Purification and synthesis of eosinophilotactic tetrapeptides of human lung tissue: Identification as eosinophil chemotactic factor of anaphylaxis

(leukocyte chemotaxis/leukocyte deactivation/anaphylactic mediators/acidic peptides of lung)

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ABSTRACT Preferential eosinophil chemotactic activity exhibiting a molecular weight comparable to that released from sensitized human lung fragments challenged with specific antigen and designated eosinophil chemotactic factor of anaphylaxis has been isolated from extracts of human lung fragments by sequential purification on Sephadex G-25, Dowex-1, Sephadex G-10, and paper chromatography. Two eosinophilotactic tetrapeptides of amino acid sequence Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu were recovered from the extracts in 4-12% overall yield of the low molecular weight peak from Sephadex G-25. Purified eosinophil chemotactic factor of anaphylaxis and the synthetic tetrapeptides were maximally active in amounts from 0.1 to 1.0 nmol per chemotactic chamber, and the activity was dependent on both the NH2-terminal and the COOH-terminal residues. Both natural and synthetic peptides were preferentially chemotactic for eosinophils and rendered them unresponsive to a subsequent stimulus.

The eosinophil chemotactic factor of anaphylaxis (ECF-A) was discovered in 1971 as a mediator released during immediate hypersensitivity reactions in guinea pig (1) and human (2) lung slices. ECF-A was subsequently recognized to be present totally preformed in rat mast cells in association with the granules (3), human leukemic basophils (4), and mast cell-rich tissues such as human lung and nasal polyps (3, 5). The release of ECF-A from human tissue by IgE-dependent mechanisms has biochemical requirements and is modulated by the intracellular levels of cyclic nucleotides in a manner comparable to histamine release from the same tissues (5, 6).

The activities of ECF-A are preferentially directed to eosinophils as compared to neutrophils and mononuclear leukocytes and include chemotaxis (1, 2, 7), chemotactic deactivation (8), stimulation of the hexose monophosphate shunt (8), and release of granular enzymes (9). ECF-A obtained by extraction or IgE-dependent reactions of human lung tissue or rat mast cells exhibited a molecular weight of approximately 500 by filtration on Sephadex G-25 (3), was inactivated by digestion with subtilisin or Pronase but not with trypsin or chymotrypsin (10), and was composed of two anodal peaks of activity on high voltage electrophoresis at neutral pH (7). Low molecular weight acidic peptides with ECF-A activity have been isolated from human lung tissue for compositional and sequence analysis and, based on their apparent structure, synthetic peptides have been prepared that possess specific ECF-A-like functions at comparable concentrations.

MATERIALS AND METHODS

Dowex AG-1-X8, 200-400 mesh, and Bio-Beads S-X1 chromomethylated resin, 200-400 mesh (Bio-Rad Laboratories, Richmond, Calif.), Sephadex G-25, Sephadex G-10, Ficoll and dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), sodium diatrizoate (Hypaque, Winthrop Lab., N.Y.), sodium metrizoate (Nyegaard and Co., Oslo, Norway), Hanks' balanced salt solution and Medium 199 with phenol red (Microbiological Associates, Inc., Bethesda, Md.), ovalbumin five times recrystallized (Miles Labs, Inc., Kankakee, Ill.), chromatography paper (3 MM, Whatman, W & R Balston Ltd., England), electrophoresis paper (Savant Instrument Co., Hicksville, N.Y.), carboxypeptidase A (treated with diisopropyl fluorophosphate), and l-dimethylamino-naphthalene-5-sulfonyl (dansyl) chloride (Sigma Chemical Co., St. Louis, Mo.), dansyl-amino-acids (Seikagaku Biochemicals, Tokyo, Japan), polyamide sheets (Cheng Chin Trading Co., Ltd., Taiwan), phenylisothiocyanate (sequenation grade) and anhydrous trifluoroacetic acid (redistilled in vacuo) (Eastman Kodak Co., Rochester, N.Y.), periodic acid (G. Frederick Smith Chemical Co., Columbus, Ohio), amionopeptidase M (Henley and Co., N.Y.), t-butoxycarbonyl (BOC)-amino acids (Beckman Instruments, Inc., Palo Alto, Calif.), 3,5-dinitrophenyl-glutamic acid (Cyclo Chemical Co., Los Angeles, Calif.), and Rhodamine 6-G and N,N'-disopropylethylamine (Matheson, Coleman and Bell, East Rutherford, N.J.) were obtained as specified. All other solvents were either reagent grade from Eastman Kodak Co., or Fisher-certified (Fisher Scientific Co., Medford, Mass.) and were redistilled before use. Methylene chloride was further purified by chromatography on a column of alumina (alumina adsorption, Fisher Scientific Co.). N,N'-Dimethylformamide was passed through a column of 4A molecular sieves (Matheson, Coleman and Bell, East Rutherford, N.J.) and stored over fresh sieves for 72 hr before use (11).

Chemotaxis. Blood from normal subjects or patients with peripheral blood hypereosinophilia of 20-95% was incubated for 45 min at 37°C with citrate anticoagulant and dextran to sediment the erythrocytes (12). The leukocyte-rich supernatant plasma was removed and centrifuged at 100 x g for 10 min at room temperature. The leukocyte pellet was either washed and suspended in Medium 199 made 0.4% in ovalbumin and 0.01 M in Tris-HCl pH 7.4 (Medium 199-ovalbumin) and used directly in the chemotactic assays, or was used as a source of a specific leukocyte population. Eosinophils were enriched by centrifugation on metrizoate cushions (13), and neutrophils and mononuclear leukocytes were purified by centrifugation on Ficoll-Hypaque cushions (14), then washed and resuspended in Medium 199-ovalbumin. Chemotaxis was assessed by a modification (12) of the Boyden microprobe filter technique (15). One ml of cell suspension in Medium 199-ovalbumin containing 3.5 ± 0.5 x 10⁶ eosinophils, 2.5 ± 0.5 x 10⁶ neutrophils, or 3.0 ± 0.5 x
10^6 mononuclear leukocytes was introduced into each polystyrene disposable chemotactic chamber (Adams, Inc., Dedham, Mass.) fitted with a 3-μm pore filter for assessment of eosinophil and neutrophil chemotaxis, and an 8-μm pore filter for mononuclear leukocyte chemotaxis; the filters separated the cells from the chemotactic factors contained in the lower compartment in 1 ml of Medium 199-ovalbumin (1, 3). After a 2 ½ to 3 hr incubation at 37°C, the filters were removed, fixed, and stained as described (1, 12). Leukocytes that migrated into the filters were counted microscopically in 10 high power fields (hpf), five from each of duplicate filters, at a fixed depth in the range of 50–90 μm from the cell source. The counting depth was selected to achieve a background count of 2 to 8 leukocytes/hpf in the absence of chemotactic stimulation, and the chemotactic responses were expressed as the net leukocytes/hpf after correction for the background counts. The loss of chemotactic responsiveness of leukocytes pretreated with a chemotactic agent for 30 min at 25°C was expressed as % deactivation = [1 – (chemotaxis after deactivation/chemotaxis of untreated cells) × 100]% (9).

ECF-A Purification. Human lung surgical specimens were processed to fragments in 100 g lots as described (16) and extracted twice with 100 ml of either butanol/glacial acetic acid (10:1) or alkaline Tyrode’s buffer-0.05 M Tris-HCl (pH 8.2) by homogenization with a tissue grinder (Polytron, Brinkmann-Kinematica, Lucerne, Switzerland) and sonication for 15 min at 4°C (Ultrasone Instruments International, Inc., Farmingdale, N.Y.). The extracts were centrifuged in 40-mL portions to obtain a clear supernatant fraction. Lung fragments, passively sensitized with IgE-rich serum from a ragweed-sensitive individual and challenged with antigen E (16), were used as a source of immunologically released ECF-A. Both the extracted and immunologically released ECF-A were applied to a Sephadex G-25 column equilibrated and eluted with 0.01 M acetic acid for the extracts and Hanks’ salt solution for the immunologically released material. Descending Sephadex G-25 and G-10 gel filtration were carried out with a capillary pump (Laboratory Supplies Co., Inc., Hicksville, N.Y.) producing flow rates of 3–4% of bed volume per hr at 4°C. Dowex AG-1 ion exchange chromatography used a linear gradient of ~0.3 pH units per 4 bed volumes in the pH range from 5 to 2. High voltage paper electrophoresis was performed on a Savant Instruments Co. (Hicksville, N.Y.) horizontal electrophoresis apparatus model FP-50A equipped with an aluminum cooling plate and operated at 50 volts per cm at 10°C using 0.05 M pyridine-acetate pH 7.4 buffer. Descending paper chromatography was performed with a Scientific Manufacturing Industries (Emeryville, Calif.) chromatocob A-125 utilizing the organic layer of a mixture of water:butanol:acetic acid (5:4:1) (17). All papers were prewashed for 24 hr with the appropriate solvents. Strips of paper 2 cm wide were eluted with 5–4 ml of 0.01 M NH₄OH, and the samples were relyophilized twice from 2 ml of distilled water. Ninhydrin determination of portions of eluates from Dowex-1 or paper followed a standard method (18). Treatment of ECF-A with 1.5 × 10⁻⁴ M periodic acid in 0.001 M NH₄ acetate, pH 7.2, for 1 hr at 25°C was followed by the addition of sodium ascorbate to a final concentration of 3.0 × 10⁻⁴ M before assay (19).

Amino Acid Analysis and Sequence Determination. Amino acid analyses were done according to the method of Spackman et al. (20) with a Beckman model 120C automatic amino acid analyzer which demonstrates a precision of ±25% in the 2–10 nmol range (21). Peptides were hydrolyzed with 0.25 ml of 6 M HCl in sealed evacuated tubes at 105°C for 30 hr. Prior performic acid oxidation or the addition of phenol was utilized to seek sulfur-containing amino acids, and tyrosine, respectively (22, 23).

The amino-acid sequence of purified peptides was analyzed by dansylation of the amino-terminal residue of a portion of each specimen with dansyl chloride according to the method of Gray (24) and Edman degradation of the remainder of the sample to expose the next amino acid for dansylation. Dansyl-peptides were hydrolyzed with 6 M HCl in 5 × 50 mm tubes flushed with N₂ and sealed and the dansyl amino acids were identified by two-dimensional chromatography on polyamide sheets (25, 26). Sequential Edman degradation and dansylation were carried out until either the quantity of dansyl-amino acid was too small for identification or multiple dansyl-amino acids were seen. Sequence analysis from the COOH-terminus was conducted by either hydrazinolysis (27) or carboxypeptidase A digestion with identification of the amino acids liberated with the Beckman amino-acid analyzer. Selective tritiation of the COOH-terminal residue followed by hydrolysis and chromatographic identification of the labeled amino acid was also used (28).

Peptide Synthesis. Reaction vessels, shaker, and other aces for the manual solid phase synthesis of peptides were obtained from Schwarz/Mann (Orangeburg, N.Y.). Three grams of chloromethylated resin (0.89 mmol of chloride per g) were reacted with a 2-fold molar excess of the cesium salt of BOC-Glu-O-benzyl ester, and then BOC-Ser-O-benzyl ester and BOC-Gly were successively coupled to each deprotected NH₂-terminus with a 3-fold molar excess of the next BOC-amino acid and dicyclohexylcarbodiimide in CH₂Cl₂ (29). Each cycle was performed as described (29, 30) except that the coupling reaction was carried out for 12 hr, the deprotection reaction used 25% (v/v) trifluoroacetic acid–CH₂Cl₂ for 30 min, and the washing after deprotection consisted of 33% dioxane in CH₂Cl₂ in alternate washes with CH₂Cl₂. The completeness of deprotection and coupling was assessed by determination of total resin-free amine with chloride titrations (31). The 2.15 mmol of BOC-Gly-Ser-Glu-resin synthesized was divided into three equal portions, of which one was coupled with BOC-Val, one with BOC-Ala and one left unreacted, after which each was cleaved from the resin with HBr-trifluoroacetic acid (29). The peptides were freed of volatile contaminants by stepwise evaporation from CH₂Cl₂ ethanold, 0.1 M acetic acid, and water, purified on Sephadex G-10 in 0.01 M NH₄OH and on Dowex-1 as for ECF-A, and analyzed for amino-acid composition.

RESULTS

Extraction and purification of human lung ECF-A

The extraction of human lung fragments with either acidicbutanol or alkaline Tyrode’s buffer yielded large quantities of eosinophil chemotactic activity with a range for four samples of 710–1080 net eosinophils/hpf per g of lung tissue with acidic-butan t and 420–760 net eosinophils/hpf per g of lung with alkaline Tyrode’s buffer. Sephadex G-25 gel filtration of extracts obtained by either procedure revealed considerable heterogeneity in molecular size of the eosinophil chemotactic activity. The major peak of activity of approximate molecular weight 300–1000 coincided with the peak of preferential eosinophil chemotactic activity released by IgE-mediated reactions (3, 7). Extraction of lung tissue with an acidic aqueous solution (0.1 M acetic acid) or a basic organic solvent system (butanol-pyridine, 10:1 v/v, pH 9.0)
Immunology: Goetzl and Austen

Amino-acid composition and sequence analyses

Compositional analysis of ECF-A purified by Sephadex G-25, Dowex-1, Sephadex G-10, and descending paper chromatography gave a total recovery of 3.0-9.9 nmol of peptide in each of the two peaks on six consecutive purifications, representing an overall yield of 3.6-12.5% of the low-molecular-weight eosinophil chemotactic activity. The mean residue values were low for serine, attributable to losses during acid hydrolysis (20), and for alanine and valine due to cross-contamination of peptides with these two NH₂-terminal residues (Fig. 2). No basic amino acids were encountered, and the only other residues noted in the analyses of both peptides were aspartic acid and leucine with mean residue values ±1 SD of 0.19 ± 0.23 and 0.27 ± 0.32, respectively; these amino acids were not noted after high voltage electrophoresis. Glutamic acid was recognized by total enzymatic digestion (32) of the peptides, ruling against the presence of glutamine.

The amino acid sequence of the ECF-A peptides (Fig. 2) was surmised from a composite of data obtained with half the material from the six preparations. The limited supply and low yields prevented identification of the COOH-terminal residue by sequential Edman-dansyl processing or concurrent analysis of the NH₂-terminal and COOH-terminal residues for any one preparation of peptides. Aminopeptidase M digestion of a preparation of the more acidic pool of ECF-A with 10 μg of enzyme in 0.04 M sodium phosphate, pH 7.8, for 2 hr at 37° released an average of 0.9 nmol of alanine and 2.7 nmol of valine, representing a 92% recovery of the NH₂-terminal residue in the composition while reducing the eosinophil chemotactic activity by 77%. Carboxypeptidase A digestion of the preparation of ECF-A with 10 μg of enzyme in 0.1 M ammonium bicarbonate, pH 7.8, for 2 hr at 37° released an average of 3.4 nmol of glutamic acid, representing an 87% recovery of COOH-terminal residue in the composition with a concomitant 65% loss of eosinophil chemotactic activity. Treatment of four of the above samples and five additional preparations of highly purified ECF-A with periodic acid had no effect.

Synthesis of tetrapeptides with eosinophil chemotactic activity

The valine and alanine tetrapeptides (Fig. 2) and their common COOH-terminal tripeptide were synthesized and then purified by the same procedures used for natural ECF-A, with the omission of the initial Sephadex G-25 step. The valyl-peptide was more acidic than the alanyl-peptide on Dowex-1 and paper electrophoresis, and exhibited greater hydrophobicity on paper chromatography (Fig. 1). Both peptides cochromatographed on Sephadex G-10, Dowex-1

![Diagram](image_url)

FIG. 1. Descending paper chromatography of partially purified alkaline-Tyrode's extract ECF-A and synthetic peptides. Dowex-1 pools pH 3.0-2.8 and 2.6-2.2 after Sephadex G-10 filtration, and 300 nm each of the alanyl and valyl tetrapeptides were spotted on paper with a Dnp-glutamic acid standard that ran 20 cm. Ten percent of each fraction was assayed for eosinophil chemotactic activity; the background eosinophil count was 2.6 eosinophil/hp.f.

![Table](table_url)

FIG. 2. Amino-acid compositions and sequences of eosinophil chemotactic peptides of human lung tissue. Six samples were analyzed for the alanyl peptide and three for the valyl peptide, as the latter was not recovered in sufficient quantity for analysis on three occasions. The mean number of nmol ± 1 SD and the range were 6.02 ± 2.88, 3.00-9.94 for the alanyl peptide and 7.88 ± 0.54, 7.30-8.36 for the valyl peptide. The nmol recovery of glycine was arbitrarily assigned a value of 1.00 in assessing the contribution of individual residues. The residue value for valine in the alanyl peptide was 0.10 ± 0.15 and for alanine in the valyl peptide was 0.36 ± 0.16. Sequence procedures were: --, dansyl-Edman reaction; ---, hydrazinolysis; *, carboxypeptidase A digestion.
Preferential eosinophil chemotactic activity by standardized when the paper, and co-electrophoresed on paper in the same region as natural ECF-A when assessed functionally. Both tetrapeptides were chemotactic for human eosinophils with maximum activity in the $10^{-6}$-$10^{-7}$ M range (Fig. 3), whereas the tripeptide was marginally active. The presence of an equal concentration of peptides on both sides of the filter eliminated the eosinophilotactic response.

### Preferential eosinophil chemotactic activity of purified ECF-A and synthetic peptides

When the concentrations of chemotactic factors were standardized to comparable neutrophil chemotactic activity, ECF-A from lung extracts and synthetic valyl- and alanyl-tetrapeptides demonstrated preferential eosinophil chemotactic activity relative to other chemotactic factors (Table 1). Plasma kallikrein exhibited preferential neutrophil chemotactic activity, whereas C5a was equally chemotactic for all three types of leukocytes. Deactivation assessed by residual responsiveness of leukocytes to their preferential chemotactic stimulus showed a similar extent of neutrophil deactivation for all factors consistent with their introduction at comparable neutrophil chemotactic doses. Although selectivity of ECF-A for leukocyte deactivation was not demonstrated at the concentrations depicted in Table 1, preferential activity was apparent at a $10^{-8}$ M level where deactivation of neutrophils, eosinophils, and mononuclear leukocytes, respectively, was 62%, 91%, and 48% for ECF-A; 49%, 88%, and 36% for alanyl-tetrapeptide; and 67%, 95%, and 53% for valyl-tetrapeptide.

### DISCUSSION

ECF-A extracted or released by antigen challenge of IgE-sensitized human lung tissue has been purified by sequential Sephadex G-25 filtration, Dowex-1 anion exchange chromatography, Sephadex G-10 filtration, and descending paper chromatography (Fig. 1). Although the eosinophilotactic activity extracted by extensive sonication in acidic-butanol or alkaline-Tyrode's solvents was heterogeneous on initial Sephadex G-25 chromatograms, material in the 300-1000 molecular weight range was selected for further purification since the activity released immunologically and designated ECF-A filtered within this region (1, 3). Whereas, Sephadex G-25 peaks of ECF-A obtained by either extraction or IgE-dependent release gave broad peaks of activity on either Dowex-1 or paper chromatography, subdivision of the activity eluted from Dowex-1 revealed that the more acidic pool showed greater hydrophobicity (Fig. 1). Amino-acid composition and sequence analysis revealed that the more acidic and hydrophobic material was predominantly a tetrapeptide Val-Gly-Ser-Glu, and that the less hydrophobic overlapping

### Table 1. Preferential eosinophil chemotactic activity of purified ECF-A and synthetic peptides

<table>
<thead>
<tr>
<th></th>
<th>ECF-A</th>
<th>Alanyl-tetrapeptide</th>
<th>Valyl-tetrapeptide</th>
<th>Kallikrein</th>
<th>C5a</th>
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</thead>
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<tr>
<td>Neutrophils Chemotaxis (net leukocytes/hpf)</td>
<td>69</td>
<td>72</td>
<td>74</td>
<td>67</td>
<td>74</td>
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<td></td>
<td>Deactivation (%)</td>
<td>86</td>
<td>100</td>
<td>96</td>
<td>100</td>
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<tr>
<td>Eosinophils Chemotaxis (net leukocytes/hpf)</td>
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<td>84</td>
<td>97</td>
<td>13</td>
<td>67</td>
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<tr>
<td></td>
<td>Deactivation (%)</td>
<td>91</td>
<td>92</td>
<td>100</td>
<td>34</td>
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<tr>
<td>Mononuclear leukocytes Chemotaxis (net leukocytes/hpf)</td>
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<td>18</td>
<td>16</td>
<td>14</td>
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<tr>
<td></td>
<td>Deactivation (%)</td>
<td>71</td>
<td>52</td>
<td>67</td>
<td>38</td>
</tr>
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</table>

Enriched preparations of 95% neutrophils, 69% eosinophils, and mononuclear leukocytes, 17% monocytes, and 72% lymphocytes were used. The concentrations of principals for chemotaxis and deactivation were: ECF-A, $10^{-6}$ M; alanyl peptide, $10^{-6}$ M; valyl peptide, $5 \times 10^{-7}$ M; kallikrein capable of generating 65 ng of bradykinin from 0.2 ml of heated plasma, and C5a produced by tryptic digestion of 5 μg of purified human C5 per chamber (7). The stimuli for deactivated leukocytes were kallikrein for neutrophils, ECF-A for eosinophils, and C5a for mononuclear leukocytes at the same concentrations as above.
peak of activity was attributable to a structurally related tetrapeptide Ala-Gly-Ser-Glu (Fig. 2). The presence of lipid eosinophilic factors in the mixture of purified peptides was unlikely in view of the failure of Rhodamine G-C to stain any relevant area of paper chromatograms or electrophoresis patterns (33). Periodic acid treatment of preparations of ECF-A failed to modify eosinophil chemotactic activity, thereby eliminating a functional contribution of carbohydrate moieties (19). The overall yield of 4–12% of the activity from the low-molecular-weight region of the initial Sephadex G-25 chromatogram reflects cumulative losses at each purification step since no minor peaks representing functional heterogeneity were encountered. No differences were seen between the extracted material and that released immunologically and originally described as ECF-A.

The valyl- and alanyl-tetrapeptides were synthesized and shown to have eosinophil chemotactic and deactivating activity and specificity comparable to that of natural ECF-A (Fig. 3, Table 1). Purified ECF-A preparations were active at 0.6–0.7 nmol per chamber, and 10^{-6} M synthetic tetrapeptides which provide 1 nmol per chamber gave similar activity (Fig. 3). Purification of the synthetic peptides and extracted ECF-A revealed that the peptide peaks and eosinophilic activity had the same characteristics on Dowex-1, Sephadex G-10, and paper electrophoresis and chromatography.

The inactivation of ECF-A and synthetic tetrapeptide eosinophil chemotactic activity by limited digestion with aminopeptidase M reveals that the NH_2-terminal sequence is critical to ECF-A activity. This conclusion is supported by the marginal activity of the COOH-terminal tripeptide (Fig. 3). The COOH-terminal glutamic residue is also necessary since carboxypeptidase A digestion reduces the chemotactic action of ECF-A. The ninhydrin positivity of eosinophil peaks during ECF-A purification, the susceptibility to aminopeptidase M digestion, and the reactivity on dansylation of the NH_2-terminus provide evidence that ECF-A has a free NH_2-terminus.

Purified and synthetic ECF-A share features with the small peptide chemotactic factors, bacterial soluble factors (34), and formyl methionyl peptides (35), such as maximum activity in nanomolar amounts, high-dose inhibition, and acidic and hydrophobic amino-acid residues (34, 35). In apparent contrast to these other peptide factors, ECF-A does not have a blocked NH_2-terminus, preferentially attracts eosinophils, and can be deactivating eosinophils to a subsequent chemotactic stimulus (8). Since the alanyl- and valyl-tetrapeptides have a hydrophobic NH_2-terminus and an acidic COOH-terminus, it is possible that the NH_2-terminus recognizes an eosinophil membrane receptor placing the acidic residue close to the cell surface. Such charge localization may perturbate the membrane to lead to pseudopod formation and directed mobility. Definition of ECF-A structure makes feasible the preparation of analogs with residue substitutions and size variations for further analysis of the structural requirements for eosinophil stimulation and possible separation of responses.

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