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Synthesis, Conformation, and Immunoreactivity of New Carrier Molecules Based on Repeated Tuftsin-Like Sequence

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Received 30 September 2003; accepted 27 October 2003
Published online 8 March 2004 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20024

Abstract: *Sequential oligopeptides based on a pentapeptide (TKPKG) derived from tuftsin with different lengths were synthesized by stepwise solid phase methodology. These highly soluble oligomers were nontoxic on mouse spleen cells, and other biological data suggested that tuftsin-like properties were also presented. The $(TKPKG)_n$ (n=2,4,6,8) oligopeptides were not immunogenic; however, they increased sheep red blood cells (SRBC) antigen specific antibody response in mice, demonstrating their immunostimulatory effect. Chemotactic activity was also found on J774 monocyte cells, while MRC5*

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Contract grant sponsor: Hungarian National Science Fund
Contract grant number: T032425 and T037719

Biopolymers, Vol. 73, 645–656 (2004)
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fibroblasts were chemotactically nonresponders to the tested forms of tuftsin. These oligomers showed unordered and flexible structure by CD measurements, confirmed by computer modeling studies indicating also a fairly good accessibility of the ϵ-amino group of each lysine residue. Data suggest that these new oligotuftsin derivatives can be considered as promising carriers for synthetic vaccine.
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Keywords: *oligotuftsin; carrier molecule; sequential oligopeptide; immunomodulatory activity; chemotaxis*

INTRODUCTION

Development of synthetic vaccines requires the presence of protective B-cell epitopes combined with appropriate T-cell determinants.[1] In view of the lack of suitable carriers devoid of any side effects, the use of synthetic peptide antigens in vaccines for human has some limitations. Conjugation of synthetic peptide fragments of a protein to carrier can lead to the formation of potent antigens, as well as to generation of site-specific antibodies against the connected sequence in the native protein.[2] However, usual carriers like keyhole limpet hemocyanine (KLH), bovine serum albumin (BSA), tetanus toxoid (TT), and purified protein derivatives (PPD) are no longer applicable in humans because of their epitopic suppression, cross-reactivity, and tolerance toward the epitope.[3–5]

To overcome various drawbacks arising from these protein–conjugates different synthetic carrier molecules were developed and applied in preparation of artificial antigens. Polymeric polypeptides like branched polypeptides based on polylysine backbone[6] as well as polytuftsin[7] proved to be efficient as a part of synthetic antigens that produced high titer of antibodies against attached epitopes without significant antibody response to the carrier moiety.[8–11] Because of the polymeric nature (difficulties in characterization, reproduction) of these conjugates, the application of discrete molecules as carriers has become more preferred in the last decade. Tam et al. have described and used with success conjugates of multiple antigenic peptide (MAP) with a branching lysine core.[12,13] However, in some cases the solubility of MAP conjugates are poor and their purification is difficult. Tsikaris et al. has designed a sequential oligopeptide carrier (SOC) containing the sequence of Ac–(Lys–Aib–Gly)$_n$–OH (n=2–7) with regular secondary structure (3_{10}-helix).[14] In this constructs the antigenic peptides attached to the ϵ-amino group of lysine residues would not interact each other or with the carrier and would thus retain their original active conformation.[14,15]

Polytuftsin built up from repeat unit of the naturally occurring tetrapeptide tuftsin (TKPR).[16] Tuftsin is a fragment (289–292) of the IgG Fc heavy chain that is known to modulate the antigen-presenting capacity of macrophages.[17] Due to its immunomodulatory role in the immune system[18,19] tuftsin-like carriers became attractive choice for preparation of synthetic vaccine. Several studies indicated that conjugates with polytuftsin retain tuftsin-like effects and increase the epitope specific antibody production.[20,21]

These results prompted us to prepare a group of new tuftsin-like biodegradable and immunostimulatory synthetic carriers, which is available for attachment of epitopes in multiple copies resulting in well-characterized conjugates. For these sequential oligopeptides with Thr–Lys–Pro–Lys–Gly (TKPKG) sequence were selected. TKPK, representing the canine tuftsin sequence[22] differs from human tuftsin only in the C-terminal amino acid, where arginine is replaced by lysine having also basic character. This change has no significant effect on biological activities.[23] However, the second lysine residue in the sequence can offer a new functional group for connection of an additional epitope or other biologically active compound. A glycine residue to the C-terminal of TKPK was added to achieve racemization free condensation of fragments to larger oligomers. It was also found that TKPRG, a human tuftsin derived pentapeptide, was more effective in stimulating in antigen processing of macrophages than tuftsin itself.[17]

In the present work we report on the synthesis and conformational properties of tuftsin-like oligomers with different lengths. We also describe our results on the analysis of these compounds in respect to their toxicity, immunomodulatory activity, immunogenicity, and chemotactic activity.

EXPERIMENTAL

Synthesis

Peptide amides with the sequence of H–[Thr–Lys–Pro–Lys–Gly]$_n$–NH_2 (n= 1, 2 [T10], 4 [T20], 6 [T30], 8 [T40]) were prepared manually by stepwise SPPS (solid phase peptide synthesis) MBHA (4-methylbenzhydrylamine) resin

(Nova Biochem, Läufelfingen, Switzerland) with a capacity of 1.1 mmol/g. Boc/Bzl (*tert*-butyloxycarbonyl/benzyl) strategy was applied for the synthesis using the following amino acid derivatives: Boc–Gly–OH, Boc–Pro–OH, Boc–Lys(ClZ)–OH and Boc–Thr(Bzl)–OH (all from Reanal, Budapest, Hungary). The synthetic protocol was as following: (a) resin wash with DCM (dichloromethane) (3×0.5 min); (a) cleavage of the Boc group with 33%TFA(trifluoroacetic acid)/DCM (2+20 min); (c) resin wash with DCM (5×0.5 min); (d) neutralization with 10% DIEA(diisopropylethylamine)/DCM (3×1 min); (e) resin wash with DCM (4×0.5 min); (f) coupling with DCC(dicyclohexylcarbodiimide)/HOBt (1-hydroxybenzotriazole) activated Boc-amino acid derivatives (3 equiv of each to the capacity of the resin) for 60 min; (g) resin wash with DMF (dimethylformamide) (2×0.5 min) then with DCM (3×0.5 min); (h) monitoring by ninhydrine assay[24] or isatin test.[25] Anhydrous hydrogen fluoride (10 mL in the presence of 0.5 g *p*-cresol) was used for the removal of side-chain protecting groups as well as for the cleavage of the peptide from the resin. Tuftsin (TKPR) and its N-terminal shortened inhibitor tripeptide (KPR) were prepared in our laboratory earlier.[26] All chemicals were Fluka products (Buchs, Switzerland), while the solvents were purchased from Reanal (Budapest, Hungary).

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Waters (Nihon Waters Ltd., Tokyo, Japan) HPLC system using a Phenomex Jupiter C_{18} column (250×4.6 mm ID) with 5 μm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B [0.1% TFA in acetonitrile–water (80:20, v/v)] was used at a flow rate of 1 mL/min at ambient temperature. Peaks were detected at λ=214, 254, and 280 nm. The samples were dissolved in eluent A. The crude products were purified on a semipreparative Phenomex Jupiter C_{18} column (250×10 mm ID) with 10 μm silica (300 Å pore size) (Torrance, CA). Flow rate was 4 mL/min. The same eluents with a linear gradient from 0% B to 30% B in 30 min were applied.

Amino Acid Analysis

The amino acid composition of peptides was determined by amino acid analysis using a Beckman Model 6300 analyzer (Fullerton, CA, USA). Prior to analysis samples were hydrolyzed in 6*M* HCl in sealed and evacuated tubes at 110°C for 24 h.

Mass Spectrometry

Positive ion electrospray ionization mass spectrometric (ESI-MS) analyses were performed on a PE API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). Spray voltage was set to 4.8 kV, and 30 V orifice voltage was applied. Samples were dissolved in methanol–water (1:1 v/v) mixture containing 0.1% acetic acid, and 5 μL of sample was injected with a flow rate of 100 μL/min. The instrument was used in Q_1 scan mode in the range of *m/z* 400–1700, with a step size of 0.3 amu and a dwell time of 0.5 ms.

CD Spectroscopy

CD spectra were recorded on a Jobin Yvon Mark VI dichrograph (Longjumeau, France) at room temperature in quartz cells of 0.02 mm path length. The spectra were averages of three scans between 185 and 280 nm. NMR grade trifluoroethanol (TFE) (Aldrich, Budapest Hungary) and double distilled water were used as solvents. The CD measurements were also performed in water solutions of various pH values (2.5–11.5) and of ionic strength (0.02–2.0*M* NaCl). The concentration of the samples was 0.5–1 mg/mL. CD band intensities are expressed in mean residue ellipticity ($[\Theta]_{MR}$ in deg cm^2 $dmol^{-1}$).

Molecular Modeling

The conformational analysis has been carried out using our conformational search algorithm based on high-energy molecular dynamics simulations in vacuum.[27] Simulation time was 0.2 μs at a temperature of 600 K using the Gromacs force field.[28,29] Geometry optimizations were performed on selected structures, taken from the high-energy trajectory, to obtain a representative set of low energy conformers.

In order to characterize conformational behavior in realistic conditions, parallel molecular dynamics simulations were started from the 43 lowest energy conformer of the selection. Each conformer was surrounded with 1893 explicit water molecules, the simulation temperature was 300 K, at duration of 0.34 ns for each structure resulting 14.62 ns of total simulation time.

In Vitro Cytoxicity Assay

The spleens of BDF_1 mice were removed and placed, ice-cold, in RPMI-1640 medium containing 10% fetal calf serum, then homogenized.[30] The washed spleen cells were filtered through sterilized gauze, and the concentration of the cells was adjusted to 1.5×10^6 cells/mL. Tuftsin and oligotuftsin derivatives were dissolved in RPMI-1640 medium. Cells were incubated with varying amounts (1.5, 15, 50 mg/mL) of compounds in rapidly shaking centrifuge tubes for 1 or 4 h at 37°C. Control tubes were incubated with medium only. Viability of pretreated spleen cells was measured by trypan blue exclusion assay.[31] Six parallel measurements were carried out with each compound at each concentration, and at least 200 cells were counted for staining with trypan blue. The percentage viability±*SD* was calculated from the mean of six measurements.

Immunization

Balb/c (H-2[d], Igh-I[a]) and CBA (H-2[k], Igh-I[c]) mice (5 animals/group) were immunized with 200 μg of oligopeptides T20, T30, or T40. One hundred micrograms of oligotuftsin was administered i.v.; and 100 μg was emulsified in complete Freund's adjuvant and was administered by s.c. injections. Three weeks later (day 21) the primed mice were boosted with the same amount (100 μg) of antigen: 50 μg i.v. was applied and 50 μg emulsified in incomplete Freund's adjuvant was administered s.c. Blood samples were taken on day 7 and day 28, after the first and second immunization, respectively.

Quantitation of the Antibody Level

The relative level of antigen specific immunoglobulin M (IgM) and immunoglobulin G (IgG)-type serum antibodies was measured by solid phase indirect immunoassay. Oligopeptides were coated to the solid phase at $c=10$ μg/mL. After washing and blocking with 10% FCS (fetal calf serum) in PBS (phosphate buffered saline), different dilutions of pooled immunosera were added. The bound antibodies were detected by horseradish peroxidase-labeled antimouse specific IgM or IgG antibodies (Southern, UK). As negative control, normal serum samples taken from unimmunized mice (before antigen administration on day 0) were also titrated.

Plaque Forming Cell (PFC) Assay

Sheep red blood cells (SRBC obtained from Philaxia, Hungary) were used as immunogens. Before immunization, the cells were washed three times with physiological saline, counted, and the concentration of the cells was adjusted to 1×10^8 cells/0.2 mL. Ten- to twelve-week-old BDF_1 mice were immunized intraperitoneally (i.p.) with 10^8 erythrocytes. Three mice were used per group. Nonimmunized control mice were injected with 0.2 mL of physiological saline. Treatments with tuftsin and oligotuftsin derivatives were carried out at doses of 0.1–10 mg/kg body weight for dose-response evaluation and injected i.p. with SRBC antigen simultaneously.

For the hemolytic plaque (PFC) assay,[32] the spleens were obtained 4 days after immunization/treatment. The spleens were pooled, homogenized in RPMI-1640 medium containing 10% calf fetal serum. The washed cells were filtered and adjusted to proper concentrations for plating ($4–5\times10^6$/mL). After mixing Noble agar solution with freshly washed SRBC and spleen cells concentrates, the plates were incubated for 60 min at 37°C, and 2.5 mL guinea pig complement diluted with balanced salt solution was added. After 30 min incubation, hemolyzed plaques in the agar were counted in triplicate and analyzed blind.

Chemotaxis Assay

In the study of chemotactic responsiveness of cells, J774 monocyte cells and MRC-5 fibroblasts were tested. J774 cultures were grown in RPMI-1640 medium (Sigma Ltd., St. Louis, MO, USA) containing 10% FCS. MRC-5 cultures were grown in Dulbecco MEM (low glucose) medium (DMEM) (Sigma Ltd., St. Louis, MO, USA) completed with nonessential amino acids (NEA) and 10% FCS. Both types of cultures were kept at 37°C and 5% CO_2, 16 mg/100 mL gentamycine was added to the cultures.

Chemotactic behavior of cells was evaluated by a modified Boyden chamber technique in a Neuro Probe chamber (Neuro Probe, Cabin John, USA).[33] This technique provides the analysis of chemotactic responsiveness of 96 identical samples. The upper chamber of the system was filled with the suspension of cells [density of cells was 10^5/mL (J774) and 5×10^4/mL (MRC5)], lower chamber — wells of a microtitration plate — were filled with the dilutions of the tuftsin derivatives. The following tuftsin derivatives were tested in the chamber: H–TKPR–OH, H–KPR–OH, H–TKPKG–NH_2, Ac–TKPKG–NH_2, and oligomers of TKPKG (T20, T30, T40) at the 10^{-12}–$10^{-6}M$ concentration range. Adhesive, PC filters (5 μm) were used to separate the two chambers. For dilution of the test substances, fresh culture media (RPMI-1640 or DMEM) were used. Cells were incubated in an optimal environment (37°C and 5% CO_2), for 3 hr for J774 and for 4 h for MRC5 cells. After incubation, the plates were centrifuged at 2000 rpm for 5 min, then 150 mL supernatant was removed.

The number of cells was determined by the 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay.[34] MTT was obtained from Sigma (St. Louis, MO, USA). This assay is based on the measurements of mitochondrial succinyl dehydrogenase activity. Samples were evaluated with Multiscan ELISA reader (Labsystems), at $\lambda=$ 540 and 620 nm.

RESULTS AND DISCUSSION

Polytuftsin derivatives used as carriers so far were based on human tuftsin sequence (Thr–Lys–Pro–Arg) and produced by polymerization. These polymers were built up using the active esters of tetrapeptide with Arg (Thr–Lys–Pro–Arg) or Pro (Arg–Thr–Lys–Pro) at the C-terminal.[7,11] The latter one was prepared to prevent racemization during the polymerization. However, no data were published on the enantiomer composition of the products. But in both cases polymers with a range of different size were produced. Also, the methods (e.g., application of glutaraldehyde) did not result in well-defined conjugates. In this study our goal was to prepare oligotuftsin derivatives with well-characterized structures, and to compare the biological activities of oligomers with different length. For building up the oligomers the Thr–Lys–Pro–Lys–Gly as pentapeptide unit was chosen. The sequence of Thr–Lys–Pro–Lys, a human tuftsin analogue was isolated from the serum of dogs.[22] The

corresponding peptide was found to be similar in immunoreactivity to that of its human counterpart.[23] Based on this observation and considering the presence of two lysine residues in the sequence, with selective side-chain protection, it is suitable for incorporation even two different chemical entities. In such a way, epitopes/drug and target molecules can be separately attached to carriers through different type of chemical bonds. To built up a long sequence by fragment condensation without racemization, the C-terminal of Thr–Lys–Pro–Lys was elongated by a glycine.

Synthesis of Oligotuftsin Derivatives

All peptide-amides were synthesized with stepwise solid phase methodology using MBHA resin. Standard Boc/Bzl strategy was followed in the synthesis of the pentapeptide (TKPKG) and its dimer, 4-mer, 6-mer, and 8-mer versions. Even in case of the 8-mer peptide containing 40 amino acid residues peptide the synthesis was simple without any problem and mostly single coupling was enough for incorporation of amino acid derivatives. After HF cleavage, the crude products showed a single main peak corresponding to the requested peptide and resulted in easy purification. The yield of the purified peptides was 60–80%. The characterization of tuftsin and oligotuftsin derivatives by mass spectrometry and amino acid analysis confirmed the expected composition (Table I).

Solution Conformation

CD spectra of oligomers were measured in buffer at different pH levels (pH 2, 7, or 11) and in the presence or absence of NaCl (0.02, 0.2, and 2*M*) as well as in TFE and in a TFE–water 1:1 (v/v) mixture. In all cases the CD spectra indicated the presence of a random coil conformation. The change of pH or ionic strength of the solution has no influence on the spectrum (Figure 1). Also, the length of oligomer chains did not have a significant effect. Some ordered conformers were detected in TFE; however, still characteristics of the random structures were predominant (Figure 2).

To learn more about the accessibility of the ϵ-amino group of lysine residues, we have performed a molecular modeling study using the Gromacs 3.0 package as described in the experimental section. Details will be published somewhere else. As shown in Figure 3, the calculation confirmed a flexible structure for T20, and also demonstrated the fairly good accessibility of the ϵ-amino group of each lysine residue.

Table I Characterization of Tuftsin and Oligotuftsin Derivatives

Sequence	Code	Yield (%)	Relative Molecular Mass		Amino Acid Analysis[b] (found/calculated)				HPLC[c]
			Calc.	Found[a]	Thr	Gly	Pro	Lys	R_t (min)
H–TKPKG–NH_2	TKPKG	80	528.6	529.5 (+1)	0.95 (1)	1.03 (1)	1.00 (1)	2.02 (2)	19.5
H–$(TKPKG)_2$–NH_2	T10	75	1040.2	1041.0 (+1), 521.1 (+2)	1.94 (2)	2.06 (2)	1.96 (2)	4.05 (4)	20.0
H–$(TKPKG)_4$–NH_2	T20	71	2062.3	1032.2 (+2), 688.1 (+3), 516.1 (+4)	3.89 (4)	4.11 (4)	3.90 (4)	8.09 (8)	20.9
H–$(TKPKG)_6$–NH_2	T30	64	3084.9	1029.8 (+3), 771.9 (+4), 617.6 (+5), 514.9 (+6)	5.81 (6)	6.17 (6)	5.88 (6)	12.15 (12)	21.8
H–$(TKPKG)_8$–NH_2	T40	60	4107.5	1028.0 (+4), 822.3 (+5), 685.2 (+6), 587.4 (+7)	7.75 (8)	8.22 (8)	7.82 (8)	16.20 (16)	22.5

[a] Electrospray–mass spectroscopy, $[M+nH]^{n+}$ because of number of lysine residues rather the multiple charged peaks were detected.
[b] Samples were hydrolyzed in 6*M* HCl at 110°C for 24 h.
[c] Column: Phenomenex Jupiter C_{18} (250×4.6 mm, 5 μ, 300 Å); eluent A: 0.1% TFA/H_2O, B: 0.1% TFA/acetonitrile–H_2O (80:20, v/v); gradient: 0 min 0% B; 5 min 0% B; 50 min 90% B; flow rate: 1 mL/min.

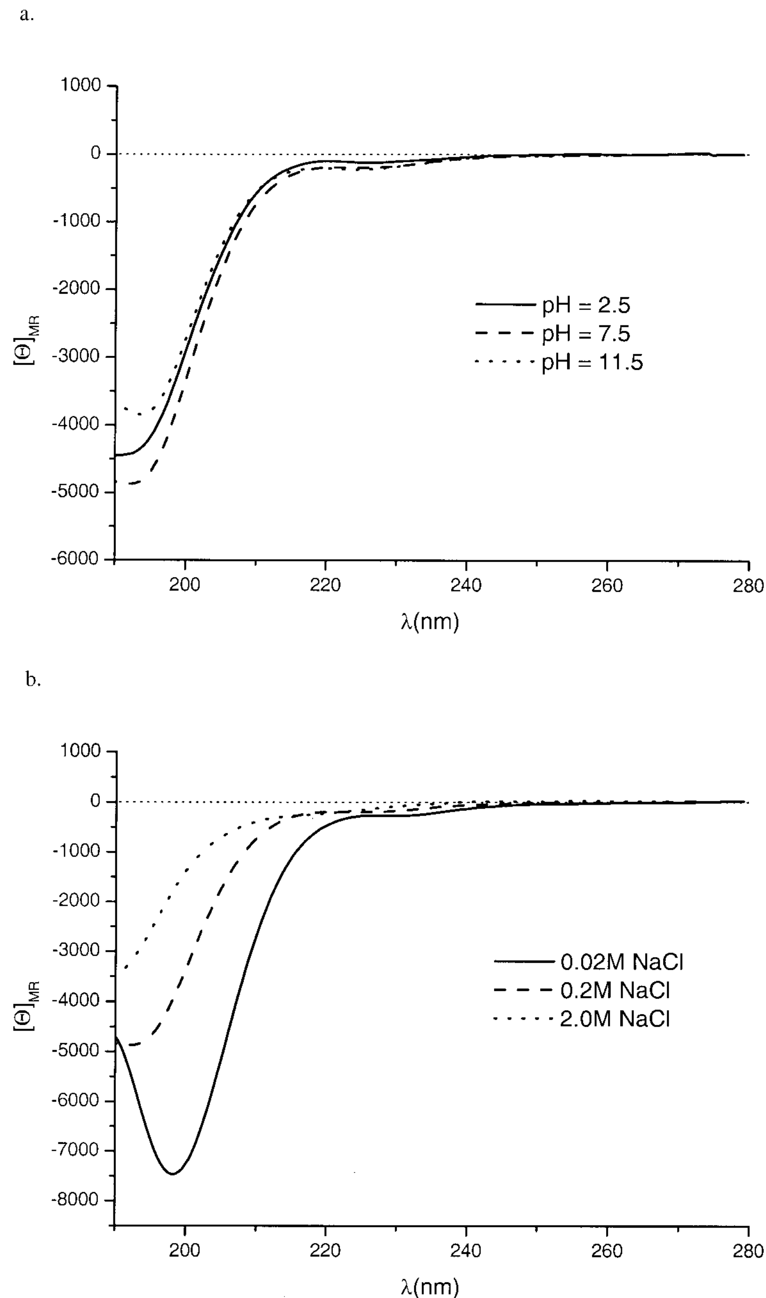

FIGURE 1 CD spectra of oligotuftsin derivative T30 at different pH values in 0.2*M* NaCl concentration (a) and at different NaCl concentrations at pH 7.5 (b).

In vitro Toxicity

The effect of tuftsin and oligotuftsin derivatives on the viability of isolated spleen cells was studied at various concentration (1.5, 15, and 50 μg/mL). Spleen cells are considered to be a highly sensitive indicator of in vitro cytotoxicity. Data are summarized in Table II, and no toxic effects of oligotuftsin derivatives was observed even at the highest concentration (50 μg/mL) after incubation for 4 h.

Antibody Response Against Oligotuftsin Derivatives

Balb/c and CBA mice were immunized by oligotuftsin derivatives T20, T30, and T40. Antigen specific IgM and IgG levels were measured after the first and second immunization. Two independent sets of experiments were performed with 5 animals/group. According to the results, no significant antibody responses were observed neither in Balb/c nor CBA

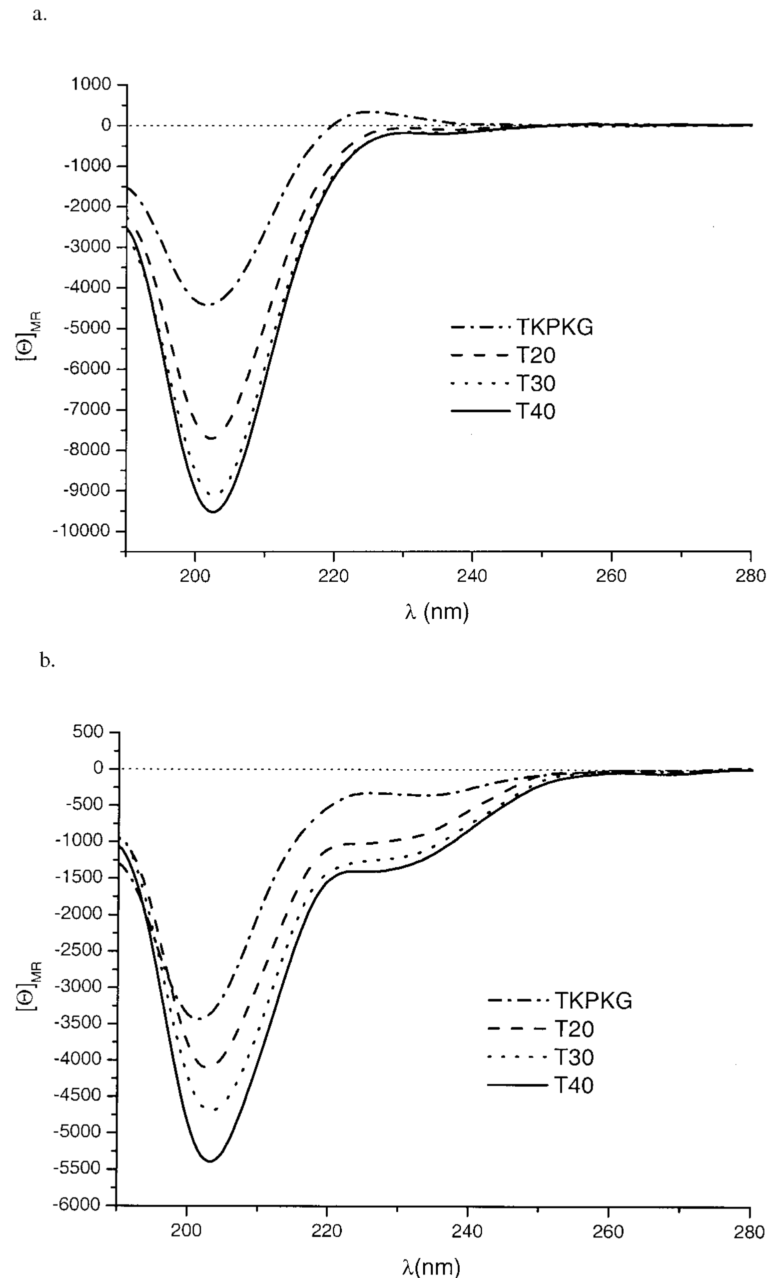

FIGURE 2 CD spectra of TKPKG and oligotuftsin derivatives T20, T30, and T40 in water (a) and in TFE (b).

strains (Table III). Even the highest IgG concentration detected in one of the experiments with T40 is considered negligible.

Immunomodulatory Effect

The immunomodulatory effect of tuftsin variants and oligotuftsin derivatives on the humoral immune response to SRBC antigens in mice were studied and evaluated by the PFC assay.

Figure 4 shows the dose dependence of the immunomodulatory effect of tuftsin analogues and oligotuftsin derivatives in a dose interval of 0.1–10 mg/kg body weight. Oligotuftsines T20 and T30 produced dose-dependent stimulatory effect on the SRBC-specific antibody response. Significant immunostimulatory effect was observed at 1 and 10 mg/kg, whereas the low dose treatment (0.1 mg/kg body weight) resulted in moderate changes. T40 increased the number of PFC/10^6 spleen cells at the dose of 10 mg/kg body

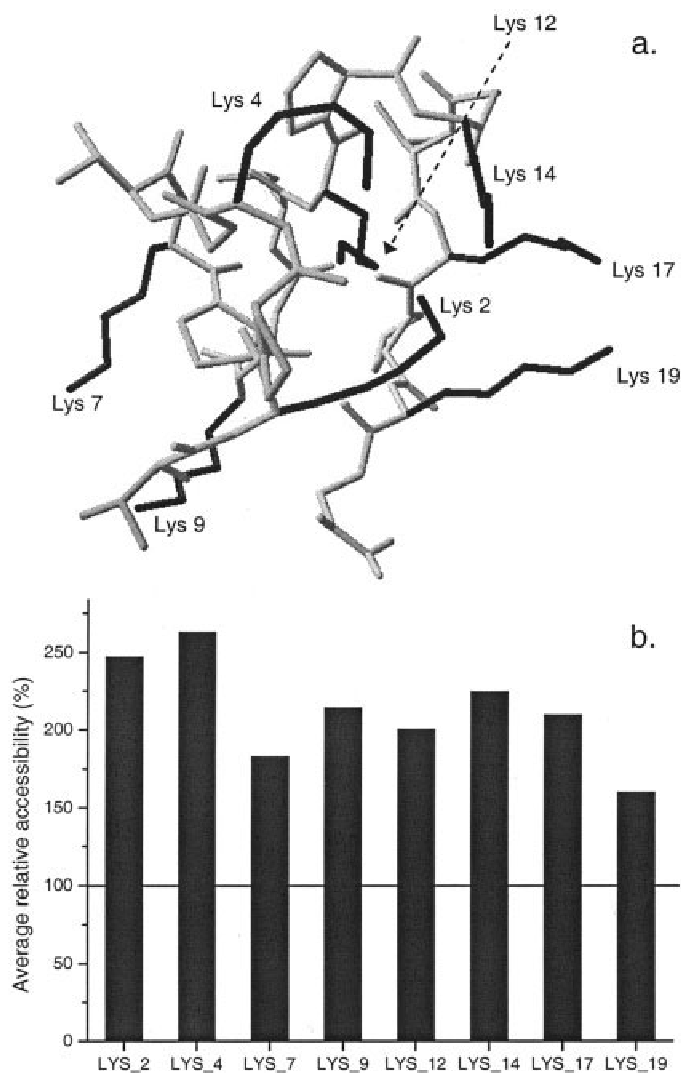

FIGURE 3 Structure of the "realistic" simulation from the lowest energy structure. Black color shows the Lys side chains (a). The calculated average relative solvent accessibility for the eight Lys ϵ-amino groups in T20 (b). The average accessibility of the whole molecule is taken as 100%.[37]

weight, and marked reduction was detected after treatment with 1.0 and 0.1 mg/kg body weight. In contrast to the T20, T30, and T40 oligotuftsins, the number of PFC/10^6 spleen cells was significantly increased after a low dose treatment with TKPR (human tuftsin) peptide (0.1 mg/kg). The canine tuftsin variant (TKPKG) produced a moderate, but concentration independent immunostimulating activity in studied range.

The data suggest that the immunomodulatory capacity of oligotuftsin peptides depends on the number of repeat unit present in the sequence. Compounds with 4 or 6 TKPKG unit exhibit limited activity at low, but pronounced immunomodulation at high (1.0 mg/kg or 10 mg/kg) concentrations. However, oligotuftsin with 8 monomer unit has stimulatory effect only at the highest dose studied (10 mg/kg). The concentration independent stimulation effect of the monomer peptide (TKPKG) is very interesting and provides a platform for a hypothesis. The degradation of oligotuftsin analogues may result in the active monomer and potentiate the immune response for example against an oligotuftsin carrier attached epitope.

Chemotaxis

Positive effect of tuftsin on chemotactic behavior of human monocytes and granulocytes was described in the literature previously. These data showed that tuft-

Table II Effect of Tuftsin Analogues on the Viability of Mouse Spleen Cells[a]

		Viability			
			Peptide concentration (mg/mL)		
Peptide	Treatment time (h)	Control	0.0015	0.015	0.05
TKPR	1	98.7 ± 0.58	98.5 ± 0.91	97.2 ± 1.72	98.9 ± 1.27
	4	96.5 ± 2.18	95.6 ± 0.99	94.3 ± 1.79	95.2 ± 2.82
T20	1	98.7 ± 0.58	98.1 ± 1.18	98.5 ± 1.05	98.3 ± 2.93
	4	96.5 ± 2.18	91.7 ± 3.56	93.7 ± 2.04	92.2 ± 1.83
T30	1	98.7 ± 0.58	98.5 ± 0.95	98.5 ± 1.55	96.3 ± 2.32
	4	89.8 ± 2.44	91.3 ± 2.59	91.2 ± 5.02	88.3 ± 4.12
T40	1	98.7 ± 0.58	96.5 ± 1.36	95.6 ± 1.14	97.6 ± 2.84
	4	89.8 ± 2.44	90.7 ± 1.64	87.5 ± 4.03	90.6 ± 2.19

[a]Six parallel measurements were carried out with each compound at each concentration. Data are given as mean percentage (±*SD*) of viable spleen cells.

sin and some of its analogues possess chemoatractant properties and stimulate the chemotaxis of defective monocytes derived from SLE[34] and Hodgkin patients.[35] Preincubation of monocytes with these peptides also enhance their random migration.[36]

Based on these observations we have studied the chemotactic responsiveness of two nondefective cell lines: in J774 monocytes and MRC5 fibroblasts. For these studies two groups of compounds were used: tuftsin and its derivatives (TKPR, KPR, TKPKG-amide, and its acetylated variant) and oligotuftsin derivatives (T20, T30, and T40). The dynamics of the chemotaxis of the two cell types differs essentially: J774 monocytes are considered as "professional" migratory cells, while fibroblasts express more reduced chemotactic abilities.

Comparative analysis of our results gained in the two model cells shows that tuftsin (H–TKPR–OH) had a weak chemoattractant effect on monocytes in concentrations tested, while fibroblasts were nonresponders to this compound (Figure 5). In the case of the N-terminally truncated tripeptide derivative (H–KPR–OH), we could detect an increased chemotactic ability as compared to the TKPR (Figure 5). This character showed a concentration dependence on monocytes (effective range 10^{-12}–$10^{-8}M$) with the maximal effect at $10^{-12}M$, and the ligand was chemoattractant also on fibroblasts at $10^{-12}M$. Increased chemotactic ability of KPR vs TKPR points to the presumable neutralizing potency of the N-terminal Thr in this molecule. In the case of canine tuftsin (H–TKPKG–NH_2) and its acetylated derivative (Ac–TKPKG–NH_2), we could detect some variance, too (Figure 5c,d). Monocytes expressed a significant positive chemotactic behavior to the acetylated form in the 10^{-12}–$10^{-8}M$ range, while the nonacetylated derivative was neutral in concentrations tested. In fibroblasts we could also detect the effect of N-acetylation; however, Ac–TKPKG–NH_2 was neutral and the non–acetylated H–TKPKG–NH_2 had a wide range (10^{-12}–

Table III Antigen-Specific Titer of Oligotuftsin Derivatives

	Balb/c				CBA			
	IgM		IgG		IgM		IgG	
Compounds	Primer	Secunder	Primer	Secunder	Primer	Secunder	Primer	Secunder
T20 exp. 1	21 ± 4	25 ± 7	<10	<10	17 ± 3	16 ± 4	<10	<10
T20 exp. 2	29 ± 3	<10	<10	<10	41 ± 7	854 ± 27	<10	<10
T30 exp. 1	19 ± 3	12 ± 4	<10	60 ± 2	<10	<10	<10	<10
T30 exp. 2	<10	72 ± 15	<10	76 ± 14	<10	42 ± 4	<10	<10
T40 exp. 1	39 ± 5	67 ± 4	<10	82 ± 6	28 ± 2	<10	<10	<10
T40 exp. 2.	22±	<10	<10	479 ± 55	<10	<10	<10	80 ± 5

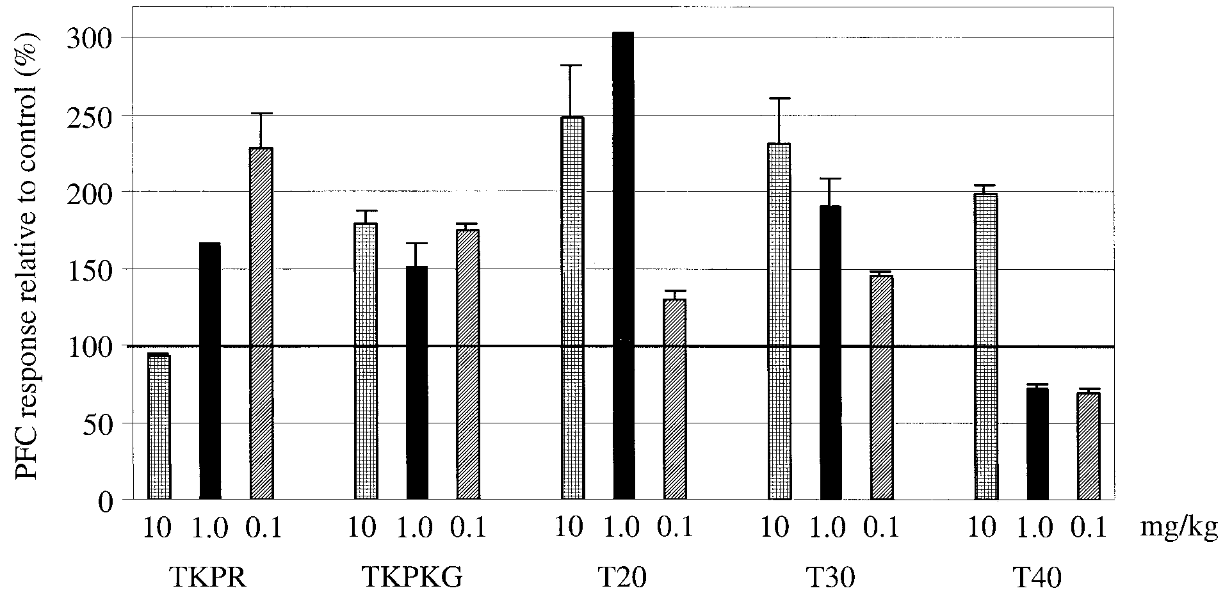

FIGURE 4 Dose-dependent effect of tuftsin and tuftsin analogues on the humoral immune response to sheep red blood cell (SRBC) in normal BDF_1 mice. Peptides and SRBC antigen were applied simultaneously i.p. in saline.

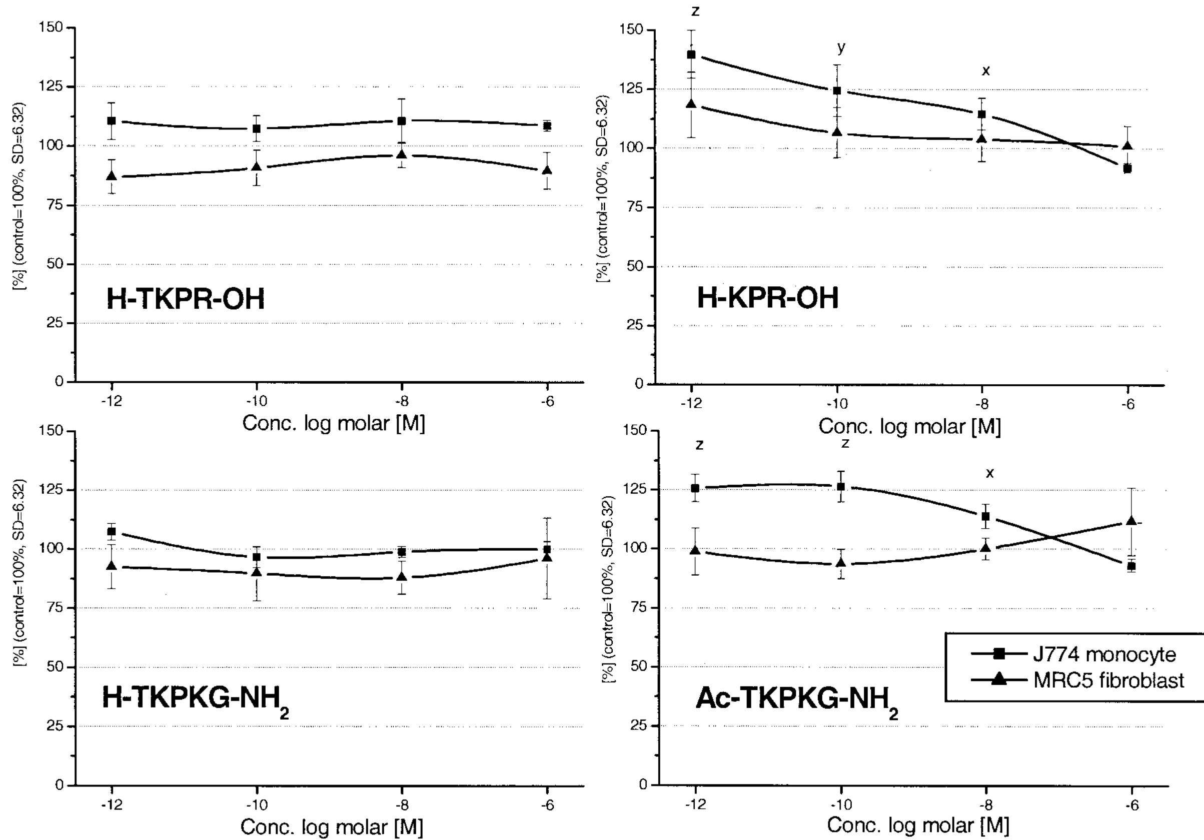

FIGURE 5 Chemotactic effect of tuftsin H–TKPR–OH (a), and its derivatives (b) H–KPR–OH, (c) H–TKPKG–NH_2 and (d) Ac–TKPKG–NH_2 on J774 monocytes and on MRC5 fibroblasts. (x − $p<0.05$; y − $p<0.01$; z − $p<0.001$).

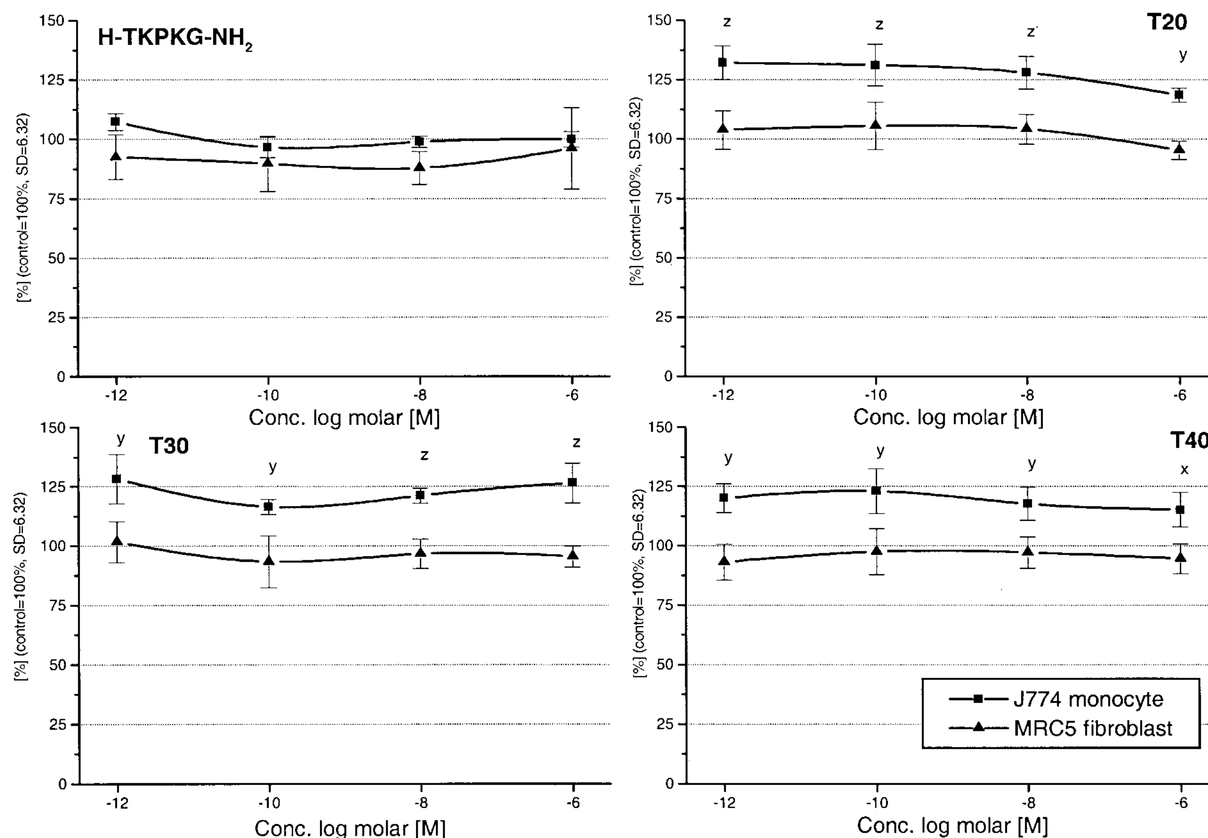

FIGURE 6 Chemotactic effect of H–TKPKG–NH_2 (a), and its oligomers (b) T20, (c) T30, (d) T40, on J774 monocytes and on MRC5 fibroblasts. (x − p<0.05; y − $p\leq0.01$; z − p<0.001).

$10^{-8}M$) weak chemorepellent effect on the fibroblasts.

Oligomers of the TKPKG peptide (T20, T30, T40) with repetitive clusters of the pentapeptide sequence also had characteristic and disparate effects in the two types of cells (Figure 6a–d). In contrast, the neutral TKPKG monomers, all three oligotuftsin derivatives, elicited characteristic, significant, and concentration-independent chemoattractant effect on monocytes. In fibroblasts, contrary to the positive chemotactic responsiveness of monocytes, the elongation of the monomer unit resulted in only a mild elevation of the profile from chemorepellent level to the neutral one. However, we observed no correlation between the size of the oligotuftsin samples and the elicited chemotactic response.

Data summarized above show that human and canine tuftsin and their close derivatives have characteristic effects on the chemotactic behavior of cells. Mild alterations of the structure, i.e., N-terminal truncation (H–KPR–OH) or acetylation (Ac–TKPKG–NH_2), can result in chemoattractant ligands. This observation might suggest that physicochemical conditions of the N-terminus of the molecule are decisive in interaction with the chemotaxis receptors of target cells. Our present results demonstrate that alteration of N- or C-termini of relatively short peptide ligands could result in characteristic changes in biological activity.

The detected differences in activation of various cell types could be also significant in application of tuftsin and oligotuftsines as parts of synthetic vaccines.

CONCLUSION

The new sequential oligotuftsin derivatives prepared with discrete molecular mass show tuftsin-like effects in biological assays used in these experiments. The immunostimulatory activity of the smaller oligomers and monomer unit as well as the length independent chemotactic effect on monocytes together with their nontoxic and nonimmunogenic behavior can be considered as crucial requirements of carrier molecules. In addition to these beneficial biological characteristics, the multivalent nature of oligotuftsin derivatives

due to the presence of ε-amino group of lysine residues and of the amino terminus can offer a great number of variations in synthesis of epitope/drug conjugates. The composition of conjugates with multiple copies of the attached entities from these well-characterized carriers can also be chemically controlled easily by mass spectrometry or/and amino acid analysis.

We thank Zsolt Skribanek (Central Institute of Chemistry, Hungarian Academy of Sciences) for mass analyses and Dr. H. Medzihradszky-Schweiger for amino acid analyses. These studies were supported by grants from the Hungarian National Science Fund (OTKA nos. T032425 and T037719).

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Reviewing Editor: Dr. Murray Goodman