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Synthesis of Linear and Comb-like Peptide Constructs Containing up to Four Copies of a T Cell Epitope and their Capacity to Stimulate T Cells

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Abstract: Polypeptide constructs containing up to four copies of the T cell epitope 306-318 of influenza virus haemagglutinin have been synthesized on solid phase. Between the copies, a non-natural PEG-based spacer amino acid has been introduced. The oligomeric epitopes were analysed by RP-HPLC and ES-MS. The arrangement of the epitopes within the peptide constructs was either linear or comb-like. The proliferative response in a T helper cell assay induced by these oligomerized epitopes has been tested, showing that the linearly arranged epitopes are more effective than the comb-like oligomers. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: epitope oligomers; haemagglutinin; solid phase peptide synthesis; spacer; T cell response

INTRODUCTION

The recognition of MHC-bound peptidic epitopes by T lymphocytes plays a key role in the cellular immune response. MHC-class II glycoproteins are loaded with peptides inside cellular vesicles, and then migrate to the cell surface where they present peptides with defined sequence motifs [1] to T cells. The T cells recognize the complex between MHC and peptide with their T cell receptors [2]. The T cell response seems to be triggered by crosslinking of T cell receptors, as T cells are stimulated by divalent antibodies directed against their T cell receptors, but not by monovalent Fab fragments of these antibodies [3]. In the case of MHC-class II, the peptides can extend out both ends of the binding groove. If this extension contains further copies of the peptide, multiple MHC-class II molecules can be bound, and adjacent T cell receptors may be crosslinked. Hence, an immune response may be triggered at low peptide concentrations, as crosslinking of receptors on cell surfaces is a common mechanism of signal transduction [4,5].

The most prominent method for the preparation of peptides containing several copies of an epitope is the concept of multiple antigenic peptides (MAPs), where a matrix of lysine residues bears radially branching peptide epitopes (Figure 1) [6]. MAPs have been used as multivalent antigens for antibodies, and were particularly effective as antigens in ELISA [7,8]. For the use as immunogens, MAPs with

Abbreviations: Aca, 6-aminocaproic acid; EC, effective concentration; Fmoc-Atg-OH, α-(*N*-Fmoc-β-aminopropionyl)-ω-(carboxymethylcarbamoyl)-tetra(oxyethylene); Fmoc-Ats-OH, α-(*N*-Fmoc-β-aminopropionyl)-ω-(β-carboxypropionyl)-tetra(oxyethylene); FT-ICR-MS, Fourier-transform ion cyclotron resonance mass spectrometry; HA, haemagglutinin; HLA, human leukocyte antigen; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PEG, polyethylene glycol; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel-electrophoresis.

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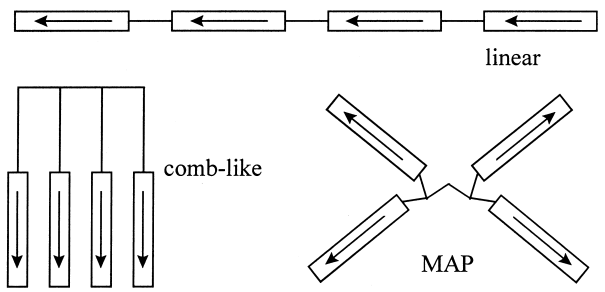

Figure 1 Schematic drawing of arrangements of synthetic epitope tetramers. Each box means an epitope, → indicates the direction C to N of the epitope.

four to eight copies of the epitope turned out to be optimal to obtain a strong immune response. However, MAPs are often heterogeneous synthetic products, and the high peptide density can also be a disadvantage, as residues close to the matrix may not be accessible for immune stimulation [9]. Oligomers of epitopes with comb-like arrangement [10,11] (Figure 1) are *in vitro* as effective as the MAP oligomers [12]. In the comb-like arrangement, each epitope is attached to a *linear* peptide segment. When this connection consists of a long spacer, the peptide density is not as high as in the MAP. Epitopes can also be arranged linearly, like a chain. Epitope oligomers with linear arrangement have been prepared recombinantly, and are potent stimulators of CD4 positive T cells [13]. In this study, it could also be shown that peptides containing a higher number of epitope copies induce a stronger response. SDS-PAGE proved that linear epitope oligomers can associate with more than one soluble HLA-DR1 molecule, supporting the mechanism of membrane protein crosslinking with respect to the MHC proteins.

The aim of the work presented here was the chemical synthesis of defined oligomers of the T cell epitope 306-318 of influenza virus haemagglutinin PKYVKQNTLKLAT (HA306-318) [14]. HLA-DR1 is the MHC class-II molecule with a high affinity to HA306-318 [15]. The response of CD4 positive HLA-DR1-restricted T cells to linear and comb-like arranged epitope oligomers was compared. For this purpose, the T cells were stimulated by PBMCs expressing HLA-DR1 in the presence of the synthetic epitope oligomers.

MATERIALS AND METHODS

Peptide Synthesis and Analysis

All amino acids, reagents and solvents were purchased from Merck (Darmstadt, Germany) and Novabiochem (Läufelingen, Switzerland). The spacer amino acids Fmoc-Ats-OH and Fmoc-Atg-OH were synthesized [16]. Peptides were built up on a multiple peptide synthesizer SMPS 350 (Zinsser Analytic, Frankfurt, Germany) with software Syro (MultiSynTech, Bochum, Germany), applying Fmoc/*t*Bu chemistry. For the preparation of linear epitope oligomers, 2-chlorotritylchloride resin (200 mg, capacity 1.0 mmol/g) was treated with Fmoc-Ats-OH (118 mg, 0.2 mmol) and *N*,*N*-diisopropylethylamine (61 μL, 0.4 mmol) in dichloromethane for 1 h. Methanol was added for another 30 min to cap unreacted trityl groups. Loaded resin (30 mg, loading ~0.5 mmol/g) was used for each peptide to be synthesized. In the standard cycle of the synthesizer, Fmoc-amino acids (tenfold excess) were activated *in situ* by DIC/HOBt. After 90 min, the Fmoc protective group was removed by treatment with piperidine/DMF (1:4) for 25 min. The resin was washed with DMF (6 ×) after each coupling and each deprotection step. The coupling of Fmoc-Atg-OH was performed either in the standard cycle of the synthesizer or in threefold excess for 10 h.

Synthesis of comb-like epitope trimers was achieved by attachment of Fmoc-Lys(Dde)-OH to Rink amide resin in the standard cycle of the synthesizer, followed by Fmoc-deprotection and stepwise synthesis of the oligopeptide segments Fmoc-Lys(Fmoc)-β-Ala-Aca-β-Ala and Fmoc-Lys(Fmoc)-Ser(*t*Bu)-Pro-Ser(*t*Bu)-Gly, respectively. Dde and Fmoc groups were removed by a solution of 3% hydrazine hydrate in DMF for 1 h. Stepwise coupling of Fmoc-Atg-OH and of the Fmoc-amino acids of the epitope was performed on the multiple synthesizer.

After completion of the syntheses, all resins were washed with dichloromethane (3 ×) and diethyl ether (3 ×) and dried *in vacuo*. The peptides were cleaved off and simultaneously side-chain deprotected with 1 mL of reagent K (82.5% TFA, 5% thioanisol, 2.5% EDT, 5% phenol and 5% water) for 3 h. The resin was removed and the peptide precipitated by addition of cold diethyl ether. The precipitated peptide was collected by centrifugation and resuspended in cold ether. This procedure was repeated two times. Then the product was dissolved in *tert*-butyl alcohol/water (4:1) and lyophilized.

A System-Gold HPLC apparatus (Beckman, Scan Ramon, USA) equipped with autosampler 507, pump unit 126 and diode array detector 126 was used to examine the purity of the peptides. The samples were analysed on a Nucleosil 300 C_{18} column (250×2 mm) using a linear gradient from 0% to 70% B (A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile; flow-rate: of 0.3 mL/min; $\lambda = 214$ nm). Semipreparative purifications were performed on a Waters 600 multisolvent delivery system equipped with a Waters Lambda-Max Model 481 LC spectrometer ($\lambda = 214$ nm) and a Nucleosil 300 C_{18} column (5 μm packing material, 250×8 mm; flow-rate: 4 mL/min). Purity of all peptides used in the T cell assay was $> 90\%$ according to RP-HPLC.

Electrospray mass spectra were obtained on a API III TAGA 6000E Triple Quadrupole mass spectrometer with ion spray ionisation (Sciex, Tornhill, Ontario, Canada). Fourier transform ion cyclotron resonance-MS was performed on a Bruker Daltonik Apex II 4.7 (Bruker Daltonik, Bremen) equipped with electrospray ionisation source (Analytica of Branford Inc., Branford, CT). Samples were dissolved in water/acetonitrile (1:1; $c = 10$ μM).

For comparison, oligomerized T cell epitopes were also produced in *E. coli*, utilizing recombinant techniques, as previously described [13]. Endotoxin and other impurities were removed from the polypeptide oligomers by separation on a reversed-phase C4-HPLC column (Vydac).

Cell Assay

Cell proliferation assays [13] were carried out with the HLA-DR1-restricted CD4 positive T cell line PD2. The line is specific for the HA306-318 epitope, and was generated by successive rounds of stimulation of peripherial blood monocytes (PBMC) with the peptide. The assays were performed in 96-well round bottom plates by using 50000 T cells and 150000 radiated (6000 rad) HLA-DR1-expressing PBMC per well in RPMI 1640 medium/10% human serum. The cells were incubated with the indicated amounts of antigen. [^{3}H]Thymidine (1 μCi/well) was added after 48 h, and the assay was harvested after 72 h, and counted in a microbetaplate reader (Wallac, Gaithersburg, MD).

RESULTS AND DISCUSSION

Linear peptide oligomers ([G-(HA306-318)-Atg]$_n$-G-(HA306-318)-Ats; $n = 0, 1, 2, 3$, Figure 2(a)) containing up to four copies of the HA306-318 epitope could be synthesized by SPPS, using Fmoc/*t*Bu strategy according to standard procedures. For the linear oligomers, 2-chlorotritylchloride resin was chosen. The resin was loaded with Fmoc-Ats-OH (Fmoc-NHCH$_2$CH$_2$COO(CH$_2$CH$_2$O)$_4$COCH$_2$CH$_2$CO-OH, Figure 2(c)). *C*-terminal attachment of Fmoc-Ats-OH was done in order to benefit of TentaGel-like

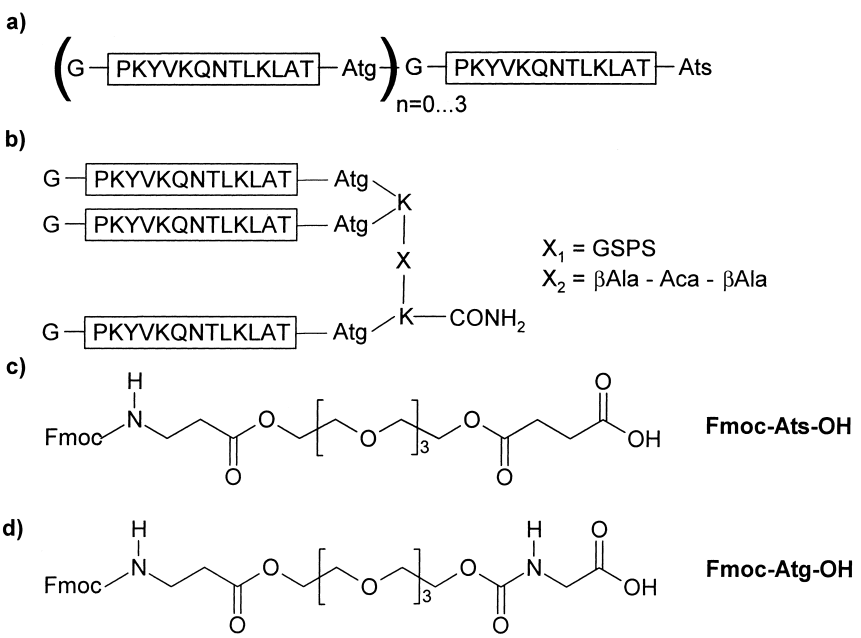

Figure 2 Structures of linear (a) and branched (b) epitope constructs (Aca = 6-aminocaproic acid) and of the spacer amino acids Fmoc-Ats-OH (c), Fmoc-Atg-OH (d).

properties. For the spacing between two epitope copies, a combination of Fmoc-Atg-OH (Fmoc-$NHCH_2CH_2COO(CH_2CH_2O)_4CONHCH_2COOH$, Figure 2(d)) and glycine were coupled. Here, Fmoc-Ats-OH was replaced by Fmoc-Atg-OH because Fmoc-Ats-OH led to imide formation when coupled to an amino group [16]. As Atg offers a backbone of 21 bonds, replacing at least seven amino acids, Atg-Gly replaces at least eight amino acids. As the backbone of Atg does not include an amide bond, this flexible and hydrophilic spacer should also be stable against enzymes cleaving amide bonds.

For the synthesis of comb-like epitope trimers (Figure 2(b)), two rather different peptide segments Fmoc-Lys(Fmoc)-β-Ala-Aca-β-Ala-Lys(Dde) and Fmoc - Lys(Fmoc) - Ser(*t*Bu) - Pro - Ser(*t*Bu) - Gly-Lys-(Dde) have been built up on Rink amide resin. The quasi-orthogonal Dde protecting group was chosen, as the handling is very convenient and can also be carried out during the automated synthesis. After simultaneous Dde and Fmoc deprotection, these backbones offer three amino groups of the Lys residues for attachment of Atg as spacer and subsequent synthesis of the HA306-318 peptide (Figure 3). Attachment of Atg and HA306-318 could be achieved by stepwise coupling of Fmoc-Atg-OH and Fmoc-amino acids.

Integrity of the products was analysed by RP-HPLC and ES-MS. The oligomeric epitopes were obtained in purities $>90\%$ according to RP-HPLC ($\lambda = 214$ nm) after a single preparative RP-HPLC purification step on a C_{18} column. RP-HPLC and ES-MS of the linear epitope tetramer [G-(HA306-318)-Atg]$_3$-G-(HA306-318)-Ats are shown in Figure 4. For this construct, with a molecular mass of about 7.5 kD consisting of 52 protein amino acids and eight spacer amino acids, the standard synthesis, purification and analysis methods come to a limit: while analytical RP-HPLC indicates a uniform product, ES-MS detects some impurities, but of rather low percentage. Assuming similar extinction coefficients and ionization probabilities, this fourfold epitope also shows a purity of more than 80%.

For the accurate mass determination of peptides with molecular mass >3 kD, an FT-ICR mass spectrum has been recorded. The high resolution of this spectrometer (up to 350000) allowed base-line separation of the isotopic pattern of, for example, the tenfold protonated ion $[M + 10H^+]$ of [G-(HA306-318)-Atg]$_3$-G-(HA306-318)-Ats (Figure 4(c)). According to the isotopic patterns, an exact mass determination of the monoisotopic mass of 7577.24 amu can be obtained in good agreement with the expected mass of 7577.20 amu for the formula $C_{341}H_{575}N_{83}O_{109}$ (calculated for ^{12}C, ^{14}N). Averaged masses are compared in Table 1.

The antigenicity of the epitope oligomers was evaluated by a T cell assay. Upon incubation of CD4 positive T cells with the HA306-318 peptide in the presence of antigen presenting cells expressing HLA-DR1, the T cells initiate an immune response indicated by proliferation and the release of cytokines [13]. Effectivity of the oligomers of HA306-318 in the T cell proliferation assay is judged in relation with monomeric HA306-318 (Figure 5). In order to compare the concentration of segments of HA306-318, the *mass* concentration has been used for the x-axis. We found that the epitope oligomers of HA306-318 induce a strong T cell response at a

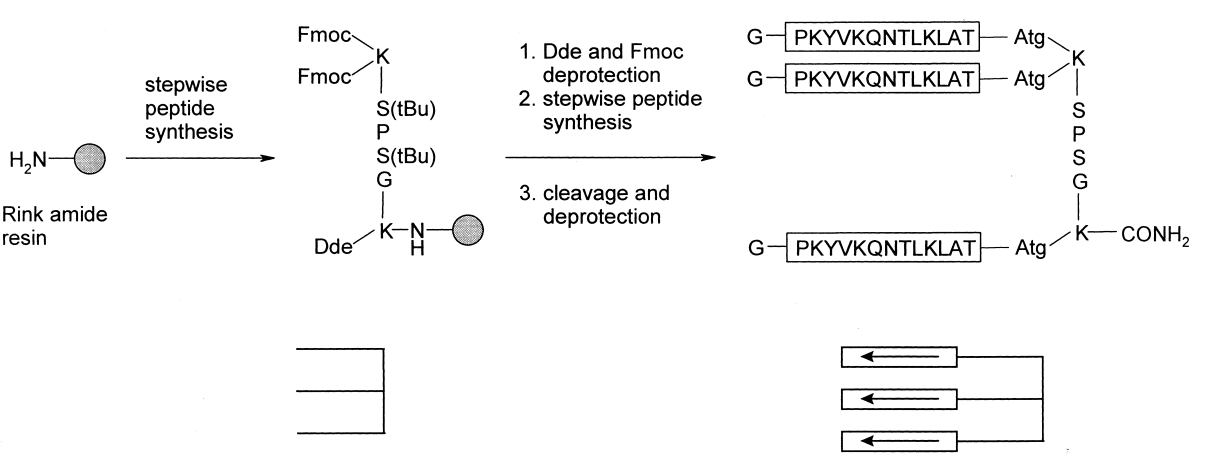

Figure 3 Solid phase synthesis of a comb-like epitope trimer.

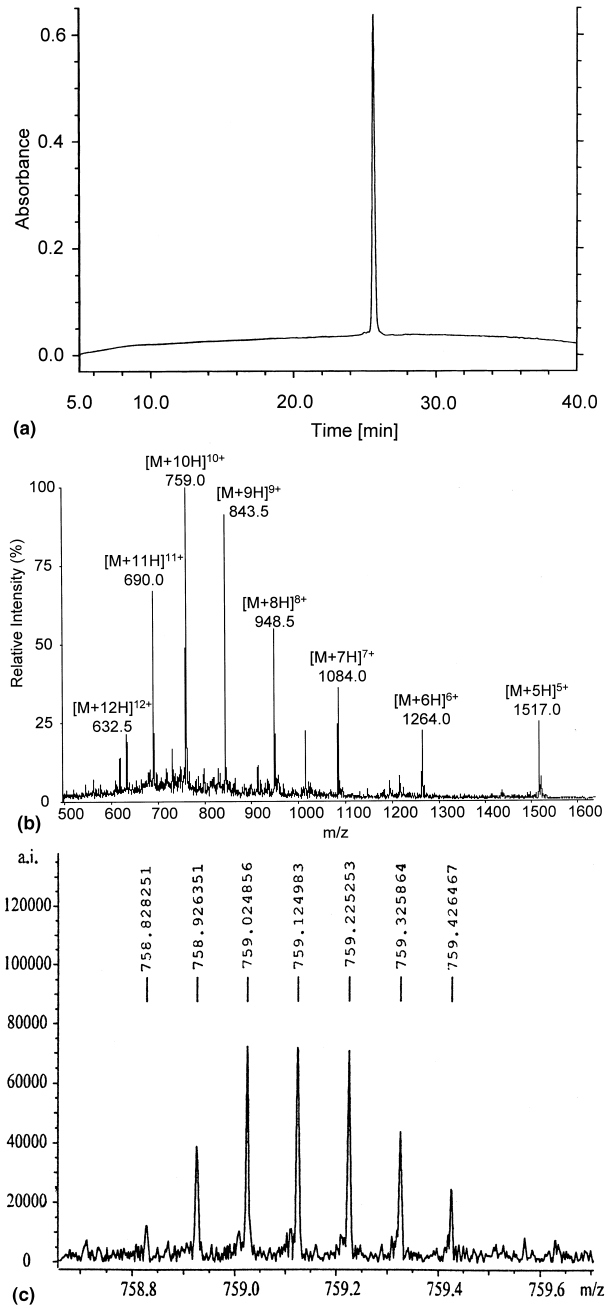

Figure 4 Analytical data of [G-(HA306-318)-Atg]$_3$-G-(HA306-318)-Ats: (a) analytical RP-HPLC (0% B to 70% B (A: water/0.1% (v/v) TFA; B: acetonitrile/0.1% (v/v) TFA), $\lambda = 214$ nm); (b) ES-MS; (c) isotopic pattern of the [M + 10H]$^{10+}$ signal, recorded with FT-ICR-MS (monoisotopic calc. 7577.20, exp. 7577.24).

lower dosage than HA306-318 itself. Stimulation is initiated at increasingly lower concentrations with every additional epitope copy in the peptide construct. This result confirms that antigenicity of a peptide antigen can be enhanced by linear oligomerization of the epitope. For the linear epitope tetramer, EC_{50} is decreased by two orders of magnitude. As a control experiment, the Tyr308 in the *N*-terminal epitope of the dimer [G-(HA306-318)-Atg]-G-(HA306-318)-Ats was substituted by an Asp residue during the synthesis. In this epitope, PK*D*-VKQNTLKLAT, an important anchor position for HLA-DR1 is deleted, virtually abolishing binding of this epitope to the MHC molecule [15]. This dimer G-PK*D*VKQNTLKLAT-Atg-G-PK*Y*VKQNTLKLAT-Ats required the same concentration for T cell activation as HA306-318 (not shown).

The improved response to epitope oligomers was also seen when the T cell line PD2 was replaced by other HLA-DR1-restricted T cells, such as the clone HA 1.7 (not shown; the clone HA 1.7 was generated by J. Lamb). Similar results were also obtained with HLA-DR4-restricted T cell hybridomas specific for HA306-318.

The PD2 T cell line and the HA 1.7 clone were also stimulated at lower concentrations by oligomeric epitopes of the formula (HA306-318-Sp)$_n$, $n = 4$, 8, 12, 16, 32, produced by recombinant techniques [13]. In a recombinantly produced oligomer, the spacer between two copies of the epitope has to consist of natural amino acids. Repetitive units of the amino acid sequence GPGG have been chosen. With these oligomeric epitopes, it could be shown that a relatively short spacer (12 amino acids, Sp = $(GPGG)_3$) results in stimulation at lower concentrations than longer spacers (24 or 36 amino acids, Sp = $(GPGG)_6$ or $(GPGG)_9$) [13]. The results for two epitope oligomers with the $(GPGG)_3$ spacer are depicted by reciprocal EC_{50} values (Figure 6(a)), where the lowest concentration required leads to the highest value. In comparison with the recombinant tetramer, the synthetic epitope tetramer is more effective. As the recombinant tetramer is of high purity also [13], this must be owing to the different types of spacers between the epitope copies, either because of differences in length, or because of the different nature of the spacer, resulting, for example, in an improved proteolytic stability of the synthetic oligomer.

According to Figure 6(b), the reciprocal EC_{50} value of the two comb-like epitope trimers is reduced to circa 50% when compared with the linear peptide construct containing three copies of the epitope. But both trimeric comb-like constructs are more active than the linear epitope dimer. The difference in EC_{50} between the comb-like oligomers (segments Lys-β-Ala-Aca-β-Ala-Lys-NH_2 or

Table 1 Experimental and Calculated Masses of Peptide Constructs Containing Copies of HA306-318

Peptide	n[a]	Formula	Calculated (M)	Experimental (M)
HA306-318		$C_{69}H_{118}N_{18}O_{19}$	1503.8	1503.9
G-(HA306-318)-Ats	0	$C_{86}H_{146}N_{20}O_{26}$	1908.2	1908.5
G-PK*D*VKQNTLKLAT-Atg-G-(HA306-318)-Ats		$C_{166}H_{285}N_{41}O_{56}$	3751.3	3751.5
G-(HA306-318)-Atg-G-(HA306-318)-Ats	1	$C_{171}H_{289}N_{41}O_{55}$	3799.4	3799.3
[G-(HA306-318)-Atg-]$_2$G-(HA306-318)-Ats	2	$C_{256}H_{432}N_{62}O_{82}$	5690.6	5690.4
[G-(HA306-318)-Atg-]$_3$G-(HA306-318)-Ats	3	$C_{341}H_{575}N_{83}O_{109}$	7581.8	7581.8
G-(HA306-318)-K[Atg-(HA306-318)-G]-SPSG-K[Atg-(HA306-318)-G]-NH_2		$C_{280}H_{476}N_{72}O_{89}$	6275.3	6275.4
G-(HA306-318)-K[Atg-(HA306-318)-G]-βAla-Aca-βAla-K[Atg-(HA306-318)-G]-NH_2		$C_{279}H_{477}N_{71}O_{86}$	6202.3	6202.7

[a] Number of repetitions of HA306-318, see also Figure 5.

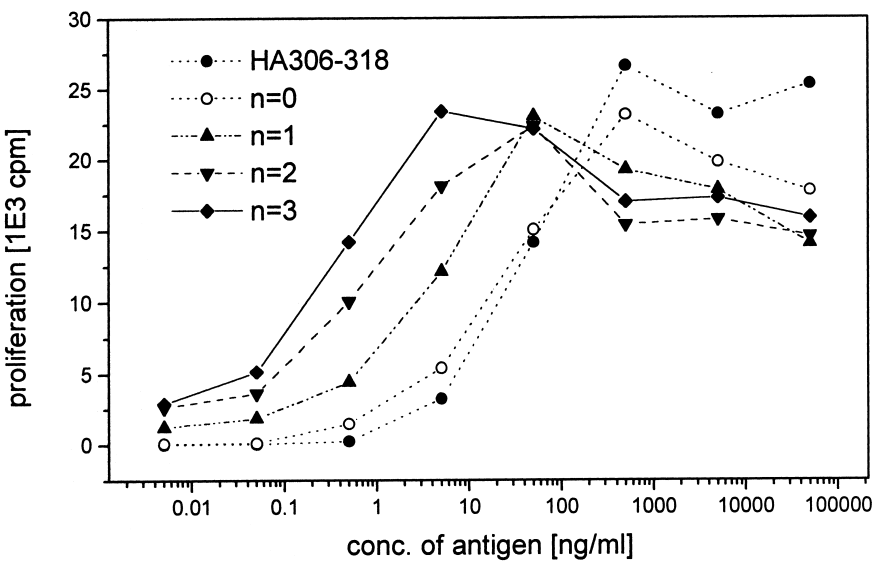

Figure 5 Proliferation of the T cell line PD2 challenged with HA306-318 or [G-(HA306-318)-Atg]$_n$-G-(HA306-318)-Ats (n = 0, 1, 2, 3) in the presence of HLA-DR1 expressing PBMCs as antigen presenting cells. The experiments were carried out in duplicates.

Pro-Ser-Gly-Lys-NH_2) lies within the experimental error of the T cell assay. As a result, it can be concluded that stimulation by the linear epitope trimer is superior to stimulation by a comb-like trimer. As the *in vitro* reactivity of comb-like and MAP oligomers was reported to be identical [12], our linear arrangement is also more effective than the MAP. A reason for this result could be that the chain-like arranged linear oligomers offer the highest degree of freedom to associated proteins. Therefore, linear epitope oligomers may allow the MHC class-II molecules to adopt favourable positions for crosslinking, leading to the initiation of the signal transduction through the TCR. The less flexible MAP arrangement may prevent some of the epitope copies to bind to MHC molecules which are only mobile within the two-dimensional cell membrane, and might also cause some steric hindrance for the interaction of the TCR with the MHC/peptide complex. A potential disadvantage of the linear epitope oligomers may be that enzymatic cleavage near the centre of the construct would lead to two fragments with only half the number of epitope copies of the original, whereas cleavage of one epitope copy in MAP or comb-like arrangements only reduces the number of copies in the construct by one.

For the preparation of T cell epitope oligomers, solid phase organic synthesis and recombinant

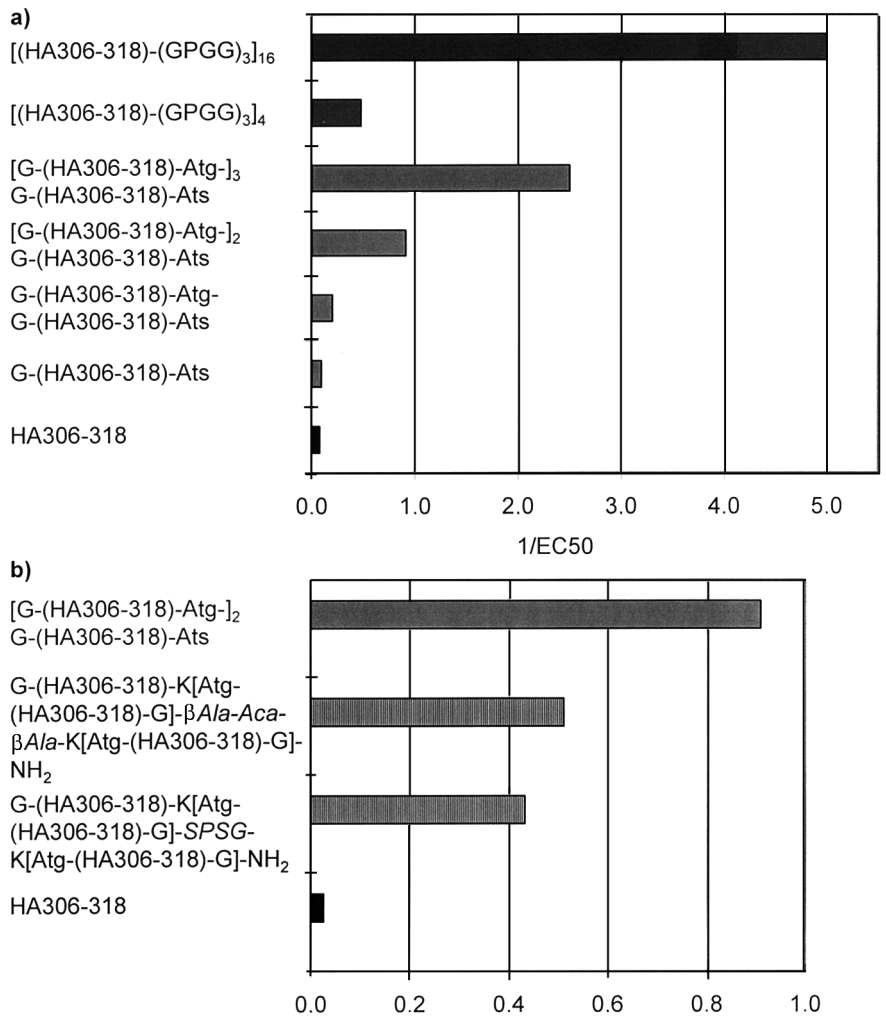

Figure 6 Reciprocal EC_{50} values for the stimulation of the T cell line PD2 by (a) synthetic and recombinant linear epitope oligomers of the T cell epitope HA(306-318); (b) synthetic epitope trimers in linear or comb-like arrangement. The experiments were carried out in duplicates. The EC50 concentration describes the concentration of antigen, which triggers half-maximal proliferation of the respective T cell.

techniques were complementary. Recombinant techniques prevail when a high, but distinct, number of copies of an epitope is to be included in a polypeptide or protein. On the other hand, the biological activity of T cell epitope units linked by the stable and flexible PEG-based amino acid spacer was significantly higher than in the recombinant constructs with more vulnerable amino acid spacers. Furthermore, the recombinant oligomer constructs are produced in bacteria and their purification from impurities arising from the expressing microorganism is cumbersome. With regard to the latter aspects, the synthetic approach is superior.

CONCLUSION

We were able to prepare peptides containing one to four copies of the influenza virus haemagglutinin epitope 306-318 in linear or comb-like arrangement with non-peptidic spacers between the copies. The synthesis of such constructs can be completely run in an automated multiple synthesizer followed by a one-step RP-HPLC purification. *In vitro*, the immune response to the linear epitope tetramer was initiated at an epitope concentration two orders of magnitude lower than required for the epitope. Furthermore, the linear arrangement is superior to a comb-like arrangement or an MAP. Oligomerized

epitopes are also attractive tools for diagnostic purposes, as their increased avidity can lead to a more stable labelling of MHC molecules.

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