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APPROACHES TO SOME BIOCHEMICAL MECHANISMS OF ACTION OF TUFTSIN AND ANALOGUES

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Abstract—Tuftsin, T-K-P-R, is a phagocytosis-stimulating peptide described as a natural immunostimulant. Four analogues of this peptide were synthesized. These compounds were assayed for their ability to compete with [^{3}H]tuftsin for its specific receptor from thioglycollate-elicted mouse peritoneal macrophages. They were also tested for their ability to change level in intracellular cGMP and to stimulate phagocytosis through the nitroblue tetrazolium reduction measurement. Surprisingly, all the analogues were poor competitors of [^{3}H]tuftsin binding but possess potent tuftsin-like activities.

Tuftsin is a humoral basic tetrapeptide, of the sequence T-K-P-R [1], which is an integral part of the C_H2 domain within the Fc fragment of the heavy chain of immunoglobulin G (IgG). Tuftsin possesses a wide range of activities exerted on the phagocytic cells, polymorphonuclear leukocytes, monocytes and macrophages, from human as well as from other mammalian species [2]. This peptide was initially found to enhance the phagocytic response of these cells. The effect of tuftsin is initiated by its binding to distinct and specific plasma membrane receptors. Direct binding studies revealed unique and single population of sites for the peptide on phagocytic cell surface [3, 4]. The multitude and diversity of activities of tuftsin raise, however, the question whether only one receptor is responsible for the transduction of the signals generated by the peptide into potentiation of the corresponding functions. Furthermore, unlike other immunostimulant agents already used in pharmacopea which are high molecular weight compounds unreachable by total chemical synthesis, tuftsin as a small peptide can be varied in its structure by synthesis. Structure–function studies with numerous synthetic analogues of tuftsin have demonstrated that rather strict structural integrity of the peptide should be preserved in order to achieve full biological potency [5].

We have synthetized analogues of tuftsin in order to establish structure–activity relationships and to find compounds more active on the *in vitro* immune responses. Two types of modifications were introduced: (i) to the N terminus by structural modification or replacement of threonyl residue (T), (ii) within chain by substitution of proline (P). In the present study, we examine the action of four tuftsin analogues on macrophage populations induced by stimulation with thioglycollate on the specific binding of [^{3}H]tuftsin. To obtain insight into the mechanism of tuftsin derivative action, we have explored the possibility that these compounds affect intracellular levels of cGMP, potentiate macrophage phagocytosis mediated by the Fc receptor, and several of their enzymatic systems involved in the pentose shunt (as assessed by enhancement of the nitroblue tetrazolium reduction test).

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MATERIALS AND METHODS

Synthesis of tuftsin analogues

Tuftsin was obtained from Senn Chemical Bachem. Its analogues (Table 1) were obtained as acetic acid salt by liquid phase coupling as described in our patent [6], and purified by high performance liquid chromatography. Their structures were ascertained by NMR and FAB-MS studies. Analytical results were within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded with a Bruker AM 400 spectrometer at 400 MHz. The chemical shift values were reported in ppm. Mass spectra analysis were determined on a NERMAG R10-10C. All tests were performed with lyophilized samples of 1 μmole each.

Harvest and culture of macrophages

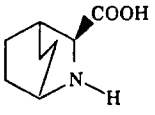
BDF1 (C57Bl/6 × DBA2) male (20–22 g weight, Charles River, France) mice were injected intraperitoneally with 3 mL of thioglycollate broth (Difco). On the fifth day, the mice were killed by cervical dislocation and injected intraperitoneally with 10 mL of RPMI 1640 medium (Gibco). Peritoneal exudate cells (PEC) were aseptically collected. The peritoneum was gently massaged and the peritoneal fluid was aspirated with a 10 mL syringe. After filtration through nylon gauze, the cell suspension was centrifuged at 300 *g* for 5 min. The supernatant was discarded and the cells were resuspended in the same medium, counted in neutral red and adjusted to a final concentration of 10^6 peritoneal cells per mL. The suspensions contained approximately 90% macrophages and 10% lymphoid cells. Peritoneal cells (200 μL) were distributed in each well of a culture microplate (Microtest III,

Table 1. Tuftsin and synthetic analogues

		Structure
Tuftsin	T-K-P-R	Thr-Lys-Pro-Arg
	K-P-R	Lys-Pro-Arg
	T-K-ABO-R	Thr-Lys-ABO[(1)]-Arg
	cyT-K-ABO-R	cyclo Thr[(2)]-Lys-ABO-Arg
	F-K-ABO-R	PheΨ(CH_2-NH)Lys-ABO-Arg
	AHPA-K-ABO-R	AHPA[(3)]-Lys-ABO-Arg

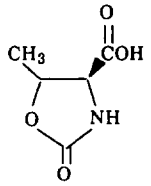
Tuftsin analogs were synthesized as described previously [6, 12].

(1) ABO



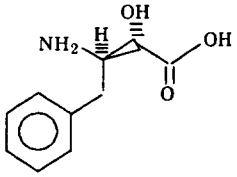
(3*S*)2-Aza bicyclo [2,2,2] octane 3-carboxylic acid.

(2) CycloThr



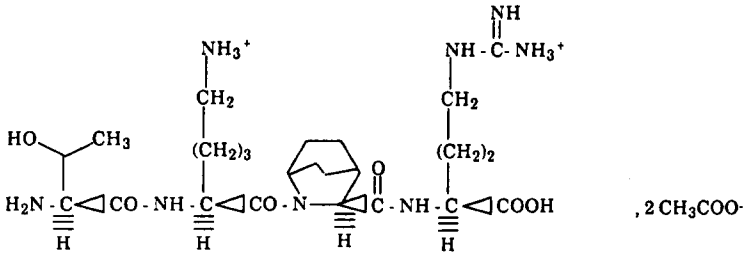
(4*S*,5*R*)5-Methyl-2-oxo-oxazoline-4 carboxylic acid.

(3) AHPA



(2*S*,3*R*)3-Amino-2-hydroxy-4-phenylbutyric acid.

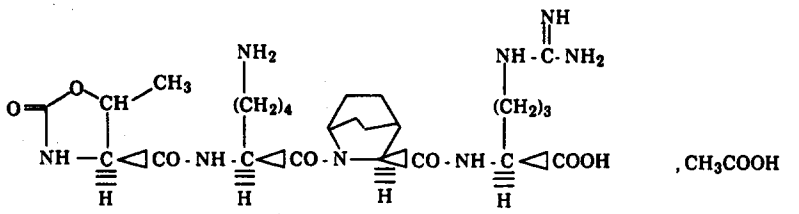
T-K-ABO-R



Anal $C_{28}H_{52}N_8O_{10}$	Calc	C 50,90	H 7,93	N 16,96
	Found	C 50,82	H 8,13	N 17,28
Mass spectrum	FAB+	[M+H]+	m/z : 541	
	FAB-	[M - H]-	m/z : 539	

NMR (DMSO-d6) δ 1,04 (2H,d,CH_3), 2,94 (1H,d,-CH-), 2,74 (2H,m, $-CH_2NH_3+$), 2,18 (1H,m,-CH-), 3,02 (2H, m, CH_2-NH-CNH-NH_3+), 4,05 (1H,m,-CH-), 4,80 (1H,m,-CH-)

cyT-K-ABO-R

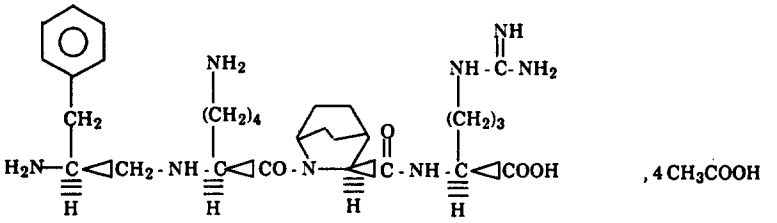


, CH_3COOH

Mass spectrum FAB+ [M+H]+ m/z : 567
FAB- [M - H]- m/z : 565

NMR (DMSO-d6) COSY δ 1,32 (3H,d,-CH_3), 1,4-1,7 (9H,m,-CH_2-), 1,5 (6H,m, -CH_2-), 1,6 (2H,m,-CH_2-), 1,75 (1H,m,-CH_2-), 2,18 (1H,m,-CH-), 2,72 (2H,m,-CH_2-NH_2-), 3,00 (2H,m,-CH_2-NH-), 3,81 (1H,m,-CH-), 3,90 (1H,m,-CH-), 4,05 (1H,m,-CH-), 4,40 (1H,m,-CH-O), 4,65 (1H,m,-CH-).

F-K-ABO-R



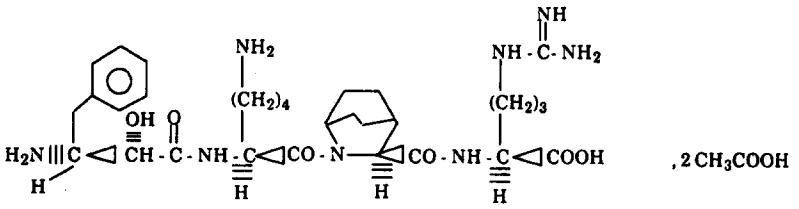
, 4 CH_3COOH

Anal $C_{37}H_{64}N_8O_{12}$ Calc C 54,67 H 7,94 N 13,78
Found C 55,05 H 7,74 N 13,24

Mass spectrum FAB+ [M+H]+ m/z : 573

NMR (DMSO-d6) COSY δ 1,5 -1,8 (18H,m,-CH_2-), 2,3-2,5 (2H,m,-CH_2-Ar-), 2,70 (2H,m,-CH_2-NH_2), 2,75 (2H,m,-CH_2-NH-), 3,00 (2H, m,-CH_2-NH-), 3,1 (1H,m,H_2N-CH), 3,45 (1H,m,-CH-), 3,85 (1H,m,-CH-), 4,05 (1H,m,-CH-), 7,3 (5H,m,Ar).

AHPA-K-ABO-R



, 2 CH_3COOH

Anal $C_{34}H_{56}N_8O_{16}$ Calc C 55,42 H 7,66 N 15,21
Found C 55,73 H 7,44 N 15,41

Mass spectrum FAB+ [M+H]+ m/z : 617
FAB- [M - H]- m/z : 615

NMR (DMSO-d6) COSY δ 1,3-1,8 (18H,m,-CH_2-), 2,55-2,75 (2H,m,-CH_2-Ar-), 2,70 (2H,m,-CH_2-NH_2-), 3,00 (2H,m,-CH_2-NH-), 3,12 (1H, m,-CH-), 3,75 (1H,m,-CH-OH), 3,82 (1H,m,-CH-), 4,75 (1H, m,-CH-), 4,08 (1H,m,-CH-), 7,3 (5H,m,-Ar)

3072, Falcon) and incubated 3 hr at 37° in a humidified atmosphere.

Competitive binding assay

All binding studies were performed in PBS at 0° for 2 hr to avoid down regulation of the occupied tuftsin receptor. Macrophage suspensions were washed three times in PBS buffer and incubated in a final volume of 0.5 mL with 5.3×10^{-8} M tritiated tuftsin ([^{3}H-propyl]tuftsin; sp. act. 1.10–1.45 GBq/mM, CEA, France) and 1.3×10^{-4} M unlabelled tuftsin, to obtain the non-specific binding.

In parallel, the same incubation was realized in the presence of various concentrations of tuftsin analogues. The specific binding was determined by the ability of analogues to compete with [^{3}H]tuftsin for tuftsin receptor sites. Binding was stopped by dilution with a sucrose buffer (0.25 M sucrose in PBS) followed by centrifugation and removal of the supernatant by aspiration. The cell pellets were then dissolved in 0.5 mL sodium dodecyl sulfate (SDS, 0.1%) overnight at 4°. Radioactivity was measured in a liquid scintillation spectrophotometer. Each point of the resulting binding plots derived from triplicate tubes.

cGMP assay

This assay was realized in parallel with the binding assay and the same macrophage suspensions were used. Macrophages (10^7 cells) were resuspended in 0.8 mL acetate buffer and incubated 10 min at 37°. The tested peptides in 0.2 mL medium or medium alone were then added and incubated 25 min at 37°. After the incubation, the samples were centrifuged (5000 *g*, 5 min) and the pellet resuspended in 0.5 mL acetate buffer. The samples were sonicated in ice followed by rapid freezing in liquid nitrogen. After thawing at 45°, the samples were vortexed for 1 min and refrozen. This procedure was repeated three times and the samples were then centrifuged (5000 *g*, 5 min). The cGMP was determined using cGMP dosage radioimmunoassay kits (NEN) following the supplier's instructions. The standard curve determined was linear. Each sample was dosed in triplicate.

Phagocytosis assay

Preparation of erythrocytes target cells. Sheep red blood cells (SRBC, Pasteur Institute) (7.5×10^8 SRBC/mL) were labelled with 0.35 mL of [^{51}Cr]-Na_2CrO_4 (sp. act. 9.25–18.50 GBq/mg, Amersham) during 2 hr at 37°. SRBC were precoated with monoclonal antobody against sheep erythrocyte (IgG_1, MAS014C, Sera-Lab) by incubating the SRBC in a 1/100 dilution of the antiserum during 45 min (SRBC-IgG).

Assay for macrophage phagocytosis. Macrophage monolayers were washed three times in RPMI 1640 medium and were incubated with tuftsin or analogues during 15 min at 37°. Subsequently, ^{51}Cr-labelled erythrocytes coated with IgG_1 (^{51}Cr–SRBC–IgG) were incubated with macrophages at 37° for 1 hr. Phagocytosis of target cells was calculated by the following formula from the radioactivity in detergent lysates of macrophages which had been treated with NH_4Cl to remove extracellular ^{51}Cr–SRBC–IgG.

$$\% \text{ Phagocytosis} = \frac{\text{Macrophage lysate (cpm)}}{\text{Maximum release (cpm)}} \times 100,$$

where the maximum release is the release of radioactivity from ^{51}Cr–SRBC–IgG incubated in the absence of macrophages.

NBT reduction assay

This assay was essentially performed as described by Pick [7]. Macrophage suspensions [2.5×10^5 cells in Hank's medium supplemented with 5% FCS (Gibco, France)] were seeded in a microtest plate (Falcon, Microtest III, 4072). Subsequent to 2 hr of incubation at 37° in a humidifed atmosphere (5% CO_2), non-adherent cells were removed by three washings in the same medium. The tested peptides were added at specified concentrations in a volume of 0.5 mL. Control wells received the same volume of Hank's medium. The NBT solution (2 mg/mL) was added (0.5 mL) in each well and the mixture then incubated for 30 min at 37° in a humidifed atmosphere. The plates were then placed in a reader fitted with a 550 nm filter, and the blanking procedure was performed on the row containing cells incubated with NBT and iodoacetamide in the absence of stimulant. Results were expressed as the difference in the measurement at 550 nm per well (2.5×10^6 cells) per 30 min of reduced NBT between stimulated tubes and blanks.

RESULTS

As shown in Fig. 1, stimulation of peritoneal cells by thioglycollate increases the tuftsin sensitivity of mouse peritoneal cells. The maximum specific binding of tuftsin is reached 5 days after elicitation and is around 190,000 binding sites per cell. The incubation of cells in the presence of tuftsin was performed at 4° to avoid further down regulation of

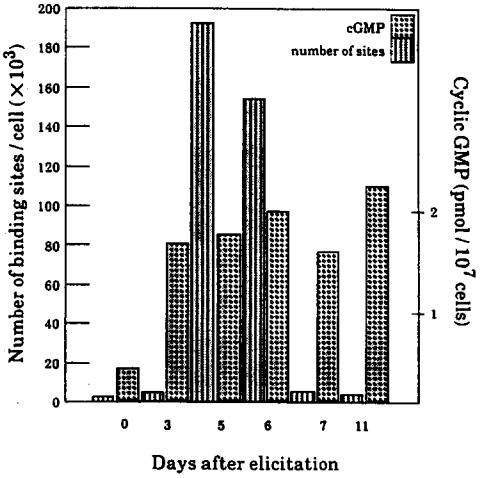


Fig. 1. Number of tuftsin binding sites and intracellular level of cGMP in thioglycollate elicited macrophages in presence of 10^{-6} M of [^{3}H]tuftsin. The amount of cGMP and the number of binding sites per cell were realized in parallel as described in Materials and Methods. Each value is the mean of triplicate determinations.

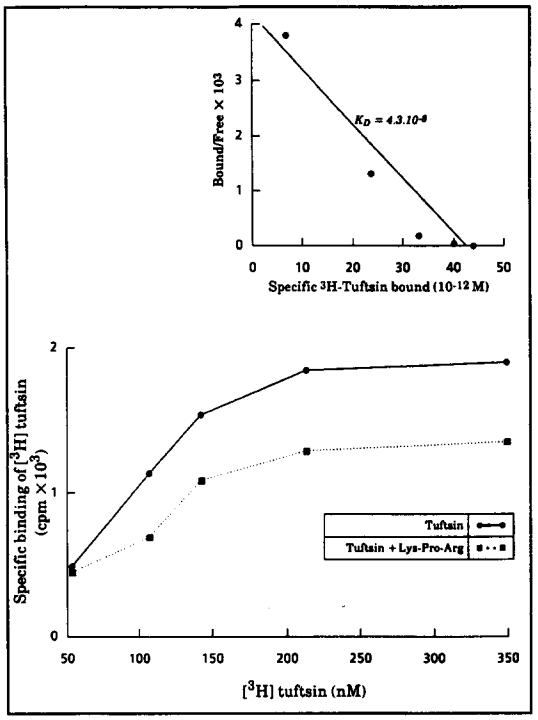


Fig. 2. Specific binding of [^{3}H]tuftsin to thioglycollate-stimulated macrophages and displacement of [^{3}H]tuftsin binding by its tripeptide derivative.

the occupied tuftsin receptor. In parallel, we measured the amount of intracellular cGMP which increases 3 days after elicitation and is maintained up to, at least, the eleventh day.

Therefore, all the experiments were realized 5 days after elicitation of peritoneal macrophages by thioglycollate to have the highest availability of tuftsin receptors. Evaluation of the specific binding of [^{3}H]tuftsin to thioglycollate-elicited macrophages revealed unique receptors for tuftsin. Saturation of binding sites occurred at a peptide concentration of 215 nM (Fig. 2). This binding was specific and saturable. The calculated equilibrium dissociation constant, K_d, is 4.3×10^{-8} M. This value is of the same order as published data [3]. Figure 3 represents a series of inhibition curves for the [^{3}H]tuftsin binding to thioglycollate-stimulated macrophages using various tuftsin (T-K-P-R) analogues. The tripeptide K-P-R which competes efficiently with T-K-P-R (Fig. 2) inhibits 50% of the [^{3}H]tuftsin binding (IC_{50}) at 2×10^{-7} M. The IC_{50} values are 4×10^{-5} M, 10^{-5} M, 2×10^{-4} M and 10^{-4} M for cyT-K-ABO-R, T-K-ABO-R, F-K-ABO-R and AHPA-K-ABO-R, respectively. These results show that all tuftsin analogues are poor competitors of [^{3}H]tuftsin binding.

Incubation of mouse peritoneal macrophages with T-K-P-R results in an increase of 70% in cGMP level ($P \leq 0.001$) (Table 2). Among the analogues, only cyT-K-ABO-R provokes a significant increase in cGMP level (+63%, $P \leq 0.001$).

As shown in Fig. 4, T-K-P-R enhances phagocytosis to SRBC–IgG maximally by 30% at 10^{-8} M ($P \leq 0.025$). AHPA-K-ABO-R stimulates phagocytosis by 47% at 10^{-8} M ($P \leq 0.025$), T-K-P-R and cyT-K-ABO-R stimulate phagocytosis by 47% ($P \leq 0.01$) and 55% ($P \leq 0.025$), respectively, at 10^{-9} M. F-K-ABO-R has no significant effect on phagocytosis.

The biological activity of T-K-P-R and the

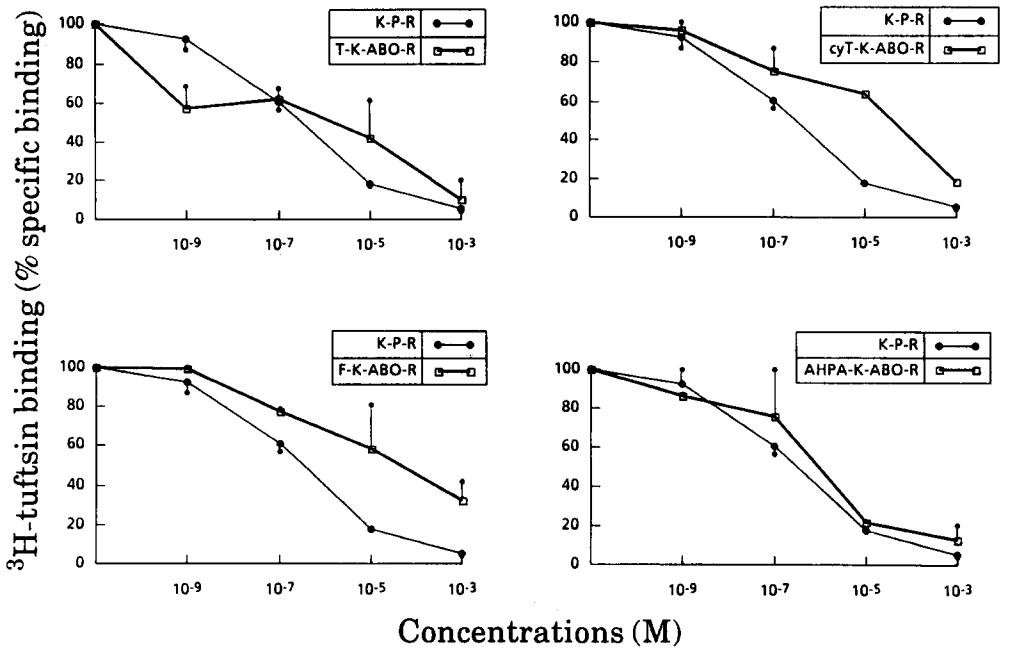


Fig. 3. Displacement of [^{3}H]tuftsin binding to mouse peritoneal cells by unlabelled tuftsin analogues. The tritiated peptide (5.3×10^{-3} M) was incubated with various concentrations of tuftsin analogues for 2 hr at 0° as described in Materials and Methods. Each point is the value (± SE) of triplicate determinations.

Table 2. Effect of tuftsin and analogues on intracellular cGMP levels

	cGMP pmol/10^7 cells			
	Control	Compounds	Δ (%)	Significance Student's *t*-test
T-K-P-R (10^{-6} M)	0.57 ± 0.06	0.89 ± 0.13	+56	$P \leq 0.05$
K-P-R (10^{-6} M)	1.17 ± 0.11	1.05 ± 0.05	−10	NS
T-K-ABO-R (10^{-6} M)	1.59 ± 0.20	1.87 ± 0.26	+18	NS
cyT-K-ABO-R (10^{-6} M)	0.55 ± 0.08	0.85 ± 0.06	+54	$P \leq 0.001$
F-K-ABO-R (10^{-6} M)	1.54 ± 0.26	1.89 ± 0.34	+23	NS
AHPA-K-ABO-R (10^{-6} M)	0.69 ± 0.10	0.92 ± 0.20	+33	NS

Each value ± SE is the mean of 6 to 14 experiments. Experimental data are compared to control by the Student's *t*-test.
NS, non significant.

$$\Delta\,(\%) = \frac{\text{cGMP level in presence of compound}}{\text{cGMP level in control}} \times 100.$$

analogues was evaluated by studying their effect on the reduction of the dye nitroblue tetrazolium (NBT) to corresponding formazan, by thioglycollate-elicited mouse macrophages (Table 3). This is a common assay which evaluates one of the intracellular events, namely the hexose monophosphate shunt activity. T-K-P-R stimulates the NBT reduction significantly up to 10^{-8} M ($P \leq 0.05$), T-K-ABO-R and F-K-ABO-R show the same effect up to 10^{-8} M. AHPA-K-ABO-R stimulates the NBT reduction only at 10^{-6} M ($P \leq 0.01$) and cyT-K-ABO-R has no effect on the reduction of NBT.

DISCUSSION

It has been recognized for a long time that modulation of the immune system, by various agents, might be crucial for the management of infectious and neoplastic diseases. The development of immunopotentiators, agents which enhance the host's own immune system against cancer cells or infectious pathogens, is essential to complement aggressive therapies.

We focused our studies on some derivatives of tuftsin which is a natural low molecular agent capable of potentiating the immunological network [8]. Our primary concern was to give a strong biochemistry background to our pharmacological research in order to understand tuftsin action at the molecular level before going on by tuftsin receptor studies.

Two types of modifications were done on the structure of the tuftsin (T-K-P-R) tetrapeptide. The first modification took place in the amino acid sequence, at the third position by replacement of

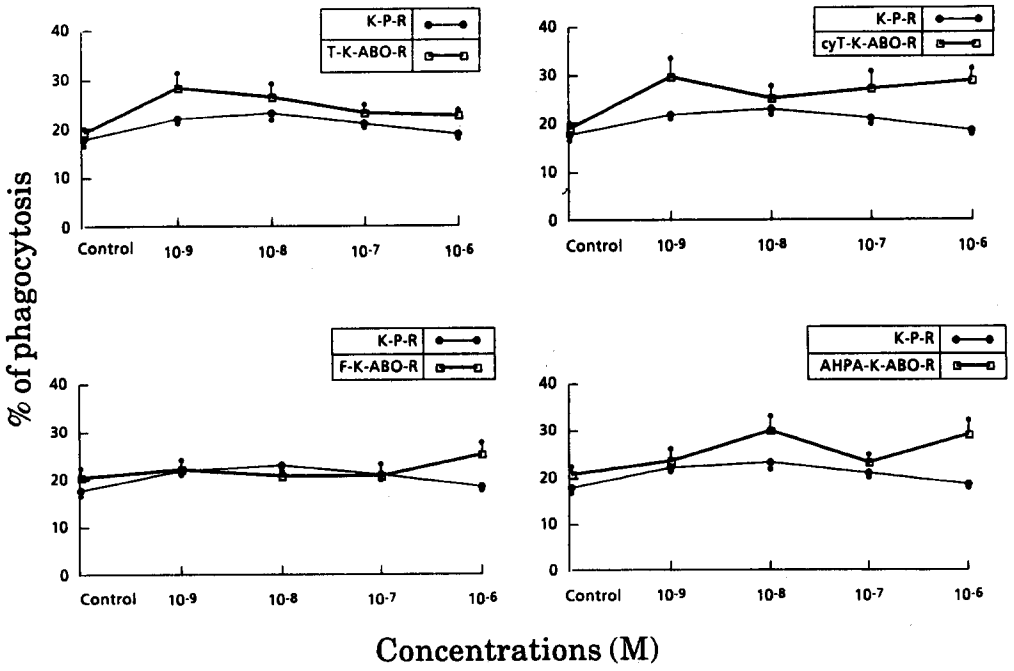


Fig. 4. Phagocytosis-stimulating activity of tuftsin and analogues. The ingestion of IgG–SRBC labelled with ^{51}Cr by macrophages was described in Materials and Methods. Each point is the mean (± SE) of triplicate determinations.

Table 3. Effect of tuftsin and analogues on the hexose monophosphate shunt activity as measured through the NBT reduction test

	$\Delta DO^* \times 10^{-2}$				
		Concentration (M)			
	Control	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Tuftsin	4.6 ± 0.3	4.9 ± 0.3 NS	5.2 ± 0.4 NS	5.6 ± 0.5 $P \leq 0.05$	5.6 ± 0.3 $P \leq 0.05$
T-K-ABO-R	4.2 ± 0.4	4.8 ± 0.3 NS	5.3 ± 0.3 $P \leq 0.01$	5.2 ± 0.4 $P \leq 0.05$	5.2 ± 0.3 $P \leq 0.01$
cyT-K-ABO-R	3.9 ± 0.4	4.4 ± 0.3 NS	4.6 ± 0.4 NS	4.6 ± 0.4 NS	4.6 ± 0.4 NS
F-K-ABO-R	4.4 ± 0.2	4.6 ± 0.3 NS	5.1 ± 0.2 $P \leq 0.01$	4.9 ± 0.3 $P \leq 0.05$	5.0 ± 0.2 $P \leq 0.01$
AHPA-K-ABO-R	4.7 ± 0.3	4.9 ± 0.5 NS	5.3 ± 0.5 NS	5.2 ± 0.5 NS	5.5 ± 0.5 $P \leq 0.05$

* ΔDO, difference in optical density between NBT reduced by resting and stimulated cells.
Each value ± SE is the mean of 5 to 7 individual data. Experimental data are compared to control by the Student's *t*-test.
NS, non signficant.

proline (P) by ABO, a non natural amino acid. The second modification was located at the N terminus by substitution or cyclization of the threonyl residue with the goal (i) to prevent the formation of the corresponding tripeptide which is a potent tuftsin inhibitor [9, 10] and may be generated from tuftsin by enzymatic splitting at the T-K peptide bond [11] and (ii) to obtain more lipophilic compound, therefore potentially gaining in cellular take up. We have conserved the same tetrapeptide structure in the analogues in order to obtain molecules with a similar steric shape.

The action of tuftsin on peritoneal cells elicited by thioglycollate was previously studied by Bar-Shavit *et al.* [3]. The increase of tuftsin receptor number and therefore the increase of tuftsin binding are concomitant to an increase in the intracellular metabolism as assessed by the cGMP concentration measurement. The high levels of cGMP observed in the presence of tuftsin for at least 11 days after administration of thioglycollate suggest that tuftsin binding to cell receptors initiated a cascade of events involving complex cell to cell interactions. The better the phagocytic stimulation, the higher the sensitivity to tuftsin occurs through receptor availability. In these conditions, the analogues were assayed for immunoregulation of phagocyte function.

The four tetrapeptide analogues were poor competitors of [^{3}H]tuftsin binding. The modification of the third amino acid, P, by a non natural amino acid, ABO, which has already been shown to be a suitable bioisostere of proline [12] was sufficient to lead to a compound which is a poor competitor of [^{3}H]tuftsin binding to its membrane receptor. Previous studies have clearly shown that structural alterations of the tuftsin molecule may lead to a marked loss in its ability to bind to phagocytic cells [13]. In particular, P-R determinants are directly associated with the binding and activity of analogues [14]. Nevertheless, T-K-ABO-R, cyT-K-ABO-R and AHPA-K-ABO-R enhanced the phagocytosis of IgG-coated SRBC. F-K-ABO-R, which was without effect on that particular test, stimulated the hexose monophosphate shunt as measured through the nitroblue tetrazolium reduction. *In vitro*, the replacement of the threonyl (T) by a phenyl alanyl (F) residue, leading to a more hydrophobic N terminus, suggests that structure requirement for activity may vary according to the cell function affected. This modification could allow an enhanced bioavailability due mainly to a better cell membrane penetration of this compound. Nevertheless, the T to F substitution was without effect both on specific phagocytosis and on intracellular level of cGMP. Stabinsky *et al.* [15] described that tuftsin might enhance phagocytosis via its effect on intracellular cyclic nucleotide levels. This enhancement of phagocytosis is not bound in our study to the enhancement of cGMP. Threonine in the first position seems not to be essential for the stimulating activity of macrophage phagocytosis [16]. Surprisingly, all the analogues were poor competitors of [^{3}H]tuftsin binding but possess potent tuftsin-like activities. This observation strongly suggests that tuftsin could exert its effects through mechanisms not dependent of its receptor or that the analogues may bind to a cell surface receptor different from the tuftsin receptor itself. Another hypothesis would be that easy penetration of the less polar analogues through the membrane would permit the compounds to reach intracellular targets, as suggested by the work of O'Brian *et al.* [17] on an acylated pentapeptide inhibitor of protein kinase C.

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REFERENCES

1. Najjar VA and Nishioka K, Tuftsin: a natural phagocytosis stimulating peptide. *Nature* **228**: 672–673, 1970.
2. Fridkin M and Najjar VA, Tuftsin: its chemistry, biology and clinical potential. *Crit Rev Biochem Mol Biol* **24**: 1–40, 1989.

3. Bar-Shavit Z, Stabinsky Y, Fridkin M and Goldman R, Tuftsin-macrophage interaction: specific binding and augmentation of phagocytosis. *J Cell Physiol* **100**: 55–62, 1979.
4. Bump NJ, Lee J, Wleklik M, Reichler J and Najjar VA, Isolation and subunit composition of tuftsin receptor. *Proc Natl Acad Sci USA* **83**: 7187–7191, 1986.
5. Najjar VA, Bump NJ, Lee J and Wleklik M, Tuftsin (Thr-Lys-Pro-Arg) a natural drug for immunostimulation: structure and function relationships. In: *New Methods in Drug Research* (Ed. Makriyannis A), Vol. 2, pp. 215–231, 1988.
6. Vincent M, Remond G and Cudennec CA, New peptide tuftsin analogues—useful as immunostimulants. European Patent 190058, 1986. C.A. 107, P7867h, 1987.
7. Pick E, Charon J and Mizel D, A rapid densitometric microassay for Nitroblue Tetrazolium Reduction and application of the microassay to macrophages. *J Reticuloendothel Soc* **30**: 581–593, 1981.
8. Ruszala-Mallon V, Lin YI, Durr FE and Wang BS, Low molecular weight immunopotentiators. *Int J Immunopharmac* **10**: 497–510, 1988.
9. Fridkin M and Gottlieb P, Tuftsin, Thr-Lys-Pro-Arg. Anatomy of an immunologically active peptide. *Mol Cell Biochem* **41**: 73–97, 1981.
10. Florentin I, Bruley-Rosset M, Kiger N, Imbach JL, Winternitz F and Mathe G, *In vivo* immunostimulation by tuftsin. *Cancer Immunol Immunother* **5**: 211–216, 1978.
11. Tzehoval E, Segal S, Stabinsky Y, Fridkin M, Spirer Z and Feldman M, Tuftsin (an Ig-associated tetrapeptide) triggers the immunogenic function of macrophages: implications for activation of programmed cells. *Proc Natl Acad Sci USA* **75**: 3400–3404, 1978.
12. Vincent M, Remond G and Cudennec CA, New tripeptides tuftsin analogues containing poly-cyclic aminoacid group useful as immunostimulant. European Patent 282374, 296059, 1988. C.A. 111, 39867y, 1989.
13. Dagan S, Gottlieb P, Tzehoval E, Feldman M, Fridkin M, Yasumura K, Okamoto K and Yajima H, Tuftsin analogues: synthesis, structure–function relationships and implications for specificity of tuftsin's bioactivity. *J Med Chem* **29**: 1961–1968, 1986.
14. Segal S, The function of tuftsin and similar sequences in other proteins. *Ann NY Acad Sci* **419**: 168–176, 1983.
15. Stabinsky Y, Bar-Shavit Z, Fridkin M and Goldman R, On the mechanism of action of the phagocytosis-stimulating peptide tuftsin. *Mol Cell Biochem* **30**: 71–77, 1980.
16. Florentin I, Chung V, Martinez J, Maral J, Le Garrec Y and Mathe G, *In vivo* immunopharmacological properties of tuftsin (Thr-Lys-Pro-Arg) and some analogues methods. *Find Exp Clin Pharmacol* **8**: 73–80, 1986.
17. O'Brian CA, Ward NE, Liskamp RM, De Bont DB and Van Boom JH, *N*-Myristyl Lys-Arg-Leu-Arg: a novel protein kinase C inhibitor. *Biochem Pharmacol* **39**: 49–57, 1990.