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Neutrophils as a Novel Source of Eosinophil Cationic Protein in IgE-Mediated Processes

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The production of eosinophil cationic protein (ECP) in IgE-mediated diseases has been associated mainly with eosinophils, although no IgE-dependent ECP release has been observed in these cells. Because there is increasing evidence of neutrophil participation in allergic processes, we have examined whether human neutrophils from allergic patients were able to produce ECP by an IgE-dependent mechanism. After challenge with specific Ags to which the patients were sensitized, ECP release was detected in the culture medium. Furthermore, intracellular protein was detected by flow cytometry, immunofluorescence staining, and Western blotting. Expression at both mRNA and de novo protein synthesis was detected, respectively, by RT-PCR and radio-labeling with 35S. Ag effect was mimicked by cell treatment with anti-IgE Abs or Abs against FerR1 and galectin-3 (FcER1I-galectin-3), but not against FcεRII. These observations represent a novel view of neutrophils as possible source of ECP in IgE-dependent diseases. The Journal of Immunology, 2007, 179: 2634–2641.

Eosinophil cationic protein (ECP) is a highly cationic protein, belonging to the RNase superfamily, with potent cytotoxic properties against mammalian and nonmammalian cells such as parasites, bacteria, and viruses. In addition to being cytotoxic, ECP has a number of noncytotoxic activities, including regulation of immune and epithelial cells, complement, coagulation, and fibrinolysis (1–3). Despite this strong association between eosinophils and allergy, the mechanisms of cellular activation are largely unknown. Expression of IgE receptors on eosinophils had been reported in cells from patients with marked eosinophilia (4–7), but not in cells from healthy donors and/or subjects with mild-to-moderate eosinophilia. In addition, IgE-dependent functional responses, such as ECP release, were not observed in blood eosinophils from healthy and allergic individuals, whereas these eosinophils responded vigorously to IgG (8–10). mAbs against ECP have been used to detect total eosinophils. Immunostaining techniques evidenced that the number of ECP+ cells was higher than the number of eosinophils. It has been shown that neutrophils also contain ECP (11–18). Furthermore, the ECP was specifically associated with neutrophils in allergic-asthmatic processes (19–24). There is increasing evidence of neutrophil participation in asthma and the allergic process (25). For instance, peripheral blood neutrophils are activated during active asthma (26), after exercise-induced bronchospasm (27), and during both early and late allergen-induced asthmatic reactions (28). Several studies have reported evidence linking the presence of neutrophils with airway damage and dysfunction (29–31). IgE can bind on neutrophils at the high-affinity receptor for IgE (FcεRI) (32), the low-affinity receptor for IgE (FcεRII/CD23) (33, 34), and galectin-3 (35, 36). We have previously shown that specific Ags were able to activate functional responses by neutrophils from allergic patients sensitized to Ags of the same type as those which produce clinical allergic symptoms. For example, allergens induce in neutrophils the release of myeloperoxidase, IL-8, and elastase, the down-modulation of L-selectin, and the respiratory burst and intracellular increase of Ca2+ levels (37–41). If eosinophils are unable to release ECP by an IgE-dependent mechanism, can neutrophils do so? The present work was undertaken to analyze this question.

Materials and Methods

Chemicals and reagents

The Ags were commercially available Ag extracts, including D1 (Dermatophagoides pteronyssinus), G3 (Dactylis glomerata), T9 (Olea europaea), and W9 (Artemisia vulgaris). They were purchased from Bial-Aristegui. Goat anti-human IgE Ab, goat anti-human IgG Ab, and the Fix-and-Perm cell kit were obtained from Caltag Laboratories. Goat or mouse IgG, 1-α-phosphatidylcholine, platelet-activating factor (PAF) (β-acetyl-y-O-alkyl), and LPS were from Sigma-Aldrich. Ficol-Hypaque, PBS, RPMI 1640, FBS, and penicillin/streptomycin were purchased from BioWhittaker. PCR primers and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Anti-human CD9 Ab, anti-human CD16 Ab, and the Fix-and-Perm cell kit were obtained from Caltag Laboratories. Goat or mouse IgG, 1-α-phosphatidylcholine, platelet-activating factor (PAF) (β-acetyl-y-O-alkyl), and LPS were from Sigma-Aldrich. Ficol-Hypaque, PBS, RPMI 1640, FBS, and penicillin/streptomycin were purchased from BioWhittaker. PCR primers and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Anti-human CD9 Ab, anti-human CD16 Ab, and the Fix-and-Perm cell kit were obtained from Caltag Laboratories. Goat or mouse IgG, 1-α-phosphatidylcholine, platelet-activating factor (PAF) (β-acetyl-y-O-alkyl), and LPS were from Sigma-Aldrich. Ficol-Hypaque, PBS, RPMI 1640, FBS, and penicillin/streptomycin were purchased from BioWhittaker. PCR primers and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Anti-human CD9 Ab, anti-human CD16 Ab, and the Fix-and-Perm cell kit were obtained from Caltag Laboratories. Goat or mouse IgG, 1-α-phosphatidylcholine, platelet-activating factor (PAF) (β-acetyl-y-O-alkyl), and LPS were from Sigma-Aldrich. Ficol-Hypaque, PBS, RPMI 1640, FBS, and penicillin/streptomycin were purchased from BioWhittaker. PCR primers and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Anti-human CD9 Ab, anti-human CD16 Ab, and the Fix-and-Perm cell kit were obtained from Caltag Laboratories.
were purchased from Abcan and eBioscience, respectively. [35S]Iodinelabeled (15S) was obtained from Nucler. Kodak X-OMat AR films were obtained from Kodak. Mouse mAb specific for human anti-β-actin (sc-8432) was purchased from Santa Cruz Biotechnology. All cultured reagents had endotoxin levels of ≤0.01 ng/ml, as tested by the Limulus lystate assay (Coastest; Chromogenix).

Patients and controls
The studied group included adult atopic patients with intermittent bronchial asthma and healthy adult nonatopic volunteer controls (42). The group of asthmatic patients had positive skin-prick test (Bial-Aristeguiu) and specific IgE (HYTEC 288; Hycor Biomedical-IZASA) to at least one common Ag. The subjects received no specific hyposensitization. The patients were not allowed to take any bronchodilators within the 8 h before challenge of cells in vitro. Oral bronchodilators were withheld for 24 h, and none of the subjects had taken corticosteroids, disodium cromoglycate, or nedocromil sodium in the previous week. The healthy controls had no history of allergy or bronchial symptoms, and had negative skin-prick test (Bial-Aristeguiu) and specific IgE (HYTEC 288) to a battery of inhaled Ags (house-dust mites, pollens, molds, and animal danders). The hospital ethics committee approved the study and each subject gave informed consent.

Preparation of neutrophils and eosinophils
Human neutrophils were isolated as previously described (37–41). Neutrophil preparations were further purified using a MACS by incubation with mouse anti-human CD9, anti-human CD203c, and anti-human CD14 Abs, and then with anti-mouse IgG micromagnetic beads. The purity of neutrophils was on average ≥99%. To prepare mRNA for RT-PCR, neutrophils were purified even further by repeating the procedure above thrice more, using anti-CD9, anti-human CD203c, and anti-human CD14 Abs each time, which reduced contaminating eosinophils to 0.001–0.004% of the final cell population. Human eosinophils were analogously purified by MACS, using anti-CD16, anti-CD203, and anti-human CD14 Abs as previously described (43).

Neutrophil and eosinophil culture: treatment with different agents
Neutrophils and eosinophils were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in an atmosphere of 5% CO2 and 95% O2. To prepare mRNA for RT-PCR, neutrophils were purified further by repeating the procedure above thrice more, using anti-CD9, anti-human CD203c, and anti-human CD14 Abs each time, which reduced contaminating eosinophils to 0.001–0.004% of the final cell population. Human eosinophils were analogously purified by MACS, using anti-CD16, anti-CD203, and anti-human CD14 Abs as previously described (43).

ECP release
ECP release was evaluated in the culture supernatants by ELISA (CAP system immunoassay; Pharmacia) according to the manufacturer’s instructions.

ECP expression analysis by flow cytometry
Neutrophils (2 × 10⁶, cultured at a density of 6.6 × 10⁶/ml) were cooled on ice with washed cold PBS. Cells were incubated with PE-conjugated anti-CD66b Ab or the IgG1-PE isotype control Ab, for 15 min. After washing with PBS, cells were fixed and permeabilized using the Fix-and-Perm cell kit, following the manufacturer’s instructions. Next, cells were incubated for 20 min with anti-human ECP (1 μg/ml), washed with PBS, and pelleted by centrifugation. Then, cells were incubated with FITC-conjugated goat anti-mouse IgG at 4°C in the dark for 30 min, washed, and finally resuspended in PBS. In the isotype control, the primary Ab was omitted. Flow cytometry analysis was performed using an Epics XL-MCL system (Coulter-IZASA). For each sample, 10,000 events were analyzed for fluorescence intensity. Results were expressed as percentage of fluorescence-positive cells (%).

Immunofluorescence staining
After treatment, 5 × 10⁶ cells (cultured at a density of 6.6 × 10⁶/ml) were harvested, washed with PBS, and smeared onto poly-L-lysine-treated glass slides. The slides were fixed at room temperature for 10 min with 2% paraformaldehyde. After washing with PBS, cells were blocked for 15 min with 1% BSA in PBS containing 0.1% Triton X-100, then incubated with primary mouse anti-human ECP Ab (2 μg/ml) for 1 h, washed extensively, and stained for 30 min with secondary FITC-conjugated goat anti-mouse IgG Ab (1:100). After extensive washing, coverslips were mounted on the slides using mounting medium (10% PBS, 90% glycerol). Immunostained cells were observed and photographed using a Nikon EFD-3 microscope (Nikon-IZASA). In negative controls, the primary Ab was not added.

Western blotting analysis
Cells (10⁶, cultured at a density of 6.6 × 10⁶/ml) were pelleted, lysed in 50 μl of ice-cold lysis buffer A (50 mM Tris-HCl (pH 7.9), 10 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and the following protease-inhibitor mixture: 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml N-tosyl-l-phenylalanyl-chloromethyl ketone, 10 μg/ml captopril, 1 mM PMSF, 1 mM benzamidine, and 10 mM iodoacetamide), and vortexed for 10 s following sonication for 10 s; insoluble material was pelleted by centrifugation at 15,000 × g for 2 min. The supernatants were used for protein measurement. Proteins (80 μg/lane) were separated on 15% polyacrylamide-SDS gels under reducing conditions, and electrophoretically transferred to PVDF membranes using a semidyeye device. Membranes were probed without need of prior blocking (45) with anti-human ECP Ab (1:500), at room temperature for 2 h, in PBS containing 1% BSA and 0.2% Tween 20. Next, membranes were washed with PBS, and incubated at room temperature for 30 min with horse anti-mouse IgG Ab linked to HRP (1:5000). After washing with PBS, immunoreactive bands were visualized by ECL (46). Reprobing of blots with anti-human β-actin Ab was conducted to verify even protein loading throughout lanes.

Dissociation of neutrophil-bound IgG
Ig molecules were dissociated from the surface of neutrophils as described previously (39, 40). Briefly, after isolation, neutrophils were resuspended in 1 ml of cold acetate buffer (50 mM sodium acetate (pH 4), 85 mM NaCl, 5 mM KCl, supplemented with 0.03% human serum albumin) and incubated on ice for 3 min. An equal volume of gelatin veronal buffer (1.8 mM sodium barbital, 3.1 mM barbital acid, 0.1% gelatin, 0.05 mM MgCl2, 141 mM NaCl, 0.15 mM CaCl2 (pH 7.4)) was then added to the treated cells, and the mixture was centrifuged at 500 × g for 10 min. After the treatment, neutrophils were cultured with the different agents.

RNA extraction and RT-PCR analysis
Total RNA from 2 × 10⁶ highly purified neutrophils or eosinophils (cultured at a density of 6.6 × 10⁶/ml) was isolated using the guanidine phenol method (47). Total RNA (1 μg) was reverse transcribed into cDNA using random primers. The first-strand cDNA was amplified with primer sets for human ECP (accession number: X55990): 5′-GCACATACGTCTGACAAAACCCTTCG-3′ and 5′-TAGAACTTCTCTCTGCTGTCT-3′, GAPDH (accession number: J04038): 5′-CCACCCATGCGAATTCCATGGA-3′ and 5′-TCTAGCAACGTGCTCCATTCCC-3′, Charcot-Leyden crystal protein (CLC) (accession number: NM_001828): 5′-AGGGAGCAACATGTTCCGT-3′ and 5′-TCCACAGCTTCAGTGTATT-3′. The reaction was performed by 40 cycles each of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR products were electrophoresed through agarose gels, and bands were visualized by ethidium bromide staining (ECP: 280 bp, GAPDH: 600 bp, CLC: 373 bp).

De novo synthesis of ECP
Cells (2 × 10⁵, cultured at a density of 6.6 × 10⁶/ml) were cultured in methionine- and cysteine-free medium (RPMI 1640 medium containing 10% (v/v) dialyzed PBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 250 μCi/ml [35S]methionine and [35S]cysteine ([35S]Iodinelabeled [35S]). Cells were incubated for 18 h, at 37°C, in an atmosphere of 5% CO2 and 95% O2. Following incubation, cells were harvested and centrifuged (12,000 × g for 10 min, at 4°C), and cell culture supernatants were collected and kept at −80°C. Pelleted cells were lysed in 1 ml of cold buffer (50 mM Tris (pH 7.4), 10 mM EGTA, 50 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 100 μM phenylarsine oxide, and the following protease-inhibitor mixture: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml N-tosyl-l-phenylalanlyl-chloromethyl ketone, 10 μg/ml captopril, 1 mM PMSF, 1 mM benzamidine, and 10 mM iodoacetamide). After 10 min, cells were vortexed for 10 s, and sonicated for 10 s, and centrifuged at 12,000 × g for 2 min at 4°C. The supernatants obtained after that centrifugation constituted whole cell extracts. Whole cell extracts (1 ml/1 mg) and cell culture supernatants previously obtained (1 ml) were incubated with 2 μg/ml anti-human ECP Ab for 18 h, at 4°C, and then with 75 μl of IgG-coupled protein G-Sepharose for 4 h at 4°C. Next, Sepharose was pelleted, and washed twice with 20 ml of PBS and 2 ml of magnesium phosphate buffer (pH 7.0) containing the previous protease-mixture inhibitor. Immunoprecipitated proteins were subjected to SDS-PAGE by boiling the Sepharose particles in Laemmli buffer.
Following electrophoresis, the gels were dried, mounted on Kodak X-Omat AR films with an intensifying screen, and exposed for 1–5 days at 80 °C.

Statistical analysis

Data are expressed as means ± SEM. A Student t test or one-way ANOVA was used to make comparisons between groups. A level of p \leq 0.05 was considered significant.

Results

Specific Ags induce release of ECP from human neutrophils, but not from human eosinophils

The potential ability to release ECP was evaluated in highly purified neutrophils and eosinophils from allergic asthmatic patients. For this, the cells were cultured in the presence of Ags to which the patients were sensitized, or of agonists, including PAF and LPS, and the release of ECP was measured in the cell culture supernatants. A Student t test or one-way ANOVA was used to make comparisons between groups. A level of p \leq 0.05 was considered significant.

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donors, or cells from asthmatic patients, incubated with an Ag to which patients were not sensitized \( (p < 0.001) \). No statistical differences were observed between unstimulated cells, cells from healthy donors, and cells from allergic subjects, cultured in the presence of Ags to which the patients were not sensitized (Fig. 2B).

**Role of IgE in ECP release by human neutrophils**

We have previously reported the ability of neutrophils from allergic patients to bind Ag-specific IgE on their cell surface (39, 40). In this regard, we further investigated the participation of IgE in Ag-induced ECP release. First, we studied whether anti-human IgE Ab treatment could mimic the effect observed with Ags. Fig. 3 shows that anti-IgE Ab, like Ags, induced ECP release in a time- (Fig. 3A) and dose- (Fig. 3B) dependent manner, with a maximal production at 18 h, at 10 \( \mu \)g/ml anti-IgE Ab. An additive effect similar to that with Ags was observed in the presence of PAF (Fig. 3A). In no case was ECP release found when neutrophils from allergic patients were treated with nonspecific goat IgG Ab. In addition, goat IgG did not affect the PAF-dependent ECP release.

**FIGURE 3.** Anti-IgE Ab time- and dose-dependent ECP release by human neutrophils. *A.* Neutrophils from allergic patients were untreated or treated with anti-IgE Ab (α-IgE; 10 μg/ml), PAF (5 μg/ml), PAF (5 μg/ml) plus anti-IgE (10 μg/ml), goat IgG (IgG; 10 μg/ml), PAF (10 μg/ml) plus goat IgG (10 μg/ml), or anti-IgG (α-IgG; 10 μg/ml), where indicated for the indicated times. *B.* Neutrophils from allergic patients were untreated or treated with anti-IgE Ab or goat IgG at the indicated doses for 18 h. Cells from an allergic patient were left untreated or were treated to elute the surface Ig and then challenged with an Ag to which the neutrophil donor was sensitized (10 μg/ml), or PAF (5 μg/ml) as a positive control of ECP release, for 18 h. Levels of ECP release in cell culture supernatant were determined by ELISA. Data shown are the mean ± SEM from seven separate experiments in which measurements were performed in duplicate.

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by neutrophils. Furthermore, when neutrophils from allergic patients were cultured with anti-IgG Abs, no ECP release was detected (Fig. 3A). Moreover, ECP release was not detected when IgE molecules were stripped from the neutrophil surface before the Ag challenge (39) (Fig. 3C). The presence of the three forms of IgE-receptor/IgE-binding molecule (FceRI, FceRII/CD23, and eBP/mac-2/galectin-3) has previously been reported in neutrophils (32–36). The next experiments were performed to identify the types of IgE receptor/IgE-binding molecule involved in IgE-dependent ECP release. Fig. 4A illustrates a higher ECP release by neutrophils from an allergic patient, incubated with CRA1 Ab (directed against the FcεRI α-chain), compared with neutrophils treated with A3A12 Ab (directed against galectin-3). However, when neutrophils were challenged with 9P.25 Ab (directed against FceRII/CD23), the amount of ECP released was similar to that in the case of unstimulated cells. In no case was ECP release found when neutrophils from allergic donors were treated with nonspecific mouse IgG1 Ab. In contrast to neutrophils, eosinophils responded only to the treatment with LPS (Fig. 4B). Galectin-3 activity is affected by lactose, which is a saccharide ligand recognized by galectin-3 (53). To further confirm the involvement of this IgE-binding protein in IgE-dependent ECP release by human neutrophils, experiments were next done to clarify whether lactose could inhibit the Ag-dependent ECP release. Fig. 4C shows a partial inhibition of Ag-dependent ECP release by lactose, while preincubation with glucose did not affect the Ag-dependent ECP release.

Intracellular expression of ECP in neutrophils

Additional experiments were performed to analyze whether, in addition to releasing ECP, human neutrophils were able to express ECP protein. To this end, we studied the intracellular ECP expression in human neutrophils. After two-color flow cytometry analysis, we detected that a low number of nonstimulated neutrophils (CD66b+ cells) displayed intracellular ECP expression (Fig. 5A). However, this expression was clearly enhanced after 18 h of incubation with Ag to which the patient was sensitized, anti-IgE Ab, and PAF. As is shown, PAF additively enhanced the effect of Ag

![Figure 5](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 5.** Analysis of neutrophil intracellular ECP expression. A. Neutrophils isolated from an allergic patient were cultured for 18 h in the absence of stimulus (a) or in the presence of goat IgG (IgG; 10 μg/ml) (b), Ag (10 μg/ml) (c), PAF (5 μg/ml) (d), anti-IgE Ab (α-IgE; 10 μg/ml) (e), PAF (5 μg/ml) plus anti-IgE Ab (10 μg/ml) (f), PAF (5 μg/ml) plus Ag (10 μg/ml) (g), or PAF (5 μg/ml) plus goat IgG (10 μg/ml) (h) as indicated in the figure. Intracellular ECP and cell surface CD66b expression was subsequently analyzed by flow cytometry. The results from a representative experiment of three with similar findings are shown. The numbers to the right of each plot represent the percent of double-positive cells (CD66b+/ECP+). B. Neutrophils from an allergic patient were processed as indicated in Materials and Methods for light microscopy (left column) or intracellular ECP detection by fluorescence microscopy (right column). Untreated cells (a) and cells after 18 h of treatment with goat IgG control Ab (10 μg/ml) (b), PAF (5 μg/ml) (c), anti-IgE (10 μg/ml) (d), PAF (5 μg/ml) plus anti-IgE Ab (10 μg/ml) (e), or PAF (5 μg/ml) plus goat IgG (10 μg/ml) (f) are shown. Two separate experiments were performed with similar results. C. Neutrophils from an allergic donor were immediately lysed after isolation (basal) or were left untreated or treated, with indicated, with goat IgG (10 μg/ml), anti-IgG Ab (10 μg/ml), PAF (5 μg/ml), anti-IgE (10 μg/ml), PAF (5 μg/ml) plus anti-IgE (10 μg/ml), or PAF (5 μg/ml) plus goat IgG (10 μg/ml) for 18 h. Cells were lysed, and the proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and sequentially probed with anti-human ECP and anti-human β-actin. Three separate experiments were performed, with similar results.
Anti-IgE (10 μg/ml) obtained in two additional experiments. Results from amplification of the GAPDH housekeeping gene transcript are shown in the three panels. Similar results were analyzed in parallel with eosinophils (E) for the amplification of the CLC transcripts (C). Results from amplification of the GAPDH housekeeping gene were analyzed by conventional RT-PCR, using specific primers for the amplification of ECP transcript (E). mRNA amplification of the CLC was also assayed to exclude eosinophil and basophil contamination (48–52) (Fig. 6C). Results from amplification of the GAPDH housekeeping gene transcript are shown in the three panels. Similar results were obtained in two additional experiments.

and anti-IgE Ab. Further analysis performed using immunofluorescence staining yielded similar results (Fig. 5B). Additional analysis by Western blotting (Fig. 5C) showed that ECP protein was only weakly detected in untreated cells; however, ECP was detected after challenge with anti-IgE Ab, PAF, or PAF plus anti-IgE Ab. In no case was ECP expression found when neutrophils from allergic donors were treated with nonspecific goat IgG Ab or anti-IgG Ab. In addition, goat IgG did not affect the PAF-dependent ECP expression by neutrophils.

IgE-dependent ECP mRNA expression in neutrophils from allergic patients

Although the presence of ECP protein has previously been found in neutrophils (11–18), its mRNA expression has never been detected in these cells. Therefore, we next studied the observed effects of anti-IgE Ab, Ags, and PAF upon ECP expression at mRNA levels, using RT-PCR. Because RT-PCR is a very sensitive method, it was important to isolate RNA from extremely pure cells to avoid false-positive results. Neutrophil and eosinophil populations were close to 100% purity (see Materials and Methods). Fig. 6 shows the absence of ECP mRNA in unstimulated neutrophils (Fig. 6A) and its presence in unstimulated eosinophils (Fig. 6B). Bands of amplification product corresponding to ECP were present in neutrophils stimulated with PAF, anti-IgE alone, or anti-IgE Ab together with PAF (Fig. 6A). Unspecific goat IgG was taken as negative control, and did not exert any effect on ECP mRNA levels (Fig. 6A). mRNA amplification of the CLC was also assayed to exclude eosinophil and basophil contamination (48–52) (Fig. 6C).

De novo synthesis of ECP by human neutrophils

For further confirmation, in addition to the presence of ECP in human neutrophils, ECP de novo synthesis was analyzed in these cells. Human neutrophils were cultured with anti-IgE Ab plus PAF for 20 h in a medium containing [35S]methionine and [35S]cysteine. Then, cellular lysates or supernatants were immunoprecipitated using a mAb against ECP. Results presented in Fig. 7 show that treatment of neutrophils with these agents induced de novo synthesis of ECP. In addition, ECP synthesis was detected in both cellular lysates and cell culture supernatants, indicating that ECP release, at least in part, was originated in de novo synthesis of the protein.

Discussion

The results described here show, for the first time, that the IgE-dependent mechanism is an effective stimulus for synthesis and release of ECP by human neutrophils. Recent studies show that ECP is not only a distinctive eosinophil protein, but has been found in neutrophils (11–18). In this regard, it has been reported that these cells are able to take up ECP from the bloodstream after phagocytosis and store it, but not to synthesize it (13). In agreement with these reports, our results indicate that in resting (unstimulated) neutrophils, no ECP mRNA was detected, and only a small amount of intracellular or released protein was found. However, after cellular stimulation, ECP was synthesized. This was verified by several lines of evidence: 1) after neutrophil stimulation, bands of PCR product corresponding to ECP mRNA were detected; 2) de novo biosynthesized protein was detected by 35S radiolabeling; 3) an increase of intracellular ECP protein was observed by flow cytometry, fluorescence microscopy, and Western blotting; and 4) an accompanying ECP release was detected by ELISA.
The results described here provide the first evidence of a new mechanism of ECP induction in human neutrophils. This mechanism, elicited by Ags, has been previously described in neutrophils from allergic patients (39, 40); 2) anti-IgE Ab challenge triggered an equivalent response to that obtained with the Ags, and this response was not detected after IgE stripping from the cell surface; 3) anti-IgG Ab challenge did not induce ECP expression or release by human neutrophils; and 4) ligation of FcεRI and galectin-3 with specific Ab evoked an ECP release.

In line with our results, IL-8 liberation by these cells following stimulation with anti-FcεRI Ab has been reported previously (32); moreover, galectin-3 stimulates superoxide production (36). Although anti-FcεRI/CD23 Ab failed to induce ECP, we have previously reported that activation of neutrophils through this receptor down-modulated L-selectin from their surface (41). The involvement of galectin-3 in the allergen-induced release of ECP was demonstrated by the fact that preincubation of neutrophils with lactose, but not with glucose, abolished the ECP release by allergens (Fig. 4C).

Our results are exclusively due to neutrophils, and cannot be ascribed to a possible contamination by eosinophils for several reasons: 1) the two cell types had a different course of ECP release. As previously described (44, 54), eosinophils released ECP after 30 min of cell stimulation; neutrophils released protein only after 3–18 h. 2) In agreement with previous reports (54, 55), PAF failed to induce ECP release by eosinophils, whereas it induced ECP release by neutrophils. 3) LPS induced ECP release from eosinophils (44), which is in agreement with our results, but not from neutrophils. 4) Ag-, anti-IgE-, anti-IgE plus PAF-, anti-FcεRI-, and anti-galectin-3-dependent ECP production was observed in neutrophils, but not in eosinophils. In this regard, previous report have shown an absence of IgE-dependent ECP release from human eosinophils (8–10). 5) ECP mRNA was detected in unstimulated eosinophils (54), but not in unstimulated neutrophils. 6) CD66b is a specific marker for neutrophils (56) and we found that these cells (CD66b+) are in fact highly expressing ECP. 7) Finally, CLC is a marker of eosinophils and basophils but not of neutrophils (48–52) and we did not find its transcript in our neutrophil preparations. These findings are greatly in support of no contamination of eosinophils and/or basophils in our neutrophil samples.

We have previously described an IgE-dependent mechanism releasing elastase (39) and lactoferrin (unpublished data from our laboratory) from human neutrophils. These neutrophil granule contents have been described as stimuli for eosinophil activation, including ECP release (57, 58). For this reason, it can be speculated that the neutrophil may be an eosinophil-regulatory cell in IgE-mediated hypersensitivity inflammation. Based on our experiments, we hypothesize that ECP released by neutrophils has a biologic role in IgE-induced reactions. We are carrying out additional studies to verify this hypothesis.

Disclosures

The authors have no financial conflict of interest.

References

4. ECP PRODUCTION BY HUMAN NEUTROPHILS

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