualized on 1% agarose gels stained with ethidium bromide.

As the primers amplifying ECP might also amplify EDN, all the ECP PCR products were digested with ECP-specific *Cla*I. The results indicated that only ECP had been amplified (data not shown).

Specific protein assays

Cells analysed for ECP content were lysed in 0.5% N-Cetyl-N, N, N-trimethyl-ammonium bromide (CTAB) at room temperature for 1 h. Cell debris was spun down and the supernatant was stored at –20 °C. Gradient fractions were analysed for ECP or MPO content or stored at –20 °C until analysis. The ECP and MPO protein contents were measured with commercially

available radio-immuno assays (Pharmacia Diagnostics) according to the instructions supplied by the manufacturer (For ECP see [11]).

ECP labelling with Iodine-125

Protein was labelled by the ChloramineT method essentially as described in [19], but substituting Iodine-131 for Iodine-125 (^{125}I). To a tube of 10 µg ECP (purified in the authors laboratory), 0.5 milli curie ^{125}I (Amersham Biosciences) and 10 µg ChloramineT (1 mg/mL) was added and the solution was mixed for 45 s. Subsequently, 24 µg sodium metabisulfite (2.4 mg/mL) was added to stop the labelling reaction and the solution was again mixed for 45 s. The labelling reaction was purified using a Nap 10 column (Amersham Biosciences) where 250 µL fractions were eluted with a phosphate buffer. Radioactivity was measured by a gamma counter.

ECP uptake by neutrophils as measured by flow cytometry

Leucocytes were isolated from heparinized blood by a modification of a method previously described [20]. Briefly, 3 mL of heparinized blood was mixed with 15 mL of PBS-2% NBS and centrifuged for 5 min at 200 g. Plasma and platelets were removed and the remaining cell suspension was reconstituted to 3 mL with PBS-2% NBS. Erythrocytes were lysed by addition of 40 mL 0.83% NH_4Cl in 0.01 M Tris-HCl buffer, pH 7.4, and incubation for 15 min at 37 °C. After centrifugation for 5 min at 200 g the supernatant was removed and the remaining leucocytes were washed twice with PBS. The concentration of granulocytes in the leucocyte suspension was calculated and 1×10^6 granulocytes were mixed with 4.5 µg purified ECP [21] in 50 µL PBS and incubated for 30 min at 37 °C. Subsequently, the cells were washed four times with PBS and fixed for 5 min at 4 °C by adding an equal volume of 4% PFA. After fixation the cell samples were washed with PBS containing 0.5% BSA. The cell samples were first incubated with a permeabilizing buffer consisting of PBS, 0.1% saponin and 0.5% BSA for 5 min, then FITC-labelled-EG2 antibodies or FITC-labelled control mouse monoclonal antibodies (X927, Dako) were added and the incubation continued for 30 min [22]. After washing with permeabilizing buffer, cell samples were resuspended with PBS-0.5% BSA and analysed by flow cytometry. Neutrophils and eosinophils were separated by their different forward-side scatter pattern induced by permeabilization [23]. The mean fluorescence intensity of neutrophils incubated with and without ECP was measured and the values of mean fluorescence intensity were converted to Mean Equivalents of Soluble Fluorochromes (MESF) by the use of DAKO FluoroSperes (Dako).

Subcellular fractionation

Granulocytes (purified neutrophils or eosinophils or mixtures of both, as indicated in each case, total number of cells used are stated in the methods or figure legends) were suspended in 1.5 mL of hypotonic sucrose (6% w/w sucrose in 10 mM Pipes) and incubated on ice for 15 min. Subsequently the cells were sonicated (Soni Prep sonicator) for 15 s at an amplitude of 8 µm. Tonicity of the solution was restored by addition of 185 µL of 34% (w/w) sucrose. Remaining intact cells and nuclei were spun down at 1000 g for 5 min, at 4 °C, obtaining a postnuclear supernatant (organelle containing cell cytoplasm).

Sucrose gradients were prepared by overlaying consecutively a 60% (w/w) sucrose cushion with 55, 50, 46, 42, 38 and

34% (w/w) sucrose solutions in 10 mM Pipes, pH 7.4. To produce continuous gradients, sucrose diffusion was allowed for 3 h at room temperature. The gradients were overlaid with postnuclear supernatants and subjected to overnight centrifugation at 85 500 *g* (r_{av}), in a Beckman ultracentrifuge (SW28.1 rotor), at 4 °C. Forty fractions of 425 μ L were each collected from the top of the gradients by upward displacement with 60% sucrose and stored at -20 °C until analysis.

Fraction densities were determined by using a refractometer (Bellingham & Stanley Ltd, Tunbridge Wells, UK). The peak densities corresponding to the two subpopulations of neutrophil primary granules (1.21 and 1.23 g/mL [24]) and to eosinophil-specific granules (1.25 g/mL [25]) are indicated in the graphs shown.

Subcellular localization of internalized ECP

Subcellular fractionation studies (Fig. 1) Granulocyte suspensions (2.5×10^6 cells) containing 92.2% neutrophils and 7.6% eosinophils were incubated for 30 min without or with ECP (9 or 90 μ g/mL) at 37 °C in 50 μ L. [^{125} I]ECP (1 μ g, 50 μ L, 2×10^8 CPM/ μ g) was then added and incubations continued for 1 h. Cells were then washed, disrupted by sonication and fractionated by sucrose gradients as described above.

Chase experiments (Fig. 2) Granulocytes (2.5×10^6 cells, 97% neutrophils and 3% eosinophils) were first incubated for 30 min at 37 °C with [^{125} I]ECP (1 μ g, 50 μ L, 2×10^8 CPM/ μ g) and then cold ECP (50 μ L, final concentration 45 μ g/mL) or PBS (controls) was added. Incubations were continued for 5 or 30 min, after which the cells were washed, disrupted and fractionated as described above.

Temperature dependency of internalization Granulocytes (2.5×10^6 cells, 93% neutrophils and 7% eosinophils) were incubated with 90 μ g/mL ECP for 30 min at either 4 °C or 37 °C, in 50 μ L. [^{125} I]ECP (1 μ g, 50 μ L, 2×10^8 CPM/ μ g) was then added and incubations continued for 1 h at the same temperatures as before. Cells incubated at each of the temperatures were washed, disrupted and fractionated as described above.

Secretion experiments (Fig. 3) Granulocytes (2.5×10^6 cells, 98% neutrophils and 2% eosinophils) were incubated for 30 min with 90 μ g/mL ECP, at 37 °C in 50 μ L. [^{125} I]ECP (1 μ g, 50 μ L, 2×10^8 CPM/ μ g) was then added and incubations were continued for 1 h. Cells were washed and one portion was left on ice and later used as a control. Another portion was resuspended in HBSS-extra (Hank's balanced salt solution supplemented with 0.74 mM Ca^{2+} and 1 g/L HSA). Serum-opsonized Sephadex G-15 particles were added to a final concentration of 55.5 mg/mL (total volume, 2.5 mL). The final cell concentration was of 1×10^6 /mL [10]. After 20 min at 37 °C, the cells were washed with PBS. The G-15 treated cells and the control cells were disrupted and fractionated as described above.

Results

Presence of ECP within neutrophils

Radioimmunoassay A specific RIA determined the cellular concentration of ECP in purified neutrophils. The cell

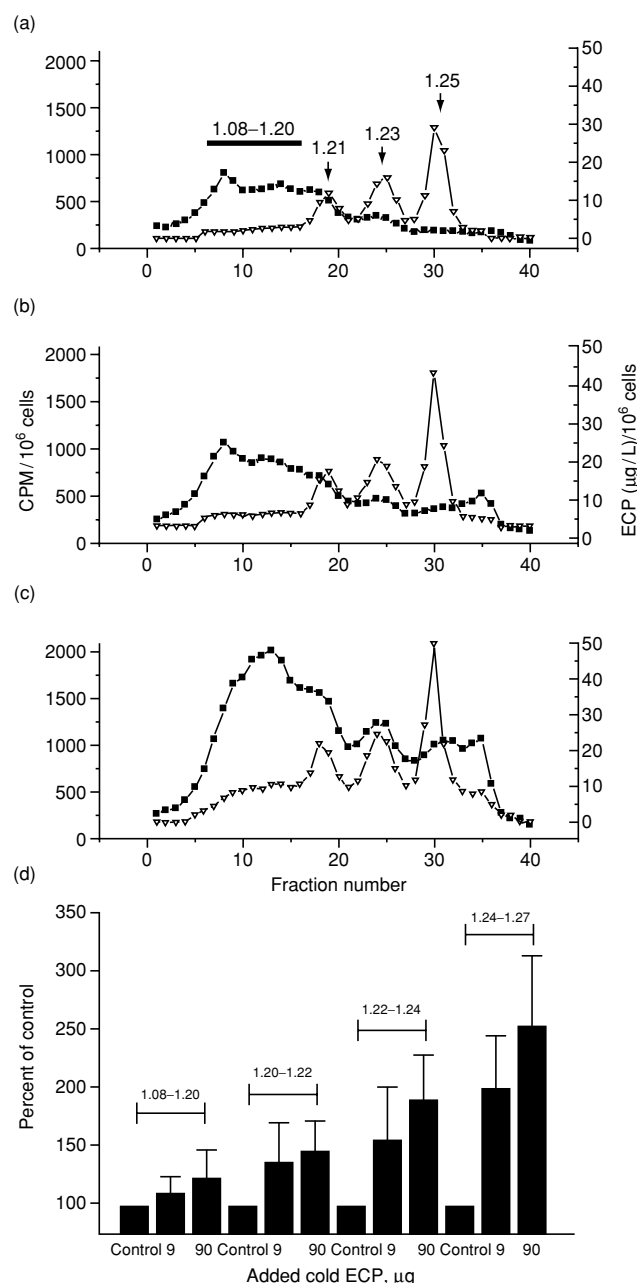


Fig. 1. Subcellular localization of internalized ECP. Granulocyte suspensions (2.5×10^6 , 92.2% neutrophils and 7.6% eosinophils) were incubated at 37 °C for 30 min in the absence (a) or presence (b) of 9 μ g/mL, or 90 μ g/mL (c) purified ECP, in a total volume of 0.05 mL. After adding [^{125} I]ECP (1 μ g, 2×10^8 CPM/ μ g) to each of the three mixtures, incubations were continued for 1 h. Cells were disrupted and fractionated as described in Methods. Results are expressed as ECP concentrations μ g/L (∇) or CPM ($\blacksquare$) in each gradient fraction, both normalized to 10^6 cells. Arrows indicate the equilibrium densities of neutrophil light (1.21 g/mL) and dense (1.23 g/mL) azurophilic granules and of eosinophil specific granules (1.25 g/mL). The horizontal bar in (a) marks the gradient region including fractions of equilibrium densities between 1.08 and 1.20 g/mL. (d) Percent internalization of radioactive ECP into different subcellular compartments as a function of increasing the addition of cold ECP. No ECP added is 100%. Results from three experiments, normalized to total amount of radioactive ECP added in each experiment. The percent ECP uptake of the fractions contained within the density intervals 1.08–1.20 g/mL (fractions 6–16), 1.20–1.22 g/mL (low density neutrophil primary granules, fractions 17–21), 1.22–1.24 g/mL (high density neutrophil primary granules, fractions 22–27) and 1.24–1.27 g/mL (eosinophil specific granules, fractions 28–33) is shown.

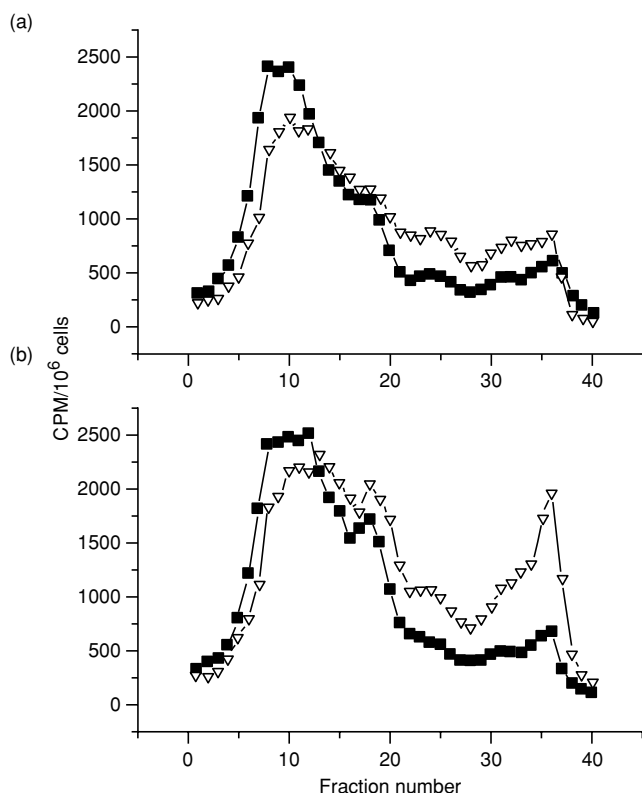


Fig. 2. Chase of internalized ECP. Granulocytes (2.5×10^6 cells, 97.2% neutrophils and 2.8% eosinophils) were first incubated at 37°C for 30 min with [^{125}I]ECP ($1 \mu\text{g}$, 2×10^6 CPM/ μg). Cold ECP (final concentration $45 \mu\text{g}/\text{mL}$) (■) or PBS (▽) was then added and incubations were continued for 5 min (a) or 30 min (b). Cells were washed, disrupted and fractionated as described in Methods. Results are expressed as CPM per gradient fraction, normalized to 10^6 cells.

preparations consisted of 98.5–100% neutrophils (the rest being eosinophils) and their ECP concentrations ranged from 123 to $342 \text{ ng}/10^6$ cells (Table 1). This value is about 1–2% of the content of ECP in eosinophils, which is on average $13.5 \mu\text{g}/10^6$ cells [26].

Immunocytochemistry The intracellular localization of ECP in neutrophils was detected by light microscopy after immunostaining with the anti-ECP monoclonal antibody EG2 (Figs 4a and b). For comparison we show the staining of an eosinophil, which was, as expected, much stronger than that of neutrophils. In Fig. 4(b), a neutrophil and an eosinophil are in close proximity and the ECP staining in the neutrophil is stronger in the vicinity of the cell contact surface.

Confocal microscopy We confirmed the localization of ECP in granules of eosinophils and neutrophils by confocal microscopy (Fig. 4c). It is well apparent that the amount of ECP is much higher in eosinophils. The use of an irrelevant FITC labelled antibody (anti- α -fetoprotein) did not show any staining in either eosinophils or neutrophils (data not shown).

Immuno electron microscopy The localization of ECP within neutrophils was estimated by immunogold staining using MAb 611 as the first antibody. Most granules were

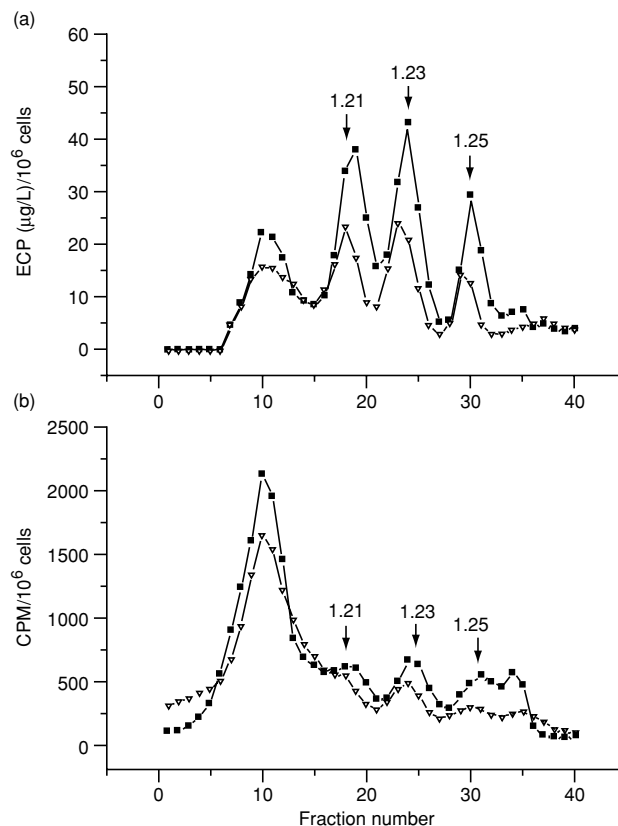


Fig. 3. Secretion of internalized ECP. Two portions of granulocytes (2.5×10^6 cells each, 98% neutrophils and 2% eosinophils) were incubated for 30 min with $90 \mu\text{g}/\text{mL}$ ECP, at 37°C . [^{125}I]ECP ($1 \mu\text{g}$, 2×10^6 CPM/ μg) was then added and incubations were continued for 1 h. Cells were washed and one portion was left on ice as untreated control (■), the other portion was resuspended with HBSS and subsequently added serum-opsonized G-15 particles (▽). After 20 min at 37°C , cells were washed, and both portions were disrupted and fractionated as described in Methods. ECP concentrations (a) and CPM (b) of the gradient fractions were measured. Data are normalized to 10^6 cells. Arrows indicate the equilibrium densities of neutrophil light (1.21 g/mL) and dense (1.23 g/mL) azurophilic granules and of eosinophil specific granules (1.25 g/mL). The results shown are representative of three independent experiments.

Table 1. ECP content in neutrophils

Experiment no.	Eosinophil contamination (%)	ECP (ng/ 10^6 cells)
1	1.5	342
2	1.1	190
3	0.2	210
4	0.05	209
5	0	123

Neutrophils were obtained by either 67% Percoll gradient centrifugation of blood from normal donors and subsequent negative selection by depletion of $\text{CD}9^+$ eosinophils (experiment 1–3 and 5) or by 67% Percoll gradient centrifugation (only) of blood from a subject with a low eosinophil count (experiment 4). Cells were lysed with 0.5% N-Cetyl-N, N,N-trimethyl-ammonium bromide (CTAB) (0.5×10^6 cells/mL in experiment 1–3 or 5×10^6 cells/mL in experiment 4 and 5). ECP concentrations were measured by RIA.

labelled for ECP, while others did not show the association of any gold particle (Fig. 4d). Some of the staining was cytosolic and a few gold particles were found associated to the cell

membrane, while no ECP was observed on Golgi structures (marked G on Fig. 4d). Control samples using only the secondary antibody or an irrelevant antibody did not show any staining (data not shown).

Cell fractionation To obtain further information about the subcellular localization of ECP in neutrophils, we studied the distribution profiles of MPO (a neutrophil primary granule protein) and ECP in granulocytes, i.e. neutrophils plus eosinophils, and compared it with those of purified neutrophils and eosinophils. The subcellular distributions of MPO and ECP, obtained after cell disruption and separation of organelles on sucrose continuous gradients, were very reproducible. The results of representative experiments are shown.

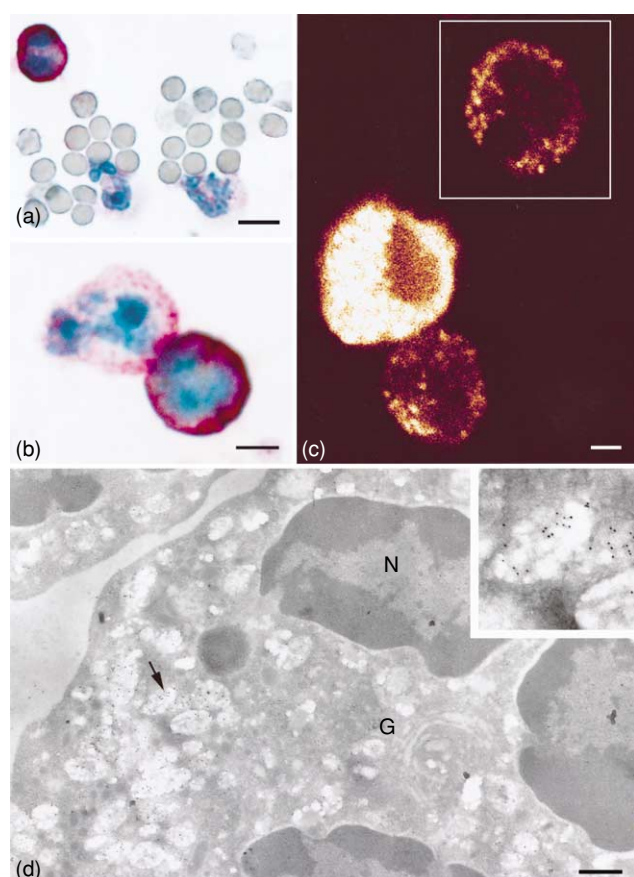


Fig. 4. ECP localization. (a) Immunocytochemistry I (light microscopy). Neutrophils and eosinophils were immunostained by the monoclonal ECP antibody, EG2. The figure shows one strongly stained eosinophil and two weakly stained neutrophils. The granulocytes are surrounded by non-stained red blood cells. Bar = 10 μ m. (b) Immunocytochemistry II (light microscopy). Neutrophils and eosinophils were immunostained by the monoclonal ECP antibody EG2. One strongly stained eosinophil is shown closely adjacent to a less stained neutrophil. Bar = 5 μ m. (c) Confocal microscopy. Peripheral blood granulocytes were stained using the FITC conjugated monoclonal antibody EG2. One strongly stained eosinophil and two weakly stained neutrophils are shown (the inset shows a neutrophil). Bar = 2 μ m. (d) Immunogold EM. A peripheral blood neutrophil stained for ECP using a primary anti-ECP MAb 611 followed by a secondary antibody coupled to 10 nm gold particles. Labelling is seen in most neutrophil granules (arrow and inset) and some staining is located in the cytoplasm. Bar = 500 nm. All the pictures shown are representative examples.

We observed that, as expected, the ECP content of neutrophils is much lower than that of eosinophils, and that most of it is present in two peaks of mean equilibrium densities 1.21 and 1.23 g/mL (Fig. 5a). This distribution is identical to that of MPO (Fig. 5c), with the peaks corresponding to the two major populations of primary granules [24]. Most of the ECP in purified eosinophils is localized in a peak of equilibrium density 1.25 g/mL (Fig. 5d), the same density as that of the smaller, most dense peak observed in Fig. 5a, which is therefore likely to originate in the 1.8% eosinophils contained in the granulocyte

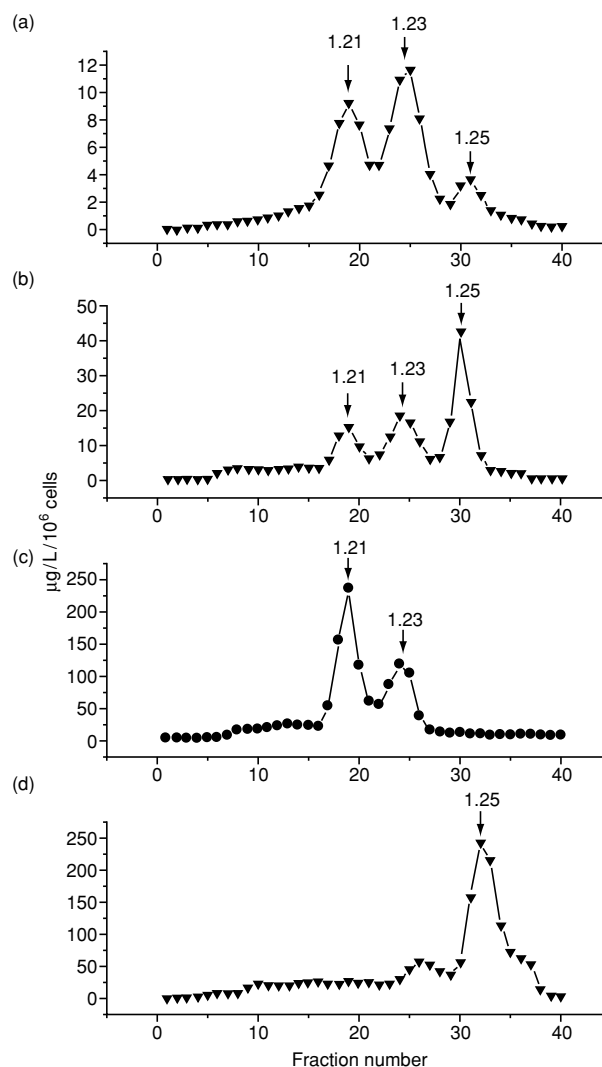


Fig. 5. Fractionation of eosinophil and neutrophil organelles by a sucrose density gradient. The figure shows the distributions of ECP and MPO in the gradients. Eosinophils and neutrophils were purified from peripheral blood. (a) ECP in neutrophils purified by CD9 negative selection (26×10^6 cells, 98.2% neutrophils, 1.8% eosinophils). (b) ECP in granulocytes (2.5×10^6 cells, 92% of neutrophils, 8% of eosinophils) obtained by separation on Percoll 67%. (c) MPO in granulocytes (2.5×10^6 cells, 92% of neutrophils, 8% of eosinophils) obtained by separation on Percoll 67%. (d) ECP in eosinophils (2.6×10^6 cells, 99.3% purity) obtained by CD16 negative selection from the same donor as in (a). The curves shown are representative of > 5 different experiments for each situation. Data are presented as concentrations (μ g/L) of ECP or MPO in the gradient fractions normalized to 10^6 cells. Arrows indicate the equilibrium densities of neutrophil light (1.21 g/mL) and dense (1.23 g/mL) azurophilic granules and of eosinophil specific granules (1.25 g/mL).

preparation. In fact, the 1.25 g/mL peak increases when the proportion of eosinophils in the granulocyte preparation increases to 8% (Fig. 5b). Therefore, we conclude that ECP is stored in the primary granules of neutrophils (1.21 and 1.23 g/mL), and that the highest density ECP peak (1.25 g/mL) corresponds to contaminating eosinophils.

mRNA Since RT-PCR is a very sensitive method, it was important to isolate RNA from extremely pure neutrophils to avoid false positive results due to contamination with eosinophils. In order to get a neutrophil population close to 100% purity, granulocytes were run three consecutive times through a MAC separation column. Total RNA was prepared from cells from each of the three rounds of purification and the integrity of the RNA was confirmed on a 1% agarose gel (Fig. 6a). Subsequently, the total RNA was subjected to RT-PCR using ECP and EPX/EDN-specific primers. EPX/EDN mRNA has been recently reported to be present in neutrophils [14] and was

therefore used as an appropriate positive control. Bands of amplification product corresponding to both ECP (514 bp) and EPX/EDN (533 bp) were present after one or two cycles of cell purification (Fig. 6b, lanes 2 and 3). RNA from cells after the third purification round (containing 0.02 or <0.005% eosinophils) gave a visible EPX/EDN RT-PCR product but no ECP bands (lanes 4 and 5). This indicated the absence of ECP mRNA in populations of extremely pure neutrophils. The amplification of β -actin, used as a control gene, showed similar amounts in all cases. Negative controls without RT did not result in any amplification products (data not shown).

A control of the sensitivity of ECP mRNA detection was performed using mixtures of fibroblasts (devoid of ECP mRNA) with eosinophils, with the latter in decreasing amounts. This established that down to at least 0.5% eosinophils give a positive signal, indicating that ECP transcripts can be amplified from low numbers of eosinophils. These results though, cannot be directly compared with those of the neutrophil/eosinophil mixtures since fibroblasts contain at least 10 times more RNA than granulocytes.

Uptake and fate of ECP

Flow cytometry Since our results up to this point had shown the presence of ECP in neutrophils although these cells do not synthesize it (absence of mRNA), we postulated that ECP might be taken up from the environment. Flow cytometry analysis of intracellular staining with the anti-ECP MAb EG2 showed that the molecules of equivalent soluble fluorochrome (MESF) value of neutrophils increased substantially after incubation with ECP, therefore suggesting the uptake of ECP by these cells (Fig. 7a). The increase of fluorescence intensity demonstrated by neutrophils after incubation with ECP was not seen in eosinophils (Fig. 7b). The reason for this result might be that the amount of ECP taken up by eosinophils was too small to be detected, compared with the already high amount present in the cells. The staining of neutrophils and eosinophils with a FITC-labelled control antibody did not show any shift in fluorescence intensity after incubation with ECP (Fig. 7b).

Subcellular localization of internalized ECP In order to study the fate of the ECP internalized by neutrophils, we incubated granulocytes (92.2% neutrophils and 7.6% eosinophils) with [^{125} I]ECP. After extensive washing, the cells were fractionated as described above. Most of the radioactivity was found distributed as a broad peak in the low density region of the gradient (1.08–1.20 g/mL, marked with a black bar in Fig. 1a). This region contains plasma membranes as well as Golgi, vesicle structures and easily mobilisable secretory organelles [25, 27, 28]. Part of the radioactivity was also present at higher densities, coinciding with neutrophil primary granules (1.21 and 1.23 g/mL) and eosinophil crystalloid granules (1.25 g/mL) (Fig. 1a). When cold ECP at two different concentrations was added prior to [^{125} I]ECP, a greater amount of radiolabelled ECP was taken up by the granulocytes (Figs 1b and c), indicating that internalization increases with higher ECP concentration in spite of the radioactivity-diluting effect of the cold ECP. The proportion of radioactive to total ECP (as determined by RIA) was greater in all gradient regions including the neutrophil primary granule peaks of densities 1.21 and 1.23 g/mL.

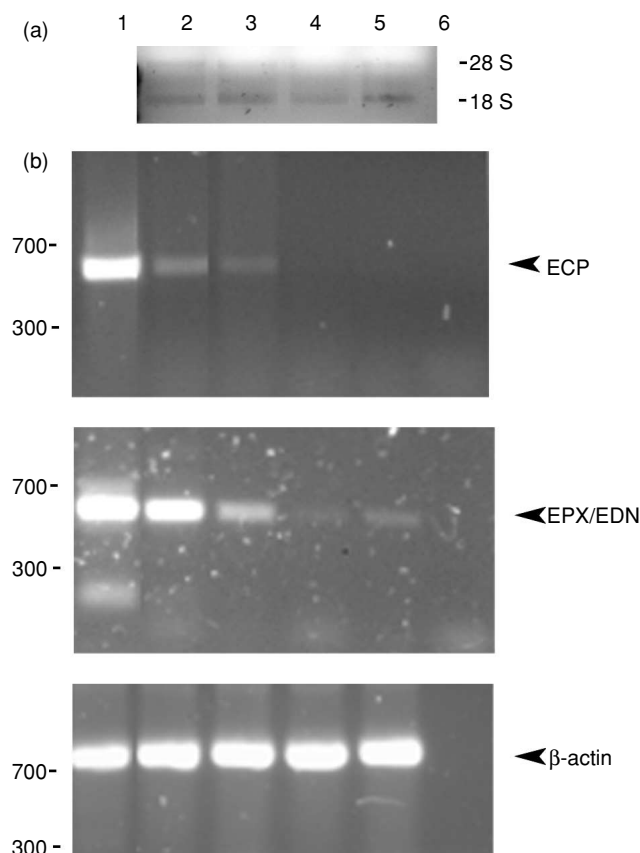
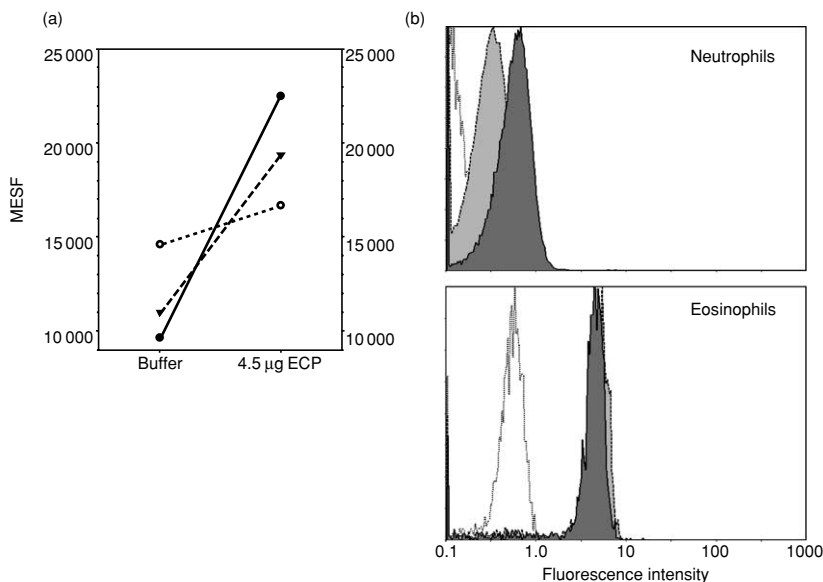


Fig. 6. Detection of ECP mRNA transcript in neutrophils as a function of cell purity. (a) 0.1 μ g of the RNA used for the RT-PCR reactions described below (lanes 2–5) was run on an agarose gel. Well-defined bands of 28S and 18S rRNA can be observed in all cases, indicating the absence of degraded RNA. (b) RT-PCR detection of a 514-bp fragment of ECP transcripts (using 0.5 μ g RNA) and a 533-bp fragment of EPX/EDN transcripts (using 0.1 μ g RNA) in: U937 cells (lanes 1), neutrophils with 0.5% contaminating eosinophils (lanes 2), 0.07% contaminating eosinophils (lanes 3), 0.02% contaminating eosinophils (lanes 4), or undetectable contamination (lanes 5). Lanes 6: Control PCR reaction without cDNA. Control cDNA reactions were performed using 0.1 μ g RNA and β -actin specific primers, obtaining 691 bp fragments. Similar results were obtained using RNA from the different stages of two independent neutrophil purifications.

Fig. 7. Analysis by flow cytometry of neutrophils after ECP internalization, using fluorescein-conjugated anti-ECP (EG2). (a) Isolated leucocytes were incubated with buffer or with 4.5 μ g ECP for 30 min at 37 °C. The mean fluorescence intensity of the neutrophils was measured and the values were converted to MESF (Molecules of equivalent soluble fluorochrome). The figure shows the individual results of three experiments performed with cells from different donors. (b) Histogram visualization of one of the experiments demonstrated above showing fluorescence intensity (logarithmic scale) on the x-axis and number of cells on the y-axis. The staining with FITC-labelled EG2 monoclonal antibody of neutrophils and eosinophils incubated with buffer (dashed line) and 4.5 μ g purified ECP (solid line) compared with the staining with a FITC-labelled control monoclonal antibody (dotted line) is demonstrated. Staining of neutrophils and eosinophils with the control monoclonal antibody (dotted line) showed the same pattern irrespective of the addition of purified ECP.



The increase of the radioactive labelled ECP of the four gradient regions mentioned above (1.08–1.20, 1.20–1.22, 1.22–1.24 and 1.24–1.27 g/mL), as a function of increasing concentrations of cold ECP added, is shown in Fig. 1(d). The figure shows three experiments with the CPM values normalized to the amount of radioactive ECP initially added to the cells. All regions in the gradient showed an increased uptake of radiolabelled ECP as a function of increasing the amount of cold ECP added. The uptake was higher in the regions with higher densities corresponding to the azurophilic granules of neutrophils and the granules of eosinophils.

In control experiments, neither radiolabelled albumin nor non-protein bound ^{125} I were taken up by the cells either in the absence or presence of cold ECP.

Chase of internalized ECP To obtain information about the intracellular trafficking of ECP, we chased internalized radiolabelled ECP with an excess of cold ECP for 5 and 30 min (Fig. 2). A displacement of [^{125} I]ECP toward denser compartments of the cells was clearly observed after a 30-min chase (Fig. 2b), again indicating the dynamic nature of the process.

Temperature dependence of ECP uptake We established the temperature dependence of internalization by determining the amount of radiolabelled ECP taken up by neutrophils and its subcellular distribution at 0 °C and 37 °C. We found that a markedly smaller amount of ECP was internalized at 0 °C than at 37 °C, indicating a process requiring cell membrane fluidity. The small amount of ECP taken up at 0 °C was not associated to any particular subcellular compartment, whereas the ECP taken up at 37 °C was found distributed in subcellular fractions of different densities, in agreement with the previous experiments reported here.

Secretion of ECP taken up To answer the question of the possible secretion of the ECP internalized by granulocytes, we stimulated cells that had taken up ECP to secrete by exposing them to serum-coated Sephadex particles. After a 20-min

period, both stimulated and unstimulated (control) cells were fractionated. As shown in Fig. 3(a), a reduction in ECP was observed in all four gradient regions, as determined by immunoassay of all gradient fractions. Regarding neutrophil primary granules, there was a decrease of 39% and 41% in the subpopulations of equilibrium density 1.21 and 1.23 g/mL, respectively, as a result of stimulation. Furthermore, radioactivity measurements of the subcellular fractions showed a reduction in the radiolabelled ECP associated to them, as a consequence of stimulation (Fig. 3b). This reduction was 20% and 27% for the neutrophil granule compartments of density 1.21 and 1.23 g/mL, and was also observed for organelles of lower density (1.08–1.20 g/mL) and eosinophil granules (1.25 g/mL). These results show that the ECP that has been internalized to low density organelles and granule fractions can be secreted from those subcellular compartments upon cell stimulation.

Discussion

The data presented in this study show that ECP is not produced by human peripheral blood neutrophils and that its presence in these cells is most probably due to an active uptake of ECP from the environment and storage within primary granules.

The cell specificity of ECP has been the focus of a number of articles during the last decade. Neutrophils and basophils have been shown to contain ECP [13,14], and preliminary results have also shown that monocytes produce small amounts of the protein [29]. Certainly, ECP can be detected by specific RIA in all these cell types. ECP has been shown to affect artificial cell membranes by making pores [30] and to be cytotoxic to parasites, bacteria and viruses [5–7].

A way of assessing the *de novo* ECP synthesis by neutrophils is by RT-PCR. We and others have demonstrated, using the same technique, that ECP is produced by mature circulating eosinophils [29,31] which have also been shown by EM to contain ECP associated to their Golgi [25]. Because RT-PCR is a very sensitive method, it was necessary to use highly purified neutrophils

to ensure that no amplification products originated from a small eosinophil contamination. Using > 99.995% pure neutrophils, no signs of ECP transcripts were observed, whereas EPX/EDN mRNA was clearly detected, as previously reported [14]. This finding, together with the fact that no ECP was detected associated to the neutrophil Golgi, as opposed to eosinophils, indicates that neutrophils do not produce this protein. We cannot completely exclude that some of the ECP found in peripheral blood neutrophils has been produced during the maturation of these cells in the bone marrow. The fact that the promyelocytic cell line HL-60 does not produce ECP [32], however, seems to argue against this possibility.

We have studied the intracellular localization of ECP by using specific antibodies combined with optical (light microscopy, EM, confocal microscopy) and subcellular fractionation techniques. Some of the staining of neutrophils for ECP could have been artefactual, as shown, e.g. by more intensely stained neutrophils adjacent to eosinophils (Fig. 4b), as this can result from the fixation procedure [33]. The rest of our data indicate however, that this cannot be the sole explanation for the presence of ECP in neutrophils.

We have found that ECP is associated to specific structures within neutrophils, and these structures are predominantly granules (Figs 4c and d).

Since there was no indication that neutrophils produce ECP, the possibility was considered that ECP was being taken up either from the bloodstream or in the bone marrow. The ECP found by immuno-EM in the cytoplasm (Fig. 4d) might be an indication that this protein is being transported to the granules (see below).

The hypothesis that ECP is taken up by neutrophils was validated by incubation of neutrophils with purified ECP. In fact, ECP was shown to be taken up by neutrophils not only by flow cytometry but also by analysis of subcellular fractions obtained from neutrophils that had been incubated with ECP.

Our results are consistent with a concentration-dependent active uptake of ECP. The internalized protein was found intracellularly associated to low density subcellular fractions and both neutrophil and eosinophil granules. Incubation of neutrophils with cold together with radiolabelled ECP resulted in a higher intracellular content of the radioactive protein, a finding difficult to explain other than by the fact that ECP has been shown to make pores in cell membranes which might facilitate the uptake [30]. However, our finding that no uptake of albumin was induced by addition of cold ECP seemed to exclude this possibility. Nevertheless, it should be pointed out that, contrary to our findings with albumin, other studies suggested that albumin was endocytosed by neutrophils or neutrophil precursors and stored in secretory vesicles from which it was secreted extracellularly upon stimulation [27]. A plausible explanation for our results could be that ECP binds to the cell membrane and subsequently becomes endocytosed or pinocytosed, and that this process is somehow enhanced by a greater concentration of ECP. This could be due to the uptake of ECP occurring through mechanisms such as cell surface proteoglycan-mediated clustering into membrane rafts, as described by Fuki et al. [34], through self association into homomultimeric complexes prior to internalization [35], or by macropinocytosis. Previous studies on angiogenin, a protein homologous to ECP, showed that this protein was actively taken up by endothelial cells and stored intracellularly [36].

We speculate that the ECP found in the low density region of the subcellular fractionation gradients (1.08–1.20 g/mL), particularly after incubation of neutrophils with radiolabelled ECP, could be vesicle-bound ECP being transported to the primary granules. Normally, very little ECP is found outside the granules, as shown by EM. The internalization vesicles would transport ECP to the primary granules of the neutrophil, where it is found colocalizing with the endogenous MPO.

The release experiments showed that the ECP that had been taken up could be secreted from neutrophils upon triggering of a receptor-mediated mechanism, similarly to the secretion of the primary granule proteins [37]. This supports the idea of ECP following ordinary trafficking pathways after being ingested; as opposed to just binding to some cell structure as it would have been the case if it had entered cells passively through membrane pores. That internalized ECP is actually inside granules and not associated externally to their membrane is indicated by the observation that the decrease in granule-associated ECP triggered by cell stimulation is not paralleled by an equivalent increase in plasma membrane-associated ECP, which should have been found in low density fractions. Indeed, only ECP present inside membrane-bound structures can disappear from cells upon stimulation.

Our results indicate that ECP may also be taken up by eosinophils. This finding, however, warrants further studies. Another eosinophil derived protein, eosinophil peroxidase (EPO) has previously been shown to be taken up by neutrophils by a receptor-coupled mechanism [38]. It was speculated that the uptake might serve to limit the tissue damage that would otherwise be the result of EPO secretion. The uptake of ECP by the neutrophils as shown here might serve a similar purpose even though the reuse of ECP is an alternative possibility.

In conclusion, our results suggest that the ECP found in the primary granules of circulating neutrophils is not produced in these cells. Instead, eosinophil-originated ECP present in the milieu is taken up by neutrophils, where it seems to follow a membrane-bound trafficking pathway that eventually ends in the primary granules.

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