STRUCTURAL DETERMINANTS OF THE EOSINOPHIL CHEMOTACTIC ACTIVITY OF THE ACIDIC TETRAPEPTIDES OF EOSINOPHIL CHEMOTACTIC FACTOR OF ANAPHYLAXIS*

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The eosinophil chemotactic factor of anaphylaxis, ECF-A,[1] was discovered in 1971 as a mediator released during immediate-type hypersensitivity reactions in guinea pig (1) and human (2) lung slices. ECF-A was subsequently established as a totally preformed mediator in human nasal polyps (3) and lung fragments (4), and its association with tissue mast cells was demonstrated by extraction from dispersed human lung mast cells after their isopycnic and isokinetic purification (5). The mast cell subcellular localization of this preformed mediator was established by its isolation from purified rat peritoneal mast cells where it was contained in the granules (4). ECF-A released by IgE-mediated immunologic mechanisms (2, 4) had a mol wt of 500 and elicited preferential eosinophil chemotaxis, and a principle with these characteristics was therefore isolated from among the various activities present in human lung extracts (6, 7).

After extraction from human lung, sequential purification of this low mol wt eosinophilotactic activity by Sephadex G-25, Dowex-1, Sephadex G-10, and paper chromatography led to the identification of two acidic peptides of sequence Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu in the active fractions (6). Both purified native and synthetic peptides were preferentially chemotactic for eosinophils in vitro in a Boyden chamber assay with peak activity at concentrations of 5×10^{-8} M to 10^{-6} M in the stimulus compartment (6). The eosinophil chemotactic activity of the synthetic peptides required a concentration gradient, elicited a wave of movement of eosinophils into the micropore filters (7), and was dependent on the integrity of the NH_2-terminal and COOH-terminal residues (6). The structural requirements for chemotactic activity of ECF-A have been further defined utilizing synthetic peptide analogues and substituents. A model of interaction between the tetrapeptide factor and eosinophil surface is proposed in which full eosinophil chemotactic activity is dependent on reversible interaction with the NH_2-terminal residue and activation by the COOH-terminal residue.

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[1] Abbreviations used in this paper: ECF-A, eosinophil chemotactic factor of anaphylaxis; HBSS, Hanks' balanced salt solution; hpf, high power field.

Materials and Methods

Dowex AG-1-X8, 200–400 mesh, and Bio-Beads S-X1 chloromethylated resin, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.), Sephadex G-10, Ficoll and dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York), hydroxyl apatite (Clarkson Chemical Co., Inc., Williamsport, Pa.), diethylaminoethyl cellulose (DE-52) (Whatman Chemicals, Balston, Maidstone, Kent, England), Hanks' balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.), ovalbumin five-times recrystallized (Miles Laboratories Inc., Elkhart, Ind.), anhydrous trifluoroacetic acid (redistilled *in vacuo*) (Eastman Kodak Co., Rochester, N. Y.), alanine amide, valine amide, and leucine amide (Nutritional Biochemicals Corp., Cleveland, Ohio), *t*-butoxycarbonyl (BOC)-amino acids (Beckman Instruments, Inc., Palo Alto, Calif.), N,N'-diisopropylethylamine (Matheson, Coleman & Bell, East Rutherford, N. J.), disposable polystyrene modified Boyden chambers (Adaps, Inc., Dedham, Mass.), and twice-recrystallized trypsin previously treated with diphenyl carbamyl chloride (Sigma Chemical Company, St. Louis, Mo.) were obtained as noted. All solvents were either Eastman Kodak Co. reagent grade or Fisher-certified (Fisher Scientific Co., Pittsburgh, Pa.) and were redistilled before use. Methylene chloride (CH_2Cl_2) was further purified by passage through a column of alumina (Alumina Adsorption, Fisher Scientific Co.). N,N'-dimethylformamide was filtered over a column of 4A molecular sieves (Matheson, Coleman & Bell) and stored with fresh sieves for 72 h before use (8). C5a was prepared by tryptic digestion (9, 10) of purified C5 prepared from human plasma (11). C5a-containing digestion mixtures were heated for 20 min at 61°C to inactivate residual trypsin and then centrifuged to remove heat-precipitated proteins (10).

Peptide Synthesis and Purification. Reaction vessels, wrist-action shakers, scrubbing and cleaving bottles, and other accessories for manual solid phase synthesis of peptides were obtained from Schwarz/Mann, Orangeburg, N. Y. For each tetrapeptide and tripeptide, 5 g of chloromethylated resin (0.89 mmol of chloride per g) were reacted for 24 h at 37°C in dimethylformamide with a twofold molar excess of the cesium salt of the BOC-derivative of the COOH-terminal residue (12). Subsequent BOC-amino acid residues were successively coupled to each deprotected NH_2-terminus during a 12-h reaction interval at room temperature and with threefold molar excess both of the next amino acid and dicyclohexyl carbodiimide in CH_2Cl_2 (12). Each cycle was performed by a standard procedure (12, 13) modified such that deprotection was accomplished with 25% (vol/vol) trifluoroacetic acid-CH_2Cl_2 for 30 min and washings, after deprotection, were carried out with 33% (vol/vol) dioxane in CH_2Cl_2 in alternate washes with CH_2Cl_2 (6). The completeness of deprotection and coupling in each cycle was assessed by determinations of total resin-associated amine with sequential chloride titrations (14). After the last coupling step, each peptide was cleaved from the resin with hydrogen bromide-trifluoroacetic acid (12). All crude peptide preparations were freed of volatile contaminants by stepwise addition and evaporation of three 80-ml portions each of CH_2Cl_2, ethanol, 0.1 M acetic acid, and water, respectively. Peptides were then dissolved in 2 ml of ethanol-0.1 M NH_4OH (1:1, vol/vol) and filtered on Sephadex G-10 equilibrated in 0.1 M NH_4OH. The peptide peaks, identified by the optical density at 215 nm of their peptide bonds, were pooled and lyophilized. The four tripeptides Ala/Leu/Phe/Val-Gly-Ser were each redissolved in 0.1 M NH_4OH, filtered again on Sephadex G-10, and pooled; a portion was removed for analysis, and the remainder was lyophilized. The tetrapeptides and the COOH-terminal tripeptide, Gly-Ser-Glu, were each purified by elution from Dowex-1 in a pyridine-formic acid gradient (6) and then filtered on Sephadex G-10 and further processed as described for the NH_2-terminal tripeptides. The purity of each peptide preparation was confirmed by comparing its amino acid composition before and after an additional descending paper chromatography step (6). Amino acid analyses of 6N HCl hydrolysates were conducted according to Spackman et al. (15) with a Beckman model 120C automated analyzer.

Leukocyte Migration. Blood from normal donors or patients with peripheral blood hypereosinophilia of 26–89% was incubated for 30 min at 37°C with citrate anticoagulant and dextran to sediment erythrocytes (16). The leukocyte-rich supernatant plasma was aspirated and centrifuged at 100 *g* for 10 min at room temperature. The leukocyte pellet from hypereosinophilic patients was washed three times and resuspended in Hanks' balanced salt solution made 0.4% in ovalbumin and 0.005 M in Tris-HCl pH 7.4 (HBSS-ovalbumin) for use in chemotactic assays. Neutrophils and mononuclear leukocytes, obtained by centrifugation on Ficoll-Hypaque (17) of the mixed leukocytes from normal donors, were washed and resuspended in HBSS-ovalbumin. The standardized

cell suspensions were within a range of concentrations of $2.2 \pm 0.5 \times 10^6$ eosinophils per ml, $2.0 \pm 0.5 \times 10^6$ neutrophils per ml, and $3.0 \pm 0.5 \times 10^6$ mononuclear leukocytes per ml. 1 ml of leukocyte suspension was employed in the cell compartment of each modified (16) Boyden (18) chemotactic chamber. A micropore filter of 3-μm pore size for eosinophils and neutrophils and 8-μm pore size for mononuclear leukocytes separated the cells in the upper compartment from the chemotactic stimuli contained in 1 ml of HBSS-ovalbumin in the lower compartment (2, 6). After a $2^{1}/_{2}$–3 h incubation period at 37°C, the filters were fixed, removed from the disposable chambers, and stained (1). Duplicate filters from chambers without a stimulus were used to determine the depth within the filters which offered a suitable background of 2–8 leukocytes/high power field (hpf) for that experiment, and all filters from stimulated and control chambers were read at this depth without knowledge of the experimental protocol. Leukocytes that migrated into the filters to this standard level were counted in 10 hpf, 5 from each of duplicate chambers, and the chemotactic responses were expressed as net leukocytes/hpf after correction for background counts. In some protocols, factors were added to the cell compartment or were used to pretreat leukocytes, and the cells were washed; the migration of such cells was determined as above and was expressed as a percent of the response of untreated leukocytes.

Results

Eosinophilotactic Activity of Analogues with NH₂-Terminal Residue Substitutions or Internal Amino Acid Inversion or Deletion. The dose responses of the chemotactic activity of synthetic tetrapeptides in which the NH_2-terminal residue was Ala, Val, Leu, or Phe, and the common COOH-terminal tripeptide was Gly-Ser-Glu were compared (Fig. 1). Eosinophils were obtained from three separate donors, and the mean responses are depicted. Valyl-tetrapeptide was consistently more active than the alanyl-tetrapeptide, with their peak responses occurring at 3×10^{-7} M and 10^{-6} M, respectively. The peak chemotactic activity for the leucyl-tetrapeptide was achieved at 10^{-8} M, while the phenylalanyl-tetrapeptide required 10^{-4} M for peak effect.

The dependence of eosinophil chemotactic activity on the spacing between the NH_2- and COOH-terminal amino acids and on the order of the two internal residues was assessed for both the ECF-A valyl-tetrapeptide and the NH_2-terminal analogue leucyl-tetrapeptide series. Inversion of the order of glycine and serine did not change the maximum eosinophil chemotactic activity or dose-response characteristics of either the valyl- or leucyl-tetrapeptides (Fig. 2). The condensed tripeptides, lacking glycine, required 10^{-6} M for a maximum activity which was approximately two-thirds of the maximum chemotactic activity obtained with the 10^{-7} M concentrations of tetrapeptides.

Chemotactic Deactivation and Cross-Deactivation of Eosinophils. Functional deactivation refers to the failure of a leukocyte to migrate along a concentration gradient of a chemotactic stimulus as a result of previous interaction with an active chemotactic factor. Deactivation has the same specificity as chemotactic activation but differs in that the concentrations of the factor interacting with the target cell are defined beacuse a gradient is not involved. 8×10^6 human eosinophils were preincubated for 2–40 min at 37°C with varying doses of valyl- or alanyl-tetrapeptide and washed three times with HBSS-ovalbumin. Deactivation is expressed as the percent residual chemotactic responsiveness of the eosinophils to 10^{-7} M valyl-tetrapeptide or C5a as compared to that of the untreated cells. Deactivation of the eosinophils by both the alanyl- and the valyl-tetrapeptides to a subsequent valyl-tetrapeptide chemotactic stimulus was rapid and dose related. Full deactivation was achieved by 10^{-10} M alanyl- or valyl-
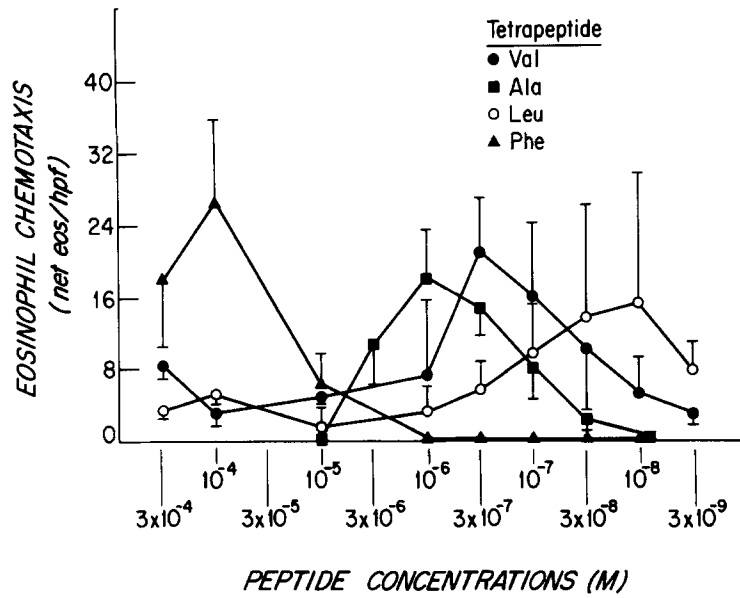
FIG. 1. Eosinophil chemotactic activity of ECF-A tetrapeptides and NH_2-terminal analogues. Values depicted are the mean ± 1 SD for three experiments with unpurified eosinophils from donors with 32, 56, and 89% peripheral blood eosinophilia.
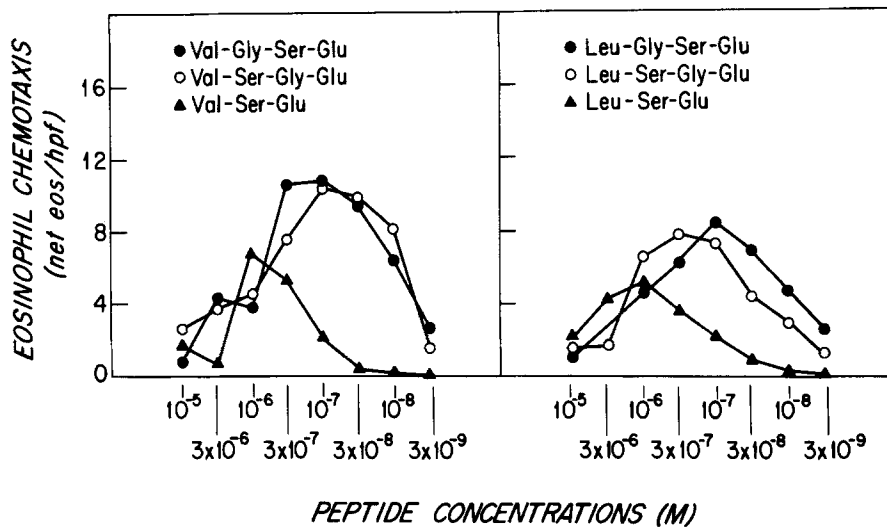

FIG. 2. Eosinophil chemotactic activity of analogues with internal amino acid inversion or deletion. Values shown are the mean of two experiments with unpurified eosinophils from donors with 38 and 76% eosinophilia.

tetrapeptide (Fig. 3) which represents 1/1,000 of the dose capable of eliciting the peak eosinophil chemotactic response (Fig. 1 and 2). Even 10^{-12} M tetrapeptide resulted in substantial deactivation. Cross-deactivation by pretreatment with synthetic tetrapeptides was dose and time related (Fig. 4). Levels of 10^{-12} M of either tetrapeptide did not decrease eosinophil responsiveness to C5a, and com-
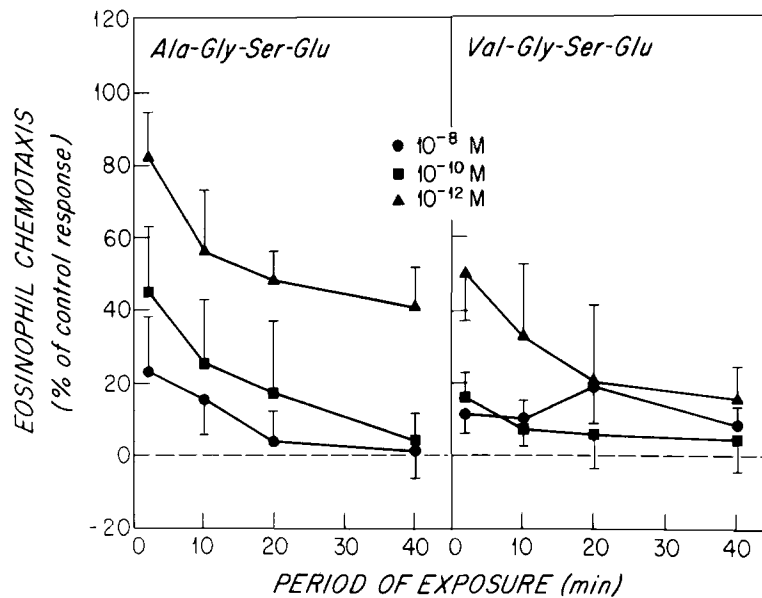
Fig. 3. Deactivation by synthetic tetrapeptides of eosinophils as assessed by their chemo-tactic response to Val-Gly-Ser-Glu. Values shown are the mean percent of control response ±1 SD for three experiments with varying periods of exposure of eosinophils to the deactivating tetrapeptide. The three donors exhibited 26, 43, and 59% eosinophilia. The control responses to 10^{-7} M valyl-tetrapeptide of these eosinophils after treatment with buffer alone were 13.8, 15.1, and 20.3 net eos/hpf, respectively; each control response was set at 100%.

plete deactivation was only found with the 10^{-8} M concentration. In experiments not shown, the leucyl-tetrapeptide analogue deactivated and cross-deactivated eosinophils with dose-response and time-course relationships similar to those observed for the alanyl- and valyl-tetrapeptides. The deactivating activity of the phenylalanyl-tetrapeptide was minimally evident at 10^{-7} M, and at 10^{-6} M was comparable to 10^{-10} M alanyl-tetrapeptide.

Modulation of Eosinophil Responses to ECF-A Tetrapeptides by Substituent Tripeptides and Amide Derivatives of Hydrophobic Amino Acids. The NH_2-terminal tripeptide, Val-Gly-Ser, and the COOH-terminal tripeptide, Gly-Ser-Glu, both of which are only marginally chemotactic, were analyzed for their effects on the eosinophil chemotactic response to the intact tetrapeptide, Val-Gly-Ser-Glu. In this study, the tripeptide was introduced into the stimulus compartment with the tetrapeptide, added to the cell compartment of the chemotactic chamber, or preincubated with the eosinophils followed by cell washing before an analysis of the chemotactic response. The NH_2-terminal tripeptide inhibited the chemotaxis of eosinophils in a dose-related fashion either when present with the stimulus or the cells (Fig. 5). When added to the 10^{-7} M tetrapeptide stimulus, Val-Gly-Ser gave 50% inhibition of the eosinophil response at approximately an equimolar concentration, while, when present on the cell side of the chamber, 10^{-7} M Val-Gly-Ser gave 75–80% inhibition. The inhibitory effect of Val-Gly-Ser on the eosinophil was reversed when the cells were washed before their response to the tetrapeptide was assayed. The COOH-
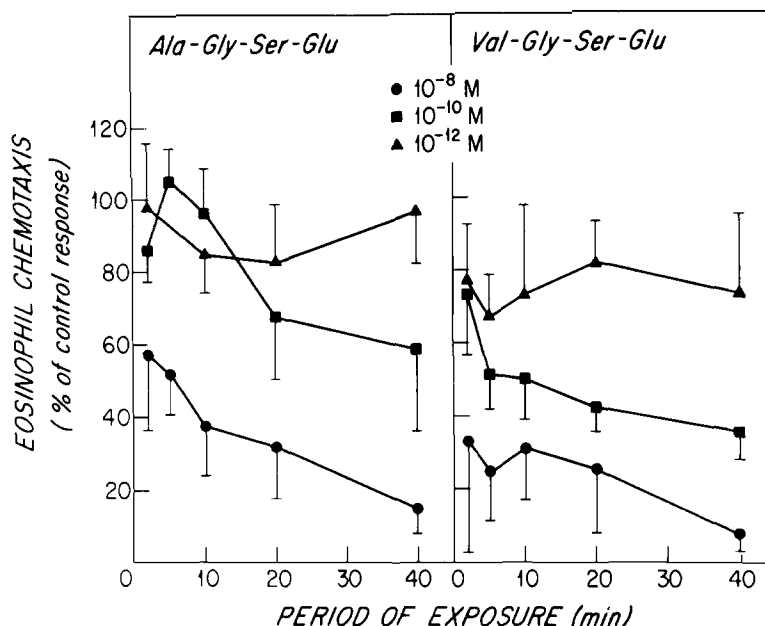
Fig. 4. Deactivation by synthetic tetrapeptides of eosinophils as assessed by their chemo-
tactic response to C5a. Values shown are the mean percent of control responsiveness ±1 SD
for three experiments with varying periods of exposure of eosinophils to the deactivating
tetrapeptide. The three donors exhibited 26, 43, and 59% eosinophilia. The control responses
to C5a, at a concentration derived from 2.5 μg equivalent of C5/ml, of these eosinophils
treated throughout with buffer were 14.9, 16.5, and 18.8 net eos/hpf, respectively; each
control response was set at 100%.

terminal tripeptide, Gly-Ser-Glu, was ineffective when a 10^{-7} M concentration
was mixed with an equimolar quantity of valyl-tetrapeptide stimulus. On the
other hand, this concentration gave 75% inhibition when present in the cell
compartment, and the inhibitory effect of Gly-Ser-Glu was not reversed by
washing the cells. The expression of the NH$_2$-terminal tripeptide effect from the
stimulus side and its reversibility suggests competition with the chemotactic
factor. In contrast, the irreversible inhibitory action of the COOH-terminal
tripeptide after its interaction with the cell and its failure to act as efficiently
from the stimulus side of the compartment is compatible with cell-directed
suppression.

The capacity of 10^{-7} M NH$_2$-terminal and COOH-terminal tripeptides to
suppress the eosinophil response to C5a was examined with the same protocol
employed above with the valyl-tetrapeptide stimulus. In two experiments in
which the NH$_2$-terminal tripeptide was added with the stimulus, there was 47
and 61% inhibition of the valyl-tetrapeptide effect, but only 18 and 29% inhibi-
tion of the C5a-stimulated response of the cells. In three experiments, pretreat-
ment of the eosinophils with 10^{-7} M COOH-terminal tripeptide followed by
washing gave 74, 62, and 48% inhibition of their response to 10^{-7} M valyl-
tetrapeptide and 71, 53, and 37% inhibition of their response to C5a. Since the
concentration of C5a utilized was selected for comparable chemotactic activity to
10^{-7} M valyl-tetrapeptide, the relative inability of the NH$_2$-terminal tripeptide
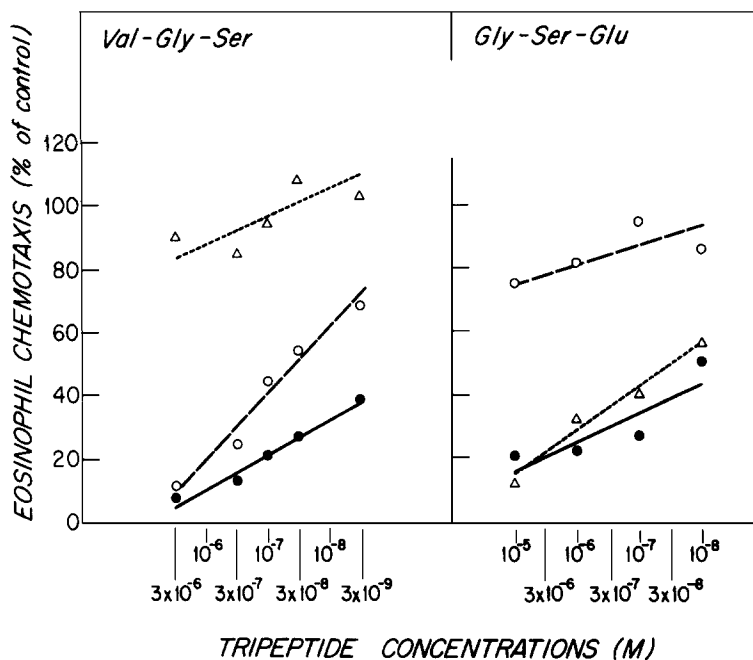
FIG. 5. Modulation of eosinophil chemotaxis to Val-Gly-Ser-Glu by substituent tripeptides. Values depicted are the mean percent of control response of two experiments in which the tripeptides were present in the stimulus compartment (O), cell compartment (●), or employed to pretreat the cells followed by washing (△). The two donors exhibited 76 and 89% eosinophilia. The control responses to 10^{-7} M valyl-tetrapeptide of eosinophils preincubated 20 min at 37°C with buffer alone were 10.5 and 14.3 net eos/hpf; the mean control response was set at 100%.

in the stimulus compartment to inhibit the chemotactic activity of C5a as compared to the valyl-tetrapeptide may indicate differences in either the molar concentrations of the stimuli or the target cell receptors.

The amide derivatives of valine, alanine, and leucine, shared with Val-Gly-Ser the ability to block the eosinophil chemotactic-stimulating activity of valyl-tetrapeptide (Table I). Approximately 50% inhibition of eosinophil chemotaxis to 10^{-7} M valyl-tetrapeptide resulted from the addition to the stimulus of 10^{-7} M leu-amide, or 10^{-6} M val-amide or ala-amide. No cell-directed inhibitory activity persisted after the pretreated eosinophils were washed; and thus the inhibitory effect of the amides, like that of the NH_2-terminal tripeptides, was reversible.

The hypothesis that the NH_2-terminal tripeptides are capable of reversible competition with the eosinophilotactic tetrapeptides was further tested by requiring the tripeptides to protect the target eosinophils from deactivation by the intact tetrapeptides. The capacity of 10^{-8} M valyl-tetrapeptide to render the eosinophils completely unresponsive to a subsequent stimulus of 10^{-7} M tetrapeptide was prevented in a dose-related fashion by the presence of the NH_2-terminal tripeptide with the deactivating stimulus (Table II). The tripeptide at a level of 10^{-7} M was completely protective, while the equimolar concentration achieved approximately 40–50% protection. The specificity of this protection toward the tetrapeptide was examined using the COOH-terminal tripeptide as a

TABLE I

*Modulation by Amides of NH_2-Terminal Amino Acids of the Eosinophil Chemotactic Activity of Valyl-Tetrapeptide**

		Amide concentration (M)			
	Protocol	10^{-5}	10^{-6}	10^{-7}	10^{-8}
		% of control response			
Val-amide	Present with stimulus‡	21.0 ± 19.7	55.0 ± 8.7	75.7 ± 14.2	83.5 ± 7.8
	Cell pretreatment§	93.4 ± 7.1	96.2 ± 1.9	86.4 ± 12.3	93.8 ± 8.2
Ala-amide	Present with stimulus	36.9 ± 21.7	45.3 ± 31.4	71.4 ± 29.0	88.0 ± 16.3
	Cell pretreatment	− ‖	86.1 ± 13.4	90.5 ± 11.8	−
Leu-amide	Present with stimulus	31.3 ± 7.0	41.7 ± 4.7	58.3 ± 12.9	86.3 ± 17.1
	Cell pretreatment	−	80.9 ± 11.4	91.6 ± 7.2	−

* Values indicate the mean percent of control response ±1 SD for three experiments utilizing donors of 86, 37, and 48% peripheral eosinophilia. The control responses of these eosinophils to 10^{-7} M valyl-tetrapeptide were 10.1, 12.3, 14.8 net eosinophils per hpf, respectively.

‡ Amides were present with the valyl-tetrapeptide stimulus.

§ Preincubation of eosinophils with amides for 20 min at 37°C, followed by washing three times before assessment of residual responsiveness to 10^{-7} M valyl-tetrapeptide stimulus.

‖ Not done.

TABLE II

*The Capacity of the NH_2-Terminal Tripeptide to Protect Eosinophils from Deactivation by the Valyl-Tetrapeptide**

	Concentrations of Val-Gly-Ser with pretreatment factor				
Pretreatment factor	0	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
	% of control response				
Valyl-tetrapeptide 10^{-8} M	−12.7	− ‡	124.9	39.2	−18.2
COOH-terminal tripeptide 10^{-7} M	54.4	36.7	28.1	47.6	−

* The pretreatment period was 20 min at 37°C, and the eosinophils were washed three times before assessing chemotactic responsiveness to 10^{-7} M valyl-tetrapeptide. Values are the mean percent of control responses for two experiments utilizing donors of 37 and 86% peripheral blood eosinophilia. The control responses to 10^{-7} M valyl-tetrapeptide of eosinophils exposed to buffer alone were 8.1 and 11.7 net eos/hpf. A negative value represents suppression below the control unstimulated level of migration.

‡ Not done.

cell-directed inhibitory factor (Table II). The NH_2-terminal tripeptide at an equimolar concentration or at a 10-fold excess afforded no protection against the COOH-terminal tripeptide, indicating that the NH_2-terminal tripeptide effect was directed against the interaction with the eosinophil of the NH_2-terminal portion of the intact tetrapeptide.

Since the eosinophilotactic tetrapeptides also elicited directed migration of neutrophils, the inhibitory actions of the NH_2- and COOH-terminal tripeptides on this response were evaluated. In two experiments with neutrophils purified to 98% and eosinophils of 51 and 68% purity, the chemotactic responses were 16.9

and 21.4 neutrophils/hpf, and 12.7 and 15.3 eosinophils (eos)/hpf, respectively. The NH$_2$-terminal tripeptide added to the stimulus gave 49 and 54% inhibition of the response of neutrophils and 53 and 64% inhibition of the response of eosinophils to an equimolar concentration of valyl-tetrapeptide. The COOH-terminal tripeptide irreversibly inhibited the neutrophil response by only 33 and 28%, as compared to 61 and 74% inhibition of the eosinophil response to 10^{-7} M valyl-tetrapeptide.

Eosinophil Chemotactic Activity of Mixtures of Alanyl- and Valyl-Tetrapeptides. To interpret the eosinophil response to mixtures of ECF-A tetrapeptides, it was essential to recognize where the molar concentration of any mixture would fall on the dose-response curve of an individual tetrapeptide. To examine both ends of the dose-response curve, equal increments of the alanyl- or valyl-tetrapeptide were added to a minimally or maximally active alanyl-tetrapeptide concentration (Fig. 6). The results obtained with each mixture were compared to the calculated additive effect of the tetrapeptides assayed separately in order to determine whether the mixtures were inhibitory, additive, or synergistic. Using a minimally active quantity of 3×10^{-8} M alanyl-tetrapeptide, the first two stepwise additions of both peptides were essentially additive, while the final 10-fold addition yielded a mixture which was less effective than the calculated additive response. When a 10^{-7} M concentration of alanyl-tetrapeptide which yielded a peak response was employed, each increment of either peptide gave a mixture that was less effective than the additive sum of their individual actions. The fact that the combinations were either additive or inhibitory indicates that there was no cooperative effect among receptors occupied by these tetrapeptides.

Discussion

The capacity of the tetrapeptides of ECF-A, Ala/Val-Gly-Ser-Glu, to stimulate directed migration of eosinophils in vitro can be modified by deletions or substitutions of single amino acid residues and modulated by nonchemotactic substituent peptides. Substitution of the NH$_2$-terminal residue led to alterations in potency and maximum activity which are defined, respectively, as the concentration of chemotactic peptide eliciting a peak eosinophil chemotactic response and the magnitude of that response. The leucyl-tetrapeptide was the most potent tetrapeptide exhibiting peak activity at 10^{-8} M–10^{-7} M, while peak activity for the ECF-A valyl- and alanyl-tetrapeptides was achieved at 10^{-7} M and 10^{-6} M, respectively (Fig. 1). In contrast, the introduction of a six-carbon aromatic ring, employing phenylalanine as the NH$_2$-terminal residue, produced a tetrapeptide eliciting a peak eosinophil chemotactic activity at 10^{-4} M. The magnitude of the eosinophil response was not related to potency in that the phenylalanyl-tetrapeptide elicited the greatest maximum response and the leucyl-tetrapeptide the least. Inversion of the order of the internal residues, glycine and serine, did not lead to an alteration of potency or maximum activity of the synthetic valyl- or leucyl-tetrapeptides (Fig. 2). However, elimination of the glycine residue from either tetrapeptide, producing condensed tripeptides of the sequence Val/Leu-Ser-Glu, was associated with a distinct reduction in potency and a modest decrease in peak activity. Thus, the nature of the NH$_2$-terminal residue as well as its distance from the COOH-terminal residue
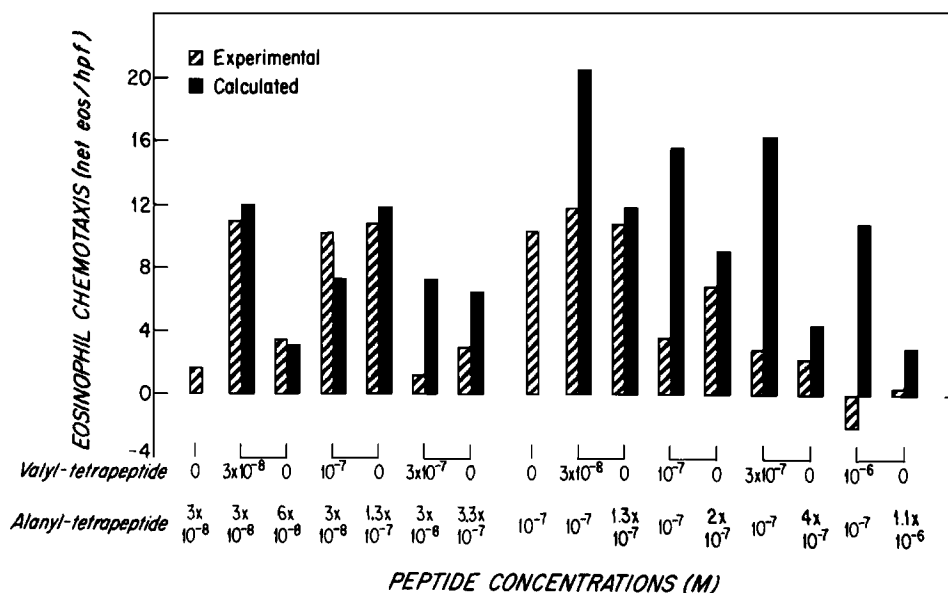
FIG. 6. Eosinophil chemotactic activity of mixtures of alanyl- and valyl-tetrapeptides. The response to the alanyl-tetrapeptide alone and to the mixtures is depicted by the height of each cross-hatched bar; all values are the mean of two studies. The fixed concentration of the alanyl-tetrapeptide was 3×10^{-8} M in the left-hand series and 10^{-7} M in the right hand series. Each black bar depicts the mean calculated sum of the individual responses to the fixed dose of alanyl-tetrapeptide and the varying concentrations of valyl- or alanyl-tetrapeptides. The experimental response to the varying concentrations of the alanyl- or valyl-tetrapeptide alone may be derived by subtracting the response to the fixed dose of alanyl-tetrapeptide from the calculated sum of the individual responses.

influences both the potency and maximum activity of the eosinophilotactic peptides.

The exposure of eosinophils to the alanyl- and valyl-tetrapeptides, followed by washing the treated cells, markedly reduced their chemotactic responsiveness to the valyl-tetrapeptide (Fig. 3) and to C5a (Fig. 4). This time- and dose-related irreversible process, termed functional deactivation, has been previously described for human neutrophils (19) and eosinophils (20), as well as rabbit neutrophils (21, 22). Deactivation of human neutrophils by C5a obtained by tryptic digestion of C5 and by partially purified kallikrein generally has not been detected at factor levels less than 1/10 the minimal chemotactic concentration (19, 23), while human eosinophil deactivation by partially purified human lung ECF-A was observed after 30 min with as little as 1/160 the minimal chemotactic concentration (20). Deactivation of eosinophils by the synthetic peptides was essentially complete after 20 min at a concentration 1/100 and after 40-100 min at a concentration 1/10,000 the minimal chemotactic dose (Fig. 3). The extent of deactivation at a given time and dose of tetrapeptide was greater to a chemotactic stimulus of valyl-tetrapeptide than to a heterologous C5a stimulus (Fig. 3 and 4). Whether the high degree of susceptibility of the human eosinophil to deactivation by the tetrapeptides is a unique feature of this class of principles or a characteristic of the target cell can only be determined by further

analyses utilizing other leukocyte types and completely purified or synthetic chemotactic factors.

The high-dose inhibition observed with ECF-A tetrapeptides, the NH_2-terminal-substituted analogues, and the condensed tripeptides lacking an internal glycine (Fig. 1 and 2) is a phenomenon shared with some other small peptide chemotactic factors acting on rabbit neutrophils (24, 25). When tetrapeptides are mixed at concentrations at which each alone is well within the ascending portion of its dose-response curve, the response to mixtures is approximately the sum of the responses to each component (Fig. 6). At higher peptide concentrations, the response to mixtures may be less than additive because the combined molar concentrations fall on an inhibitory portion of the dose-response curves for ECF-A tetrapeptides. This interpretation presumes that the two eosinophilotactic tetrapeptides are independently recognized by the target cells, and that the effect of their combined molar concentrations is similar to what would be observed with a comparable molar concentration of either alone.

The substituent NH_2-terminal and COOH-terminal synthetic tripeptides, which lack significant chemotactic activity, are capable of inhibiting the eosinophil chemotactic response to the intact stimulus. The NH_2-terminal tripeptide, Val-Gly-Ser, gave a dose-response inhibition when present on the stimulus side with 10^{-7} M valyl-tetrapeptide which was 50–60% at equimolar concentrations (Fig. 5). The inhibition by Val-Gly-Ser was somewhat greater when present with the cells as compared to the stimulus side, but the effect on the cells was reversed by washing. The inhibitory action of the NH_2-terminal tripeptide when present on the stimulus side and the reversibility of its inhibitory effects when present on the cell side suggest a direct competition with the eosinophilotactic tetrapeptide at the eosinophil surface. This concept is substantiated by the capacity of the NH_2-terminal tripeptide to protect the eosinophil from deactivation by the valyl-tetrapeptide with 40–50% protection at equimolar concentrations (Table II). The entire tripeptide was not required for effective competition with the valyl-tetrapeptide in that amide derivatives of the NH_2-terminal residues, which were devoid of chemotactic and irreversible cell-directed inhibitory activities, gave dose-response inhibition when present on the stimulus side (Table I). 50% inhibition by the amide derivatives required a 10-fold higher concentration than for the corresponding tripeptide which implies that the seryl-residue enhances the interaction of the tripeptide with the eosinophil and potentiates its competitive capability. Further, the inhibitory action of the NH_2-terminal tripeptide could also be observed on the chemotactic response of neutrophils and equimolar concentrations again reduced the response to 10^{-7} M valyl-tetrapeptide by approximately 50%. The capacity of the NH_2-terminal tripeptide to inhibit the chemotactic stimulation of neutrophils as well as eosinophils and to prevent deactivation of eosinophils by the intact tetrapeptide indicates a stimulus-specific competitive action.

The COOH-terminal tripeptide irreversibly suppresses the eosinophil chemotactic response to 10^{-7} M valyl-tetrapeptide in a dose-response manner. Full suppression at 20 min required COOH-terminal tripeptide concentrations of 10^{-6} M–10^{-5} M (Fig. 5) which are more than 10,000-fold higher than the doses of intact tetrapeptide required for complete deactivation (Fig. 3). The COOH-

terminal tripeptide did not compete with the tetrapeptide from the stimulus side (Fig. 5). Further, the cell-directed inhibitory action of the COOH-terminal tripeptide, in contrast to that of the intact tetrapeptide, was not blocked by the NH₂-terminal tripeptide (Table II).

A hypothetical model is proposed for a tetrapeptide chemotactic receptor based on the functional data derived from the eosinophilotactic activity of the structural analogues of the ECF-A tetrapeptides and the modulation of tetrapeptide activity by substituent sequences. The chemotactic activity of the tetrapeptide is dependent on both the hydrophobic NH_2-terminal residue, which interacts with a hydrophobic domain in the chemotactic receptor, and the highly-charged COOH-terminal residue which is presumed to initiate eosinophil movement by perturbing a polar domain in the same receptor. The spatial requirement for effective interaction with both domains in the receptor is revealed by the lower potency and activity of the condensed tripeptides lacking glycine. The 10-fold greater potency of NH_2-terminal tripeptide compared to the amide derivatives of NH_2-terminal amino acids in reversibly inhibiting the intact tetrapeptides suggests a role for serine in binding to a portion of the receptor, possibly by hydrogen bonding. The COOH-terminal substituent tripeptide irreversibly suppresses eosinophil chemotaxis by a cell-directed action possibly reflecting its capacity to perturb the polar domain; this effect, resembling deactivation, requires higher concentrations than needed for deactivation by the tetrapeptide.

Summary

The acidic tetrapeptides of ECF-A, Ala/Val-Gly-Ser-Glu, exhibit peak in vitro chemotactic activity for human eosinophils at concentrations of 3×10^{-8} M to 10^{-6} M, and rapidly deactivate eosinophils to homologous and other stimuli at concentrations as low as 10^{-10} M. The analogue Leu-Gly-Ser-Glu reaches peak activity at 10^{-8} M-10^{-7} M, while Phe-Gly-Ser-Glu requires 10^{-4} M to elicit a peak response. Although inversion of the order of glycine and serine does not alter the eosinophil chemotactic activity of the tetrapeptides, deletion of glycine increases by 10-fold the concentration required for peak chemotactic activity, indicating the critical nature of the spacing between NH_2- and COOH-terminal residues. The substituent COOH-terminal tripeptide, which is only marginally chemotactic, irreversibly suppresses eosinophil chemotactic responsiveness at a concentration 10,000-fold higher than concentrations necessary for deactivation by the intact tetrapeptide. The high concentration of tripeptide required for this cell-directed effect, which is assumed to be analogous to deactivation, is attributed to the absence of the NH_2-terminal residue which would facilitate effective interaction with the eosinophil. A substituent NH_2-terminal tripeptide and amides of the NH_2-terminal amino acids, which are devoid of chemotactic and deactivating activities, reversibly inhibit the tetrapeptide stimulus in a dose-response fashion. The additional finding that the NH_2-terminal tripeptide protects the eosinophil from deactivation by the intact tetrapeptide confirms that the competitive interaction is stimulus specific.

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