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International Journal of Pharmaceutics 317 (2006) 90–100
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Cationic liposomes as potential carriers for ocular administration of peptides with anti-herpetic activity

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Received 29 August 2005; received in revised form 24 February 2006; accepted 27 February 2006
Available online 5 April 2006

Abstract

In the present study the preparation, characterization and activity of cationic liposomes containing the secretory form of herpes simplex virus type 1 (HSV-1) glycoprotein B (gB1s) or two related polylysine rich peptides, namely DTK1 and DTK2, were described. The immunotherapeutic potential of these HSV antigens containing liposomes was examined with a rabbit ocular model of HSV-1 infection. Our study indicates that the liposomes (i) are able to encapsulate quantitatively gB1s and around 30% the DTK peptides, (ii) are characterized by dimensions compatible with ocular applications and (iii) can release the peptide comparably to the free solution. In addition, neutralization studies demonstrated that an anti-DTK specific polyclonal antiserum can inhibit HSV-1 infection, indicating that such peptides could be a good immunogen/antigen in an anti-HSV vaccine formulation. Although the vaccination protocol did not induce protection against the eye disease, a significative protection against a lethal ocular challenge was detectable together with the absence of reactivation episodes from latency on the survived animals. In this respect, the use of cationic liposomes coupled to gB1s and DTK peptides, as a local ocular vaccine, could represent an interesting approach in order to obtain a possible efficacy in protecting animals against a subsequent HSV-1 ocular challenge.

Keywords: Ocular delivery; Liposomes; HSV-1 vaccine; Peptide administration

1. Introduction

Human herpes simplex virus (HSV) infections are extremely common, and cause a wide range of symptoms, from non-apparent to life-threatening encephalitis (Bernstein and Stanberry, 1999). Among eye infections, HSV is the most common cause of viral infection and infectious blindness in developed countries, with more than 90% of ocular HSV infections caused by HSV type 1 (HSV-1) (Bernstein and Stanberry, 1999). Antiviral drugs can control the virus activity during active replication, but they are not able to control latency or latency reactivation processes. Therefore, the sole approach for limiting the diffusion of the disease could be typified by the therapeutic or prophylactic vaccination (Bernstein and Stanberry, 1999). Most vaccine strategies have focused on the development of subunit vaccines consisting of one or more HSV glycoproteins, which have been proven efficient in various animal models (Manservigi et al., 1990a; Ghiasi et al., 1996; Rajcani et al., 1995; Peng et al., 1998). However, there is still a need to develop vaccines against HSV suitable for both prophylactic and therapeutic use. Furthermore, recent results suggest that, although still poorly studied, the induction of local immunity may play an important role in limiting HSV infections (Kuklin et al., 1997; O'Hagan et al., 1999). The benefits of vaccination have been observed globally for many years. A major drawback with current vaccination regimes lies in the method of administration, which generally uses the parenteral route. Factors such as the need for trained personnel, needle-stick injuries, needle disposal and cost, complicate the wide-spread use of this method in less developed nations and areas of the population

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doi:10.1016/j.ijpharm.2006.02.050

which may be financially deprived. As a consequence alternative routes of vaccine administration, such as mucosal surfaces including the nasal, oral and vaginal route, are being sought.

With particular regard to ocular infections, local periocular vaccination was shown to be equally or even more effective than systemic vaccination in protecting rabbits from primary infection (Nesburn et al., 1994, 1998; Caselli et al., 2001). In fact rabbits represent the ideal model for studies of primary and recurrent ocular HSV-1 infections, since they share many characteristics with the naturally occurring infections in humans. Previous results obtained by the group of Prof. Manservigi (Chiarantini et al., 1997; Manservigi et al., 1990a,b) have shown that the immunization with a recombinant secreted form of HSV-1 glycoprotein B (gB1s) obtained in human cells could induce a strong protective immunity in mice and rabbits. Therefore, it was interesting to verify if intraocular administration of liposome-carrying either gB1s or gB1s immunogenic peptide epitopes would be effective in protecting animals against a subsequent HSV-1 ocular challenge.

Despite extensive research in the field, the major problem in the ocular drug delivery domain is the rapid precorneal drug loss and poor corneal permeability. One of the approaches recently developed is the drug incorporation into cationic submicronic vectors which exploit the negative charges present at the corneal surface for increased residence time and penetration, such as liposomes, emulsions and nanoparticles (Meisner and Mezei, 1995; Velpandian et al., 1999; Kaur et al., 2004; Rabinovich-Guilatt et al., 2004).

Among the cationic colloids developed for ophthalmic delivery, cationic liposomes could be interesting in order to create an electrostatic interaction with the negative charges of mucin lying on the eye surface and to allow the maintaining at the target site of the vesicles for longer periods, with respect to conventional eye drops. Taking into account these considerations, the removal of the active molecules from eye natural protection systems (i.e. weeping, winking) could be reduced and a possible liposome adjuvant effect could be reached.

Aims of the present study were to (a) synthesize and characterize two different peptides, namely DTK1 and DTK2 (DTKs), mimicking the amine terminal portion of the recombinant glycoprotein B (gB1s). This is a polylysine rich (pK) region involved in the recognition of a cellular receptor, namely heparan sulfate (HS) proteoglycans, and in the subsequent attachment of the virus to the cell surface (Shieh et al., 1999; Laquerre et al., 1998); (b) evaluate the immunogenic and protective activity of the produced peptides; (c) produce and characterize cationic liposomes containing both DTKs and gB1s; (d) evaluate the activity of cationic liposomes as adjuvants for the administration of both peptides and gB1s as vaccine in the ocular infection rabbit model.

2. Materials and methods

2.1. Materials

Soy phosphatidyl choline was purchased from Lipid Products (Surrey, England). All other materials and solvents of the high purity grade were from Sigma Chemical Co.

2.2. Synthesis, characterization and purification of DTKs peptides

DTK1 and DTK2 peptides, differing for the presence of the last aminoacidic residue (namely, proline) (Fig. 1), were synthesized as C-terminal amide according to published methods using standard solid-phase synthesis techniques (Atherton and Sheppard, 1989) with a Milligen 9050 synthesizer (Millipore, USA). Protected amino acids and chemicals were

	peptide	MW	R
DTKPKKNKKPKNP-R	DTK1	1717.35	PP
	DTK2	1618.92	P

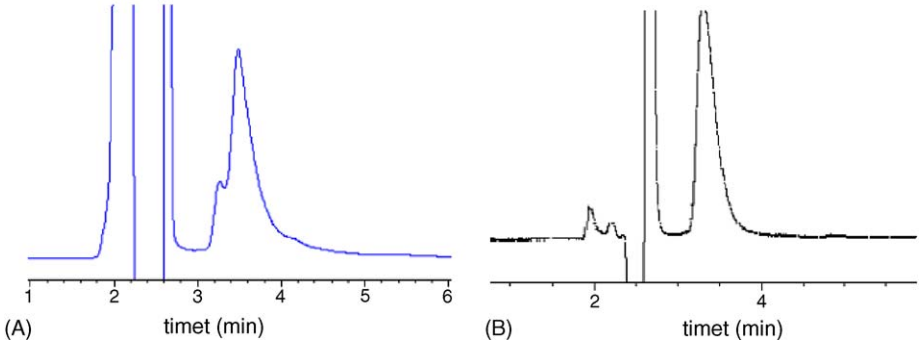

Fig. 1. Sequence, characteristics and HPLC spectra of DTKs peptides. Panel A: HPLC chromatogram of DTK1. Panel B: HPLC chromatogram of DTK2.

purchased from Bachem, Novabiochem or Fluka (Switzerland). The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid] on the polyethyleneglycol/polystyrene support, (Fmoc–PAL–PEG–PS) was from Millipore (Waltham, MA, USA). Fmoc derivatives of amino acids were used in the coupling reactions and all lateral amino acid protections were trifluoroacetic acid labile. Fmoc-PAL-PEG-PS resin, (0.21 mmol/g, 0.5 g in all synthesis) was treated with piperidine (20%) in DMF and the Fmoc amino acid derivatives (four-fold excess) were sequentially coupled to the growing peptide chain using [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) (Carpino, 1993) (four-fold excess) in DMF, and the coupling reaction time was 1 h. Piperidine (20%) in DMF was used to remove the Fmoc group at all steps. After deprotection of the last Fmoc group, the peptide resin was washed with methanol and dried in vacuo to yield the protected peptide–PAL–PEG–PS–resin. All protected peptides were cleaved from the resin by treatment with TFA/H_2O/phenol/ethanedithiol/thioanisole (reagent K) (82.5:5:5:2.5:5 v/v) 10 ml/0.2 g of resin at room temperature for 1 h (King et al., 1990). After filtration of the exhausted resin, the solvent was concentrated in vacuo and the residue triturated with ether. The crude peptides were purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 cm × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 ml/min with solvent A (2% v/v, acetonitrile in 0.1% aqueous TFA), and a linear gradient from 0 to 20% of solvent B (50% v/v, acetonitrile in 0.1% aqueous TFA) over 25 min was adopted for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 125 liquid chromatograph fitted with a Alltech C_{18} column (4.6 mm × 150 mm 5 μm particle size) and equipped with a Beckman 168 diode array detector. Analytical purity and retention time (t_R) of each peptide were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 ml/min using a linear gradient from 5% to 30% B over 25 min. Molecular weights of compounds were determined by a MALDI-TOF (matrix assisted laser desorption ionization-time of flight) analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix.

2.3. Peptide characterization: NMR spectroscopy

NMR spectroscopy has been performed on a Bruker Advance spectrometer operating at 400.13 MHz on a 1 mM sample of the peptide in water solution with 10% D_2O at 298 K and pH 4.5. TOCSY and ROESY experiments have been performed suppressing the water peak by Watergate procedure using TPPI phase cycle (Marion and Wuthrich, 1983; Piotto et al., 1992; Braunschweiler and Ernst, 1983; Bax and Davis, 1985). Different mixing times have been applied for the two experiments, i.e. 30 and 60 ms for TOCSY and 100 and 150 ms for ROESY. Typically 512 experiments were performed accumulated over 2K of memory with 90 and 128 scans for the two experiments respectively. A data manipulation was applied to FID before FT transformation using a sinebell shifted of $\pi/33$ in both dimensions.

The processing of the NMR data was performed on a SGI workstation O_2 using the NMRpipe and NMRview programs (Delaglio et al., 1995).

2.4. HPLC analysis of DTKs

The HPLC determination of DTKs was performed using a Jasco (Japan) gradient chromatographic pump, a Rheodyne 7125 sample injection valve (equipped with a 50-μl loop) and a Jasco UV detector. Samples were eluted on a 150 mm × 4.6 mm reverse-phase stainless steel column packed with 5 μm particles (Model BDS C18 Hypersil, Hewlett Packard, USA). The elution was isocratically performed at room temperature with a mobile phase constituted for 86% of water, 14% acetonitrile and 0.15% TFA at a flow rate of 0.8 ml/min. DTKs were monitored at 225 nm, the λ_{max} characteristic of both peptides.

2.5. Preparation of positively charged liposomes

Empty, gB1s and DTKs containing liposomes were prepared as follows: 8 mg of egg-PC/CH/DDAB$_{18}$ mixture (8:2:1, mol/mol/mol), dissolved in 1.0 ml of a methylene chloride/methanol mixture (1:1 v/v) were placed in a 100 ml round bottom flask. After solvent evaporation by vacuum-dried under nitrogen using a rotary evaporator, the resulting dried lipid-film was re-hydrated with 2.0 ml of warm (60 °C) phosphate buffer pH 7.4 with or without DTK (0.5 mg/ml) then the mixture was vortexed and sonicated at 60 °C for 10 min in a bath sonicator (Branson 2200, Branson Europe, The Netherlands) to obtain a more homogeneous sized vesicles population (Perkins et al., 1988; Cortesi et al., 1998). The obtained liposome preparation were left at room temperature under continuous stirring (180 mot/min) for 16–18 h in order to give a higher encapsulation efficiency (Leong et al., 2002).

The determination of encapsulation efficiency was performed by ultrafiltration. 300 μl of the liposome preparation was filtered through 10 000 NMWL pore filters (Micron YM-10, Millipore Corporation, Bedford, USA) by centrifuging at 10,000 rpm for 30 min in a Beckman Microfuge 18 centrifuge. The drug concentration in the preparation (P_T) and in the ultrafiltrate (P_U) was assayed by HPLC as previously described. Thus, the equation for the encapsulation efficiency in the liposomes was as follows:

$$\text{encapsulation efficiency}(\%) = [(P_T - P_U)/P_T] \times 100$$

2.6. Dimensional and zeta potential analysis of liposomes

The determination size and surface charge of liposomes was performed using a Zetasizer 3000 PCS (Malvern Instruments, Malvern, UK) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements of size analysis were made at 25 °C at an angle of 90°. Data were interpreted using the Contin software.

2.7. Evaluation of K_D between liposomes and DTKs

The DTKs-liposomes affinities were evaluated calculating the dissociation constant (K_D) of their binding equilibrium, as follows. Aqueous solutions containing six increasing concentration of DTK2, ranging from 0.07 to 3.5 mg/ml (from 44 μM to 2.2 mM), were singularly incubated with a 10 mg/ml (12.8 mM) of empty liposomes (PC:CH:DDAB18, 8:2:1 mol/mol/mol) suspension for 6 and 24 h at 180 rpm using a KS250 basic orbital stirrer (IKA Labortechnik, Staufen, Germany). The incubation volume was 300 μl. Afterwards, each sample was filtered through 10,000 NMWL pore filters (Micron YM-10, Millipore Corporation, Bedford, USA) and then analysed using the HPLC system above described, in order to evaluate the content of free peptide [P]. The concentrations of the peptide bound to the liposomes [LP] were obtained by difference of the total concentrations of peptide used for each sample with the corresponding [LP] values. The concentrations of free liposomes [L] were obtained by difference between their total concentration and the [LP] values. Dissociation constants were calculated as $K_D = [L]\,[P]/[LP]$ for each concentration of DTK2 introduced in the system after 6 or 24 h of incubation. The mean ± S.E.M values, referred to 6 or 24 h of incubation, were obtained from the K_D values corresponding to the six different concentrations of DTK2 incubated.

2.8. In vitro studies: peptide release and stability

In vitro DTKs release profile was obtained by dialysis method. The release experiments were conducted at 37 °C. Typically, 1 ml of DTKs containing liposomes (0.5 mg/ml of peptide) was placed into a dialysis tube (molecular weight cut off 10,000–12,000; Medi Cell International, UK), then placed into 20 ml of 0.1 M phosphate buffer, pH 7.4 under orbital stirring. 150 μl of sample were withdrawn at regular time intervals from 1 to 24 h and analysed by HPLC for DTK content.

In vitro DTKs stability was performed incubating at 35 °C for 30 and 60 min, 100 μl of free or liposomal DTKs in the presence of 11.5 U.I. of human lysozyme. After incubation each sample was centrifuged using 10,000 MNWL filters (Micron YM-10, Millipore Corporation, Bedford, USA) and analysed by HPLC for peptide content.

2.9. Anti-DTK1 serum production

Two New Zealand White rabbits aged 9–10 weeks were immunized subcutaneously on the back with 0.5 mg of DTK1 peptide emulsified with Freund's complete adjuvant for the first injection and then twice at three weeks intervals with the peptide in Freund's incomplete adjuvant. The animals were bled 10 days after the last injection, and sera from animals which reacted with gB1s, as detected by Western blotting analyses, were pooled.

2.10. Cells and viruses

Vero cells (ATCC, Bethesda, MD) were maintained at 37 °C in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and were used to propagate all the HSV-1 viral strains. The mutant KgBpK$^-$ HSV-1 virus, derived from strain KOS, and the LV HSV-1 variant, were previously described (Laquerre et al., 1998; Tognon et al., 1985).

2.11. Immunogens

For protein-based immunization, gB1s was obtained from human 293 cells constitutively expressing the secreted form of the glycoprotein (Manservigi et al., 1990b). The antigen was collected from the culture medium where it is released, and subsequently purified by affinity chromatography to a purity degree of 80%, as determined by SDS-PAGE and Western blot analyses (Caselli et al., 2001; Chiarantini et al., 1997; Manservigi et al., 1990b).

2.12. Western blotting analysis

KOS and KgBpK$^-$ Vero infected cell lysates were separated by SDS-PAGE and analyzed by Western blotting using an anti-DTK1 polyclonal serum or, as a control, an anti-gB1s polyclonal antibody made in our laboratory, both diluted 1–400 into hybridization solution.

2.13. Virus neutralization assay

KOS and KgBpK$^-$ viruses were incubated for 3 h at 37 °C with shaking, with 5 μl of rabbit complement and with or without 50 μl of the polyclonal anti-DTK serum. As a control, viruses were incubated with 5 μl of rabbit complement and with 30 μl an anti-gB1s polyclonal antibody made in our laboratory. The neutralized virus preparations were used to infect confluent Vero cell monolayers in six-well plates for 1 h and 30 min at 37 °C with shaking. After removing the inocula, the cell monolayers were treated for 1 min with 1 ml of low-pH glycine buffer (0.1 M glycine, pH 3.0) to inactivate non-penetrated virus. The cell monolayers were then washed three times with complete medium, overlaid with medium containing 0.2% human γ-globulins, and virus plaques were allowed to form at 37 °C. Cells were then fixed and stained with crystal violet to visualize and count viral plaques.

2.14. Animals and immunizations

Nine to 10-week-old female New Zealand White rabbits (Nossan, Milan, Italy) were used for the experiments. All animal protocols were approved by the local committee on animal welfare of the University of Ferrara, and adhered to the guidelines prescribed by the Italian Government (PM 28:1:94 N29:94A; art.7 DL27:1:92 N16). Rabbits were divided into three groups of nine animals each (group A, B and C), receiving three inoculations of immunogens (group B and C) or mock vaccines (group A) at 3-week intervals (days 0, 21, 42). For intraocular vaccination, rabbits were immunized by placing 33 μl of the liposome preparation bilaterally into the conjunctival cul-de-sac. At the first vaccination, each eye

received 4 μg of purified gB1s/liposome preparation. At boost vaccinations, group C received in each eye 4 μg of purified gB1s/liposome preparation, and group B received in each eye DTK1 or DTK2 peptide/liposome preparation. The control group A received mock vaccine, consisting of pure liposome preparation.

2.15. Virus and viral challenge

A highly pathogenic morphologic variant of HSV-1 (LV variant) was used (Tognon et al., 1985). The variant was plaque purified, grown on Vero cell monolayer (ATCC CCL81) and stored in aliquots at −80 °C until used. Titers were measured in Vero cells and expressed as plaque forming units (PFU)/ml. Three weeks after the final vaccine boost (day 64), vaccinated and mock-vaccinated rabbits were bilaterally infected with scarification, using a 5-mm cornels thephine, by placing 10^5 PFU of HSV-1 (LV), in a total volume of 0.1 ml, into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s. In naive rabbits, this dose of virus resulted in the death of 100% of animals within 15 days.

2.16. Measurement of titers

HSV serum neutralizing antibody titers were measured as previously described (Caselli et al., 2001), using a HSV-1 plaque reduction neutralization assay in the presence of added complement with two-fold serum dilution. The reported titer is the reciprocal of the serum dilution required to inhibit the cytolysis of a confluent monolayer of Vero cells by 50%.

HSV serum enzyme-linked immunosorbent assay (ELISA) titers were determined as previously described (Caselli et al., 2001), using threefold dilutions of serum and recombinant gB1s as the capture antigen. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

For titrating HSV in tears, tears were collected with a microcapillary pipette, then tear soluble immunoglobulin A (sIgA) ELISAs were performed as described above, using recombinant gB1s as the capture antigen.

2.17. Determination of clinical eye disease

Clinical eye disease patterns were determined by examining the rabbit eyes in a masked fashion on day 3, 5, 7, 10 and 14 post infection for scoring the incidence of severity of conjunctivitis, iritis, dendritic and geographic ulcers characteristic of HSV (epithelial keratitis), and acute transient stromal keratitis and edema (corneal clouding), as previously described (Caselli et al., 2001).

2.18. Virus reactivation

Virus reactivation procedure was performed as described by Rajcani et al. (1995). In this experiment a single iontophoresis procedure was combined with a single epinephrine injection. Briefly, under anaesthesia the cathode was attached to the wet ear and the anode was applied to corneal limbus of the rabbit. Epinephrine (0.1%) was repeatedly dropped onto gently scarified cornea and the iontophoresis was allowed to proceed for 8 min. under 2 mA current. This treatment was followed by an i.m. injection of 0.4 ml of epinephrine (diluted 1:20). Conjuctival swabs were taken before starting the procedure and daily for a period of 14 days since the beginning of the stimulation procedure.

2.19. Statistical analysis

The Student's *t*-test and the Fisher exact test were used to statistically analyse antibody levels in rabbit sera and rabbit protection experiments. Results were considered statistically significant when the P value was <0.05.

3. Results and discussion

3.1. Synthesis, characterization and purification of DTK peptides

As described in Section 2, DTK1 and DTK2 peptides (Fig. 1) were prepared by a solid-phase method. The stepwise syntheses were carried out by Fmoc chemistry, the functionalized Fmoc–PAL–PEG–PS was condensed step by step with the other aminoacid residues. The acylating residue was used in a fourfold excess with respect to the degree of derivatization of the resin. At the end of the synthesis, each DTK was removed from the resin by treatment with reagent K at room temperature. After isolation DTKs were purified by HPLC showing > 97% purity when monitored at 220 nm. Fig. 1 reports the chemical structure, some physicochemical characteristics and typical chromatographic profile of DTKs. During the formulation study, DTKs quantitation was performed by HPLC. Under the conditions described in Section 2, the retention time of both DTKs was 3.46 min. Analytical properties of the compounds obtained by a MALDI-TOF displayed the following results: DTK1 t_R min 13.86, $[M+H]^+$ calculated 1717.0 found 1717.1; DTK2 t_R min 13.47, $[M+H]^+$ calculated 1619.9 found 1619.7.

3.2. NMR spectroscopy of DTKs

NMR spectroscopy has been carried using both TOCSY and ROESY experiments. The assignments led to identify the spin systems (Jeener et al., 1979; Wüthrich, 1986) of those resonances which are clearly distinguishable from the others in the NMR spectrum. In fact the low dispersion of the NMR resonances clearly indicate the absence of marked sequential and or conformational effects on the resonances thus leaving their values very similar to those of the unstructured aminoacid (Wüthrich, 1986; Wishart et al., 1991). The fingerprint region of TOCSY spectrum of DTK1, reported in Fig. 2A, clearly shows that all the resonance of similar residues are close one another. These observations are in agreement with the absence of sequential NOE effects in the fingerprint region and also in that region diagnostic of helical secondary structure characterized by the presence of contacts between NH protons (Fig. 2B).

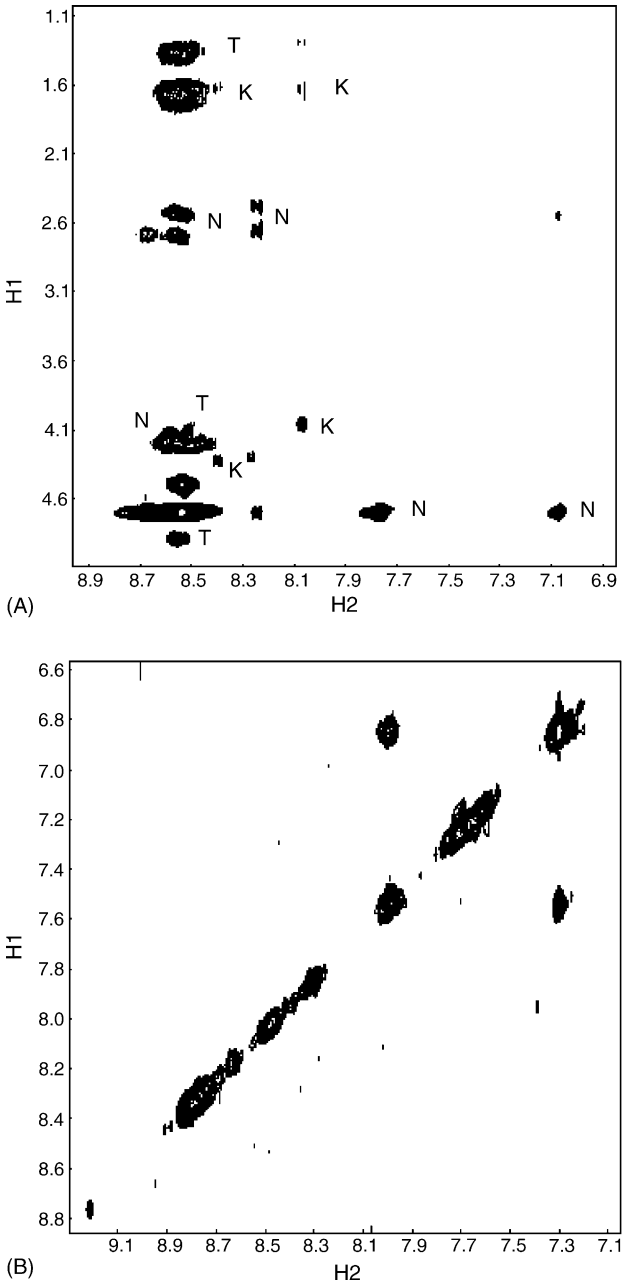

Fig. 2. Fingerprint region of TOCSY spectrum of DTK1 in the presence (panel A) and with absence (panel B) of NOEs.

The assignments of DTK1 are also reported in Table 1. The values found perfectly correspond to the values statistically reported in protein residues in a random coil conformation (Wishart et al., 1991). The line of the resonances found in the NMR spectrum and the absence of any dependence on concentration are compatible with the absence of aggregation effects.

Table 1
Chemical shift (ppm) of the NMR resonances of DTK1 spectrum

Residue	NH	CH-α	CH-β	Others
Asn 7, 12	8.62	4.72	2.6; 2.78	NH_2 = 6.95
Asp 1	8.62	4.22	2.4; 2.6	–
Lys 3, 5, 6, 8, 9, 11	8.9	4.38	–	–

From the assignment of the resonances of DTK1 resulted that the composition of the peptide corresponds to the sequence that has been synthesized. The results of NMR spectroscopy clearly indicate that in our experimental conditions the peptide lacks of any defined secondary structure conformation and, then, is present in water in random coil conformation.

As expected, in reason of the similar aminoacidic structure of the two DTKs, NMR spectra of DTK2 are superimposable to that of DTK1 (data not shown).

Taking into account the obtained results, the study of the solution structure of DTKs carried out by NMR spectroscopy, revealed that: (a) the peptides are soluble and not aggregated, (b) the assignment of the resonance of the NMR spectrum of both peptides are in agreement with the sequence composition and (c) in water solution no elements of secondary structure are evident.

3.3. Preparation and characterization of liposomes containing DTKs

Peptide containing liposomes were prepared by direct hydration and sonication as described in Section 2. In particular, to allow the full hydration of the sample, a slow and warm aqueous buffer was used to hydrate the phospholipid thin layer. In addition a overnight gentle shaking, at room temperature, was performed in order to get an enhancement of peptide encapsulation. Unencapsulated peptide was removed using the ultrafiltration method as described in Section 2. Samples prepared without overnight shaking showed a DTKs encapsulation around 2%, namely 1.97% ± 0.3% for DTK1 and 2.30% ± 0.2% for DTK2. On the other hand, after overnight incubation, the encapsulation efficiency reached 28.6% ± 1.2% and 32.4% ± 1.8% for DTK1 and DTK2, respectively, showing an increase of peptide encapsulation of 14.51 and 14.08-fold for DTK1 and DTK2. The obtained results are in agreement with respect to other liposome preparations containing hydrophilic molecules, since drugs are encapsulated within liposomes in percentages generally below 25–35% (Betageri et al., 1993).

DSC experiments were performed in order to evaluate the interaction between DTKs and vesicle bilayers since it is well known that this methodology can simply and precisely provide information about the interaction between a drug and phospholipid membranes (Jain, 1988). The obtained results (data not shown) indicated that neither DTK1 or DTK2 induced a change of the thermotropic parameters of the liposomes and Tm variations generally remained within the experimental error range. In addition, also the ΔH values were not subjected to significant changes. These results give rise to the almost complete absence of interaction between hydrophilic DTKs and liposomes lipid domains.

The analysis of the binding equilibrium between DTK2 and empty liposomes allowed us to find dissociation constants (K_D) in the high millimolar range. The K_D values appeared very similar at different incubation times, namely 51 ± 5 mM after 6 h and 55 ± 7 mM after 24 h. According to these values, we have evi-

Table 2
Characteristics of liposome containing DTKs

Peptide	Encapsulation efficiency (%) ± S.D.	Mean size (nm) ± S.D.	Polydispersity	Zeta potential
None (#1, #2, #3)	–	327.6 ± 30.5	0.34 ± 0.07	19.6 ± 1.2
DTK1 (#1a, #2a, #3a)	28.6 ± 1.24	314.8 ± 12.9	0.24 ± 0.04	23.8 ± 2.1
DTK2 (#1b, #2b, #3b)	32.4 ± 1.76	322.6 ± 26.8	0.35 ± 0.06	26.3 ± 1.9

Results are the mean of five independent experiments ± S.D. on three different batches. #: batch.

denced that 10 mg/ml (12.8 mM) empty liposomes can bind from 18% to 23% of peptide amounts ranging from 0.07 to 3.5 mg/ml (44 μM–2.2 mM). Moreover, the high millimolar range of K_D values indicates the presence of extremely weak interactions between DTK2 and liposomes, supporting the results obtained by the DSC analysis.

These overall information suggest that peptide containing liposomes may show considerable amounts of the drug in their external surface, allowing therefore DTK2 to be promptly available in the administration site (Table 2).

The liposome size distribution as determined by photon correlation spectroscopy (PCS) showed that the presence of both DTKs seems not to interfere with liposome size. In fact, the mean dimension of the vesicles slightly decreases passing from 398.2 ± 16.8 to 314.8 ± 12.9 nm for DTK1- and from 402.2 ± 12.5 nm to 322.6 ± 26.8 nm for DTK2-containing liposomes.

3.4. In vitro studies: peptide release and stability

The liposomes containing DTKs were subjected to in vitro experiments in order to get information about the efficiency of release of DTKs from liposomes and the stability of the peptide in the presence of the ocular enzymatic system typified by the presence of lysozyme. The determination of peptide release was performed by dialysis method as described in Section 2. As reported in Fig. 3, the release of both DTKs from liposomes are slightly reduced with respect to those obtained by DTKs in aqueous solution. It is to be underlined that this reduction of release can be quantified, in the overall times investigated, around the 20%. This value seems dependent on the peptide ability to bind the liposomes at the external surface, in agreement with the above described results where, under similar experimental conditions, almost the 20% of DTK2 is able to bind the empty liposomes. In this view the presence of liposomes could be intended to protect and possibly to facilitate (as adjuvant) the peptide internalisation within the eye.

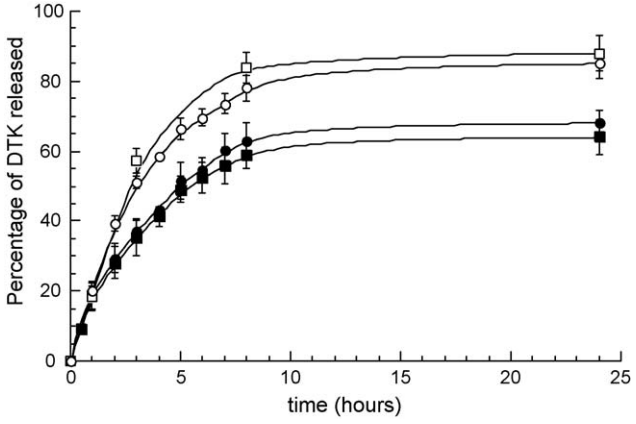

Fig. 3. Release profile of DTK1 (●) and DTK2 (■) encapsulated in liposomes. The releases were determined by dialysis method. As comparison DTK release from aqueous solution is also reported (open symbols). Data represents the average of three independent experiments ± S.D.

Concerning the test of stability performed on DTKs both in the free and liposomal form, the peptide have demonstrated to be unaffected by lysozyme activity after 30 or 60 min of incubation in the presence of such enzyme.

3.5. Anti-DTK1 polyclonal antiserum production and characterization

In order to determine the specificity of the anti-DTK1 serum for the lysine-rich (pK) gB1 epitope, two different kind of assays were performed. First, the antiserum was used to reveal the HSV-1 gB protein by Western blot analysis. Vero cells infected with

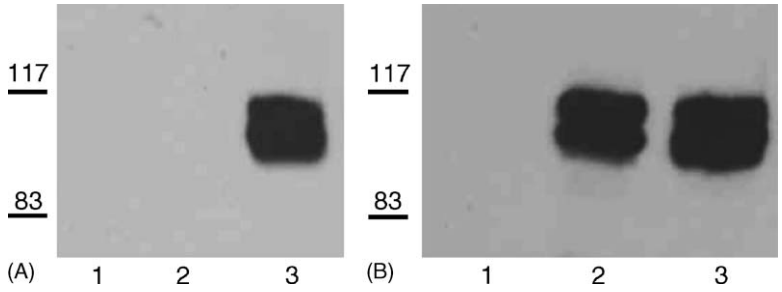

Fig. 4. Western blot analysis of Vero cells lysates derived from mock (lane 1), KgBpK$^-$ (lane 2), and KOS (lane 3) infected cells: (A) anti-DTK polyclonal antiserum; (B) anti-gB1s polyclonal antiserum.

Table 3
Neutralization activity of anti-pK and anti-gB1s polyclonal antisera

	Percentual reduction in number of plaques	
	Anti-pK antibody (%)	Anti-gB1s antibody (%)
KOS	47	80
KgBpK$^-$	18	75

HSV-1 KOS wild type strain or with KgBpK$^-$, a previously described HSV-1 mutant (Laquerre et al., 1998) in which the pK region of gB was deleted, and mock infected cells were collected 24 h post infection and cell lysates were analysed by Western blotting using the anti-DTK1 antiserum or, as a control, using an anti-gB1s antiserum produced in our laboratory. As it is possible to observe in Fig. 4A, the anti-DTK1 antibody is able to reveal both the mature and immature forms of the wild type gB protein (lane 3), but it cannot detect any protein in the KgBpK$^-$ infected cells (lane 2), consistently with the deletion in the pK region of gB, or in mock infected cells (lane 1). As a control, the anti-gB1s antibody is able to detect both the mature and immature forms of the wild type gB and of the gBpK$^-$ proteins (Fig. 4B, lanes 3 and 2, respectively) and, as expected, it does not reveal any protein in mock infected cells (Fig. 4B, lane 1).

To assess whether anti DTK1 specific antibodies could inhibit in vitro viral infection, a neutralization assay was performed using either the anti DTK rabbit polyclonal antibody or the rabbit polyclonal anti-gB1s specific antibody. As shown in Table 3, when using the anti DGT1 antiserum, the infectivity of wild type strain KOS was inhibited by 47% compared to the 18% inhibition of the pK$^-$ mutant virus, while an anti gB1s antiserum, used as a positive control, inhibited both KOS wt and pK mutant at the same level (between 75% and 80%). This data is consistent with the deletion in the pK region of gB in the KgBpK$^-$ virus. Taken together these findings demonstrate that DTK1 peptide is exposed on the viral surface and is sensitive to inhibition by specific antibodies, supporting the hypothesis that such peptide could be a good immunogen/antigen in an anti-HSV vaccine formulation.

3.6. Vaccine immunogenicity

All rabbits were bled 3 weeks after the final vaccination, just prior to challenge with HSV-1, strain LV. Neutralization and ELISA (against HSV-1 and gB1s protein) were done individually on all sera. Six out of nine animals in the two vaccinated groups (B and C) showed detectable HSV-1 neutralizing antibody titers with respect to the liposomes administered control. Those animals that were demonstrated positive in the neutralization studies, showed also detectable ELISA titers against gB1s with respect to the other vaccinated and control rabbits (Table 4). No sIgA were detected in all the three different groups of rabbits (data not shown).

3.7. Rabbits vaccination and resistance to viral challenge

Since previous results obtained in our and other laboratories (Nesburn et al., 1994; Caselli et al., 2001) have shown that local periocular gB1s administration, in presence of incomplete Freund's adjuvant, was effective in protecting animals against a subsequent HSV-1 ocular challenge in a rabbit model, we planned to produce a protein delivery system for topical use able to: (i) reduce toxicity problems related to the presence of the adjuvant; (ii) induce protective immune response; (iii) use a lower amount of protein or an antigenic peptide, in substitution of the protein, for the immunostimulation.

To this purpose, two groups of nine rabbits each (groups B and C) were vaccinated by placing bilaterally into the conjunctival cul-de-sac the liposome formulation containing gB1s purified protein. Control rabbits (group A) were immunized with the liposome preparation alone. After the first inoculation, rabbits were boosted twice at 3-week intervals. Group C received the liposome/gB1s preparation as immunogen, while group B received DTK1 or DTK2 peptide/liposome preparation in the right or the left eye, respectively (Table 4). As expected, the vaccine formulation did not induce conjunctival inflammation of the eyes.

Three weeks after the last boost, vaccinated and mock-vaccinated rabbits were bilaterally infected with a highly

Table 4
Protection against lethal HSV-1 challenge and antibody response in rabbits immunized by intraocular (i.oc.) route with gB1s- or gB1s plus-DTK peptide-containing liposomes

Group	Immunogen	Number of survivors/total[a] (%)	ELISA titers[b]		Neutralization titers[c]	
			Dead animals	Survived animals	Dead animals	Survived animals
A	Empty liposomes	0/9 (0%)	0 (0.033 ± 0.018)	–	<1:4	–
B	gB1s + DTK/liposomes	6/9 (66.6%)	1:100 (0.185 ± 0.02)	1:600 (0.369 ± 0.093)	<1:4	1:73
C	gB1s/liposomes	6/9 (66.6%)	1:200 (0.145 ± 0.05)	1:800 (0.486 ± 0.065)	<1:4	1:112

[a] Three groups of nine animals each were immunized at 3-week intervals and challenged by ocular scarification with HSV-1 (LV) strain (see text for details). Data were statistically significant as determined by Fisher exact test ($p = 0.037$).

[b] All rabbits were bled 3 weeks after the final vaccination, prior to challenge, and the sera were assayed by ELISA, using gB1s as the Ag (see text for details). Results are expressed as the mean end point ELISA titer of the responder animals. In parentheses are listed the mean $O.D._{450\,nm}$ values ± S.D. of responder animals at the lower serum dilution (1:50).

[c] Sera of the bleeding were tested for their capability to neutralize HSV-1 cytopathic effect in a plaque reduction assay. Results are expressed as the serum titer required to inhibit cytolysis of 50% of the control value, determined as the mean plaque number obtained by infecting Vero cells with 100 PFU of virus preincubated with sera derived from mock vaccinated rabbits.

pathogenic HSV-1 variant (LV variant). The analysis of the clinical signs of HSV-1 of ocular infection revealed no significant differences in the average severity of corneal lesions among mock vaccinated and vaccinated rabbits, on any days examined, and the average peak severity was in the range of 4–5 at 7 days for both groups of animals (data not shown).

However, as shown in Table 4, the vaccination protocol resulted in a significative protection from mortality determining the survival of six rabbits for group B and six for group C (66.6% of survival in both groups). It was noteworthy to observe that the rabbits showing detectable neutralization antibody titer, were those able to survive at the lethal challenge.

3.8. Reactivation test

To test the possibility that the liposome formulated vaccine could protect rabbits from viral reactivation, the survived animals were subjected to the epinephrine iontophoresis. The cornea were stimulated with a single iontophoresis procedure and the conjunctival sacs were swabbed daily for a period of 14 days in order to detect shedding of reactivated virus. Under our experimental conditions, none of the rabbits were shown to release infectious virus upon epinephrine iontophoresis (data not shown).

4. Conclusion

In the present study the preparation, characterization and activity of cationic liposomes containing gB1s and its immunogenic DTK peptides for vaccine delivery was described. Cationic liposomes represent an established vehicle for the delivery and expression of exogenous genes into tissue of the adult rabbit eye (Jones et al., 1994; Masuda et al., 1996). Moreover, ocular delivery of positively charged liposomes resulted in an increase of acyclovir corneal absorption that led to a better resolution of HSV-1 infection (Law et al., 2000). However, there is very few information about their use in experimental vaccine formulation to prevent infectious diseases (Li et al., 2004). This could be due to the fact that the transcorneal selected antigen transport is normally limited by the intrinsic permeation characteristics of the corneal epithelium (Streilein et al., 2002; Akpek and Gottsch, 2003). Our study indicates that these liposomes (i) are able to encapsulate (or bind) almost quantitatively gB1s and around 30% both the peptides, (ii) are characterized by dimensions compatible with ocular applications and (iii) can release the secreted protein and the peptide comparably to the free solution.

The use of gB1s and its peptides as a vaccine component comes from different considerations: (i) the protection of rabbits against HSV-1 ocular infection when a secreted form of gB1 was inoculated either systemically (Manservigi et al., 1990a) or periocularly (Caselli et al., 2001). Particularly, both local and systemic administration elicited a neutralizing antibody response, reduced ocular symptoms and completely prevented the death of rabbits from encephalitis; (ii) the mediation of the first binding of the virion to the cell by DTK peptide representing the pK-gB1 epitope involved in the recognition of HS present on the cell surface (Shieh et al., 1992; Laquerre et al., 1998). Mutants deleted for this sequence are impaired in binding and penetration of the virus into the cell (Laquerre et al., 1998); (iii) the induction by DTK peptide of neutralizing antibody when injected into rabbits (Table 4), for this reason DTK can be considered a promising immunogen for the development of a new formulated vaccine against HSV infection.

The above reported considerations together with the analysis of the data obtained in the present study can allow the resuming of the following points: (i) the gB1s antigen and the immunogenic DTK peptides can be delivered to the eyes as liposome vehiculated antigens; (ii) the vaccine does not protect the animals from the eye disease, but it confers a significative protection against a lethal challenge with a neurovirulent HSV-1 strain; (iii) the protected rabbits show detectable ELISA and neutralization titers. At this regard, incorporating antigens into liposomes has been already shown to enhance immunogenicity, at least for antibody production (Li et al., 2004; Streilein et al., 2002; Akpek and Gottsch, 2003; Peppard et al., 1998); (iv) no sIgA were detected in the tear film.

In conclusion both vaccines did not provide significant protection against any of the four parameters measured (conjunctivitis, iritis, epithelial keratitis, and corneal clouding). However, it is important to notice that animals raising a good anti-gB immune response, were protected against HSV lethal infection and, above all, against viral reactivation from latency. Although in this trial it was not possible to clearly evaluate viral reactivation from latency due to the death of all mock vaccinated animals, all our attempts to reactivate the virus in the survived vaccinated rabbits were unsuccessful. At this regard, it should be stressed that the goals for an HSV vaccine are different from those of other common infections, because an ideal vaccine would prevent not only the acute disease produced by initial infection, but also the establishment of the latent infection and the reactivation of virus from latency. These preliminary observations will press us to investigate in future animal trials the therapeutic capability of this liposome formulated gB1s antigen, when administered as ocular vaccine, using lower doses of HSV-1 for challenge in order to have latently infected animals suitable to be tested for viral reactivation and evaluation of recurrences.

Another parameter that could be evaluated for future experiments in order to possibly increase the peptide absorption at ocular level, is typified by the liposome composition in term of phospholipid type and concentration. The choice of the phospholipid could in fact originate vesicles able to bind in different ways (i.e. using different physico-chemical interactions) the cornea surface allowing a longer time of retention and possibly giving to the peptide more chances to penetrate the corneal barrier. In addition, the different phospholipid composition of the liposomes could heavily affect the percentage of drug encapsulation of the peptide, allowing the administration of more concentrated doses of antigen that could be important for the enhancement of drug absorption at ocular level. Moreover, to favour antigen presentation we plan to use the sub-conjunctival route for

the periocular vaccination. This routinely utilized method in clinical ophthalmology should facilitate the use of liposomes and ensure the delivery and retention of the vaccine at local site.

Acknowledgements

The authors are grateful to the group of Prof. M. Paci, University of "Roma Tor Vergata" for NMR analysis. The authors thank the National Research Council of Italy (CNR "Progetto Finalizzato Biotecnologie") and the Ministry of University, Scientific Research and Technology of Italy (MIUR-FIRB-2001 RBNE0127YS_002, COFIN-2002, PRIN 2004 and 2005 ex 60%) for financial support.

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