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Peptide Based Vaccine Design: Synthesis and Immunological Characterization of Branched Polypeptide Conjugates Comprising the 276–284 Immunodominant Epitope of HSV-1 Glycoprotein D

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Abstract: The importance of the length and conjugation site of a protective epitope peptide (^{276}SALLEDPVG^{284}) from glycoprotein D of herpes simplex virus in branched polypeptide conjugates has been investigated. A new set of peptides, with a single attachment site and truncated sequences, was prepared. The immunogenicity of conjugates and the specificity of antibody responses elicited were investigated in BALB/c, C57/Bl/6 and CBA mice. It was found that the covalent coupling of the peptide comprising the 276–284 sequence of gD through its Asp residue at position 281 did not influence the immunogenic properties of the epitope, while involvement of the side chain of Glu at position 280 almost completely abolished immunogenicity. These results clearly indicated that the conjugation site of the epitope peptide influenced the intensity and specificity of antibody responses. Comparison of the immunological properties of conjugates containing truncated gD peptides revealed the presence of two epitopes within the 276–284 region. One of the proposed epitopes is situated at the *N*-terminal (276–281) region, while the other is located at the *C*-terminal end of the sequence (279–284). Binding data demonstrated that some of the peptides comprising these epitopes induced gD-specific responses in their conjugated form and also elicited an immune response that conferred protection against lethal HSV-1 infection. The correlation of peptide- and gD-specific antibody responses with the protective effect of the immune response is discussed.

Keywords: epitope-conjugates; synthetic antigens; HSV-1 epitope peptide; protection against HSV-1 infection; antibody recognition

Abbreviations: Abbreviations for amino acids and their use follow the revised recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, entitled Nomenclature and Symbolism for Amino Acids and Peptides. *Eur. J. Biochem.* 1984; **138**: 9–37. All chiral amino acids are of the L-configuration. AK, poly[Lys(DL-Ala$_m$)]; CFA, complete Freund's adjuvant; DP$_n$, number average degree of polymerization; EIA, enzyme immunoassay; gD, glycoprotein D; HSV-1, herpes simplex virus serotype 1; ICFA, incomplete Freund's adjuvant; i.p., intraperitoneal; s.c. subcutaneous.

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INTRODUCTION

In order to investigate the role of a carrier in inducing epitope-specific, immune responses against proteins, artificial antigens with polymeric branched polypeptide carrier [1,2] or α-conotoxin host protein [3,4] and B-cell epitopes of viral origin have been designed. It has been concluded that peptide immunization can result in the generation of virus-specific antibodies if the synthetic peptide mimics the orientation and conformation of the epitope within the original native protein [5,6].

For the present studies, antibody epitope sequences were selected from glycoprotein D of herpes simplex virus. Herpes simplex virus-1 (HSV-1) and HSV-2 are members of the herpes virus subfamily of enveloped viruses that infect both humans (HSV-1, HSV-2) and animals (pseudorabies virus, bovine herpes virus) [7]. The virion envelope of HSV contains at least 10 glycoproteins [8]. Glycoprotein D (gD) is a highly conserved glycoprotein with >98% identity among HSV strains, and represents a major target of both humoral and cellular immune responses of the human host [9]. A large number of gD-specific monoclonal antibodies exhibit high virus-neutralizing activity [10]. Glycoprotein D of HSV-1 is a glycoprotein of 369 residues in length with an *N*-terminal ectodomain of 316 residues and with three *N*-linked oligosaccharide attachment sites. The x-ray crystal structure of a soluble ectodomain of gD, truncated at residue 285, was published recently [11].

In three regions of gD (1–44, 260–314, 340–369), continuous B cell epitopes were identified using monoclonal antibodies and HSV seropositive sera [reviewed in 12]. Based on prediction analysis of the primary structure, the 268-284 region was selected for further epitope mapping studies. Peptides covering this region were prepared, conjugated to the branched polypeptide poly[Lys(DL-Ala_m)], (AK) where $m \sim 3$, and used as synthetic immunogens to analyse the antibody response, and for protection experiments in HSV-1 infected BALBc and C57/Bl/6 mice. These studies indicated the presence of a protective antigenic determinant at the *C*-terminal part of this region [13–18]. These results were obtained with AK-conjugates in which the peptide ^{276}SALLEDPVG^{284} (276–284) was connected at position 280 or 281 through the ω-COOH group of Glu or Asp, respectively.

In order to analyse the importance of the conjugation site of the peptide epitope in (276–284)-AK conjugates on immunogenicity and antibody specificity, we prepared a new pair of compounds with single conjugation sites. For further identification of the location and minimal size of epitope(s) within the 276–284 sequence, we also investigated the immunological properties of the polypeptide conjugates with truncated peptides corresponding to the 276–284 region. Here we report on the synthesis and the immunogenicity of the conjugates, as well as on the fine specificity of antibodies and the protective effect of immune responses induced by the gD-derived peptide-AK conjugates.

MATERIALS AND METHODS

Materials

The solvents acetonitrile (MeCN), dichloromethane (DCM), *N*, *N*-dimethylformamide (DMF), dimethylsulphoxide (DMSO) were purchased from Reanal, Hungary. Other chemicals, trifluoroacetic acid (TFA), 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yl-oxy-tris-dimethylamino phosphonium hexafluorophosphate (BOP), *N*, *N*′-diisopropylcarbodiimide (DIC), *N*, *N*′-dicyclohexylcarbodiimide (DDC), *N*, *N*-diisopropylethylamine (DIEA); *m*-cresol, 1,2-ethanedithiol (EDT), trimethylsilyl trifluoromethanesulphonate (TMSOTf), anisole and thioanisole, were from Fluka, Buchs, Switzerland. CMC, *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide methyl *p*-toluene sulphonate was from Sigma, Budapest, Hungary. Boc-amino acid derivatives were purchased from Novabiochem, Laufelfingen, Switzerland. 4-methyl benzhydrylamine resin (1.2 mequiv/g) and Merrifield resin (0.45 mequiv/g) were from Bachem, Switzerland. Truncated glycoprotein D (gD) from HSV type 1 containing the 1–319 amino acid residues was produced in Sf21 cells, and was a kind gift of Professors Systke Welling-Wester and Gjalt W. Welling, Department of Medical Microbiology, University of Groningen, The Netherlands.

Synthesis

Synthesis of HSV-1-gD peptides. All the peptides were synthesized by solid phase methodology on 4-methylbenzhydrylamine or Merrifield resin as described [18]. Briefly, Boc/Bzl strategy was utilized for the preparation of peptide-amides on 4-methylbenzhydrylamine resin (1.2 mequiv/g). Activation and coupling were performed using the DCC/HOBt method, in DCM/DMF (4 : 1 v/v, except for Gln and Asn where 1 : 4 v/v was applied). Asp,

Glu and Ser side chains were protected with Bzl groups. The coupling efficacy was monitored by ninhydrin [19] or bromophenol blue [20] assays. Boc-groups were removed by 35% TFA in DCM, while neutralization was performed with 10% DIEA in DCM. After removal of the *N*-terminal Boc-group, peptides were deprotected and cleaved from the resin with 1 M TMSOTf-thioanisole/TFA mixtures containing EDT and *m*-cresol. After gel filtration on a Sephadex G-25 M column in 10% acetic acid, the crude products were analysed and purified by reverse phase HPLC as described below.

Synthesis of epitope peptide – branched polypeptide (AK) conjugates. Peptides were covalently attached to poly[Lys(DL-Ala_m)], AK by using a water-soluble *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide methyl *p*-toluene sulphonate (CMC) as described earlier [14] or by *in situ* active ester method applying the BOP reagent [21]. Fifteen mg (20.5 μmol) AK was dissolved in 1 ml deionized water, and this solution was diluted with 3 ml DMF. H-SALLENPVG-NH_2 (9.2 mg, 10.25 μmol) (0.5 mol calculated for side chains of the polymer) dissolved in 3 ml DMF was added to the solution of AK. Following the addition of 9.1 mg (20.5 μmol) BOP reagent in 1 ml DMF, the pH of the reaction mixture was adjusted to pH 7.5 with *N*, *N*-diisopropylethylamine (477 μl, 82.0 μmol). The mixture was stirred overnight at room temperature. The solution was placed in Visking tubing (molecular mass cut-off 12000–14000 Da) and dialysed extensively against deionized water for 3 days. Finally, the conjugate was isolated by lyophilization. AK-conjugate with H-SALLQDPVG-NH_2 was also prepared according to this protocol.

Analysis

High-performance liquid chromatography. Analytical RP-HPLC was performed using a Delta Pak C_{18}, column (300 × 3.9 mm I.D., with 15 μm particle and 300 Å pore size, Spherical, Nixon Waters Ltd, Tokyo, Japan) as a stationary phase. The following linear gradients were applied: 10%–40% B in 35 min; 30%–95% B in 30 min; 5 min 0%, 30 in 35% B or 5 min 5%, 30 min 55%. As eluent A 0.1% TFA in water and as eluent B 0.1% TFA in acetonitrile–water (80 : 20, v/v)] were used as the mobile phase at a flow rate of 1 ml/min, at ambient temperature and with detection at $\lambda = 215$ nm. Purification of crude peptides was performed on a Delta Pak C_{18}, column (7.8 mm × 30 cm; 15 μm silica, 300 Å pore size) and using gradient elution with the same eluents. Homogeneity of the pure peptides was confirmed by analytical RP-HPLC.

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

Fast atom bombardment mass spectrometry (FAB-MS). Fast atom bombardment mass spectrometry measurements were carried out on a Fisons (UK) VG-ZA-2SEQ tandem mass spectrometer equipped with a Cs^+ ion gun (30 keV). The peptide samples were dissolved in DMSO and mixed with a glycerol matrix.

Immunoreactivity

Immunization. C57/B1/6 (H-2^b, Igh-1^b), CBA (H-2^k, Igh-1^e) and BALB/c (H-2^d, Igh-1^a) mice were immunized with equimolar amounts of peptides, calculated on the basis of the molar substitution ratio corresponding to the AK-conjugated peptide (Table 2). The first injection was administered s.c. in complete Freund's adjuvant (CFA) with conjugates corresponding to 0.03 mM HSV-1 peptide on day 0. Three weeks later (day 21), the primed mice were boosted with the same amount of antigen emulsified in incomplete Freund's adjuvant (ICFA). A third challenge was given in PBS on day 35. Blood samples were taken as indicated in the Figure legends.

Detection and quantification of the antibody response. Detection of antigen-specific serum antibodies was performed by solid phase indirect enzyme immunoassay (EIA) using antigen-coated plates as described previously [16]. Recombinant gD, AK or peptide-AK conjugates were coated on the surface of PVC plates at 10 μg/ml concentration. After the washing and blocking procedures, graded dilutions of the immune sera were added. The bound antibodies were detected by horseradish peroxidase-labelled anti-mouse IgG antibodies (Sigma, St Louis, MO). Preadsorption of diluted sera (1:100) was performed by incubating them at 37 °C , for 2 h on carrier-coated plastic surfaces (96-well EIA microplates (Greiner, Germany) pretreated with 100 μg/ml AK) and subsequently titrated for peptide specific antibodies as described above.

Protection experiments. Mice were preimmunized with conjugates as described above. Challenge with infectious HSV-1 virus (strain KOS), grown in Vero cells (Flow Laboratories, UK), was carried out 10 weeks after the last conjugate challenge (week 15 of the experiment) by an i.p. injection with a dose of $100xLD_{50}$ determined in a preliminary experiment performed with CFLP mice.

RESULTS AND DISCUSSION

Synthesis and Chemical Structure of HSV-1-gD Peptides and Their Conjugates with Branched Polypeptide

Seven peptides related to the sequence of (276–284) of HSV type 1 glycoprotein D were prepared by solid-phase peptide synthesis. The amino acid composition of the peptides was verified by amino acid analysis and FAB mass spectrometry. Peptides were characterized also by their R_t values determined by RP-HPLC.

Peptide (276–284), which has been used in previous studies, has two free carboxyl groups utilized for conjugation reaction with the carrier molecule [16]. The two new derivatives reported here are analogous to the native fragment, but they contain only a single carboxyl group. In order to achieve this in peptide SALLENPVG (N(276–284)) the Asp^{281} residue was substituted by Asn, while in peptide SALLQDPVG (Q(276–284)) Gln replaced Glu^{280}. These peptides were used for covalent coupling to branched polypeptide, to produce conjugates with a single and well-defined attachment point. These AK-conjugates contain multiple copies of uniformly oriented oligopeptides N(276–284) or Q(276–284).

For identification of the smallest segment(s) in the 276–284 region responsible for the HSV-1 gD specific antibody response [16], four peptides containing truncated *N*-terminal (ALLEDPVG (277–284), LEDPVG (279–284) and DPVG (281–284)) or *C*-terminal (SALLED (276–281)) were also synthesized. The structural characteristics of peptides are summarized in Table 1. All the seven gD peptides were attached, by carbodiimide [16] or BOP *in situ* active ester [21] coupling, to the branched polypeptide poly[Lys(DL-Ala_m)] (AK) containing free α-amino groups. In order to avoid multiple attachments of carboxyl groups of 276–284 peptide and its shortened variants, they were activated with a 1.5 molar access of coupling reagent. This procedure resulted in iso-amide linkage formation between a ω-carboxyl group of the gD peptide (the side chain of Asp in position 281 or Glu in position of 280) and the α-amino group of a branch terminal alanine residue. These AK-conjugates contain multiple copies of uniformly or randomly attached oligopeptide at position 281 (DPVG) or at positions 280 or 281 (ALLEDPVG, LEDPVG or SALLED) oligopeptides. In the former group, the tetrapeptide is connected with the carrier through Asp^{281}, while the latter group of conjugates could have gD peptides linked by both Glu^{280} and Asp^{281} residues. Under these conditions no precipitate was detected during the synthesis. The amino acid composition, and average molar substitution ratio of conjugates (estimated from $\overline{DP}_n$ of polylysine and from the amino acid

Table 1 Structure and Analytical Data of Synthetic gD-1 Peptides

Compound	Code	M[H^+][a]		R_t[min][b]	Amino acid analysis found[c]							
		Calc.	Found		D/N	P	S	G	A	L	V	E/Q
^{276}SALLEDPVG^{284}	(276–284)	899.5	899.3	27.0[1]	1.0	0.95	0.9	1.0	0.99	2.03	1.05	1.05
^{276}SALLENPVG^{284}	N(276–284)	898.5	898.7	18.8[3]	0.96	0.99	1.03	1.09	1.09	2.01	1.02	0.91
^{276}SALLQDPVG^{284}	Q(276–284)	898.5	898.5	19.0[3]	1.04	0.9	0.95	1.0	1.04	2.03	0.96	1.11
^{277}ALLEDPVG^{284}	(277–284)	812.4	812.4	19.3[4]	0.97	1.0		1.04	1.1	1.98	0.94	1.03
^{279}LEDPVG^{284}	(276–284)	628.3	628.3	15.1[3]	0.98	0.95		1.01		1.03	0.97	1.07
^{281}DPVG^{284}	(276–284)	386.2	386.2	11.9[2]	1.02	0.98		1.02			0.98	
^{276}SALLED^{281}	(276–284)	647.3	647.3	17.1[1]	0.98		0.94		1.01	2.0		1.1

[a] Determined by FAB-MS.
[b] Column: Delta Pak C_{18}, 300 Å, 15 μm (300 mm × 3.9 mm I.D.), linear gradient elution, eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile/water (80-20, v/v), flow rate: 1 ml/min, gradient: [1]10%–40% B in 35 min, [2]5 min 0%, 30 in 35%, [3]5 min 5%, 30 min 55% or [4]0 min 30% B 30 min 95% B, $\lambda = 214$ nm detection.
[c] Acid hydrolysis (6 M HCl, 110 °C, 24 h).

Table 2 Characteristics of gD-1 Peptide–AK Conjugates

Conjugate	Code[a]	$\overline{M}_w$ (+/− 5%)	SR[b] (%)	Molar ratio of gD-1 peptide: AK (mol/mol)	gD-1 peptide: conjugate (mmol/mg) × 10^{-1}
poly[L-Lys(DL-Ala_m)]	AK	43 500	–	–	–
(^{276}SALLEDPVG^{284})-AK	(276–284)-AK	82 200	35.0[c]	42.0	5.11
(^{276}SALLENPVG^{284})-AK	N(276–284)-AK	87 900	40.4[d]	48.5	5.51
(^{276}SALLQDPVG^{284})-AK	Q(276–284)-AK	72 700	26.3[d]	31.6	4.34
(^{277}ALLEDPVG^{284})-AK	(277–284)-AK	78 000	34.5[c]	41.4	5.31
(^{279}LEDPVG^{284})-AK	(276–284)-AK	61 900	23.2[c]	27.8	4.50
(^{281}DPVG^{284})-AK	(276–284)-AK	55 400	23.8[d]	28.6	5.15
(^{276}SALLED^{281})-AK	(276–281)-AK	84 500	51.6[c]	61.9	7.33

[a] Based on one letter symbols of alanine and lysine and codes of gD-1 peptides.
[b] Average substitution ratio. Calculated from the number average degree of polymerization ($\overline{DP}_n$ = 120) of poly[L-Lys] and of the side chain composition as described in the Materials and Method section.
[c] CMC conjugation.
[d] BOP conjugation.

composition of the monomer unit) are summarized in Table 2.

The Effect of Conjugation Site in the Peptide Epitope on Immunogenicity and Antibody Specificity of the (276–284)-AK Conjugate

In this set of experiments, the role of conjugation site in inducing epitope-specific immune responses was investigated. The peptides (276–284), N(276–284) or Q(276–284) were attached to the branched polypeptide AK and used as immunogens in three inbred mouse strains. The intensity and specificity of polyclonal antibody responses (raised against the (276–284)-AK conjugate, the AK carrier and the free peptide covering the 268–284 sequence of gD) were studied. Control mice were treated by the same protocol, except PBS was used instead of the conjugates (Figure 2). Peptide-, conjugate- and carrier-specific antibody responses were measured by EIA using (276–284)-AK conjugate (Figure 2A) and free 268–284 peptide (Figure 2B) as target antigens. The results showed differences in the intensity of antibody responses developed in CBA, C57/Bl/6 and BALB/c mice. The carrier- (AK) and peptide- (268–284) specific antibody titres were low (<100), but higher than those detected in control mice of all three strains. In contrast, the intensity of (276–284)-AK conjugate induced IgG responses were mouse strain dependent. Based on the titre values obtained on solid phase bound conjugate (Figure 2A) or peptide (Figure 2B), the rank order of responsiveness was as follows: BALB/c ≫ C57/Bl/6 > CBA.

Considering these results, BALB/c mice were used for comparative analysis of fine specificity of polyclonal antibodies elicited by AK-conjugates containing uniformly (N(276–284), Q(276–284)) or randomly attached ((276–284)) oligopeptides (Figure 1). Sera obtained from mice immunized with the (276–284)-AK, N(276–284)-AK and Q(276–284)-AK conjugates were preabsorbed on solid phase bound AK to eliminate carrier-specific antibodies and then studied by EIA on conjugate- and AK-coated plates. The results summarized in Figure 3 show differences in the fine-specificity pattern of antibodies to the three conjugates. The (276–284)-AK conjugate induced polyclonal antibodies which bind to both the N(276–284)-AK and Q(276–284)-AK conjugates (Figure 3A). Antibodies of N(276–284)-AK immunized animals did not recognize the conjugates (276–284)-AK and Q(276–284)-AK on the plate (Figure 3B). The serum of Q(276–284)-AK immunized mice reacted with solid phase bound (276–284)-AK, but not with N(276–284)-AK (Figure 3C).

The fine specificity of antibodies of (276–284)-AK immunized mice (Figure 3A) indicated that the peptide 276–284 is present in the conjugate in two forms. Peptide 276–284 is attached through the side chain of Asp at position 281 or of Glu at position 280. Considering the reaction conditions, the occurrence of peptide attachment

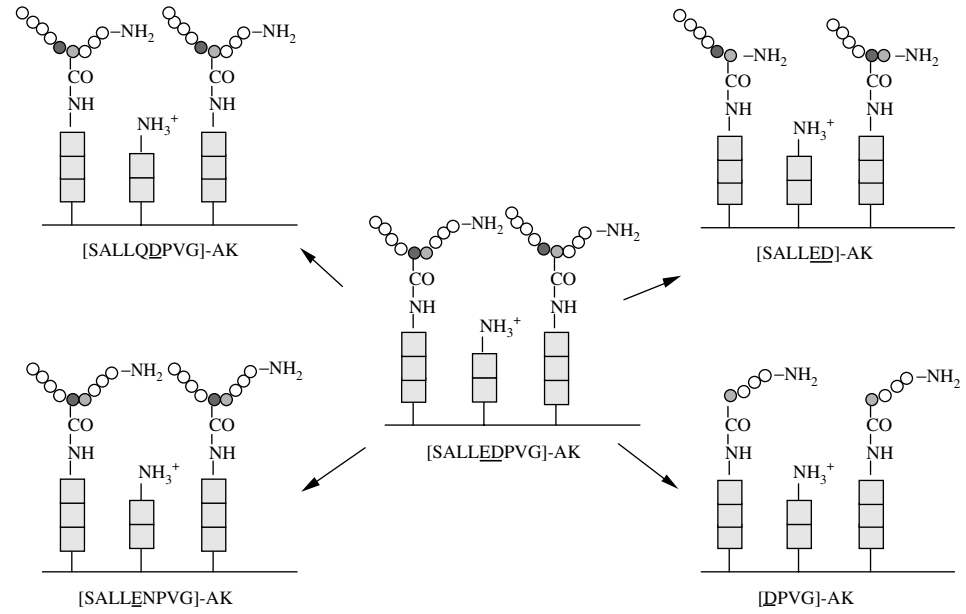

Figure 1 Schematic representation of branched polypeptide conjugates with analogue or truncated peptides from the 276–284 region of HSV-1 glycoprotein D.

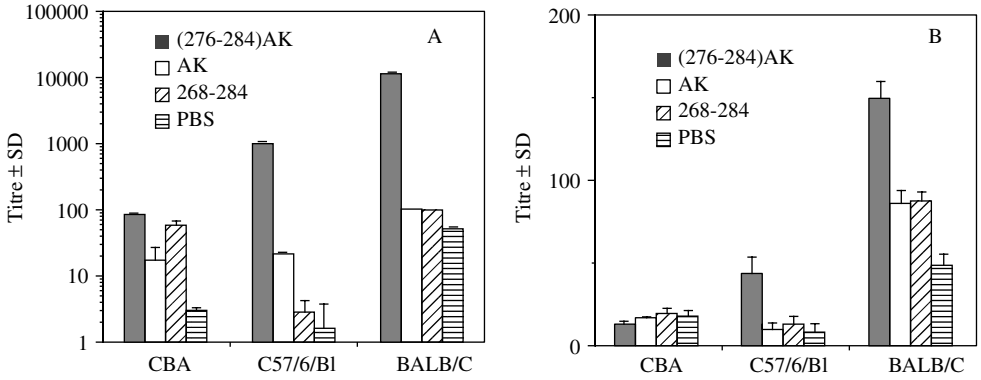

Figure 2 Magnitude and specificity of the IgG-type antibody response induced by the (276–284)-AK conjugate, free carrier AK, or peptide 268–284 in CBA, C57/Bl/6 and BALB/c mice. Serum samples were tested as described in the Experimental Section using (276–284)-AK conjugate (A) or free peptide 268–284 (B) as solid phase bound antigen. Results are expressed as titre ± SD values calculated from three titration curves.

simultaneously to both sites could be excluded, but it was difficult to estimate the ratio of peptides coupled preferentially to position 281 or 280 in the conjugation with AK. This supposition was confirmed by the data presented in Figure 3B. These indicate that the conjugate which contains the peptide N(276–284) only induced a low-level antibody response and the antibodies did not cross react with other conjugates. The involvement of the side chain of Glu at position 280 might have destroyed the immunogenicity of peptide 276–284. In contrast to this situation, the conjugate Q(276–284)-AK induced a strong cross-reactive antibody response. This indicates that covalent coupling of peptide 276–284 through its Asp residue at position 281 does not significantly influence the antigenic properties of the epitope. Consequently the antibodies induced by the conjugate (276–284)-AK are directed predominantly against peptides that are attached to AK at the side chain of Asp. These results clearly indicate that the conjugation site of the 276–284 epitope peptide has a major influence on the intensity and fine specificity of the antibody responses.

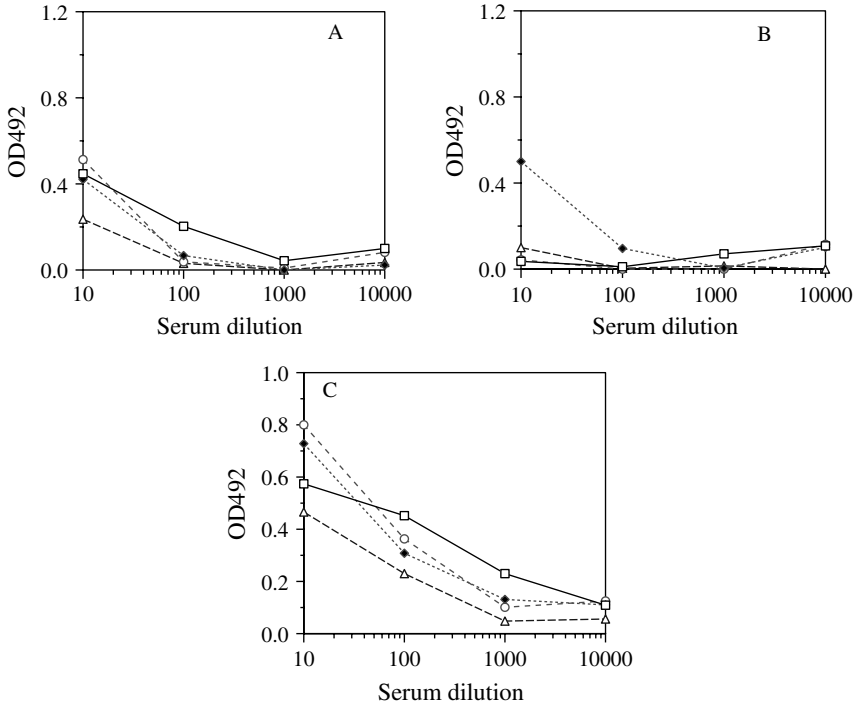

Figure 3 Cross-reactivity of polyclonal antibodies raised against (276–284)-AK (A), N(276–284)-AK (B) and Q(276–284)-AK (C) conjugates in BALB/c mice. Serum samples were absorbed on AK-coated plates and tested as described in the Experimental Section using (276–284)-AK, N(276–284)-AK and Q(276–284)-AK conjugates or free carrier AK as solid phase bound antigen. Results are expressed in OD values measured at $\lambda = 492$ nm. □ (276–284)-AK, ● N(276–284)-AK, ○ Q(276–284)-AK, Δ AK.

The Effect of Peptide Length on the Immunogenicity of, and Antibody Binding to, Peptide-AK Conjugates

Since the (276–284)-AK conjugate was able to induce significant levels of conjugate- and peptide-specific antibody responses, we extended our studies to the immunological analysis of conjugates containing short peptides corresponding to the 276–284 sequence of HSV-1 gD. Conjugates with the peptides (277–284), (279–284), (281–284), (276–281) were prepared (Figure 1, Table 2) and used together with the (276–284)-conjugate as immunogens in BALB/c, C57/Bl/6 and CBA mice. Serum samples were tested for IgG type antibodies binding to the conjugates, to the carrier, and to recombinant gD protein. Specific IgG responses were measured. Carrier-specific antibodies were removed by AK-adsorption and specific IgG responses were measured.

The cross-reactivity profile of antibodies induced by five different conjugates, is depicted in Figure 4 and summarized in Table 3. These data show that antibodies raised against (276–284)-AK, (277–284)-AK and (279–284)-AK conjugates recognize predominantly the sequence of 279–284 in the relevant compounds (Figure 4 B, C, D). Interestingly, the antibodies produced against (279–284)-AK or (281–284)-AK were capable of binding also to (277–284)-AK or (279–284)-AK, respectively (Figure 4 D,E). Antibodies raised in CBA and C57/Bl/6 mice showed a similar tendency to recognize two regions in the 276–284 region (data not shown).

Detectable binding to recombinant gD-1 protein was observed with some of the sera only (Figure 5, Table 3). Interestingly, conjugates containing slightly altered analogues of the 276–284 peptide were more efficient in the induction of antibodies (which also recognized recombinant gD) than antibodies raised against the parent peptide. The conjugate containing the sequence (277–281) was active in both BALB/c and CBA mice, while the peptide (277–284) resulted in gD-1 specific antibody production in BALB/c only. The short stretch (281–284), coupled to AK, induced a low

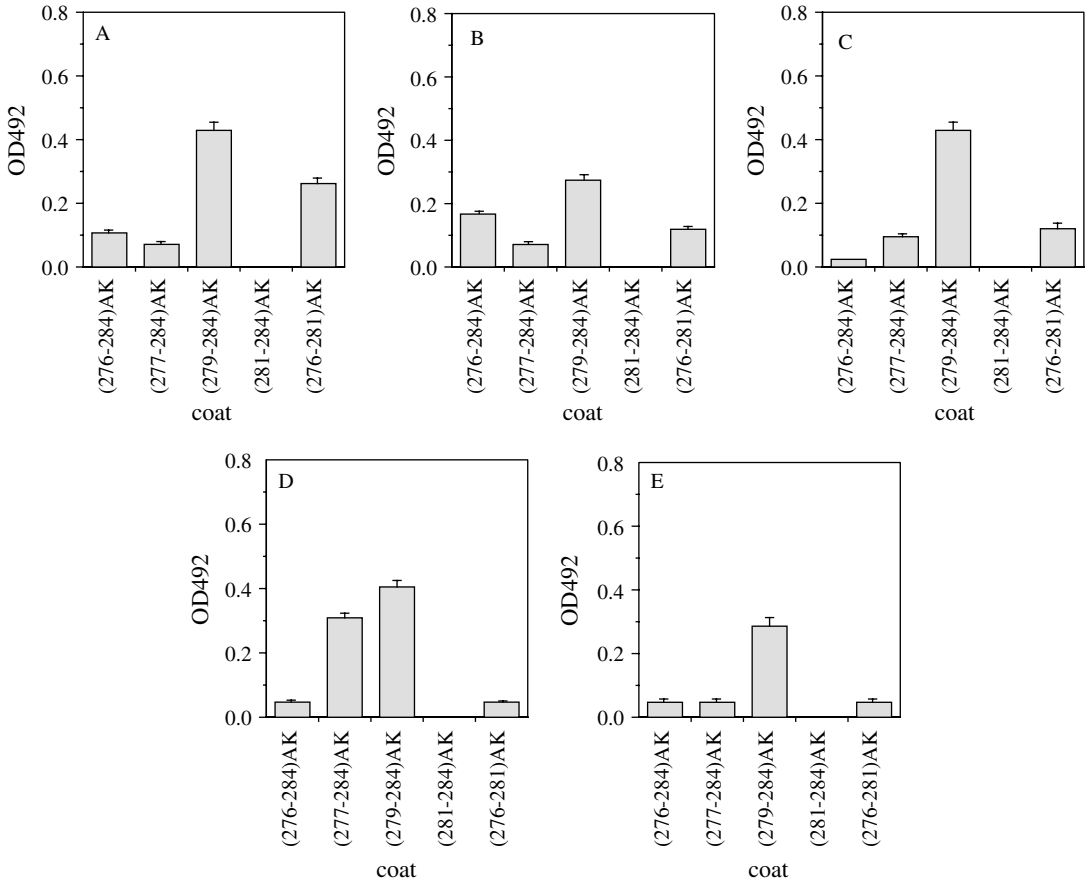

Figure 4 Magnitude and specificity of the IgG-type antibody responses induced by A: (276–281)-AK; (B): (276–284)-AK; (C): (277–284)-AK, (D): (279–284)-AK; (E): [281–284]-AK conjugate in BALB/c mice. Serum samples were absorbed on AK-coated plates and tested as described in the Experimental Section by using the immunogens as solid phase bound antigens. Results are expressed in OD values measured at $\lambda = 492$ nm.

Table 3 Immunoreactivity of gD Peptide–AK Conjugates in BALB/c and C57/Bl/6 Mice

Compound	BALB/c			C57/Bl/6		
	Peptide-specific antibodies[a]	gD-specific antibodies	HSV-1 protection	Peptide-specific antibodies	gD-specific antibodies	HSV-1 protection
PBS	−	−	−	−	−	−
AK	−	−	−	−	−	−
[276–284]-AK	+	−	−	+	+	+
[277–284]-AK	+	+	+	n.t.[b]	+	+
[279–284]-AK	++	−	+	n.t.	+	−
[276–281]-AK	+	+	+/−	n.t.	+	+/−
[281–284]-AK	−	−	+	n.t.	+/−	+

[a] Based on the binding activity of AK-absorbed sera to the immunizing conjugate as summarised in Figure 4.
[b] Not tested.

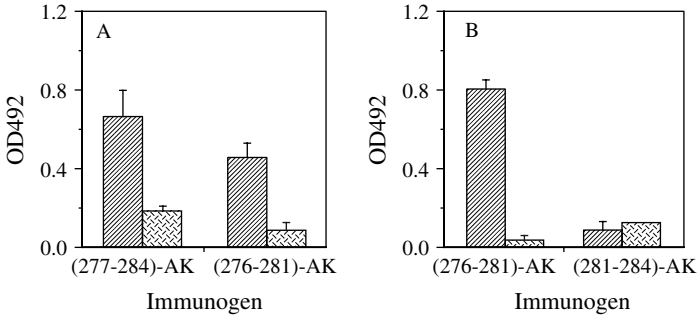

Figure 5 Binding of polyclonal antibodies induced by peptide-AK conjugates in BALB/c (A), and in C57/Bl/6 (B) mice to glycoprotein D (gD) of HSV type 1. Serum samples were tested as described in the Experimental Section using truncated gD as solid phase bound test antigen. Results are expressed in OD values measured at $\lambda = 492$ nm.

level of gD-1 reactive antibodies in C57/Bl/6 mice (Figure 5B).

Based on these results, we suggest that the optimal length of the peptide for antibody production is 279–284, and the polyclonal antibodies generated by the conjugates are able to recognize two separate epitopes within the 276–284 region. Considering the generally accepted view on the size of the linear B-cell epitope (e.g. 3–4 amino acid residues), it is not unrealistic to propose the existence of two partially overlapping epitopes, one localized in the 276–281 sequence, and the other situated at the *C*-terminal 279–284 end of the peptide. Immunization with conjugates comprising these epitopes also elicited antibodies which recognized the recombinant gD-1 protein. It is interesting to note that the conjugate containing the full size 276–284 peptide has a relative low immunogenic potential compared with the *N*- or *C*-terminally truncated analogues, which might be explained by the steric hindrance of the two epitopes in close vicinity.

Preimmunization with Carrier-peptide Conjugates Confers Protection Against Lethal HSV-1 Infection

The effect of preimmunization with various (peptide)-AK conjugates, encompassing sequences of the HSV-1 gD 276–284 region on the protection against a lethal dose of HSV-1 infection was investigated *in vivo* in BALB/c and C57/Bl/6 mice. Immunization with four conjugates containing the *C*-terminal DPVG sequence of the 276–284 region ((276–284)-AK, (277–284)-AK, (279–284)-AK and (281–284)-AK) and one compound encompassing the 276-281 *N*-terminal part were involved in these experiments. Control mice were treated according to the immunization protocol except PBS was used instead of the conjugates.

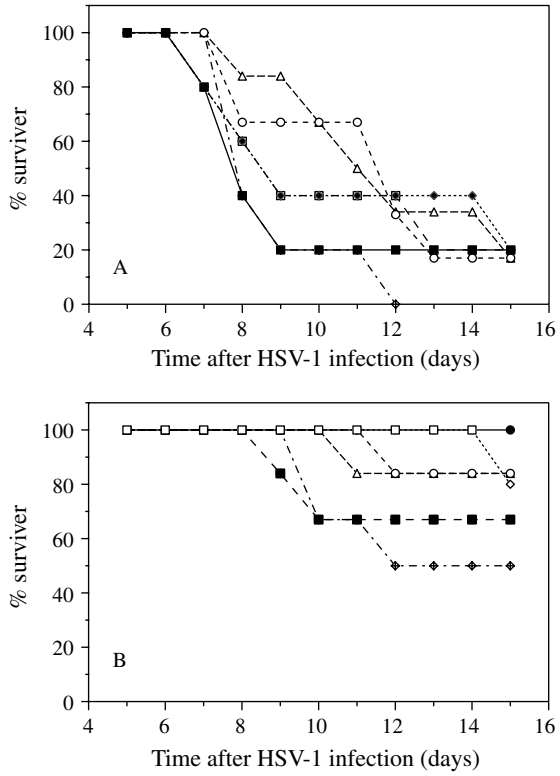

Figure 6 Survival of HSV-1 infected BALB/c (A) and C57/Bl/6 (B) mice preimmunized with peptide-AK conjugates. Mice were infected with $100xLD_{50}$ of HSV-1 after immunization as described in the Experimental Section. Control mice were injected with PBS instead of conjugates. ● (276–284)-AK, □ (277–284)-AK, ○ (279–284)-AK, Δ (281–284)-AK, ■ (276–281)-AK, + PBS.

The results presented in Figure 6 show that preimmunization with certain conjugates is able to extend the survival time of infected animals compared with controls and the efficacy of the various conjugates differed in conferring this

protective effect. Compounds containing either *C*-terminal fragments, i.e. (277–284)-AK, (279–284)-AK, (281–284)-AK or the *N*-terminal fragment (276–281)-AK of the 276–284 region were more efficient than the conjugate with the 'full size' (276–284) peptide. It is surprising that the conjugate (281–284)-AK comprising a tetrapeptide sequence was capable of provoking a protective immune response. However, the protective effect of conjugate preimmunization did not show a direct correlation with the intensity of the peptide-specific immune response (Figure 4, Table 3) or with the appearance of gD-1 cross-reactive antibodies (Figure 5). One possible explanation would be that the antigenicity of recombinant gD-1 is not identical to the native viral protein in terms of the expression of the 276–284 region. Another possibility is that T-lymphocytes, recognizing the common carrier of the various conjugates, are involved in directing immunogenicity and antibody specificity. Specific helper T-cells can provide help for antibody production in a different way if they are activated by the conjugate during preimmunization or by the virions introduced upon infection. Further experiments are required to clarify these mechanisms.

CONCLUSION

The characteristics of the antibody responses induced by various peptide-AK conjugates in inbred mice and the protective effect of conjugate preimmunization showed that the peptides spanning the 276–284 HSV-1 gD region are immunogenic when coupled to the AK carrier. The conjugates induce carrier- and peptide-specific antibodies, a fraction of which are also able to recognize recombinant gD. Preimmunization with some of the conjugates results in enhanced protection against lethal HSV-1 infection, which is at least partly mediated by polyclonal antibodies cross-reactive with the virus.

Based on our results, we also conclude that it is feasible to construct synthetic immunogens with synthetic branched polypeptide carriers, since such conjugates may be able to induce epitope-specific and protective antibody responses. Comparative studies with two sets of designed peptides attached to the same polypeptide carrier, clearly suggest that potency to elicit appropriate immune responses is highly dependent on the site of attachment and peptide length. Polypeptide-based conjugates provide an efficient and promising tool for exploring the minimal size and localization of predicted and/or partially characterized regions of pathogen-derived proteins.

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