Brief Definitive Report

Cytokine RANTES Released by Thrombin-stimulated Platelets Is a Potent Attractant for Human Eosinophils

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Summary

Thrombin stimulation of human platelets results in the release of a preformed proteinaceous human eosinophil (Eo)-chemotactic activity. By the use of different high-performance liquid chromatography techniques, two Eo-chemotactic polypeptides (EoCPs), tentatively termed EoCP-1 and EoCP-2, were purified to homogeneity. Upon SDS-PAGE analysis, these chemotaxins showed molecular masses near 8 kD. NH_2-terminal amino acid sequence analysis revealed identical sequences for both EoCP-1 and EoCP-2, which are also identical to that of RANTES, a cytokine that structurally belongs to the interleukin 8 superfamily of leukocyte selective attractants, and that is known to be a "memory-type" T lymphocyte–selective attractant. In the major Eo chemotaxin, EoCP-1, the residues 4 and 5, which in EoCP-2 were found to be serine residues, could not be identified. Electrospray mass spectrometry (ESP-MS) of EoCPs revealed for EoCP-2 a molecular mass of 7,862.8 ± 1.1 daltons, which is 15.8 mass units higher than the calculated value of RANTES, indicating that EoCP-2 is identical to the full-length cytokine, and oxygenation, probably at methionine residue number 64, has taken place. Upon ESP-MS, EoCP-1 showed an average molecular mass of 8,355 ± 10 daltons, suggesting O-glycosylation at these serine residues. Both natural forms of RANTES showed strong Eo-chemotactic activity (ED_{50} = 2 nM) with optimal chemotactic migration at concentrations near 10 nM, however, there were no significant migratory responses with human neutrophils. Chemotactic activity of RANTES for human Eos could be confirmed using recombinant material, which has been found to be as active as the natural forms. Since RANTES gene expression has been detected in activated T lymphocytes, and recombinant RANTES was shown to be a "memory" T lymphocyte–selective attractant, it is now tempting to speculate about an important role of RANTES in clinical situations such as allergene-induced late-phase skin reactions in atopic subjects or asthma, where in affected tissues both memory T cells and Eos are characteristic.

Inflammatory diseases are characterized histologically by immigration of different leukocyte subtypes, i.e., neutrophils, monocytes/macrophages, T lymphocyte subsets, or eosinophils (Eos). Tissue-oriented migration of certain inflammatory cell types assumes, apart from expression of adherence proteins and other in vivo migration-facilitating factors, the existence of locally produced cell-selective chemotactic factors. Well-described chemotaxins such as C5a or leukotriene B_4 are chemotactic for a wide variety of leukocytes and do not show this specificity. Apart from these pan-leukotactic factors, recently a number of leukocyte-selective chemotaxins have been discovered.

IL-8 represents one of these selective leukocyte attractants, which is chemotactic for neutrophils (1) and T lymphocytes (2), but not for monocytes and Eos (3, 4). IL-8 comprises a polypeptide mediator that is a member of a superfamily of structurally related low molecular weight cytokines, which contain four cysteines at identical relative positions with a conserved -Cys-X-Cys-(C-X-C) or -Cys-Cys- (C-C-) motif. Members of the C-X-C-subfamily, such as IL-8 (3–5), melanoma growth-stimulatory activity (MGSA/gro α) (6, 7), and neutrophil-activating protein 2 (NAP-2) (8), are potent chemotactic factors for neutrophils and in part are also known to be chemotactic for lymphocytes, however, not for monocytes or Eos.

Members of the C-C-subfamily, monocyte chemotactic protein 1(MCP-1) (9)/monocyte-chemotactic and -activating factor (MCAF) (10), and the cytokine RANTES (11) represent chemotactic factors for monocytes, but not for neutrophils. Other members of this subfamily, the human equivalents of

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macrophage inflammatory protein 1α and 1β, are chemotactic for T lymphocyte subsets, cytotoxic T lymphocytes and naive T lymphocytes, respectively, whereas RANTES shows an apparent selectivity for memory T lymphocytes (12).

So far members of the IL-8 superfamily have not been reported to be Eo-chemotactic factors, and hence we address the question whether the IL-8 supergene family of low molecular mass (6–10 kD) cytokines also contains Eo-selective attractants. We report here that platelets stimulated with thrombin represent a source of Eo-chemotactic proteins, which structurally belong to the same superfamily of host defense cytokines as a number of other cell-selective chemoattractant cytokines do.

Materials and Methods

Recombinant Cytokines. Recombinant cytokines RANTES and IL-8 were purchased from Pepro Tech Inc. (Rocky Hill, NJ). Purity was >98% as shown by a single line upon SDS-PAGE analysis.

Isolation of Eosinophils and Neutrophils. Blood was taken from healthy donors or subjects with a mild eosinophilia (5–10% of peripheral blood leukocytes). Eos and neutrophils were isolated from acidic citrate dextran–treated blood with the use of discontinuous Percoll density gradient centrifugation as previously described (13). Yielded cell preparations were >85% pure for Eos and >95% for neutrophils.

Production of Platelet-derived EoCPs. Platelet-rich plasma obtained from citrate/dextran blood was centrifuged at 2,000 *g* for 30 min. Platelet pellets were washed twice with PBS containing 10 mM EDTA and resuspended in PBS. Platelet suspensions were incubated at 37°C for 30 min in the presence of 2 U/ml thrombin (Sigma Chemical Co., Munich, FRG). After centrifugation at 4°C, cell-free supernatants were collected and stored below −70°C until further use.

Purification of EoCPs. EoCPs were purified by HPLC methods similar to those used for purification of NAP-1/IL-8 (3), MGSA/gro (6), and platelet-derived neutrophil attractant (NAP-4) (14). Pooled supernatants of thrombin-stimulated platelets acidified with trifluoroacetic acid (TFA) to pH 3 were concentrated over filters (YM5; Amicon Corp., Danvers, MA) and applied to a preparative wide-pore reversed-phase (RP)-8 HPLC column (300 × 7 μm C8 Nucleosil, 250 × 12.5 mm; Macherey-Nagel, Düren, FRG). Proteins were eluted using a gradient of acetonitrile containing 0.1% TFA. Fractions active in an Eo chemotaxis system off RP-8 HPLC were pooled, concentrated by lyophilization, and applied to a TSK-2000 size exclusion HPLC column (600 × 8 mm; LKB, Bromma, Sweden) previously equilibrated with 0.1% TFA. Proteins were eluted with the same solvent. Eo-chemotactic fractions off TSK-2000 HPLC were pooled and applied to a wide-pore CN-propyl RP HPLC column (5 μm, 250 × 4 mm; J. T. Baker, Gross Gerau, FRG) previously equilibrated with 0.1% aqueous TFA. Protein elution was performed with a gradient of *n*-propanol containing 0.1% TFA. Thereafter, fractions off CN-propyl-RP-HPLC, which were active in the Eo chemotaxis assay system, finally were applied to a narrow-pore RP-18-HPLC column (100 × 7 μm, C18 Nucleosil, 250 × 10 mm; Macherey-Nagel), previously equilibrated with 0.1% aqueous TFA containing 10% (vol/vol) acetonitrile, and polypeptides were eluted with a gradient of acetonitrile containing 0.1% TFA.

SDS-PAGE. SDS-PAGE analysis was performed with the Phast system (Pharmacia, Freiburg, FRG) using high-density gels according to the manufacturer's instructions. CNBr-cleavage products of myoglobin (Sigma Chemical Co.) as well as recombinant human Ser_{72}-IL-8 served as molecular mass standards.

Chemotaxis Assays. Eo chemotactic activity was measured in blind-well Boyden chambers as previously described (13). In some experiments Eos were determined microscopically using a modification of Boyden's method as previously described in detail (3, 13). Chemotactic activity is expressed as chemotactic index, calculated as: stimulated migration/random migration.

Amino Acid Sequence Analysis. Underivatized samples were analyzed using a gas phase sequencer (4701; Applied Biosystems, Inc., Foster City, CA) with on-line HPLC analysis of the phenylthiohydantoin derivatives.

Electrospray Mass Spectrometry (ESP-MS). For ESP-MS analysis, a Trio-2 quadrupole (VG Biotech-Fisons Instruments LTD, Manchester, UK) was used. The peptides were introduced into the ESP-MS ion source as a solution in 50:50:1 methanol/water/acetic acid (vol/vol/vol) at a flow rate of 5 μl/min. The electrospray needle was held at 4 kV relative to the source. As an internal mass calibrant ubiquitine was used.

Results and Discussion

To test the hypothesis that 8–10-kD Eo-specific or -selective attractants would exist, we originally used supernatants of PBMC preparations that were stimulated with a mixture of bacterial LPS and PHA, since these cell preparations are known to produce a number of IL-8-like chemotactic cytokines such as IL-8 itself (3–5, 15), MCP-1/MCAF (16), MGSA/gro α (6), and apparently also other chemotactic members of the C-C-family (12).

In initial experiments we detected 6–10-kD Eo-chemotactic activity in supernatants of stimulated PBMC (data not shown). To determine its origin purified monocytes were stimulated with LPS in one experimental series and lymphocyte preparations were treated with mitogens in another series. To our surprise, in the monocyte supernatants, which contained high amounts of neutrophil-chemotactic IL-8, no Eo-chemotactic activity could be detected. Instead Eo-chemotactic activity was found in supernatants of PHA-stimulated monocyte-depleted lymphocyte preparations (data not shown).

Because these lymphocyte preparations contained various numbers of contaminating platelets, we tested whether these cells are a possible source of Eo-chemotactic activity. Platelets are known to produce members of the IL-8 supergene family such as platelet basic protein and its truncation products, connective tissue–activating peptide III (CTAP III) as well as β-thromboglobulin (17), which by enzymatic cleavage form neutrophil-chemotactic NAP-2 (18). Moreover, platelet factor 4 (PF-4) (19), as well as a structurally related molecule we tentatively termed NAP-4 (14), are stored in platelets. Therefore, it seemed to be an attractive working hypothesis to assume that platelets also are a source of other members of the 6–10-kD cytokine family.

When lysates of platelets were tested for Eo-chemotactic activity in vitro, a high titer of the activity was observed (data not shown). To investigate whether this Eo-chemotactic activity is released by physiological stimulation, platelets were incubated with thrombin (2 U/ml) for 30 min and supernatants were analyzed for Eo-chemotactic activity. As shown

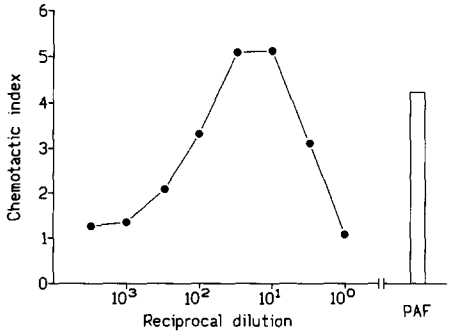

Figure 1. Eo-chemotactic activity in supernatants of thrombin-stimulated platelets. Platelets were incubated at 37°C for 15 min in the presence of thrombin (2 U/ml). Cell-free supernatants were collected, diluted in PBS/BSA, and assayed in the Boyden chamber system for Eo-chemotactic activity. The Eo chemotaxin PAF (100 nM) served as control. A typical experiment is shown.

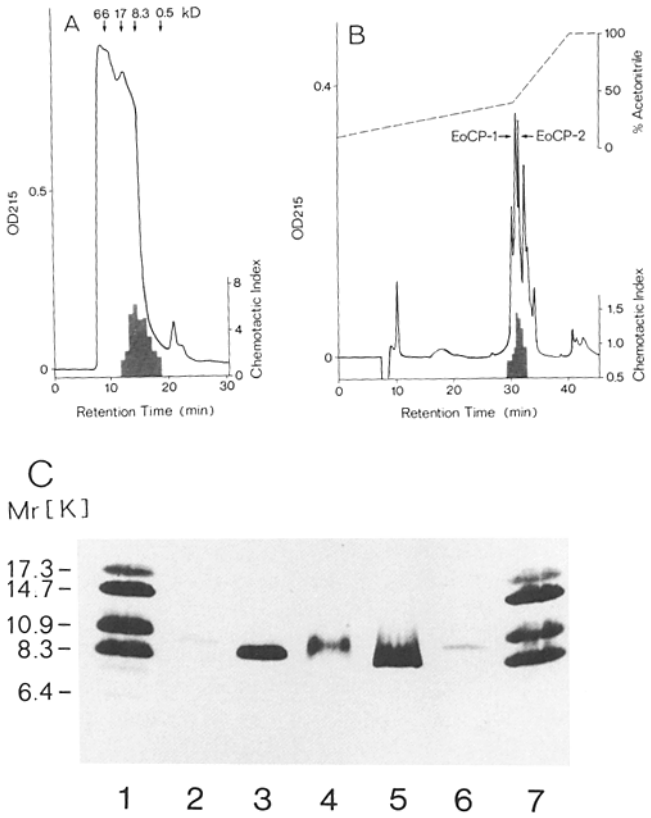

Figure 2. Purification of EoCPs. Eo-chemotactic activity detected in supernatants of thrombin-stimulated platelets was purified by a series of HPLCs. The shaded area represents Eo-chemotactic activity in HPLC fractions. Experimental conditions are detailed in Materials and Methods. (*A*) TSK-2000 size exclusion HPLC of Eo-chemotactic fractions off RP-8-HPLC. (*B*) Narrow pore RP-18-HPLC of an EoCP preparation purified by CN-RP-HPLC. A representative purification is shown. (*C*) SDS-PAGE of purified EoCPs. SDS-PAGE analysis was performed with the Phast System and proteins were silver stained. In lanes *1* and 7, CNBr cleavage products of myoglobin were applied as M_r standards, whereas lanes *2* and *6* contained 72-residue rIL-8, and lane *3* contained rRANTES. In lane *4*, EoCP-2 was applied, and lane *5* contained EoCP-1.

in Fig. 1, a dose-dependent bell-shaped Eo-chemotactic response was observed. Since platelets are known to produce PAF, a potent Eo chemotaxin of low molecular weight (20), the relative molecular mass of this Eo-chemotactic activity was determined. By TSK-2000 size exclusion HPLC, the majority of Eo-chemotactic activity was detected in fractions corresponding to the molecular mass range between 5 and 15 kD (data not shown). To purify the Eo-chemotactic polypeptide(s) (EoCPs) present in supernatants of thrombin-stimulated platelets, we used similar HPLC methods as those with which we purified NAP-1/IL-8 (3), MGSA/gro (6), and platelet-derived neutrophil attractant 4 (NAP-4) (14).

At the first step, pooled platelet supernatants were chromatographed on a preparative RP-8 HPLC column followed by a TSK-2000 size exclusion HPLC (Fig. 2 *A*). Eo-chemotactic material was further purified by wide-pore cyanopropyl HPLC and finally purified by narrow-pore RP-18 HPLC (Fig. 2 *B*). The last purification step led to a broad peak of Eo-chemotactic activity corresponding to two peaks (EoCP-1 and EoCP-2) absorbing at 215 nm (Fig. 2 *B*). The presence of a single silver-stained band upon SDS-PAGE analysis for each EoCP, which showed a mobility somewhat higher than that of authentic 72-residue IL-8 (molecular weight of 8,532), indicated that the material in these EoCP preparations are 8-kD polypeptides (Fig. 2 *C*).

NH_2-terminal amino acid sequencing revealed a single sequence of 16 or 32 residues for each EoCP:

EoCP-1, SPYXXDTTPXXFAYIA
EoCP-2, SPYSS DTTPXXFAYIARPLPRAXXXEYFYXXG
RANTES (21), SPYSS DTTPCCFAYIARPLPRAHIKEYFYTSG...

The only sequence obtained for both EoCPs in several investigations is identical to that reported for the cytokine RANTES.

The molecular weight of EoCP-1 and EoCP-2 was determined by ESP-MS (Fig. 3), and obtained values were 8,355 ± 10 and 7,862.8 ± 1.1, respectively. Although both EoCPs showed the same amino acid sequence, EoCP-1 had a molecular mass approximately 500 mass units higher than that of EoCP-2. This difference could be attributed to O-glycosylation presumably at serine residues 4 and 5, where sequence analysis of EoCP-1 failed. N-glycosylation sites are known to be absent from RANTES (21). The measured molecular mass of EoCP-2 agrees well with the calculated molecular mass of RANTES. RANTES contains four cysteine amino acids, and these are thought to exist in the reduced form, the peptide being folded with two disulphide bridges, as has been proven by ^{1}H-NMR-analysis as well as x-ray crystallographic analysis for other members of the RANTES/IL-8 family (22). This reduces the calculated molecular weight from 7,851.06 to 7,847.03. This value differs from the measured value by 15.8 units, which can be accounted for by the assumption of oxidation having taken place on the single methionine residue number 67 in RANTES. Oxidation of methionine is very common, especially in the case of samples that have been exposed to the atmosphere for any length of time before analysis.

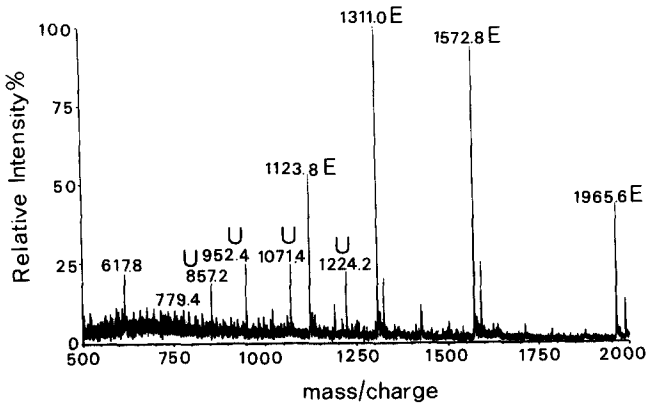

Figure 3. ESP-MS of EoCP-2. The calculated molecular mass for EoCP-2 (*E*), corrected for Ubiquitin (*U*) as an internal standard, is 7,862.8 ± 1.1 daltons. The charge states shown are +4 (1,965.6), +5 (1,572.8), +6 (1,311.0), and +7 (1,123.8).

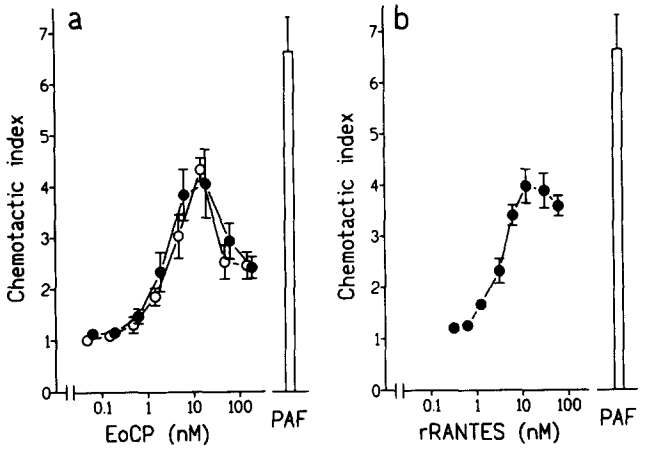

Figure 4. Eo-chemotactic activity of EoCPs and rRANTES. (*a*) Both RP-18-HPLC-purified fractions, EoCP-1 (●) and EoCP-2 (○), were analyzed for Eo-chemotactic activity. Results are expressed as mean ± SE from six (EoCP-1) and five (EoCP-2) experiments. (*b*) Eo-chemotactic activity of rRANTES. Results are expressed as mean ± SE from five experiments. PAF (100 nM) served as control.

Both EoCP-1 and EoCP-2 showed similar dose-dependent Eo-chemotactic activity in the Boyden chamber system (Fig. 4 *a*), indicating that derivatization of serine residues 4 and 5 does not affect Eo-chemotactic activity either in potency (ED_{50}) or efficacy (percent input migrating Eos upon optimal stimulation doses). Moreover, Eo-chemotactic activity of RANTES could be confirmed with recombinant material: rRANTES showed Eo-chemotactic activity at similar doses than found for both natural forms (Fig. 4 *b*). In contrast with this, neither EoCP induced significant migratory response of human neutrophils in the Boyden chamber system (data not shown).

RANTES originally was identified as an apparently T cell–specific inducible gene, which was found to be expressed by cultured T cell lines that were antigen specific and growth factor dependent (21). Furthermore, RANTES mRNA expression has been found to be inducible in PBL by antigen or mitogen stimulation. It is therefore likely, but yet not proven, that supernatants of mitogen-stimulated PBL contain Eo-chemotactic RANTES.

The Eo is one of the predominant cell types found in late-phase reactions or at the inflammatory sites in allergic diseases (23, 24). Release of cytotoxic cationic Eo granule proteins as well as synthesis of peptido-leukotrienes are believed to contribute to such hypersensitivity diseases. Apart from other cytokines such as IL-5 (25), GM-CSF (25), and "lymphocyte chemotactic factor LCF" (26), RANTES has been identified as an Eo-selective attractant in vitro and might participate in recruitment of Eos to T cell–mediated hypersensitivity reactions in vivo. Since it is known that rRANTES attracts T lymphocytes of the memory type ($CD45RO^+$) (11), RANTES may be a common mechanism in diverse immunological reactions, which culminate in the emigration of T lymphocytes of this phenotype and Eos from the circulation into sites of inflammation. The allergen-induced late-phase skin reaction in atopic subjects, in which both memory T cell infiltration and Eo accumulation are characteristic (24, 27), may represent a possible clinical example.

Our finding that platelets release upon stimulation Eo-chemotactic RANTES serves as additional evidence for the recent understanding that platelets contribute to inflammatory reactions (reviewed in reference 28). From guinea pig models it was suggested that platelets are a prerequisite component in allergic asthma, since platelet depletion reduced Eo infiltration into the lung after PAF or allergen exposure to sensitized animals (29). Moreover, bronchial Eo accumulation was reduced without a significant change in neutrophil infiltration after antigen challenge in thrombocytopenic-allergic rabbits compared with control animals (30). It is therefore tempting to speculate that RANTES released from platelets might play a role for selective Eo infiltration after antigen challenge in these situations.

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