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Peptides 25 (2004) 207–215
PEPTIDES
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Dimerization of the immunosuppressive peptide fragment of HLA-DR molecule enhances its potency

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Received 22 August 2003; received in revised form 18 December 2003; accepted 18 December 2003

Abstract

Our previous studies revealed that the nonapeptide fragment of HLA-DR molecule, located in the β chain 164–172 with the VPRSGEVYT sequence, suppresses the immune responses. The sequence is located on the exposed molecule loop, therefore it may be involved in the interactions with other proteins. We suggested that the loop may serve as a functional epitope on the HLA class II surface for intermolecular binding, and that possible mechanism of biological action of the synthesized peptides is associated with interfering of adhesion of HLA class II molecules to their coreceptors. It has been postulated that oligomerization of the coreceptors is required for stable binding to class II HLA. Based on the crystal dimeric structure of HLA-DR molecules, we designed, and synthesized molecules able to induce the putative coreceptors dimerization. The synthesized series of compounds consisted of two VPRSGEVYT sequences linked through their C-termini by spacers of different length: $(VPRSGEVYTG_n)_2K\text{-}NH_2$ ($n = 4$–6). The results demonstrate that the dimerization of the nonapeptide fragment of HLA-DR results in enhanced immunosuppressory properties.

Keywords: Superdimer of MHC class II; MHC fragments; Dimeric analogs; Immunosuppressors; Linker length

1. Introduction

MHC class II molecules present antigenic peptides to T-cells and are thus essential for the initiation of cellular and humoral immune responses (reviewed by Pieters [27], Hiltbold and Roche [14]). The cellular expression of MHC class II molecules is restricted to antigen-presenting cells such as B cells, macrophages, and dendritic cells. MHC class II molecules are heterodimers with a molecular mass of 60 kDa, consisting of noncovalently associated polymorphic α and β subunits. The α and β chains are structurally homologous to each other. Each chain is composed of two extracellular domains (designated as α1 and α2 in the α-chain and β1 and β2 in the β-chain), a transmembrane portion and a cytoplasmic tail.

The class II molecules are highly polymorphic proteins. The presence of several class II isotypes (DP, DQ and DR) further increases their diversity. At least two alleles of each of the three class II subsets are expressed in most humans. Extreme polymorphism among class II alleles leads to differential peptide binding, with disparate peptides preferentially bound by separate alleles.

Our previous studies showed that fragments located in the β164–172 loop of HLA-DQ suppress the humoral and cellular immune responses, [34,36] and inhibit some integrins [35]. The fragments contain the RGD sequence, known to be important in several proteins in mediating cell adhesion interactions. The sequence is located in a loop of the HLA-DQ molecule exposed toward the solvent and, therefore, it may be involved in the interactions with other proteins. The immunosuppressory region is situated on the opposite side of the molecule than the antigenic peptide-binding site and in a close spatial proximity to the site identified as interacting with the T-cell coreceptor CD4 [1]. The corresponding fragments of HLA-DP and HLA-DR show immunological properties similar to the HLA-DQ fragment [39]. Particularly, the nonapeptide fragment of HLA-DR (Val-Pro-Arg-Ser-Gly-Glu-Val-Tyr-Thr) is a strong suppressor of the immune response, although the fragment does not contain the RGD sequence. We also designed

Abbreviations: CSA, cyclosporine; DTH, delayed type hypersensitivity; HLA, human leukocyte antigen; MHC, major histocompatibility complex; PFC, plaque-forming cells; SRBC, sheep red blood cells

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doi:10.1016/j.peptides.2003.12.011

and synthesized constrained (by cyclization) analogs of the fragments to mimic the immunosuppressory loop of HLA class II molecules and found that the investigated peptides suppress more strongly immune responses than their linear counterparts [32,37]. We suggested that the loop may serve as a functional epitope on the HLA class II surface for intermolecular binding, and that a possible mechanism of biological action of the synthesized peptides is connected with specific interference with the adhesion of HLA class II molecules to their coreceptors, e.g., some specific integrins and/or the T-cell coreceptor CD4 [38].

The three-dimensional structure of the human MHC (HLA) class II molecule has been determined by X-ray crystallography [2]. The crystallographic structure showed that the αβ heterodimer can itself dimerize to form a four chain $(\alpha\beta)_2$ superdimer of 120 kDa. There is also evidence for the existence of the 120 kDa $(\alpha\beta)_2$ complex of the class II molecules in animal B cells [30]. It has been suggested that MHC class II molecules may interact with the T-cell receptors (TCR) and $CD4^+$ as an $(\alpha\beta)_2$ superdimer, potentially ensuring more stable and stimulatory interactions than can be provided by the simple αβ heterodimer alone [29]. It has been also evidenced, that formation or rearrangement of a T-cell receptor dimer is necessary and sufficient for initiation of T-cell signaling [6]. Self-association of CD4 coreceptor may also affect T-cell activation [26]. Therefore, substances that are able to modulate the receptors dimerization may control such a process and are potential immunomodulators. Dimeric ligands are known candidates for mediating dimerization of these types of receptors [4,5]. It has been previously found [12] that peptides that consist of two tandemly repeated epitopes joined by a flexible linker have an increased affinity for class II molecules and are more potent at inducing proliferation of T-cell clones than monomeric epitopes. These results support a role for the MHC class II dimer of heterodimers in amplifying the proliferative response of T-cells to antigen by dint of the superdimers having a higher affinity for CD4 than the nominal class II alpha beta heterodimers.

Based on three-dimensional structure of HLA-DR (Fig. 1) we attempted to design and synthesize a series of dimeric analogs able to mimic the dimeric nature of the immunosuppressory fragments HLA class II molecules. The dimeric analogs consist of two VPRSGEVYT sequences joined through their C-termini by flexible spacers of different length: $(VPRSGEVYTG_n)_2K\text{-}NH_2$ ($n = 4$–6) (Fig. 2). Three dimeric analogs were assayed in this study to examine whether their activities changed according to the length of the linker. Their immunosuppressory potencies were compared with their monomeric counterparts $VPRSGEVYTG_6\text{-}NH_2$ and VPRSGEVYT, respectively. We also tested the effect of dimerization on nonapeptide sequence chosen in regions contiguous to the HLA-DR 164–172 loop.

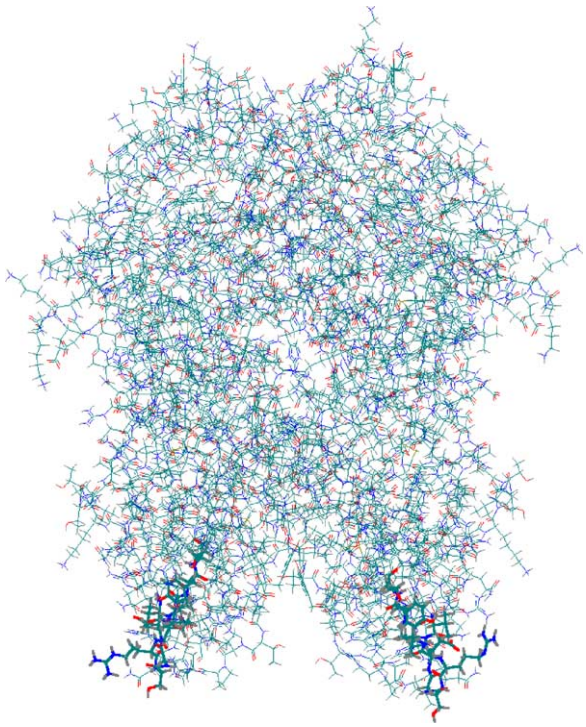

Fig. 1. View of the superdimer of human class II histocompatibility antigen HLA-DR1 [2]. The β164–172 loops with the immunosuppressive sequences (VPRSGEVYT) are in bold.

2. Materials and methods

2.1. Peptide synthesis

The derivatives of amino acids for peptide synthesis and the coupling reagent (PyBOP) were purchased from NovaBiochem. The side chain protecting groups for Fmoc-amino acids were *t*-butyl for Thr, Tyr, Glu, Ser and Pmc for Arg. MBHA-Rink Amide resin (0.55 mmol/g) was purchased from NovaBiochem. The solvents for peptide synthesis were obtained from Riedel de Haën (DMF) and J.T. Baker (methanol).

Peptides were prepared by manual solid-phase techniques. The peptide was assembled on the solid support, using standard Fmoc synthetic procedure. The first amino acid attached to the MBHA-Rink Amide resin was di-Fmoc-Lys in case of dimeric compounds **2**, **3**, **4**, and **7**. Successive amino acids were coupled simultaneously to the α- and ϵ-amino groups of the lysine residue. Single coupling by PyBOP was performed in a mixture of DCM/ DMF/*N*-methyl-2-pyrrolidone (1:1:1) with 1% Triton X-100 and 2 M ethylenecarbonate followed by acetylation with acetic anhydride at the end of each cycle. Lithium chloride was used to increase swelling properties of the resin. Peptides were cleaved from the resin using "Reagent K" (TFA/thioanisole/water/phenol/ethanedithiol 33:2:2:2:1, v/v) for 6 h, extracted with trifluoroacetic acid, followed by precipitation with diethyl ether. Final products were obtained with more than 98% purity as estimated by analytical HPLC.

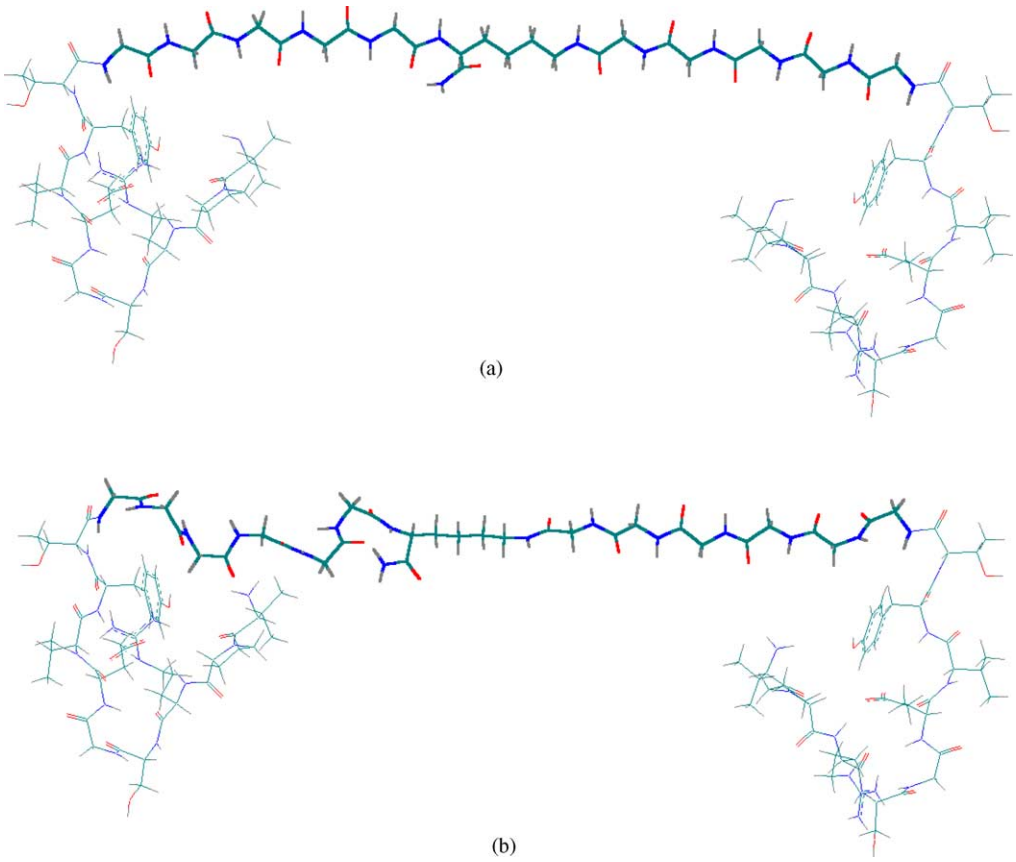

Fig. 2. The β164–172 loops connected with a linker (bold) of different lengths: the loops are oriented in space like in the superdimer structure. (a) Peptide **3** contains a 37-atom linker that is able to span the distance between the carbonyl groups of Thrβ^{172}, like in the superdimer structure. The linker structure is stretched but does not produce distortions on the immunosuppressors. (b) Forty-three-atom linker of peptide **4** is longer than necessary and may have conformational flexibility to orient the VPRSGEVYT loops in the same position like in the crystal structure.

The products were purified by preparative reversed-phase HPLC on a Vydac C4 column 22 mm × 250 mm, using a linear gradient of 2–13% acetonitrile in 0.1% trifluoroacetic acid (gradient 0.5%/min, flow rate 7 ml/min) and transformed into acetate forms. The monomeric peptides **1**, **5**, and **7** were synthesized by standard Fmoc procedure similar to one described previously [39]. The obtained products possessed correct molecular weight (confirmed on a Finnigan MAT TSQ 700 mass spectrometer equipped with a Finnigan electrospray ionization source) and amino acid composition (measured on DIONEX-AAA Direct System).

2.2. Immunological tests

2.2.1. Animals

CBA, BALB/c and C57BL, 8–12 weeks old mice were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław. Mice were fed a commercial, granulated food and filtered water ad libitum.

2.2.2. Preparation of splenocytes

Spleens were isolated aseptically and pressed through a plastic screen into Hanks' medium. The cells were centrifuged and treated for 5 min at room temperature with 0.83% ammonium chloride to lyse erythrocytes. Then, the cells were washed 2× with Hanks' medium and passed through cotton wool columns to remove cell debrids. Finally, splenocytes were resuspended in a culture medium/RMPI 1640 supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics.

2.2.3. Immunomodulatory activities

The immunomodulatory activities of the peptides were tested in the humoral (plaque-forming cells—PFC) and cellular (delayed type hypersensitivity—DTH) assays. The footpad test was used for determination of the cellular immune response. The details of the tests were described previously [3,41]. All data obtained in the biological experiments were statistically elaborated using the Student's *t*-test. To make the comparison of the data easier we included the percentage values of the inhibition of the immune responses. The values were calculated according to the equation:

$$\%\,\text{of immunosuppression} = 100\left(1 - \frac{\text{experimental value}}{\text{control}}\right)$$

In the case of statistically non-significant (NS) results the values in parentheses were given. The results of the immunological tests are summarized in Tables 1 and 2.

Table 1
The number of PFC in the spleens of CBA/Iiw mice immunized with SRBC and treated i.p. with peptides 24 h after antigen administration

Peptide	Dose (μg (nmol)/mouse)	PFC/10^6	±S.E.[a]	*P* (Student's test)	Suppression (%)
0[b]	0[c]	1382	11		
	10 (9.93)	956	14	<0.001	31
	100 (99.3)	813	14	<0.001	41
1	0	1744	45		
	10 (7.42)	1472	40	<0.01	16
	100 (74.2)	1298	55	<0.001	26
2	0[b]	1570	29		
	10 (3.88)	1180	74	<0.01	25
	100 (38.8)	865	39	<0.001	45
3	0	1570	29		
	10 (3.71)	1130	57	<0.001	28
	100 (37.1)	640	46	<0.001	59
4	0	1570	29		
	10 (3.56)	415	26	<0.001	74
	100 (35.6)	820	71	<0.001	48
5	0	888	45		
	10 (9.71)	458	57	<0.01	45
	100 (97.1)	329	34	<0.001	63
6	0	888	45		
	10 (7.95)	566	123	NS	(36)
	100 (79.5)	834	82	NS	(6)
7	0	888	45		
	10 (3.81)	659	71	NS	(27)
	100 (38.1)	1187	112	<0.05	−34
CSA	0	1570	29		
	10 (8.33)	965	66	<0.001	39
	100 (83.3)	1105	68	<0.001	30

[a] The results are expressed as a mean ± S.E. of determinations.
[b] Results for nonapeptide VPRSGEVYT were published previously [39].
[c] Control–0.9% NaCl solution.

PFC number was determined by the Jerne test modified by Mishell and Dutton [25]. Eight to ten weeks old CBA/Iiw mice were immunized with sheep red blood cells (SRBC) and treated intraperitoneally with 10 or 100 μg portions of peptides dissolved in 0.2 ml of 0.9% saline, 24 h after immunization. After 4 days the number of PFC in the spleen cells was determined. The results are expressed as a PFC number per 10^6 splenocytes.

DTH reaction was performed according to Lagrange [19] using CBA/Iiw mice. The animals were immunized by an intravenous injection of 5×10^5 SRBC in 0.9% saline and the DTH reaction was elicited 4 days later by administration of 10^8 SRBC into hind foot pads. The peptide solutions were administered 24 h after the sensitizing dose of the antigen. The results are expressed in units as an antigen specific increase in footpad thickness (1 unit = 10^{-2} cm).

2.2.4. Proliferative response of splenocytes to concanavalin A (Con A)

Splenocytes from CBA mice were resuspended at concentration 4×10^5 per well in 96-well, flat bottom microtiter plates. Con A was applied at 2.5 μg/ml and the peptides at doses: 0.1, 1.0 and 10 μg/ml. After 3-day incubation rate of the cell proliferation was determined by MTT colorimetric method. [11]. The results are presented as mean OD values from quadriplicate wells ± S.E.

2.3. CD spectroscopy

CD spectra were recorded on a Jasco J-600 spectropolarimeter. Peptides were dissolved at concentrations of approximately 70 μg/ml and the spectra were recorded in water and trifluoroethanol (TFE) with 10% water at room temperature. The spectrum of the solvent was recorded under identical conditions and was subtracted. A rectangular quartz cuvette of 1 mm path length was used. Each spectrum represents the average of eight scans. Data are presented as molar ellipticity (Θ).

3. Results

We synthesized a series of dimeric compounds consisting of two VPRSGEVYT sequences linked through

Table 2
DTH reaction (foot pad test) in CBA/Iiw mice sensitized with SRBC and treated i.p. with the preparation 2 h after administration on the sensitizing dose of antigen

Peptide	Dose (μg (nmol)/mouse)	Units[a]	±S.E.[b]	*P* (Student's test)	Suppression (%)
0[c]	0[d]	9.8	0.9		
	20 (19.87)	8.5	0.6	NS	(13)
	200 (198.7)	7.2	0.6	<0.05	27
1	0	7.5	0.6		
	10 (7.42)	5.7	0.2	<0.02	24
	100 (74.2)	4.7	0.3	<0.001	37
2	0	8.5	0.3		
	10 (3.88)	5.5	0.3	<0.001	35
	100 (38.8)	6.0	0.2	<0.001	29
3	0	8.5	0.3		
	10 (3.71)	4.1	0.2	<0.001	52
	100 (37.1)	4.3	0.2	<0.001	49
4	0	8.5	0.3		
	10 (3.56)	5.0	0.3	<0.001	41
	100 (35.6)	5.3	0.3	<0.001	38
5	0	6.9	0.4		
	10 (9.71)	7.9	0.4	NS	(−14)
	100 (97.1)	8.5	0.5	<0.05	−23
6	0	6.9	0.4		
	10 (7.95)	11.3	1.1	<0.01	−64
	100 (79.5)	11.2	0.4	<0.001	−62
7	0	6.9	0.4		
	10 (3.81)	2.9	0 4	<0.001	58
	100 (38.1)	3.9	1.0	<0.05	43
CSA	0	8.5	0.3		
	10 (8.33)	4.3	0.2	<0.001	49
	100 (83.3)	5.1	0.2	<0.001	40

[a] One unit = 10^{-2} cm.
[b] The results are expressed as a mean ± S.E. of six mice.
[c] Results for nonapeptide VPRSGEVYT were published previously [39].
[d] Control–0.9% NaCl solution.

their C-termini by flexible spacers of different length (peptides **2**–**4**) (Scheme 1). For synthetic ease, the two monomer residues were covalently linked through their C-termini, using lysine amide with attached glycine residues as spacers. We also synthesized a linear counterpart, VPRSGEVYTG_6-NH_2 (peptide **1**). The dimeric peptides were synthesized directly on solid support, using branched lysine backbone to produce antiparallel dimeric constructs. Details of the synthesis of the peptides were given in the experimental part. Structures of the purified peptides were confirmed by amino acid analysis and mass spectrometry. The final purity for each peptide, estimated by analytical HPLC (in 227 nm) was equal or greater than 98%.

1. H-Val-Pro-Arg-Ser-Gly-Glu-Val-Tyr-Thr-Gly-Gly-Gly-Gly-Gly-Gly-NH_2

2. (H-Val-Pro-Arg-Ser-Gly-Glu-Val-Tyr-Thr-***Gly-Gly-Gly-Gly)$_2$-Lys-NH_2*(31)

3. (H-Val-Pro-Arg-Ser-Gly-Glu-Val-Tyr-Thr-***Gly-Gly-Gly-Gly-Gly)$_2$-Lys-NH_2*(37)

4. (H-Val-Pro-Arg-Ser-Gly-Glu-Val-Tyr-Thr-***Gly-Gly-Gly-Gly-Gly-Gly)$_2$-Lys-NH_2*(43)

5. H-Thr-Phe-Gln-Thr-Leu-Val-Met-Leu-Glu-NH_2

6. H-Thr-Phe-Gln-Thr-Leu-Val-Met-Leu-Glu-Gly-Gly-Gly-Gly-Gly-NH_2

7. (H-Thr-Phe-Gln-Thr-Leu-Val-Met-Leu-Glu-***Gly-Gly-Gly-Gly)$_2$-Lys-NH_2*(31)

Scheme 1. Synthetic dimeric analogs of the HLA-DR 164–172 and 155–163 fragments (compounds **2**–**4** and **7**, respectively) and their monomeric counterparts. The linker structures are given in bold; numbers of atoms in the linker are in parentheses-counting only the atoms contributing to the length of the linker.

The synthesized peptides were investigated for their activities in the humoral and cellular immune response and the results are summarized in Tables 1 and 2. Their potencies were compared to that of cyclosporine (CSA) and a previously tested nonapeptide fragment of HLA-DR located in the β164–172 loop with the VPRSGEVYT sequence (**0**) [39]. As a rule, dimeric analogs showed stronger immunosuppressive potencies than their monomeric counterparts, as determined both, in the delayed hypersensitivity and humoral immune response assays.

The effects of the peptides on the humoral immune response of mice were assayed by determining the number of antibody forming cells in the spleen. The synthesized peptides suppressed the humoral immune response to various

degrees (Table 1). The potency of the linear peptide **0** was comparable to that of CSA. Introduction of heksa-glycine chain (in peptide **1**) only slightly diminished its immunosuppressory potency in the PFC test, suggesting, that the linker-forming residues do not drastically influence immunosuppressory activity. The immunosuppressory activity of the dimeric peptides depended on their linker length. A shorter linker dimeric analog (**2**) possessed the weakest potency in the series, although, slightly higher than that of its monomeric analog **1**. The dimer's longer linker analogs were very active immunosuppressants, even more effective than **CSA**. A very promising property seems to be associated with an unusually high immunosuppressive activity of peptide **4** tested at 10 μg per well. Comparison of the potencies of peptides **3** and **4** may suggest that increasing flexibility of the linker length by two additional glycine residues affected their potency.

The influence of the peptides on the inductive phase of the delayed type hypersensitivity (DTH) is shown in Table 2. Distinct dose-independent immunosuppressive effects were observed for all of the dimeric peptides. Peptides **3** and **4** were highly active and the potency of peptide **3** was even higher than that of cyclosporine, while their shorter linker analog **2** was considerably less active.

Again, it is clearly evident from that experimental model that the dimeric peptides **3** and **4** exhibited higher potencies than their linear counterparts. Therefore, the dimerization model proposed by us of the 164–172 HLA-DR-fragment affects both the humoral and cellular immune response.

We also synthesized and tested effect of dimerization on the nonapeptide sequence contiguous to the HLA-DR 164–172 loop as an external control. As can be seen from Tables 1 and 2, the nonapeptide fragment of HLA-DR molecule, located in the β chain 155–163 with the TFQTLVMLE sequence (**5**), selectively suppresses the humoral immune response. Dimerization of this compound (peptide **7**) did not enhance this effect, but completely diminished it. On the other side, peptide **7** caused significant reduction of the cellular immune response measured in the DTH assay.

The synthesized peptides were also tested in in vitro experimental models such as: mixed lymphocyte reaction (MLR) using CBA and BALB/c mouse splenocytes, presentation of antigen (lactoferrin) to lactoferrin-specific T-cell line BLFK2 and proliferative response of splenocytes to concavalin A (Con A) a T-cell-specific mitogen. We have not observed any significant immunomodulatory effect in the MLR and in the antigen presentation assay (data not shown). However, the peptides were active in the mitogen-induced cell proliferation (Table 3). Compounds **4** and **6** were inhibitory, whereas peptides **1** and **5** were strongly and **3** and **7** moderately stimulatory.

Conformational investigations of peptides **1**–**4** were performed by CD method using TFE and water as solvents. CD spectra of peptides investigated are presented in Fig. 3a and b. The shape of spectra was strongly solvent-dependent.

Table 3
Proliferative response of splenocytes to concanavalin A (Con A)

Peptide	Dose (mg (μmol)/dm^3)	OD[a] 550/630	±S.E.	*P* (Student's test)
DMSO[b]	0.1	0.313	0.006	
	1	0.311	0.015	
	10	0.321	0.02	
1	0.1 (0.074)	0.430	0.013	<0.001
	1 (0.74)	0.439	0.008	<0.001
	10 (7.4)	0.481	0.023	<0.01
3	0.1 (0.037)	0.391	0.02	<0.01
	1 (0.37)	0.331	0.01	NS
	10 (3.7)	0.346	0.011	<0.02
4	0.1 (0.036	0.285	0.005	<0.02
	1 (0.36)	0.244	0.014	<0.02
	10 (3.6)	0.298	0.009	NS
5	0.1 (0.097)	0.411	0.005	<0.001
	1 (0.97)	0.414	0.003	<0.001
	10 (9.7)	0.433	0.008	<0.01
6	0.1 (0.079)	0.259	0.012	<0.01
	1 (0.79)	0.266	0.007	<0.05
	10 (7.9)	0.298	0.007	NS
7	0.1 (0.038)	0.394	0.009	<0.001
	1 (0.38)	0.361	0.009	<0.05
	10 (3.8)	0.387	0.004	<0.02
CSA	0.1 (0.083)	0.052	0.001	<0.001
	1 (0.83)	0.043	0.002	<0.001
	10 (8.3)	0.005	0.001	<0.001

[a] Results are expressed as a mean OD value of quadriplicate determinations ± S.E.

[b] DMSO was used as a initial solvent then the compounds were diluted in culture medium. Control DMSO does corresponded to the DMSO doses contained in respective peptide concentrations.

Spectra of all the peptides dissolved in water solution (at neutral pH), were characterized by a positive band below 185 nm, a negative band at 195 nm and a very weak negative shoulder at 220 nm (Fig. 3a). The spectra were typical for unordered structures, although their intensities of this band were rather small [42]. The CD spectra in TFE/water (9:1, v/v) solution displayed two distinct negative bands. The bands were more intense for peptide **4** at 206–208 nm and near 222 nm (Fig. 3b). The spectra were somewhat similar to the CD spectrum of the α-helix. The similarity of the spectra of tested compounds in one solvent implies that the dimerization has little effect on stabilization of their structure in both water and TFE solution. The unstructured conformation of the peptides in water solution suggests that the linker may easily adopt extended conformation, which enables them to situate the immunosuppressory peptides in a desired orientation.

4. Discussion

Dimerization of an active compound often results in enhanced binding and improved pharmacological properties.

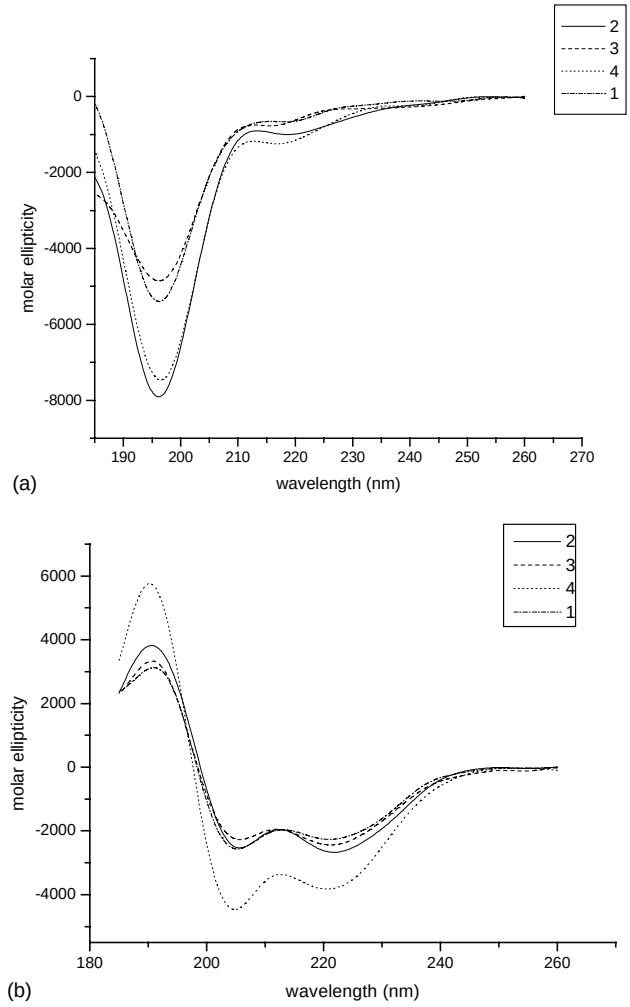

Fig. 3. CD spectra of monomeric (**1**) and dimeric (**2**–**4**) peptides (a) in water solution (neutral pH) and (b) in 10% water in TFE.

Ligands having two pharmacophores connected by a spacer (bivalent ligands) have a potential for bridging vicinal receptors. Such bridging should be manifested by a substantial increase in potency due to the high local concentration of the free pharmacophore in the vicinity of the proximal recognition site when the bivalent ligand is bound in a monovalent mode. Indeed, bivalent ligands containing peptide pharmacophores have been reported to possess enhanced agonist or antagonist potencies at a specific spacer length. This phenomenon was observed for enkephalin [8,22], ACTH [21], gonadotropin releasing hormone [17], substance P [13], dermorphin [20] and neurokinin A and B [18].

Previously we suggested that the β164–172 loop of HLA-DR molecules may serve as a functional epitope on the molecule surface for intermolecular binding, and that a possible mechanism of immunosuppressory action of the synthesized peptides with the VPRSGEVYT sequence could be associated with specific interfering with the adhesion of HLA class II molecules to their coreceptors [39]. Since the HLA-DR molecules act as superdimers, it may be expected that a properly designed dimerization of the nonapeptide VPRSGEVYT, should increase its immunosuppressive potency. Therefore, we decided to design and synthesize a dimeric analog of the VPRSGEVYT immunosuppressor, which mimics sterically the immunosuppressive regions of the HLA-DR superdimer. For synthetic ease, we selected lysine amide to facilitate positioning of the carboxy-terminal domain to a proper location for the interaction. The synthesis of the linker, as well as peptide monomers, was performed simultaneously on both amino group of lysine attached to solid support. In the past, lysine amide has been used as an effective spacer in preparation of some dimeric peptide analogs [10,31]. We chose $(Gly_n)_2-Lys\text{-}NH_2$ as a linker to minimize nonspecific hydrophobic interaction between the bridge and the receptor surface (*n* indicates the number of Gly residues). We focused herein on the linker backbone only and the linker length was expressed as the number of atoms contributing to the length of the linkers.

It has been previously evidenced that a linker length may affect dramatically biological potencies of bivalent bio-ligands [33]. The bivalent analog should consist of two monomers covalently linked by a spacer with sufficient length to be able to position them in the same orientation as in the superdimers. We began the design by examining the three-dimensional structure of the superdimers [2] (Fig. 1). The residues located in the β chain 164–172 with the VPRSGEVYT sequence do not contribute to formation of the superdimer. The distance between the carbonyl groups of $Thr\beta^{172}$ is 47 Å and the molecular modeling (results not shown) suggested that a linker consisting of 37 atoms is the smallest one to span it. To quickly determine the optimal linker length, we decided to synthesize a series of dimeric analogs containing linkers of various lengths. Whereas compound **2** contains a 31-atom linker, which is too short to orient the suppressors in the same position like in the crystal structure, its analogs **3** and **4** possess sufficient length of the linkers (Fig. 2a and b, respectively). The 43-atom linker in compound **4** seems even longer than necessary, although flexible enough to orient the peptides more precisely, without any distortion. This may explain the unusually high immunosuppressive potency of peptide **4** observed at 10 μg per well in a PFC test (Table 1).

The enhancement in the immunosuppressory activity of our dimeric analogs could be explained by a microaggregation of the receptors at the cell surface induced by the dimeric antagonists, which would prevent the access of the agonist to the receptors. Nevertheless, it is known that increased affinity of dimeric analogs of the ligands may be also independent of receptor density [24]. Their potencies may attribute to a higher concentration of pharmacophores in the proximity of their recognition sites by a simple statistical advantage. Bivalent ligands are known to produce thermodynamically more favorable binding interaction with their receptor than the monovalent binding [15].

However, the observed dependence of the immunosuppressory potency on the linker length supports rather a

possibility that dimeric structures may be capable of bridging independent recognition sites of the receptor. Thus, the 37-atom linker in peptide **3** probably facilitates positioning of immunosuppressory peptides to a proper location for the interaction with their receptors. Our results are in agreement with that reported recently by Cochran et al. [7]. The authors studied influences of intermolecular orientation within the activating T-cell receptor dimer. Dimers of class II MHC proteins coupled in a variety of orientations and topologies were able to activate $CD4^+$ T-cells, although the triggering was affected by the inter-molecular distance between MHC molecules.

The small differences between the activities of the compounds in the humoral and cellular immune response models could be interpreted by different signaling requirements leading to generation of respective types of the immune response and/or lower MHC class II density needed for development of cellular immune response [9]. It has to be underlined that the peptides demonstrated somewhat different activities in the generation of the antigen-specific immune response and in the Con A-induced splenocytes proliferation which represents a nonspecific activation of majority of T-cells with a minor involvement of MHC class II molecules [16]. Mitogen-induced cell proliferation is, however, also dependent on a presence of costimulatory molecules and costimulatory signals from accessory cells [23,28,40]. Lack of suppressory properties of the peptide dimers in the MLR and antigen presentation model, where the engagement of MHC class II plays a major role, confronted with the data from the SRBC induced humoral and cellular immune response assays, suggests that the peptides do not affect the process of antigen recognition by the T-cell receptor, rather the peptides may prevent association of MHC class II molecules on antigen presenting cells with costimulatory molecules such as CD4 [28] which require aggregation for efficient binding to MHC molecules. The studied peptides, mimicking parts of MHC molecule, could bind to CD4 membrane distal parts, preventing association with MHC molecules and consequently delivery of costimulatory signals to T-cells. Although costimulatory CD4 molecules play a role in the model of antigen presentation to antigen specific cell lines and in the mixed lymphocyte reaction, the lack of inhibition by the peptides may be due to different model used. Whereas in the generation of antigen-specific immune response, SRBC-specific T-cells are recruited from extremely rare precursors, so that costimulatory CD4 are crucial in that process, presentation of antigen to antigen-specific T-cell lines involves 100% antigen specific cells and also a vast majority of cells participate in antigen recognition in the MLR. Therefore the activation threshold derived from the costimulatory molecules in the later models may be much lover, so that the effects of the interfering peptides are minor.

In this paper we presented highly specific immunomodulation of the nonapeptide fragment of HLA-DR molecule, located in the β chain 155–163 with the TFQTLVMLE sequence (**5**). The peptide stimulates the cellular immune response in the DTH assay, but suppresses the humoral one (Tables 1 and 2). Elongation of this peptide with 4 glycine residues (**6**) resulted even in a higher stimulatory effect. The sequence of peptide **5** is contiguous to the immunosuppressive 164–172 loop and is located on a flat surface of the HLA-DR molecule, partially contributing in the αβ-heterodimer formation. Although a mechanism of the immunomodulatory action of compound **5** is unknown, our results demonstrated that the dimerization of the peptide dramatically affects its immunomodulatory specificity. The dimeric peptide **7** strongly suppresses the cellular immune response, and moderately stimulates the humoral immune response and the Con A-induced proliferation. Again, this result indicates that dimerization may serve as a tool to modify biological effects of some compounds. Although peptide **5** was constructed based on theoretically irrelevant region of the molecule it is adjacent to the immunosuppressory loop, therefore it may still affect the cell activation, particularly after dimerization.

In summary, we attempted to design a dimeric peptide to mimic the discontinuous immunosuppressive epitopes of the HLA-DR superdimers. Our results demonstrate that the dimerization of the nonapeptide fragment of HLA-DR results in enhanced immunosuppressory properties. The linker length affects the immunosuppressory effect.

Acknowledgments

This work was supported by grant No. 6 P05A 013 21 from the State Committee for Scientific Research (KBN, Poland). The authors thank Prof. Ignacy Z. Siemion for helpful discussions. This work is part of a doctoral thesis (M.B.).

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