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PEPTIDE FRAGMENTS FROM THE TUFTSIN CONTAINING DOMAIN OF IMMUNOGLOBULIN G SYNTHESIS AND BIOLOGICAL ACTIVITY

Philip Gottlieb[1], Ester Tzehoval[2], Michael Feldman[2], Shraga Segal[3] and Mati Fridkin[1]

Departments of Organic Chemistry[1] and Cell Biology[2]
The Weizmann Institute of Science, Rehovot, Israel 76100

Unit of Microbiology and Immunology, Faculty of Health Sciences,
The Ben-Gurion University of the Negev, Beer-Sheva, Israel

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SUMMARY: Peptides corresponding to sequences of the Fc-portion of immunoglobulin G (IgG) surrounding and containing the tuftsin molecule were synthesized. The compounds were assayed for their ability to compete with [^{3}H-Arg^4]tuftsin in binding to mouse peritoneal macrophages and to stimulate the cell's capacity to phagocytize. Despite the sensitivity that tuftsin has demonstrated to various chemical modifications and structural alterations which usually cause reduction or total loss of biological activity, IgG-related analogs possess potent tuftsin-like activity. The activity is not caused by enzymatic breakdown and release of tuftsin. The fact that the elongated tuftsin analogs can specifically be attached to and activate macrophages may indicate a possible connection between Fc and tuftsin's receptors.

The naturally occurring peptide tuftsin has been shown to possess a wide range of immunopotentiating activities which are exerted, primarily on phagocytic cells, *i.e.* the polymorphonuclear leukocyte, the monocyte and the macrophage (1-3). Tuftsin is a basic tetrapeptide whose sequence, H-Thr-Lys-Pro-Arg-OH, corresponds to positions 289-292 at the C_H2 domain of the Fc-segment of the immunoglobulin G protein (4). The biological activity of tuftsin appears to be mediated *via* specific receptors which have been found to exist on phagocytic cells (2). The possibility that these binding sites are related in some way to the Fc-receptor was raised by us (5) and by other investigators (6). This assumption was basically made in view of the involvement of the Fc-domain of IgG in phagocytic processes and the fact that tuftsin, originating from this fragment in leukokinin (1-3) - a specific IgG protein - is a potent stimulator of phagocytosis.

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As an approach to further exploring the tuftsin receptor-Fc receptor relationship, five peptides encompassing the tuftsin moiety and corresponding to the tuftsin's region of the Fc-fragment were synthesized. The peptides are: *H-Thr-Lys-Pro-Arg*-Glu-Gln-Gln-Tyr-OH (positions 289-296; octa-C); H-His-Asn-Ala-Lys-*Thr-Lys-Pro-Arg-OH* (positions 285-292; octa-N); H-His-Asn-Ala-Lys-*Thr-Lys-Pro-Arg*-Glu-Gln-Gln-Tyr-OH (positions 285-296; dodeca); H-His-Asn-Ala-Lys-OH (positions 285-288; tetra-N); H-Glu-Gln-Gln-Tyr-OH (positions 293-296; tetra-C). The ability of the peptides to compete with [^{3}H-Arg^{4}]-tuftsin in binding to mouse peritoneal macrophages and to stimulate cell's capacity to phagocytize was studied in comparison to that of tuftsin.

The mode of generation of tuftsin from its precursor IgG-protein leukokinin appear to involve at least two steps of specific enzymatic processings (1,3-5). The first, a cleavage at the peptide's C-terminus is carried out by the action of a splenic enzyme, while the second, at the peptides N-terminus is performed by the enzyme leukokininase which was isolated from the plasma membrane of human polymorphonuclear leukocytes. The possibility that elongated pre-tuftsin peptidic fragments are intermediate products in the "biogensis" of tuftsin has not been raised. In this respect, the stability of the above octa-C, octa-N and dodeca-peptides toward degradation by macrophages was studied.

MATERIALS AND METHODS

Synthesis: All protected amino acid derivatives were obtained from Vega Biochemicals (Tuscon, USA). Tuftsin (6) and [^{3}H-Arg^{4}]tuftsin (specific activity 20.8 Ci/mmol) (7) were synthesized as previously described. Peptides: H-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH (octa-C) and H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH (dodeca) were synthesized according to the solid phase method (8). α-Amino functional groups were protected by t-butyloxycarbonyl (t-Boc). Side-chain protecting groups were as follows: His, N^{im}-p-toluenesulfonyl; Lys, N^{ε}-benzyloxycarbonyl: Tyr, o-benzyl; Arg, N^{G}-nitro; Glu, γ-benzyl. The first C-terminal amino acid (Tyr) was attached to 2% crosslinked chloromethylated polystyrene (Chemalog, South Plainfield, USA). All coupling stages were performed with a three-fold excess of protected amino acid derivatives using N,N -dicyclohexylcarbodiimide (DCC) as a coupling agent, except for Asn and Gln residues, where a three-fold excess of o-nitrophenyl active esters were used. All stages of coupling and α-amino deprotection were monitored with ninhydrin for the presence or absence of free amino groups. H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH (octa-N) was synthesized by stepwise extension, in solution of H-Thr-Lys(Cbz)-Pro-Arg(NO_2)-OBzl (9). H-His-Asn-Ala-Lys-OH (tetra-N) and H-Glu-Gln-Gln-Tyr-OH (tetra-C) were also prepared in solution. DCC was employed as a coupling agent except for the Asn

and Gln residues where o-nitrophenyl esters were used. All peptides were deprotected by anhydrous HF for 1 h at 0°C in the presence of anisole (10). Detailed synthetic procedures are described elsewhere (11).

Peptide Purification: Purification of tetra-N, octa-N, octa-C and dodeca peptides was achieved on a cation exchanger carboxymethylated cellulose (CM-23; Whatman, Springfield Mill, England). The column was equilibrated with 50 mM ammonium acetate and peptides eluted with a linear gradient up to 0.5 M ammonium acetate at a constant pH 6.5. Peptides, including tetra-C, were further purified by high-performance liquid chromatography (HPLC). HPLC was carried out on a reversed-phase Lichrosorb RP-18 column (Merck, Darmstadt, Germany; 0.4x2.5 cm, particle size 10μ). Column was equilibrated with the starting buffer - 50 mM ammonium acetate pH 6.5, containing (v/v) 0.2% isopropanol and 0.01% triethylamine. Peptides were eluted at a constant flow rate of 1 ml/min, by linear increasing of the iso-propanol content up to 6% (v/v). The eluent was monitored spectrophotometrically at 230 nm. All solvent and samples were filtered through 0.22μ Millipore filters before use.

Macrophages: Thioglycollate-stimulated mouse peritoneal macrophages were aseptically collected from Balb/c male mice (6-8 weeks old), 4 days after an intraperitoneal injection of 3 ml thioglycollate broth (2.98 g/100 ml, Difco Laboratories). Exudate celles (containing at least 90% macrophages of viability higher than 85%) were harvested and used for the preparation of macrophage monolayers on plastic tissue culture dishes, as previously described (13,14).

Phagocytosis Assay: Sheep erythrocytes (SRBC) were used as target particles. Cells were suspended (10^9/ml) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, New York) and sensitized with rabbit anti-SRBC antibodies (7S, IgG, Cordis Laboratories) (1 ml of 1:500 dilution in DMEM) for 30 minutes at 37°C. Following three washes in DMEM, the cells were labelled (12) with ^{51}Cr (1 hr, 37°C, 15μCi), washed with DMEM and resuspended in that medium to yield $2x10^8$ ^{51}Cr-IgG-SRBC cells/ml. Macrophage monolayers ($0.5x10^6$/well) were preincubated with 0.9 ml DMEM containing tuftsin or the specified tested peptide (200 nM; 15 minutes, 37°C), and subsequently 0.1 ml target particles suspension was added. After 60 minutes incubation at 37°C, the monolayers were thoroughly washed with DMEM and particles not ingested were lysed with 0.89% NH_4Cl solution. Cells were then washed twice with phosphate buffered saline (PBS; pH 7.4), and dissolved in 0.2% aqueous sodium dodecyl sulfate. Radioactivity of solution was then measured in a Hewlett-Packard gamma-counter. The standard error of the mean did not exceed 10% in triplicate cultures.

Competitive Binding Assay: Binding assay was carried out as previously described (7,13). Essentially, macrophage monolayers ($1x10^6$ cells/well) were incubated with varying concentrations ($\sim10^{-8}$-10^{-5} M) of the tested peptide and [^{3}H-Arg^4]tuftsin (10^{-7} M) in a final volume of 0.5 ml PBS. Subsequent to 35 minutes incubation at 22°C, the cells were washed twice with PBS (22°C), dissolved in 0.5 ml sodium dodecyl sulfate (0.2%) and the solution obtained added to vials each containing 10 ml of a triton-toluene scintillation mixture. Radioactivity was measured at an efficiency of 60% using a Beckman 7500 liquid scintillation spectrophotometer. Non-specific binding was defined as the amount of [^{3}H]tuftsin binding not inhibited by 10μM of tuftsin.

RESULTS AND DISCUSSION

Five peptides which correspond to the tuftsin-containing domain at the Fc-segment of IgG were synthesized. Three of these peptides are extended sequences of tuftsin in which four amino acid residues are added either to the

C-(octa-C) or N-(octa-N) terminus or both (dodeca). Two additional synthesized peptides (tetra-C; tetra-N) are the tetrapeptides immediately flanking the tuftsin sequence. The octa-C and the dodecapeptide were synthesized *via* the solid-phase procedure, whereas all other peptides were prepared using classical solution techniques. The peptides, except for tetra-C, were purified by ion exchange chromatography (CM-23). Further purification of all five peptides was achieved utilizing HPLC (Fig. 1). Amino acid content of peptides and their mobility as homogeneous ninhydrin, Sakaguchi and Pauli positive species, in high-voltage paper electrophoresis are summarized in Table 1.

The ability of the five synthetic peptides to compete with [^{3}H-Arg^{4}]-tuftsin in binding to mouse peritoneal macrophages at 22°C, was studied. Thus, the three extended analogs (octa-C; octa-N and dodeca) of tuftsin were capable, rather similarly to each other and to tuftsin, to inhibit the binding of tritium-labelled tuftsin to cells (Fig. 2). The tetra-C peptide was similarly effective while the tetra-N derivative did so at a much lower extent (Fig. 3).

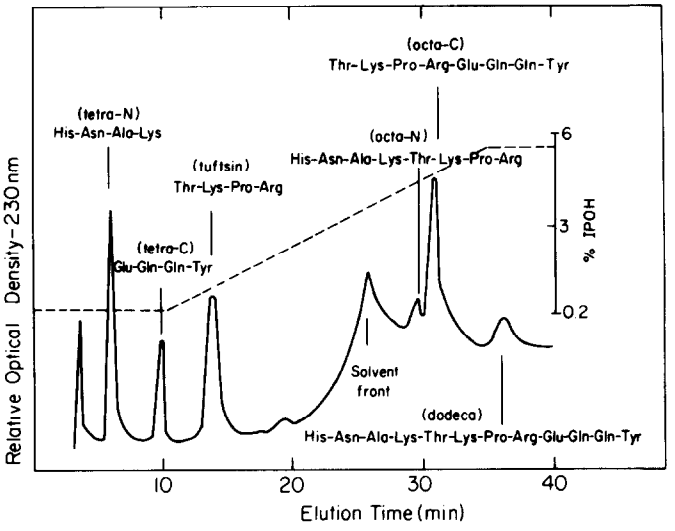

Figure 1. Reversed phase system for separation and purification of synthetic peptides surrounding and containing the tuftsin sequence. RP-18 column was equilibrated with 50 mM ammonium acetate buffer pH 6.5, containing (v/v) 0.2% iso-propanol (IPOH) and 0.01% triethylamine. The indicated gradient of IPOH was used.

Table 1. Amino acid composition and electrophoretic mobilities of tuftsin-related IgG-peptides. Peptides were hydrolyzed with 6N hydrochloric acid containing 4% phenol at 110°C for 20 h in evacuated sealed tubes. High-voltage paper electrophoresis was performed on Whatman No. 3 paper for 45 min at 60V/cm in pyridine/acetate buffer (pH 3.5) as compared to tuftsin = 1.00.

Peptide	Thr	Lys	Pro	Arg	Glu(n)	Ala	His	Asn	Tyr	Mobility
H-Thr-Lys-Pro-Arg-Glu-Gln--Gln-Tyr-OH	0.99	1.0	1.05	0.96	2.98	-	-	-	0.97	0.56
H-His-Ala-Asn-Lys-Thr-Lys--Pro-Arg-OH	1.05	2.0	1.20	1.07	-	1.05	0.90	1.00	-	1.09
H-His-Ala-Asn-Lys-Thr-Lys--Pro-Arg-Glu-Gln-Gln-Tyr-OH	0.98	2.0	1.01	0.98	2.80	1.00	0.96	1.04	0.89	0.75
H-Glu-Gln-Gln-Tyr-OH	-	-	-	-	3.00	-	-	-	0.95	0.15
H-His-Ala-Asn-Lys-OH	-	1.0	-	-	-	1.05	0.98	1.02	-	1.00

The effect of the synthetic peptides on the phagocytosis of ^{51}Cr-IgG coated sheep red blood cells by macrophages was assayed. As shown in Table 2, three peptides, octa-C, octa-N and dodecapeptide, have the ability to stimulate phagocytosis to the same degree as tuftsin, tetra-C to slightly half of that value, whereas tetra-N was devoid of tuftsin-like activity.

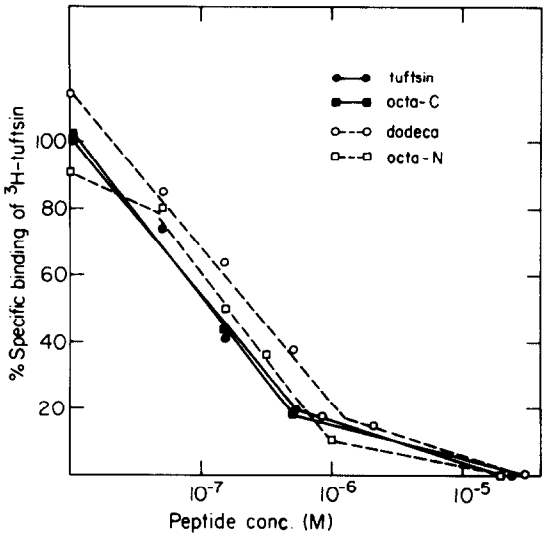

Figure 2. Effect of unlabelled tuftsin and its Fc-related extended analogs on displacement of [^{3}H-Arg^{4}]tuftsin from thioglycollate-stimulated mice peritoneal macrophages. Assay performed at 22°C with a concentration of labelled peptide of 100 nM.

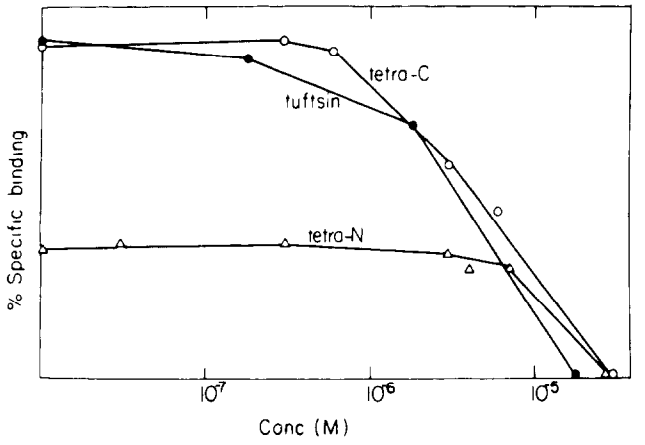

Figure 3. Effect of unlabelled tuftsin and Fc-tetrapeptides adjacent to tuftsin on the binding of [^{3}H-Arg^{4}]tuftsin to mice macrophages.

The results obtained from the above binding assays may shed some light on the nature of the tuftsin receptor. Previous studies have clearly shown that structural alterations, often even minute, of the tuftsin molecule may lead to a marked loss in its ability to bind to phagocytic cells (2). This is particularly evident when chemical modifications, such as random addition of amino acid residues, involve the N-terminus of the molecule. The finding that IgG-related extended analogs of tuftsin do bind to macrophages to the same extent as tuftsin, suggest a close relation between the Fc and the tuftsin

Table 2. The effect of tuftsin and IgG-related peptides on ingestion of ^{51}Cr-labelled opsonized sheep red blood cells by mouse peritoneal macrophages. Stimulation of phagocytosis was calculated as: 100x (radioactivity of ingested cells with peptide - radioactivity of ingested cells without peptide)/radioactivity of ingested cells without peptide. Final concentration of tested peptide was 200 nM.

Peptide	Phagocytosis (%)
Tuftsin (H-Thr-Lys-Pro-Arg-OH)	61
H-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH	56
H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH	67
H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH	55
H-Glu-Gln-Gln-Tyr-OH	26
H-His-Asn-Ala-Lys-OH	--

receptor. This assumption is strengthened by the fact that the tetra-C peptide, vastly different in its chemical characteristics from tuftsin, can efficiently inhibit, as tuftsin, [^{3}H-Arg^{4}]tuftsin binding to macrophages. Moreover, the association of the synthetic peptides, except for the tetra-N, with the macrophage cell also has functional implications. Thus, the extended analogs of tuftsin, octa-C, octa-N and the dodecapeptide can augment the cell's capacity to phagocytize opsonized sheep erythrocytes as efficiently as tuftsin. Even more noteworthy is the finding that the tetra-C peptide, which bears no resemblance to tuftsin, substantially stimulates phagocytosis.

To ascertain whether the ability of the synthetic Fc-fragments (octa-C, octa-N and dodecapeptide) to attach to and to activate macrophages is their intrinsic feature, or whether this is due to enzymatic breakdown which eventually yields tuftsin, the peptides were incubated (up to 60 minutes at both 22°C and 37°C) with cells. Careful analysis of the incubation mixtures by HPLC did not reveal the formation of tuftsin, nor the formation of either of its two N- or C-terminus extensions. As shown in Fig. 1, the method can clearly distinguish between the relevant peptide derivatives. After prolonged incubation, however, a very small extent of "non-specific" peptide degradation was noticed. Tuftsin is the final, and as yet the only detected bioactive peptidic product of leukokinin processing (1-3,5). In view of the present results, the existence of some Fc-derived tuftsin containing sequences (pre-tuftsin peptides), which may play a certain physiological role, should not be excluded.

In conclusion, it was demonstrated that the tuftsin receptor on mouse peritoneal macrophages can specifically accommodate peptides which correspond to the tuftsin-containing domain at the Fc-portion of IgG. It is suggested that the tuftsin receptor and the Fc-receptor share some common structural determinants. It is not yet clear whether the former represent a sub-class of the latter receptor. The synthetic, tuftsin-containing, IgG-peptides can augment the capacity of macrophages to phagocytize as efficiently as tuftsin. In view of this activity and peptide's stability toward degradation, it is

possible that tuftsin-like peptides are present in circulation and function as activators of phagocytic cells.

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