Biochemical Properties of the Eosinophil Cationic Protein and Demonstration of Its Biosynthesis In Vitro in Marrow Cells From Patients With an Eosinophilia

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The eosinophil cationic protein (ECP), which has been shown to be secreted both in vitro and in vivo, is a cytotoxic unique constituent of eosinophil granules. To increase the understanding of the mechanisms behind the role of the eosinophil as a cytotoxic effector in disease, a detailed biochemical characterization of ECP was performed. A considerable molecular heterogeneity was revealed when purified ECP was eluted isocratically from a high-resolution cation exchange resin; the separation, reproducibly achieved, of five components was probably due to hydrophobic interaction with the resin. These polypeptides, which reacted quantitatively with anti-ECP antiserum, showed molecular weights (mol wt) of 19,500 and 16,700 and showed almost identical amino acid compositions. The amino-terminal sequence for one of the polypeptides was (in the standard one-letter code) (R-P-X-Q-F-T-R-A-Q-W-F-A-I-Q-H-I-S-L-N-P-R-R-C-T-I-A-M-R-A-I-N-N-Y-). The biosynthesis of ECP was demonstrated in marrow cells from patients with eosinophilia using labeling with (14C)-leucine, followed by immunoprecipitation with anti-ECP, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography for visualization of labeled ECP. Biosynthesis was demonstrated of mol wt 22,000 ECP, which may represent precursor ECP, since with time some of it was processed into ECP with a mol wt of 18,000 to 19,000. Monensin, a proton ionophore, blocked the processing of mol wt 22,000 ECP. This study shows that ECP consists of a family of similar polypeptides. These may, however, have different biological activities.

MATERIALS AND METHODS

Materials. Sephadex G-75 superfine, CNBr-activated Sepharose 4B, protein A-Sepharose CL 4-B, and Mono S cation exchange columns were from Pharmacia, Fine Chemicals (Uppsala, Sweden). The Pharmacia fast-pressure liquid chromatography (FPLC) system was used for chromatography. Acrylamid/Bis 29:1 was obtained from Bio-Rad Laboratories (Richmond, Calif). L-(14C)-Leucine (342 mCi/mmoll) and En3hance were from New England Nuclear (Boston). Phenylmethylsulfonylfluoride (PMSF), Triton X-100, and Monensin were from Sigma Chemical Co (St Louis). Diaflo membranes were from Amicon (Lexington, Mass).

Purification of ECP. ECP was isolated by methods previously described. Briefly, leukocytes from a patient with chronic myeloid leukemia (CML) with 15 x 10^9 blood eosinophils per liter and leukocytes from a patient with bronchial carcinoma with 32 x 10^9 blood eosinophils per liter were homogenized in 0.34 mol/L sucrose, followed by differential centrifugation to obtain a crude granule fraction. Granules were extracted with 10 vol of 0.2 mol/L sodium acetate buffer, pH 4. After concentration by ultrafiltration on a Diaflow PM-10 membrane, chromatography was performed on a Sephadex G-75 column, and the material of the ECP-containing peak was further separated by ion exchange chromatography on E-aminoacaproic acid Sepharose. These procedures resulted in the isolation of a family of essentially pure ECPs reacting with an antibody against the major ECP component, which had been purified by electrophoresis on agarose. The antibody was produced by immunization of rabbits with the homogenous major ECP component as described previously.

The isolated ECPs were analyzed by chromatography on a 1-mL
Mono S cationic exchange column using the FPLC system. Elution was with a gradient of sodium acetate pH 5.0 at a flow rate of 1 mL/min.

Amino acid composition. Amino acid composition was determined with an automatic amino acid analyzer after hydrolysis in 6 mol/L HCl under argon at 110 °C for 24 hours.

Amino acid sequence analysis. Purified ECP was reduced and carboxymethylated with iodo(14C)acetic acid in 6 mol/L guanidine-HCl. Automated amino acid sequence analysis was carried out by the method of Edman and Begg on a Beckman 890C sequencer. Amino acids were identified by high-performance liquid chromatography (HPLC) on a 30-cm Waters u-Bondapak C18 column using stepwise elution with methanol-containing buffers for the aqueous phases and acetonitrile-containing buffers for the aqueous phases. PTH derivatives of amino acids were also identified by thin-layer chromatography. PTH-carboxymethylcysteine residues were also identified by measurements of radioactivity.

Isolation of marrow cells for biosynthetic labeling of ECP. Bone marrow cells from five patients with eosinophilia were collected in heparinized McCoy’s medium and erythrocytes removed by dextran sedimentation. The cells from the supernatant were washed and used for biosynthetic labeling. Four patients had the idiopathic hypereosinophilic syndrome with blood eosinophils in the range of 3 to 14 × 10⁶/L and a duration of the illness from one to 13 years. One patient had eosinophilia secondary to a malignant lymphoma. Marrow cells were also isolated from one patient with chronic myeloid leukemia and 12 × 10⁶ blood eosinophils per liter.

Labeling of cells. For biosynthetic labeling of ECP, marrow cells were starved in leucine-free minimum essential medium (Eagle) with 1% fetal bovine serum (FBS) for 60 minutes at 37 °C. The labeling medium was the same deficient medium with 10% FBS. The cells (3 × 10⁶/mL) were incubated with 15 μCi/mL of (14C)-leucine for one to 18 hours. In chase experiments, cells were first pulsed with 50 μCi/mL (14C)-leucine for 60 minutes, washed and suspended in RPMI 1640 medium with 10% FBS with 10⁵ cells per milliliter, followed by incubation for various chase time periods.

Fig 1. ECP, 1 mg, isolated from blood eosinophils of a patient with bronchial carcinoma using gel chromatography and E-amino-caproic acid Sepharose chromatography, was subjected to high-resolution chromatography on Mono S using the FPLC system. Elution was with sodium acetate pH 5.0 (-----) at a flow rate of 1 mL/min. The absorbance at 280 nm is monitored, as well as the ECP content of fractions determined by radial immunodiffusion (-----). The SDS-PAGE pattern of ECP eluted in various fractions is also included, with mol wt markers.

Extraction of cells. A radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 mol/L NaCl, 30 mmol/L HEPES, pH 7.3, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) was used for extraction. Approximately 1 mL of RIPA buffer with 1 mmol/L PMSF was added per 10⁷ cells and the extracts kept in ice for one hour, followed by centrifugation at 4 °C at 32,000 g for two hours. The clear supernatant was stored frozen until used for immunoprecipitation.

Immunoprecipitation of radioactive ECP. RIPA-buffer extracts, 100 to 300 μL, were mixed with 15 μL anti-ECP. After standing at 4 °C for 12 to 18 hours, 40 μL of a protein A-Sepharose in RIPA buffer (200 mg/mL) was added for collection of the immunoprecipitate by rotation at 4 °C for 60 minutes. The protein A-Sepharose was washed five times with RIPA buffer and the supernatant was carefully removed. The pellet was suspended in 50 μL H₂O with 15 μL electrophoresis sample buffer (0.4 mol/L Tris, pH 6.8, 50% glycerol, 10% SDS, and 5% β-mercaptoethanol), boiled for five minutes, and used for electrophoresis.

SDS-PAGE and fluorography. SDS-PAGE was performed on slab gels 18 cm long, 1.5 mm thick, and 16 cm wide, with ten gels in an LKB 2001 Vertical Electrophoresis unit (LKB Products, Bromma, Sweden) according to Laemmli. Samples were loaded on a linear gradient of 5% to 20% polyacrylamide gel with 3% stacking gel. The electrophoresis was run at 25 mA per gel without cooling. After electrophoresis, gels were fixed in 10% trichloroacetic acid, 10% acetic acid, and 30% methanol for at least one hour and treated with Enhance for one hour and H₂O₂ for one hour. Gels were dried on filter paper and exposed to x-ray film (Kodak X-Omat S, Eastman-Kodak Co, Rochester, NY) at −80 °C for three to six days.

Apparent molecular weights (mol wt) were determined by use of the following (14C) methylated standards (New England Nuclear): cytochrome C, 12,300; carbonic anhydrase, 30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; and phosphorylase B, 97,400.

To quantitate radioactivity, individual bands, localized on the dried gel using fluorography, were excised and treated overnight at 37 °C in 1 mL of a solution containing 95 parts 30% H₂O₂ and five
RESULTS

High resolution ion exchange chromatography of ECP. ECP isolated by gel filtration, followed by E-amino-caproic acid Sepharose chromatography from blood eosinophils of a patient with bronchial carcinoma and high eosinophilia showed two distinct components when analyzed by SDS-PAGE. The mol wt of these components were 19,500 and 16,700. All ECP was eluted from a Mono S column with approximately 1.0 mol/L sodium acetate, pH 5.0, without achieving separation of the ECP components. Surprisingly, however, a considerable heterogeneity was revealed by isocratic elution with 0.9 mol/L sodium acetate, which resulted in the resolution of five separate components (Fig 1). As demonstrated in Fig 1, all components reacted quantitatively in a radial immunodiffusion assay using an antiserum raised against one homogeneous ECP species isolated by preparative electrophoresis. Thus a marked microheterogeneity for
Eosinophil Cationic Protein

Table 1. Amino Acid Composition of ECP Components

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<thead>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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ECP components A through D were separated by high-resolution ion exchange chromatography (Figs 2 and 3). Data are given as residues/100. Cysteine and tryptophan were not quantitatively determined. Methionine was barely detectable, but the amino acid sequencing of the NH₂-terminus of component D revealed one methionine residue.

ECP molecules were found. ECP in the first two peaks eluted from the Mono S column had a mol wt of 19,500, while that of the three peaks eluted later had a mol wt of 16,700 (Fig 1). The elution pattern of ECP was reproducible, and an identical separation pattern as in Fig 1 was seen in another experiment with ECP purified from eosinophils of a patient with the idiopathic hypereosinophilic syndrome (data not shown). Eosinophils from a patient with CML were used for detailed biochemical characterization of ECP, which was obtained from the Mono S column in four peaks by isocratic elution with 0.9 mol/L sodium acetate, pH 5.0 (Fig 2). By rechromatography twice of peak B, a homogeneous protein species was obtained with a mol wt of 18,000 (Fig 3). Rechromatography once of peak D resulted in a heterogeneous polypeptide with a mol wt of 16,700 (Fig 3). ECP in peak C was difficult to isolate in a homogeneous state by rechromatography.

**Amino acid analyses.** Table 1 shows amino acid analyses of ECP components A through D isolated by high-resolution ion exchange chromatography (Figs 2 and 3). The amino acid compositions were almost identical for all ECP components.

**Amino acid sequence of ECP.** The amino acid sequence of the NH₂-terminus is shown in Fig 4. Thirty-three amino acid residues were sequenced. The third residue could not be identified.

**Biosynthesis of ECP.** Marrow cells from a patient with CML and a patient with eosinophilia were incubated with (14C)-leucine for different times, followed by extraction and immunoprecipitation with anti-ECP and SDS-PAGE. The fluorograms (Fig 5A and B) showed that ECP polypeptides were synthesized with a mol wt of 22,000. With time, CML marrow cells produced polypeptides with mol wt of 25,000 and 18,000. In addition, eosinophil marrow cells were pulsed with (14C)-leucine for one hour and the label was chased for 1, 3, 7, and 18 hours and the fluorograms obtained after SDS-PAGE show (Fig 5C). The pulse-chase labeling experiments showed that some of the newly synthesized mol wt 22,000 ECP polypeptide was slowly converted into ECP with a mol wt of approximately 19,000. An experiment was also performed with these cells in an identical manner but with inclusion of 1 μmol/L Monensin during chase (Fig 5D). Monensin, a carboxylic proton ionophore that exchanges preferentially Na⁺ ions for protons, prevented the conversion of mol wt 22,000 ECP into lower mol wt products.

The time course for incorporation of (14C)-leucine into mol wt 22,000 ECP is shown in Fig 6. Incorporation increased for at least 15 hours.

Figure 7 shows results from ECP biosynthesis studies of marrow cells in five patients with eosinophilia. In all patients, the major ECP product had a mol wt of 22,000, while a minor product had a mol wt of approximately 18,000. Monensin prevented the formation of the mol wt 18,000 ECP. However, total biosynthesis of ECP was inhibited by Monensin to a variable degree. Therefore, the conclusion that Monensin diminished the production of immunoprecipitable ECP species with mol wt of less than 22,000 is not unambiguous from these data. However, data from the pulse-chase experiment of Fig 5D clearly show that Monensin prevents formation of ECP with a mol wt of 18,000 to 19,000.

**DISCUSSION**

One approach to the understanding of eosinophil function is to study the physicochemical properties and biological actions of eosinophil granule constituents. Eosinophil cationic polypeptides of granules may be responsible for cytotoxic effects of eosinophils on parasites and cells. In this work we show that an extended separation of purified ECP revealed a considerable molecular heterogeneity. Thus by isocratic elution from a high-resolution cation exchange resin, five ECP components were resolved. The separation was probably not a result of ion exchange, but more likely a result of partition chromatography due to hydrophobic interactions between ECP and the resin. Although the different components showed slight differences in molecular mass judging from SDS-PAGE, the amino acid compositions were almost identical.

The separation pattern of ECP components on the cation exchange resin was highly reproducible, and ECP purified from different patients with eosinophilia showed an identical elution profile. Future work will show if the ECP components obtained differ in biological activities.

The sequence determination of the amino-terminal part of one ECP component revealed a unique sequence. Thus when this sequence was aligned with known protein sequences in a computerized search, no specific homologies were found.
Minor homologies with the receptor-binding site of diphtheria toxin (constituting eight of the sequenced 33 amino acids of ECP) are probably without significance.

In the present study, ECP was purified from granule extracts. Eosinophils have an ability to secrete ECP both in vitro and in vivo. It is not known, however, whether all forms of ECP, as detected in the present work, are secreted to the same extent. The secretory process has been investigated by the use of monoclonal antibodies against ECP. One antibody to ECP bound to eosinophils from both healthy individuals and patients with eosinophilia. A second antibody also recognized ECP but was specific for the secreted form of the protein. The results suggest that there are structural alterations of ECP during secretion.

In another approach to study structural modification of ECP, biosynthetic labeling was used, followed by immunoprecipitation with anti-ECP. Marrow cell specimens from patients with eosinophilia were used for these experiments. All experiments demonstrated biosynthesis of ECP with a mol wt of 22,000. This form does not correspond to any of the ECP forms purified from eosinophil granules and may therefore represent a precursor polypeptide for ECP, which is subject to processing during intracellular transport and storage in eosinophil granules. It is consistent with this interpretation that some lower-mol-wt ECP appeared with time. This was most clearly shown in pulse-chase labeling experiments. The processing of mol wt 22,000 ECP into lower-mol-wt ECP was, however, not very rapid. In CML cells, but not with nonleukemic cells, production of immunoprecipitable ECP species with higher mol wt than 22,000 were seen. The significance of this finding is not clear.

The smallest polypeptide, mol wt 16,700 of purified ECP, which was the major form, was not biosynthetically labeled by incubation of cells in vitro for 16 hours. One explanation is that processing is slow and production of mol wt 16,700 ECP takes longer than 16 hours. Another explanation, although unlikely, is that proteolytic modifications took place during extraction and purification of ECP and that the mol wt 16,700 polypeptide is a degradation form of ECP.

As judged by pulse-chase labeling, Monensin, a proton...
ionophore, blocked the processing of newly synthesized ECP. This could be explained by the known inhibitory effects of Monensin on transport of secretory or lysosomal proteins and related to Monensin’s ability to raise the normally acidic pH of the vacuolar system of the cell.21

Additional structural studies of ECP will require the use of DNA hybridization probes complementary to ECP-mRNA. Knowledge of the amino-terminal sequence of ECP can be used for synthesis of corresponding oligonucleotides to be used as probes for screening of a cDNA-library established from marrow cells of patients with an eosinophilia.

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REFERENCES


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