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Immunosuppressory activity of the cyclodimeric peptide with RGD-sequences★✪

Zbigniew Szewczuk[1]✉, Paweł Buczek[1], Piotr Stefanowicz[1], Krzysztof Krajewski[1], Zbigniew Wieczorek[2] and Ignacy Z. Siemion[1]

[1]*Faculty of Chemistry, University of Wrocław, Wrocław, Poland;* [2]*Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland*

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Our previous studies showed that the nonapeptide fragment of HLA-DQ of the sequence H-Thr-Pro-Gln-Arg-Gly-Asp-Val-Tyr-Thr-OH, located in the β164–172 loop, strongly suppresses the humoral and cellular immune responses, while its shorter analogs, H-Arg-Gly-Asp-Val-OH, H-Arg-Gly-Asp-Val-Tyr-OH and H-Gln-Arg-Gly-Asp-Val-Tyr-OH show only a weak stimulatory activity in respect to the humoral immunological response. These fragments contain the Arg-Gly-Asp (RGD) sequence, known for its importance for cellular association phenomena. Based on the crystal structure of HLA-DR1, we also designed and synthesized a cyclic analog H-Cys-Arg-Gly-Asp-Val-Tyr-Cys-OH with restricted conformation, which strongly suppresses the immune response and selectively inhibits the αvβ3 integrin, suggesting that the mechanism of the immunosuppressory action of the peptide is associated with inhibition of the integrin. In this paper we present the design and synthesis of the cyclodimeric peptide, cyclo-Arg-Gly-Asp-Arg-Gly-Asp, which is also known as a selective αvβ3 inhibitor. The synthesized peptide strongly suppresses both the humoral and cellular immune response. The results support our hypothesis that the immunomodulatory activity of HLA-DQ fragments may be connected with their interactions with some particular integrins on the cell surface.

The main role of human leukocyte antigen (HLA) proteins is the presentation of peptide fragments of foreign antigens to T cells to initiate the immune response. The proteins of HLA-II class

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✉Zbigniew Szewczuk, Wydział Chemii, Uniwersytet Wrocławski, F. Joliot-Curie 14, 50-383 Wrocław, Poland; tel: (48 71) 320 4212; fax: (48 71) 328 2348, e-mail: szewczuk@wchuwr.chem.uni.wroc.pl

Abbreviations and symbols are in accordance with recommendations of the European Peptide Society (*J. Peptide Sci.* **5**, 465–471, 1999). DTH, delayed type hypersensitivity; HLA, human leukocyte antigen; PFC, plaque-forming cells; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, SRBC, sheep red blood cells; TP-5, thymopentin.

are expressed primarily by specialized antigen-presenting cells such as macrophages, dendritic cells, and B-lymphocytes. The coreceptor molecule CD4 enhances the binding between the T-cell receptor and the class II molecules, particularly in low-affinity interactions. The class II HLA molecules are highly polymorphic. Two alleles of each of the three class II subsets, DR, DQ, and DP, are expressed in most humans.

The three-dimensional structure of the HLA-DR1 molecule, has been determined by X-ray crystallography (Brown *et al.*, 1993). HLA class II molecules are heterodimers with a molecular mass of 60 kDa, consisting of noncovalently associated α (32 kDa) and β (28 kDa) chains. The α and β chains are structurally homologous. Each chain is composed of two extracellular domains (designated as $\alpha 1$ and $\alpha 2$ in the α-chain and $\beta 1$ and $\beta 2$ in the β-chain), a transmembrane portion, and a cytoplasmic tail. The crystal structure of human HLA-DQ has not been solved yet. However, because of a high homology to HLA-DR, it should be closely related to the known structure of HLA-DR. An alignment of amino-acid sequences of class II molecules: DR, DP and DQ, has been proposed (Gorga, 1992) which enables the construction of three-dimensional model of HLA-DQ based on mutational analyses of the HLA-DR crystal structures (Paliakasis *et al.*, 1996, Szewczuk *et al.*, 1996b).

The $\beta 2$-domain of HLA-DQ contains the Arg-Gly-Asp-Val-Tyr (167–171) sequence, which is very similar to thymopentin (pentapeptide Arg-Gly-Asp-Val-Tyr, the active fragment (32–36) of thymopoietin, an immune system activator produced in thymus). The fragment is situated in a close spatial proximity to the site engaged in the interactions with the T-cell coreceptor CD4 and contains the Arg-Gly-Asp (RGD) cell adhesion sequence. Such a tripeptide fragment is present in many adhesion proteins and functions as their recognition site, which interacts with proteins of the integrin family. Most of the DQβ variants with known sequences have an RGD loop in position $\beta 167$–169 of the $\beta 2$ domain. The RGD loop may confer cell adhesion possibilities of the HLA-DQ proteins. There are three known alleles that have His instead of Arg in $\beta 167$, yielding sequences with an unknown cell adhesion function. Small RGD-containing peptides may inhibit binding of many different integrins to extracellular matrix proteins and to other adhesive proteins. Our analysis shows that the RGD sequence is located in the loop of the HLA-DQ molecule exposed toward the solvent and, therefore, it may be involved in the interactions with other proteins, but the biological role of the HLA-DQ fragment still remains unknown.

We found that a nonapeptide fragments of HLA-DQ (164–172, H-Thr-Pro-Gln-Arg-Gly-Asp-Val-Tyr-Thr-OH) strongly suppresses the cellular and humoral immune response. Shorter fragments, H-Gln-Arg-Gly-Asp-Val-Tyr-Thr-OH and H-Arg-Gly-Asp-Val-Tyr-Thr-OH, show a slightly weaker potency than the nonapeptide, whereas H-Gln-Arg-Gly-Asp-Val-Tyr-OH, H-Arg-Gly-Asp-Val-Tyr-OH, and H-Arg-Gly-Asp-Val-OH show marginal immunosuppression in the cellular response and a weak stimulatory activity in respect to the humoral immunological response (Szewczuk *et al.*, 1996a; 1997). The corresponding fragments of HLA-DP and HLA-DR show immunosuppressive properties similar to the HLA-DQ fragments (Szewczuk *et al.*, 1999). We also found that the immunomodulatory properties of the investigated peptides are connected with their conformational preferences in solution (Szewczuk *et al.*, 1996b).

Based on the crystal structure of HLA-DR1, we also designed and synthesized an analog conformationally constrained by cyclization, H-C⌈ys-Arg-Gly-Asp-Val-Tyr-Cys⌉-OH, to mimic the RGD loop of HLA-DQ (Szewczuk *et al.*, 1996b). The cyclic product strongly suppresses both the humoral and cellular immune response and was found to be a selective inhibitor of $\alpha v\beta 3$-like integrins. This result suggests that the HLA-DQ molecule may be involved in interactions with $\alpha v\beta 3$-like receptors.

In the present study, we synthesized another conformationally constrained analog of the HLA-DQ fragment with the dimeric RGD sequence to mimic the thymopentin-like loop of HLA-DQ. The objective was to obtain a peptide that may interact with hypothetical receptors of the thymopentin-like mini-region of HLA-DQ and to test our concept that the HLA class II mole-

cules may interact with $\alpha v\beta 3$-like integrins. We chose the Arg-Gly-Asp-Arg-Gly-Asp sequence because it can easily mimic the RGD fragment of HLA-DQ and, more importantly, the peptide is known as a selective inhibitor of $\alpha v\beta 3$-integrin (Burgess *et al.*, 1996). Here, we describe a simple, improved method of synthesis of the peptide and test it for immunomodulatory activity.

EXPERIMENTAL

Peptide synthesis

Protected amino acids were prepared as described by Bodanszky & Bodanszky (1985). All solvents were purified according to standard procedures, other reagents were of analytical or reagent grade. Melting points (uncorrected) were determined on a PHMK VEB Analitic (Dresden) apparatus. Optical activities were measured with a Polamat A (C. Zeiss, Jena) polarimeter. Thin-layer chromatography (TLC) was carried out on silica gel plates F254 from Merck in the following solvent systems: chloroform/methanol/water (8:3:1, by vol.) (A); n-butanol/acetic acid/water (4:1:1, by vol.) (B); chloroform/methanol (12:1, v/v) (C); chloroform/methanol (19:1, v/v) (D). The plates were developed with ninhydrin reagent (0.3% in acetone) and by incubation in iodine vapours. Column chromatography (1.7 × 100 cm) was carried out with Merck Silica gel 60 (70–230 mesh; 80–100 g silica gel per 1 g of compound) in an appropriate solvent. Elution from the column was controlled by TLC analysis. Amino-acid compositions were determined on an AAA-851 analyzer (Mikrotechna, Czechoslovakia) following hydrolysis in 6 M HCl at 100°C for 16 h. Molecular weights were determined by electrospray mass spectrometry on a Finnigan Mat TSQ 700 spectrometer. The NMR spectra were recorded on a Brucker AMX-300 MHz spectrometer.

General procedure A. Coupling reactions using DCC/HOBt. Amino component (1.0 mmol) was dissolved in DMF (12.5 ml) and neutralized with triethylamine (0.14 ml, 1.0 mmol) at 0°C. The carboxy component (1.0 mmol) and HOBt (0.14 g 1.0 mmol) were added, followed by DCC (0.23 g, 1.1 mmol). The reaction mixture was stirred for 4 h at 4°C and overnight at room temperature. The resulting dicyclohexylurea was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, washed successively with water, cold 1 M $KHSO_4$, saturated $NaHCO_3$, and saturated NaCl. The ethyl acetate layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The peptide was purified by silica gel chromatography and crystallized from ethyl acetate-hexane.

General procedure B. Removal of BOC and t-Bu groups. Peptide (1.0 mmol) in 10 ml TFA was stirred for 12 min at room temperature. After removal of the excess of the reagent *in vacuo*, the residue was re-evaporated twice from anhydrous diethyl ether. Crystals were filtered and washed twice with diethyl ether and then dried over KOH *in vacuo* for a minimum of 4 h.

General procedure C. Removal of Fmoc group. Fmoc-peptide (1.0 mmol) in 10 ml DMF was treated with 1 ml diethylamine. Progress of the reaction was monitored by TLC, being generally complete in 1 h. The solvent was evaporated *in vacuo*. The product was converted to the acetate form by adding equivalent amount of acetic acid and re-evaporated twice from anhydrous diethyl ether. The product was used for the following synthesis without further purification.

Fmoc-Arg(NO_2)-Gly-OtBu (1). Compound 1 was synthesized from Fmoc-Arg(NO_2)-OH and $HClO_4 \cdot$H-Gly-OtBu by general procedure A, followed by purification on a silica gel column (solvent system C). Overall yield 60%; R_f(A) 0.75, R_f(B) 0.93; m.p. 67–69°C; $[\alpha]^D_{25} = -4.72^o$ (c = 1, $CHCl_3$). Anal. calcd. for $C_{27}H_{34}N_6O_7$: C, 58.48; H, 6.13; N, 15.16. Found: C, 58.31; H, 5.90; N, 14.91. ^{1}H-NMR ($CDCl_3$): 1.4 (s, 9H), 1.5–2.0 (m, 4H), 3.2–3.5 (m, 2H), 3.8–4.0 (m, 2H), 4.1–4.2 (m, 1H), 4.3–4.5 (m, 3H), 6.1–6.3 (m, 1H), 7.2–7.8 (m, 11H), 8.4–8.7 (m, 1H).

Boc-Asp(cHx)-Arg(NO_2)-Gly-OtBu (2). Compound 1 was deprotected by general procedure C and coupled with Boc-Asp(cHx)-OH by general procedure A, followed by purification by silica gel chromatography (solvent system D). Yield 41%; R_f(A) 0.89, R_f(B) 0.82; m.p. 78–80°C; $[\alpha]^D_{25}$ =–18.2

(c = 1, $CHCl_3$). Anal. calcd. for $C_{27}H_{47}N_7O_{10}$: C, 51.51; H, 7.47; N, 15.58. Found: C, 51.00; H, 7.08; N, 15.04. ^{1}H-NMR: 1.2–2.0 (m, 14H), 1.4 (s, 18H), 2.7–3.0 (m, 2H), 3.2–3.5 (m, 2H), 3.8–4.0 (m, 2H), 4.5–4.6 (m, 1H), 4.6–4.8 (m, 2H), 5.8–6 (m, 1H), 7.5–7.8 (m, 4H), 8.5–8.7 (m, 1H). ^{13}C-NMR: 23.54 (2C), 24.41 (1C), 25.19 (1C), 27.98 (3C), 28.20 (3C), 29.89 (1C), 31.37 (2C), 36.47 (1C), 40.68 (1C), 41.96 (1C), 51.21 (1C), 52.10 (1C), 73.78 (1C), 80.60 (1C), 82.16 (1C), 155.62 (1C), 159.09 (1C), 168.79 (1C), 170.90 (1C), 171.55 (1C), 171.89 (1C).

Arg(NO$_2$)-Gly-Asp(cHx)-Arg(NO$_2$)-Gly-Asp(cHx) (3). Compound 2 was deprotected by general procedure B. The resulting product, TFA · H-Asp-(cHx)-Arg(NO_2)-Gly-OH (1 mmol) was cyclized in a mixture of DMF (70 ml) and methylene chloride (180 ml) at room temperature by addition of PyBOP (680 mg, 1.3 mmol) in the presence of HOBt · H_2O (200 mg, 1.3 mmol) and ethyl(diisopropyl)amine (1.1 ml, 6 mmol). The progress of the ring closure was followed by the ninhydrin test and the cyclization reaction was complete after 24 h. The solvent was removed *in vacuo*. The peptide was dissolved in 5% acetic acid in methanol (60 ml) and 10% palladium-on- charcoal catalyst (0.2 g) was added and the mixture was stirred in an atmosphere of hydrogen. After 3 days the catalyst was removed by filtration and the solvent evaporated *in vacuo*. The cyclic dimer was obtained with the yield of about 70%, estimated by analytical HPLC of the crude product. The peptide cyclo(Arg-Gly-Asp(cHx)-Arg-Gly-Asp(cHx)) was crystallized from chloroform and purified by preparative HPLC using an Alltech Econosil C-18 column. Molecular weight calculated for $C_{36}H_{60}N_{12}O_{10}$ 820.4; found 821.6 for $[M + H]^+$ and 411.5 for $[M + 2H]^{2+}/2$.

***Arg-Gly-Asp-Arg-Gly-Asp* (5).** The peptide 4 (0.1 mmol) was dissolved in TFA (2.4 ml) and trifluoromethanosulfonic acid (0.3 ml) was added. The reaction mixture was stirred for 0.5 h at 4°C and 1.5 h at room temperature, followed by precipitation by diethyl ether. The peptide was purified by preparative HPLC using an Alltech Econosil C-18 column. Yield about 30%. The peptide was shown to be pure (over 99%) by analytical HPLC using a Beckman Ultrasphere C-18 column. Molecular weight calculated for $C_{24}H_{40}N_{12}O_{10}$ 656.3, found 657.5 for $[M + H]^+$ and 329.4 for $[M+2H]^{2+}/2$. Amino acid analysis: $Arg_{1.11}Gly_{1.08}$-$Asp_{1.00}$.

Immunological tests

The immunomodulatory activities of the peptide (humoral immune response) were tested by the direct plaque-forming cell (PFC) test. The foot-pad test was used for determination of the delayed type hypersensitivity (DTH) (cellular immune response). The Jerne methodology as modified by Mishell & Dutton (1967), was used in determination of the PFC number. In the case of the DTH test, the methodology of Lagrange *et al.* (1974) was applied. The details of all tests *in vivo* and *in vitro* have been described previously (Wieczorek *et al.*, 1991; Cebrat *et al.*, 1996). The animals used were 8–10-week old 129/Iiw and CBA/Iiw mice; the antigen was sheep red blood cells (SRBC) and the solvent was 0.9% saline solution.

2.0–4.0 mg of the peptide was dissolved in 0.2–0.4 ml of 0.9% saline solution and the solution was diluted to the desired concentration with 0.9% saline. In the experiments, 0.1–0.2 ml of peptide solution was administered intraperitoneally (i.p.) or added to cell culture wells. The animals were immunized by i.p. treatment with SRBC in 0.9% saline. The peptide solution was administered twice, 3 h before and 24 h after sensitization of the animal or 1 h after the effector dose of the antigen. The results are expressed as the PFC number per 10^6 splenocytes (PFC test), and the increase of the foot-pad thickness (DTH test; 1 unit = 10^{-2} cm). All data obtained in biological experiments were statistically elaborated using the P Student's test. The results of the immunological tests are summarized in Table 1.

Molecular modelling

The general principles of molecular modelling of Arg-Gly-Asp-Arg-Gly-Asp were similar to those published by Burgess *et al.* (1996), although those authors performed their calculations using different force field (CHARM) and different method for

generation of conformers (quenched molecular dynamics). To investigate conformational preferences of the peptide we generated 4000 initial structures *via* random changes of all φ and ψ angles. After rejection of duplicating structures, the remaining 2384 were optimized using an AMBER force field (HyperChem 4.5) with dielectric constant 78 (representing water). After rejection of the structures with energy 6 kcal/mol over the lowest found (–16.8 kcal/mol), we obtained 126 low energy conformations. Within these low energy structures we searched for conformations similar to the conformation of the 165–169 fragment of the X-ray crystal structure of HLA-DR (Brown *et al.*, 1993). We found one conformer, with energy –15.7 kcal/mol (1.1 kcal/mol over the lowest found), whose backbone geometry is very similar to the HLA-DQ fragment with small deviations of the positions of peptide backbone atoms (Cα, C′ and N). RMSD (root-mean-square deviation) was found to be 0.4 Å for superimposed fragments, Asp^{1} C_{α} — Arg^{5} C_{α} of the cyclic peptide and Pro^{165} C_{α} — Glu^{169} C_{α} of the HLA-DR1 fragment (Fig. 1). The φ and ψ torsion angles for this conformer are as follows: Asp^{1}, –168, –56; Arg^{2}, –93, 43; Gly^{3}, 171, –72; Asp^{4}, –59, –48; Arg^{5}, –97, 175; Gly^{6}, –82, 63.

CD spectroscopy

CD spectra were recorded on a Jasco-600 spectropolarimeter. Peptides were dissolved at approx. 200 μg/ml. A rectangular quartz cuvette of 1 mm pathlength was used. The spectra were recorded at room temperature. Eight scans of background spectra (water blanks) and peptide spectra were acquired, and the background was subtracted from the peptide spectra. Data are presented as molar ellipticity [Θ].

RESULTS

Peptide synthesis

The synthesis of the cyclic peptide Arg-Gly-Asp-Arg-Gly-Asp (**5**) has been reported previously by Burgess *et al.* (1996). The authors achieved cyclization, by intramolecular coupling of the C-terminal Asp to the N-terminal Arg resi-

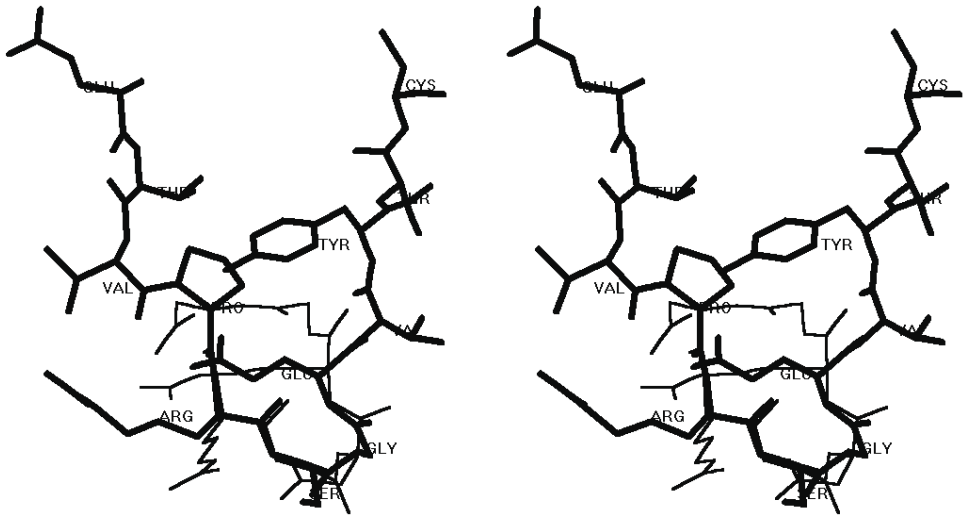

Figure 1. Comparison of the three-dimensional structures of the low energy conformer of Arg-Gly-Asp-Arg-Gly-Asp and HLA-DR.

Bold, from the X-ray crystal structure (Brown *et al.*, 1993).

due, after elongation of the intact linear hexapeptide obtained on the solid phase. The relatively high ratio of racemization at an Asp residue and the presence of other by-products, reported by Burgess *et al.* (1996), encouraged us to find another method of synthesis of the peptide.

We synthesized the cyclic peptide **5** with RGD sequences by cyclodimerization of a partially protected tripeptide. There are three possible pairs of amide bonds that could be selected for the final ring closure step *via* cyclodimerization. We chose the peptide with C-terminal glycine to prevent the possibility of racemization. The peptide intermediate, H-Asp(cHx)-Arg(NO_2)-Gly-OH, was synthesized by conventional methods in solution. The cyclodimerization was performed in a mixture of dichloromethane and DMF, using PyBOP following the deprotection. The peptide was purified by preparative reversed-phase HPLC and transformed into the acetate form. It was obtained with over 99% purity estimated by analytical HPLC. The purified cyclodimer was identified by its molecular weight using electrospray mass spectrometry as well as amino acid analysis and NMR spectrometry. The cyclic dimer was obtained with the yield of about 70%, estimated by analytical HPLC of the crude product. Mass spectrometry analysis of the purified final peptide proved that the product was devoid of any contamination containing unprotected arginine side chains. These contaminations are known to alter the immunoreactivity of synthetically prepared peptides (Schlager *et al.*, 1997).

The likelihood that the initial tripeptide substrate could form cyclic tripeptide was low, because small rings can be closed only if at least one of the peptide bond has the *cis* rather than the more stable *trans* conformation. Therefore, cyclomonomerization can be facilitated by the presence of the proline residue only, but tri-, tetra-, and pentapeptides without proline are prone to cyclodimerization, that is to the formation of rings twice the size of those obtained in cyclomonomerization. This phenomenon was first successfully utilized by Schwyzer & Sieber (1958) in the synthesis of an analog of gramicidin S, which is a naturally occurring cyclodimer.

The purified cyclodimer **5** was identified by its molecular weight using an ion-spray mass spectrometer (Fig. 3). Since the isotope peaks are separated by 1.0 and 0.5 Da consecutively for singly charged and doubly charged species, the peaks at m/z 657.5 and 329.4 correspond to the singly and doubly charged ions, respectively, of the peptide. Analysis of the isotope peaks intensity revealed that the synthesized cyclodimer was contaminated neither by the cyclomonomer nor by other oligomers. The tendency of the tripeptide to cyclodimerize can be explained by the assumption of an antiparallel arrangement of two molecules prior to cyclization, where the two chains are held together by hydrogen bonds in a β-sheet-like conformation.

Immunological properties

The synthesized cyclic peptide was investigated for its activity in the regulation of both the cellular and humoral immune responses. Each experiment was repeated at least twice, giving similar results. The influence of the peptide on the humoral immune response in mice (Table 1) was assayed *in vitro* by counting the number of spleen cells that formed plaques with sheep red blood cells (PFC-test). The results are expressed as mean ± S.E. of four wells. The tested peptide evoked a strong suppression of the humoral immune response as measured by the PFC number (Table 1). The effect does not depend significantly on the dose of the peptide. The cellular immune response (Table 2) was assayed *in vivo* by determination of the influence of the peptide on the inductive phase of the delayed type hypersensitivity (DTH). The results are expressed as mean ± S.E. of six mice. A strong immunosuppressive effect was observed when the cyclic peptide was administered at the low dose of 20 μg/mouse. At a high dose (200 μg/mouse) the peptide was less active, albeit a significant immunosuppressive potency was observed.

The cyclic peptide was designed to mimic the RGD loop of the HLA-DQ molecule. The high immunosuppressory effect in the cellular as well as the humoral immune response and, in addition

to this, the antiadhesive activities of **5** reported by Burgess *et al.* (1996) support our suggestion that the thymopentin-like region of the HLA-DQ molecule constitutes its immunosuppressory and adhesion mini-region.

CD spectroscopy

Conformational investigations of **5** were performed by the CD method using trifluoroethanol and water as solvents. The CD spectra are characterized by a positive band below 190 nm, a negative band at 200 nm, and a negative shoulder at 220 nm (Fig. 2). Essentially, the spectrum of **5** in trifluoroethanol is practically the same as in water, but the intensity of the band at 220 nm is slightly lower in the former case. This α helix-like spectrum results probably from the presence of a β-turn (type I or III) conformation in the conformational equilibrium. The spectra obtained are very similar to those reported for another peptide that is thought to contain a type-I β-turn motif (Bisang *et al.*, 1995), and also to those corresponding to the β-turn type I or III conformation (Perczel & Fasman, 1992). The CD spectrum of **5** dissolved in a potassium phosphate buffer (pH, 5.4), reported previously by Burgess *et al.* (1996), was identical with our spectra, measured in pure water as well as in trifluoroethanol. The similarities imply that the peptide molecule is rigid and the solvent does not affect its conformation.

DISCUSSION

Proteins that contain the RGD attachment site, together with the integrins that serve as their receptors, constitute the major recognition system for cell adhesion. The RGD sequence is the cell attachment site of a large number of adhesive extracellular matrix and cell surface proteins, and nearly half of the over 20 known integrins recognize this sequence in their adhesion protein ligands (reviewed by Ruoslahti, 1996). The integrin-binding activity of adhesion proteins can be inhibited by short synthetic peptides containing the RGD sequence. RGD peptides that have not been specifically designed to be selective towards certain integrins mimic a number of adhesion proteins and bind to more than one receptor. Many cyclic peptides with selected sequences around the RGD have been designed to bind selectively to only one of the RGD-directed integrins. Consequently, the cyclic RGD-peptides and mimetics

Table 1. PFC number in spleen cell cultures of CBA/Iiw mice immunized with SRBC

Dose (μg/well)	PFC/10^6	±S.E.[a]	P (Student's test)	Suppression (%)
Control[b]	1033	14.21		
1	766	11.26	< 0.001	25.8
10	699	26.99	< 0.001	32.3
100	682	10.77	< 0.001	34

[A]Mean ± S.E. (standard error) of four wells; [b]control 0.9% saline solution.

Table 2. Delayed type hypersensitivity (DTH) reaction in 129/Iiw mice sensitized with SRBC and treated i.p. with preparation 24 h after administration of the sensitizing dose of the antigen

Dose (μg/well)	Units[a]	±S.E.[b]	P (Student's test)	Suppression (%)
Control[c]	13.8	0.42		
20	7.8	0.88	< 0.001	43.5
200	10.1	0.75	< 0.01	26.8

[a]1 unit = 10^{-2} cm; [b]mean ± S.E. (standard error) of six mice; [c]control 0.9% saline solution.

can be used to probe integrin functions in various biological systems and provide a great deal of information about cell adhesion mechanisms.

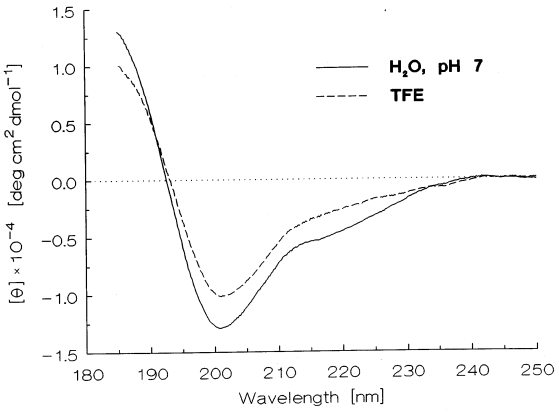

Figure 2. CD spectra of Arg-Gly-Asp-Arg-Gly-Asp in water and trifluoroethanol.

According to the three-dimensional model of HLA-DQ (Szewczuk *et al.*, 1996b) the RGD sequence is located in the loop projecting outward from the HLA-DQ molecule toward the solvent and, therefore, it may be involved in the interactions with some specific integrins. A number of integrins expressed on leukocytes (including T-lymphocytes) have been identified (Steward *et al.*, 1995). Integrins are also known to co-stimulate the TCR-mediated T cell activation (reviewed by Dianzani & Malavasi, 1995). This suggests that HLA-DQ itself may be involved in such a process in the case of T cells. It seems, therefore, possible that peptides containing the RGD motif, derived from HLA-DQ, may inhibit the binding of the histocompatibility protein to certain integrins, causing their immunosuppressive activity.

In this situation, the biologically active conformation of the synthesized peptide should be similar to that of the corresponding region of the native protein. Previously, we checked that the nonapeptide fragment of HLA-DQ selectively inhibits adhesion of platelets to fibrinogen, whereas its restricted analog, H-Cys-Arg-Gly-Asp-Val-Tyr-Cys-OH, was found to be a selective inhibitor of the $\alpha v\beta 3$ integrin (Szewczuk *et al.*, 1996b). Burgess *et al.* (1996) reported previously that the cyclic peptide **5** is also a very selective inhibitor of $\alpha v\beta 3$-integrin. The effects produced by **5** in both the humoral and cellular immune responses, presented in this paper, are similar to those evoked by

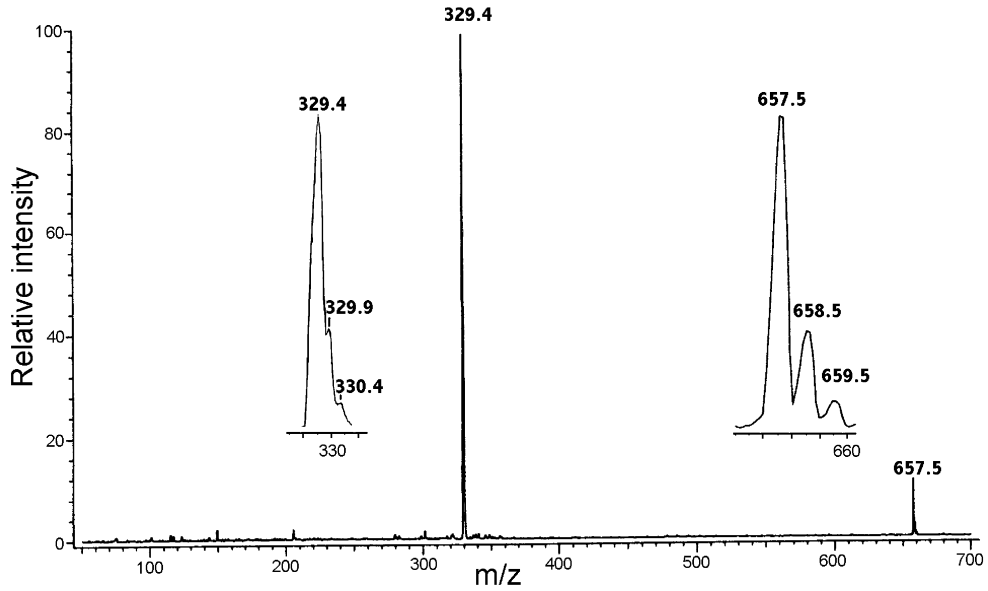

Figure 3. The electrospray mass spectrum of Arg-Gly-Asp-Arg-Gly-Asp.

The peaks at 329.4 and 657.5 represent different charge states of the same molecule. The isotopic distributions of both peaks are also presented.

H-Cys-Arg-Gly-Asp-Val-Tyr-Cys-OH (Szewczuk *et al.*, 1996b). In addition, the similar specificity of these two cyclic peptides toward integrins may indicate that the mechanism of the immunological action of these peptides is also similar.

It has been suggested that the binding site of $\alpha v\beta 3$ is narrower or more restricted than that of $\alpha IIb\beta 3$. Consequently, ligands that bind the former selectively tend to have no more than 6.7 Å between the C_β atoms of the Arg and Asp residues, whereas the optimun distance is in the range of 7.5–8.5 Å for $\alpha IIb\beta 3$ (Pfaff *et al.*, 1994). This indicates that $\alpha IIb\beta 3$ is less sensitive to variations in the RGD backbone structure and can accommodate a larger Arg–Asp distance than the $\alpha v\beta 3$ integrins.

We constructed a model of the nonapeptide fragment of HLA-DQ, based on mutational analyses of the HLA-DR crystal structure and utilizing our alignment (Szewczuk *et al,* 1996b). The model is characterized by a β-turn with the corners occupied by Gly^{168} and Asp^{169}, and the RGD sequence occupies the same positions as residues Arg^{166}, Ser^{167}, and Gly^{168}, respectively in the crystal structure of HLA-DR (Brown *et al.*, 1993). Consequently, the distance between the C_β atoms of Arg and Asp in HLA-DQ, critical for integrin recognition, should be similar to the distance between the C_β atom of Arg^{166} and *proS* hydrogen connected to the C_α atom of Gly^{168} in the HLA-DR molecule. The distance is around 6.5 Å, therefore the orientation of the arginine and aspartic acid residues in the RGD loop of the DQ molecule is favorable for interaction with $\alpha v\beta 3$. Interestingly, the distance between the C_β atoms of Arg^{166} and Glu^{169} in the β-HLA-DR molecule is also below 6.7 Å, although these residues are separated by two amino acids. All this may indicate that the synthesized peptide and HLA-DQ molecules may interact with integrins with a narrow binding site like $\alpha v\beta 3$.

The molecular modelling study as well as NMR and CD studies on peptide **5** performed by Burgess *et al.* (1996) strongly suggest that the molecule may adopt a β-turn conformation with a relatively short distance between the Asp and Arg side chains. Our conformational search points to one low energy conformer of peptide **5** in which the distance between the C_β atoms of the Arg and Asp residues is 6 Å. We compared this conformer with the X-ray crystal structure of HLA-DR (Brown *et al.*, 1993) to examine the possibility that peptide **5** may mimic the RGD loop of HLA class II. As can be seen in Fig. 1, the conformations of the backbones of the peptide and the 165–169 fragment are very similar. Despite the differences in the conformational angle values of the side chains, the general run of the peptide backbone of the tetrapeptide fragments is similar in both cases, which is evidenced by relatively small deviations of the positions of the peptide backbone atoms. Therefore, the peptide may easily mimic the RGD loop of HLA-DQ and block its interactions with potential receptors, possibly type $\alpha v\beta 3$ integrins. The high immunosuppressory activities of the peptide indicate that integrins may be involved in the mechanism of activation of lymphocytes.

The results presented here support our hypothesis that the HLA-DQ molecule can interact with some integrins on the cell surface. Substances that mimic the RGD loop may inhibit these interactions leading to immunosuppression. A better understanding of the immunological effects of RGD peptides could lead to the development of new immunosuppressive compounds.

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