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Original article

Design of dimeric peptides obtained from a subdominant Epstein-Barr virus LMP2-derived epitope

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Abstract – The latent membrane protein 2 (LMP2) is expressed in EBV-associated tumours. LMP2 is a target of HLA-A2 restricted EBV-specific CTL responses and consequently it may represent a good target for specific CTL-based immunotherapies. However, the efficacy of such therapy is limited by the poor immunogenicity of the protein that induces weak cytotoxic T lymphocyte (CTL) responses directed against the CLGGLLTMV (CLG) epitope. Indeed, the CLG peptide presents low affinity for HLA-A2 and does not produce stable complexes. Therefore we synthesized and tested CLG-dimeric analogues with the purpose of characterizing new compounds with the capacity to bind HLA-A2 molecules. By these studies we have identified a few peptides which, compared to the natural epitope, showed higher affinity for HLA-A2 molecules and superior capacity to form a complex. These dimeric peptides may have the potential to induce efficient CTL responses directed to the natural epitope. © 2000 Éditions scientifiques et médicales Elsevier SAS

dimeric analogues / Epstein-Barr virus / immunogenic peptides / solid phase synthesis / segment condensation

1. Introduction

Viruses, bacteria and protozoan parasites establish their infections inside the host's cells, where antibodies cannot reach them. The host's cells carry major histocompatibility complex (MHC) class I molecules on their surface. These MHC molecules bind and display oligopeptides, 8–10 amino acids long, deriving from processed proteins in infected cells. MHC-peptide complexes form the antigens that can be recognized by specific T-cell receptors (TCR) expressed on cytotoxic T lymphocytes (CTL). In this way CTL can identify and kill infected cells selectively, sparing healthy cells. This is a critical stage in the initiation of all immune responses, leading finally to the effective elimination of intracellular parasites.

The presentation of antigenic peptides bound to MHC class I molecules is a prerequisite for stimulation of cytotoxic T-cell responses. Molecular details of MHC/peptide association demonstrated that the peptide binding site is localized in a groove formed by the two α-helices lying across an eight-stranded β pleated sheet [1]. The groove accommodates peptides in an extended conformation and with free N- and C-terminal ends. Specific amino acid side chains interact with six defined pockets A to F. The main anchor residues, usually at positions 2 and 9, interact with pockets B and F, respectively [2–7]. All these interactions contribute to the formation of stable MHC/peptide complexes and at present, the general

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Abbreviations: CTL: cytotoxic T lymphocytes; DIPCDI: N,N'-diisopropylcarbodiimide; DMF: dimethylformamide; EBV: Epstein-Barr virus; EtOAc: ethyl acetate; Fmoc: 9-fluorenylmethoxycarbonyl-; GABA: 4-aminobutyric acid; HD: Hodgkin's disease; HLA: human leucocyte antigens; HOBt: N-hydroxybenzotriazole; HPLC: high-performance liquid chromatography; LMP2: latent membrane protein 2; MALDI-TOF: matrix-assisted laser desorption ionization time-of-flight; MHC: major histocompatibility complex; MeOH: methanol; NPC: nasopharyngeal carcinoma; PEG: polyethyleneglycol; PITC: phenyl isothiocyanate; RP: reverse phase; tBu: tert-butyl; TCR: T-cell receptor; TFA: trifluoroacetic acid; Trt: trityl; WSC: 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide.

Table I. Sequence of CLG and its dimeric analogues.

Number	Sequence
CLG	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH
1	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH
2	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-GABA-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH
3	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-NH-$(CH_2)_{10}$-CO-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH
4	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-Cys-Leu-**Ala**-Gly-Leu-Leu-Thr-Met-Val-OH
5	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-GABA-Cys-Leu-**Ala**-Gly-Leu-Leu-Thr-Met-Val-OH
6	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-NH-$(CH_2)_{10}$-CO-Cys-Leu-**Ala**-Gly-Leu-Leu-Thr-Met-Val-OH
7	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-Cys-Leu-Gly-**Ser**-Leu-Leu-Thr-Met-Val-OH
8	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-GABA-Cys-Leu-Gly-**Ser**-Leu-Leu-Thr-Met-Val-OH
9	H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-NH-$(CH_2)_{10}$-CO-Cys-Leu-Gly-**Ser**-Leu-Leu-Thr-Met-Val-OH

consensus is that stability of HLA/peptide complexes is important for determination of peptide immunogenicity [8–12].

In the present investigation we report modified epitopes of the H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH (CLG) peptide, an Epstein-Barr virus (EBV) epitope derived from the membrane protein LMP2 [13]. The CLG peptide represents a good target for the immunotherapy of EBV-associated malignancies since it is expressed and conserved in nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD) biopsies [14]. However, we found that the CLG peptide does not produce stable complexes with HLA-A2 and this may account for its low immunogenicity. We therefore synthesized and tested peptide analogues carrying amino acid substitutions at non-anchor positions to increase HLA/peptide stability. Among the analogues evaluated we identified two peptides CLAGLLTMV ([Ala^3]CLG) and CLGSLLTMV ([Ser^4]CLG) which showed higher affinity for HLA-A2 molecules and induced stable HLA/peptide complexes [15].

Now, attempting to improve the affinity of the subdominant CLG epitope, we prepared and tested for binding of dimeric peptides (*table I*). The new compounds consisted of two molecules of the original nonamer directly linked head to tail or conjugated through different spacers: the 4-aminobutyric acid (GABA) or the 11-aminoundecanoic acid (dimers **1**–**3**). Derivatives **4**–**9** contain the CLG peptide linked to the improved epitopes [Ala^3]CLG or [Ser^4]CLG.

To address the influence of epitope spacing and the presence of improved epitopes in the C-terminal heterodimer portions we tested all dimers for their ability to bind to HLA-A2 molecules and to produce stable HLA-A2/peptide complexes.

2. Chemistry

The dimeric CLG-derivatives **1**–**9** were synthesized by solid phase segment condensation (*figure 1*). This synthetic strategy utilized a unique N-terminal nonameric fragment for the preparation of all dimeric analogues.

Suitable NovaSyn® TGT [16] and Wang [17] resins were used to achieve the N- and C-terminal segments, respectively.

The stepwise synthesis was carried out by Fmoc chemistry using a continuous flow instrument with on-line UV monitoring (Milligen, Biosearch 9050).

The Fmoc-Val-Wang resin (0.2 g, 0.57 mmol/g) or Fmoc-Val-NovaSyn® TGT resin (2 g, 0.26 mmol/g) were swollen in DMF for 1 h and packed in the reaction column. Amino acids were coupled in a 4-fold excess using DIPCDI in the presence of the HOBt, always in 4-fold excess. The Fmoc group was cleaved with 20% piperidine–DMF solution. Side chain protecting groups were as follow: Ser and Thr, $tBu^{\bullet}$, Cys, Trt. Protected CLG peptide was cleaved from the acid sensitive resin by short (2 min) and repeated (10 times) treatments with 0.5% TFA in DMF.

This protected nonapeptide was then condensed to the appropriate linked resin segment (*figure 1*) in the presence of HOBt and WSC for 24 h.

Finally, each dimeric peptide was cleaved from the solid support by treatment with 10 mL of modified reagent B (88% TFA, 5% H_2O, 7% Et_3SiH) for 1 h at room temperature. The resin was removed by filtration and washed with TFA (2 × 1 mL), the filtrate and washing were combined, evaporated at 25 °C and the residue was triturated with ethyl ether (10 mL). The resulting products were collected and purified by preparative HPLC. Homogeneity of the purified CLG-dimeric peptides was assessed by analytical HPLC and structure verification

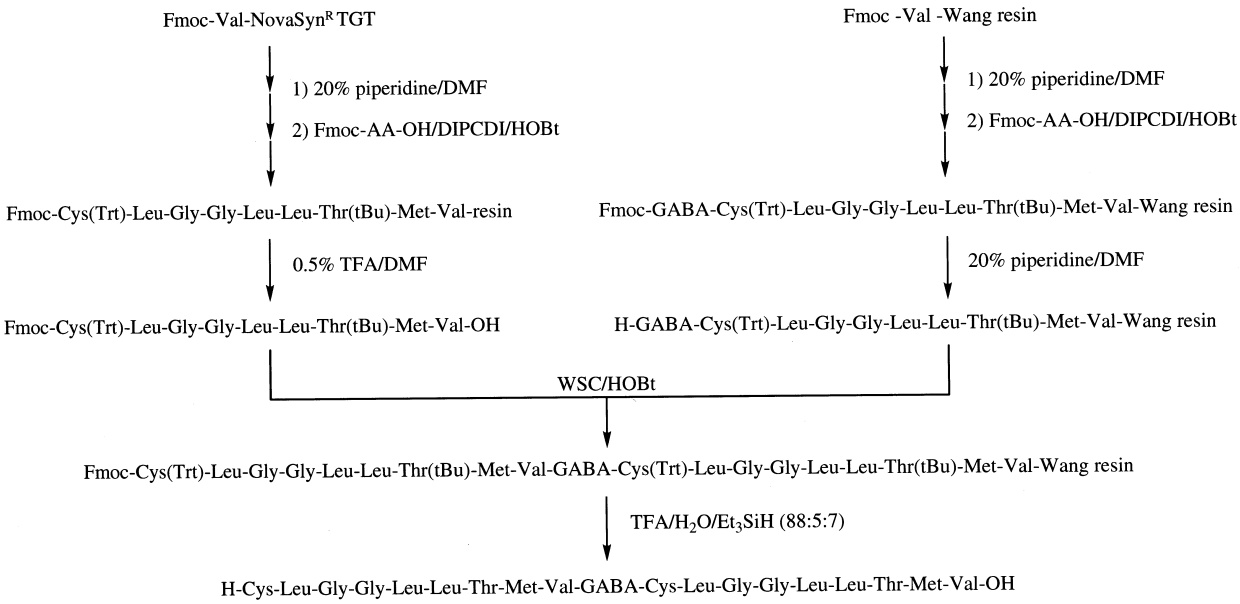

Figure 1. Solid-phase synthesis of CLG-dimeric analogue **2** by segment condensation.

was achieved by amino acid analysis and MALDI-TOF mass spectrometry. The physico-chemical data of CLG-analogues **1**–**9** are reported in *table II*.

3. Affinity analysis

All peptides were tested for their ability to associate with HLA-A2 molecules expressed at the surface of T2 cells (*figure 2*). The mutant T2 cells were treated at 26 °C for 18 h in serum-free medium to induce high levels of empty molecules at the cell surface, and then exposed to 1×10^{-4} M CLG-analogues at 37 °C for 1 h. The cells were extensively washed to remove the unbound peptide and the surface expression of HLA class I complexes was evaluated in kinetic experiments by immunofluorescence using the monoclonal antibody W6-32, which recognizes HLA class I molecules independently of the associate peptides.

4. Results and discussion

In this study we have prepared a series of homodimeric-CLG derivatives (**1**–**3**) and heterodimers (**4**–**9**) having, at the C-terminal portion, the improved [Ala^3]CLG and [Ser^4]CLG epitopes. In a first synthetic approach using

Table II. Physico-chemical and analytical data of CLG-dimeric analogues.

Number	M.p.	$[\alpha]_D^{20}$	HPLC	MS	Amino acid analysis							
	(°C)	(c = 1, DMF)	(t_R)	(MH^+)	Ala	Cys	Gly	Leu	Met	Ser	Thr	Val
1	174–178	–7.6	17.72	1765		1.89	4.08	5.87	1.74		1.92	2.00
2	153–156	–6.8	17.34	1881		1.79	4.12	6.14	1.89		1.85	2.00
3	146–149	–5.4	18.03	1978		1.86	4.05	5.76	1.78		1.88	2.00
4	175–177	–7.9	16.94	1809	1.05	1.91	3.09	5.91	1.88		1.79	2.00
5	168–171	–9.2	16.55	1895	1.01	1.85	2.98	5.83	1.81		1.84	2.00
6	158–162	–6.4	17.07	1992	1.04	1.93	3.13	6.05	1.90		1.80	2.00
7	195–199	–8.8	15.87	1825		1.78	2.88	6.21	1.85	0.93	1.93	2.00
8	182–184	–10.3	15.72	1911		1.84	2.97	6.11	1.73	0.98	1.91	2.00
9	170–174	–5.9	16.33	2008		1.88	2.90	5.93	1.76	0.93	1.89	2.00

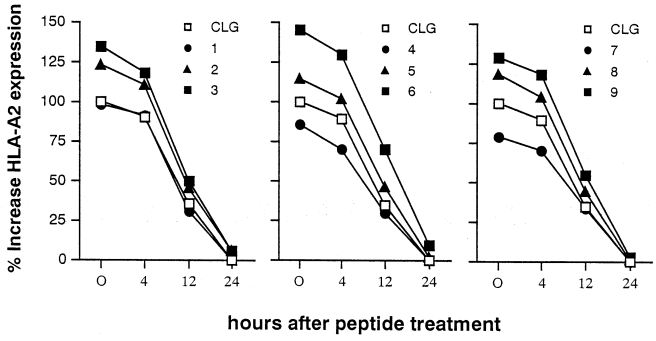

Figure 2. HLA-A2/peptide complex stability. T2 cells were cultured overnight at 37 °C in serum free medium containing 10^{-4} M of the indicated peptides. Cells were washed, treated with mitomycin C to avoid cell proliferation, divided in aliquots and maintained at 37 °C for kinetic experiments. At the indicated time points the surface expression of HLA class I molecules was detected by indirect immunofluorescence using the W6-32 mAb. Results are expressed as % increase of HLA class I expression calculated with respect to untreated T2 cells. Mean of four different experiments.

the usual solid phase step by step condensation, few sequence-related problems were encountered.

On-line monitoring of the deprotection and coupling and then crude product analysis were unsatisfactory. The spectrometric deblocking profile as chain elongation proceeded displayed an abnormally slow deprotection reaction after condensation of the eleventh and following residues (numbered in order of addition from the carboxyl terminus).

In order to overcome the above drawbacks the fragment condensation method depicted in *figure 1* was used to obtain CLG dimeric peptides. Furthermore, this synthetic approach can be strategically considered more advantageous. In fact, modifications only at the C-terminal part of **1**–**9** (*table I*) suggested a route in which the invariant N-terminal segment is first prepared and is subsequently condensed with the appropriate C-terminal portion, yielding target peptides. By this procedure, we have obtained CLG-analogues in good yields and without detectable racemization.

All dimeric epitopes were able to bind to HLA-A2 molecules, the derivatives **3**, **6** and **9**, carrying the 11-aminoundecanoic acid spacer, exhibited an increased affinity. The other analogues, constituted by nonameric units condensed head to tail or linked by GABA, associated with HLA-A2 molecules as the wild-type CLG epitope.

The affinity data prompted a favourable conformational change induced by a longer spacer and a minimal perturbation of the monomeric unit not involved in the HLA interaction.

In addition, the results reported in *figure 2* indicate that the HLA-A2/CLG-dimeric peptide complexes showed similar or better stability if compared to the CLG epitope. In fact, for the wild-type epitope, a net decrease in expression was detected after 4 h and all complexes were disrupted after 24 h. The derivatives containing the improved epitopes [Ala^3]CLG and [Ser^4]CLG at the C-terminus showed the same affinity as the corresponding homodimers, suggesting that the N-terminal sequence is directly implicated in the binding with HLA molecules.

In summary, the 11-aminoundecanoic acid containing heterodimers exhibited an improved affinity and stability for HLA-A2 molecules compared to wild-type CLG epitope. These CLG-analogues might be considered a good base for the development of new molecules, like dimers linked by a disulfide bridge and/or dimers where monomeric units are reversed, and which possess favourable structural requirements for HLA-A2 interaction.

5. Conclusion

Selection and presentation of CTL epitopes by HLA class I molecules can be studied in vitro by the use of synthetic peptides corresponding to naturally presented peptide epitopes. In addition, synthetic peptides corresponding to immunogenic epitopes may be used as vaccine components or to pulse specific antigen presenting cells to induce specific CTL responses in vivo [18].

The LMP2-derived CLG epitope may be regarded as a target of specific immunotherapies for the treatment of EBV-associated tumours, since it is a target of HLA-A2 restricted CTL responses and is conserved among EBV isolates including viruses present in NPC and HD cell biopsies. However, specific CTL therapies may be limited by the poor immunogenicity of this antigen. Because the clustering of TCR/MHC/peptide complexes apparently plays a critical role in the full activation of CTL responses, we attempted to design dimeric epitopes with the potential to trigger TCR dimerization/oligomerization. In spite of the fact that HLA class I molecules have a strong preference for binding 8–10 amino acid long peptides, we show here that the dimeric peptides derived from the CLG epitope maintain or even improve the capacity to bind to HLA-A2 molecules. These dimeric epitopes may be further studied for their immunogenicity since they are efficiently presented by the relevant HLA molecules.

6. Experimental procedures

6.1. General

Amino acid derivatives and solid support were purchased from Novabiochem AG (Laufelfingen, Switzerland). Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin Elmer (Vaterstetten, Germany) 141 polarimeter with a 10 cm water-jacketed cell. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were obtained using the HPG2025A mass spectrometer (Hewlett Packard, Palo Alto, CA, USA). HPLC analysis was performed on a Bruker (Karlsruhe, Germany) liquid chromatograph LC21-C instrument using a Vydac C 18 218 TP 5415 column (175 × 4.5 mm, 5 µm particle size) equipped with a Bruker LC 313 UV variable-wavelength detector; recording and quantification were accomplished with a chromatographic data processor (Epson computer FX80X7). Analytical determinations were carried out by a gradient made up of two solvents: A: 10% (v/v) acetonitrile in water; B: 60% (v/v) acetonitrile in water, both containing 0.1% TFA. The gradient program used was as follows: linear gradient from 0–100% B in 25 min at a flow of 1 mL/min. All analogues showed purity greater than 99% following analytical HPLC monitored at 220 nm. Preparative reversed-phase HPLC was carried out with a Water Delta Prep 3000 (Milford, MA, USA) using a Delta Pack C 18-300 A (30 mm x 30 cm, 15 µm, spherical). The gradient used was identical to that of analytical determinations. Chromatography was performed at a flow rate of 30 mL/min. Amino acid analyses were carried out using PITC methodology as the amino acid derivatization reagent (Pico-Tag, Waters-Millipore, Waltham, MA, USA). Lyophilized samples of peptides (50–l00 pmol) were placed in heat-treated borosilicate tubes (50 × 4 mm), sealed and hydrolysed using 200 µL 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 h at 150 °C. A Hypersil ODS column (250 × 4.6 mm, 5 µm particle size) was employed to separate the PITC-amino acid derivatives. TLC used precoated plates of silica gel F254 (E. Merk, Darmstadt, Germany) in the following solvent system: A: 1-butanol/acetic acid/H_2O (3:1:1); B: EtOAc/pyridine/acetic acid/H_2O (12:4:1.2:2.2); C: CH_2Cl_2/MeOH/toluene (8.5:1:0.5); D: $CHCl_3$/MeOH/benzene/H_2O (8:8:8:1). Ninhydrin 1%, fluorescamine and chlorine spray reagents were employed to detect the peptides.

6.2. Cell lines

The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T-cell line CEM [19] and was maintained in RPMI 1640 supplement with 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% heat inactivated foetal calf serum (HyClone, Utah, USA).

6.3. Detection of HLA-A*0201/peptide complex stability

Aliquots of 5×10^6 T2 cells were cultured overnight in 2 mL serum free AIM-V medium containing 1×10^{-4} M of the indicated peptides. Cells were then extensively washed, treated with mitomycin C (Sigma-Aldrich, Milan, I) to avoid cell proliferation, divided into aliquots in serum free AIM-V medium (Life Technologies, Milan, I) and maintained at 37 °C for kinetic experiments in AIM-V medium containing 1 µg/mL brefeldin A (Sigma-Aldrich) to block the egress of new class I molecules [20]. Surface expression of HLA class I molecules was detected by direct immunofluorescence using the mouse mAb W6-32, which recognizes HLA-A, -B and -C molecules irrespective of the associated-peptide. Mean log fluorescence intensity was measured with a FACS analyser. Data are expressed as % increase HLA class I expression calculated with respect to untreated T2 cells.

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