Peptide-loaded chimeric influenza virosomes for efficient in vivo induction of cytotoxic T cells

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Abstract

Virus-specific CD8+ T cells are thought to play an important role in resolving acute hepatitis C virus (HCV) infection as viral clearance has been associated with a strong and sustained CD8+ T cell response. During the chronic state of HCV infection virus-specific T cells have a low frequency and a reduced responsiveness. Based on this, a therapeutic vaccine increasing the frequency of specific T cells is a promising alternative for the treatment of chronic HCV infection. We improved an existing vaccine platform based on immunopotentiating reconstituted influenza virosomes (IRIVs) for efficient delivery of peptide epitopes to the MHC class I antigen presentation pathway. IRIVs are proteoliposomes composed of phospholipids and influenza surface glycoproteins. Due to their fusogenic activity, IRIVs are able to deliver encapsulated macromolecules, e.g. peptides to immunocompetent cells. We developed a novel method based on chimeric virosomes [chimeric immunopotentiating reconstituted influenza virosomes (CIRIVs)] combining the high peptide-encapsulation capacity of liposomes and the fusion activity of virosomes. This new approach resulted in a 30-fold increase of the amount of incorporated soluble peptide compared with current preparation methods. To study the immunogenicity of chimeric virosomes HLA-A2.1 transgenic mice were immunized with CIRIVs containing the HCV Core132 peptide. Core132-CIRIVs efficiently induced specific cytotoxic and IFNγ-producing T cells already with low peptide doses. Vaccine formulations, which include combinations of different HCV-derived CTL epitopes could be used to induce not only a strong but also a multi-specific CTL response, making them potential candidates for therapeutic and maybe prophylactic T cell vaccines in humans.

Introduction

Immunopotentiating reconstituted influenza virosomes (IRIVs) have been developed to obtain a potent and well-tolerated adjuvant for vaccines, already registered for use in humans (1–8). IRIVs are spherical, unilamellar vesicles with a mean diameter of ~150 nm. Their base is a liposome comprised of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and lipids derived from the influenza virus. Phospholipids are virtually non-immunogenic and have enjoyed a long history of use in human pharmaceutical preparations. The haemagglutinin (HA) and trace quantities of viral neuraminidase and phospholipids from influenza virus are intercalated within the phospholipid bilayer, whereby the presence of HA is necessary to enhance the immunopotentiating effect to antigens associated with IRIVs (9). The influenza HA plays a key role in the mode of action of the IRIVs. HA is a trimeric integral membrane protein (M, 220 000) comprised of an ectodomain of identical subunits, each of which contain two polypeptides, HA1 and HA2, linked by a disulphide bond (10). The two polypeptides arise from a proteolytic cleavage event and are essential for fusion activity of the virus with the endosomal...
Novel chimeric influenza virosomes for CTL induction

A conformational change occurs in the HA which is a pre-cytosis (14). At the low pH of the host cell endosome (pH ~5), virosomes into cells occurs through receptor-mediated endocytosis (14). The HA2 subunit of HA then mediates the fusion of virosomal and endosomal membranes. As a result of this fusion the contents of the virosores are released into the cytosol. Virosomes, therefore, are an elegant carrier system for the delivery of macromolecules like antigenic peptides, nucleic acids and others into the cell cytoplasm.

Synthetic peptide-based vaccines, which are designed to stimulate CD8+ cytotoxic T cells, are an attractive approach to the treatment or prevention of infectious diseases and malignant disorders. One major drawback of such peptide vaccines is the rapid degradation of the peptide by proteases (15). However, macromolecules encapsulated into virosomes are protected from enzymatic degradation (16).

Upon endosomal fusion of the virosores containing cytotoxic T cell (CTL) epitopes within the APC, the T cell epitopes are released into the cytoplasm and directed into the MHC class I antigen presentation pathway thereby leading to the induction of CD8+ T cells. It has been shown in vitro that virosomes containing a synthetic peptide are able to deliver the peptide antigen to the MHC class I presentation pathway, stimulating specific CTLs as well as rendering target cells susceptible to antigen-specific CTL-mediated lysis (17). In order to induce a CTL response, not only CTL epitopes have to be delivered but also T helper epitopes. These epitopes play a critical role in the induction of MHC class I-restricted CTL (18–21). The virosomal carrier is also able to trigger a T helper response since T helper epitopes are located on the influenza HA, the major component of IRIVs. It has been demonstrated that a synthetic peptide enclosed in virosomes is able to induce a CTL immune response in mice (22). In addition, the use of virosomes avoids the induction of tolerance, which has been observed under certain conditions after immunizing mice intra-peritoneally with soluble peptides (23).

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, such as cirrhosis and liver cancer. About 3% of the world's population is infected with HCV and ~70–80% of newly infected patients progress to chronic infection (24). Anti-viral drugs such as IFNs/pegylated IFNs, alone or in combination with ribavirin, are effective in up to 80% of patients (25, 26) but many patients do not tolerate combination therapy (24). As for many other viral infections, vaccination might be an effective tool to control disease and therefore, it is desirable to develop a vaccine for the treatment of chronic infection as well as to prevent de novo infection. Recent evidence indicates that the cellular immune response is responsible for viral clearance in HCV-infected individuals. In particular, CTL may be a major defence mechanism in HCV infection (27–30). The ability to induce cytotoxic and IFNγ-producing T cells is considered to be an important feature of a candidate HCV vaccine. Since the core protein is the best-conserved structural protein in the HCV genome (31), we used the HCV Core132 epitope (DLIMGY1PLV, aa 132–140) (32) as a model antigen.

One of the problems in current virosores technology is the lack of methods for the efficient entrapment of peptides or other molecules. For the induction of a good antibody immune response (B cell immune response), the antigens must be cross-linked to the virosores surface or integrated into the virosores bilayer via a lipid anchor. With the widely used detergent-removal procedure (4), efficient loading can be achieved for such antigens (33). In contrast, for CTL epitopes, which need to be encapsulated into virosomes, <1% of the aqueous phase can be entrapped in the virosores particle with the detergent-removal method and a given lipid concentration of 1 mM and an assumed virosores diameter of ~150 nm. Therefore, this is a rather inefficient and expensive method for the delivery of T cell antigens in vivo. On the other hand, high encapsulation efficiencies can be achieved by the freeze/thaw technique used to prepare pure lipid vesicles (34). However, liposomes lack the specific targeting to sialic acid residues on the surface of APCs and the adjuvant effect of the HA observed with virosomes. As a consequence, a method was developed that combines the positive properties of virosomes and liposomes with the result of a substantially increased entrapment of peptides or other molecules within functional virosomes. IRIVs prepared with this novel method induce a strong and dose-dependent CTL response against the peptide used.

Methods

Reagents and peptides

Octaethylenglycol-mono-(n-dodecyl)ether (OEG, C12E8), 1,2-dipalmitoyl-sn-glycero-3-phospho-rac(1-glycerol) (PG), acetonitrile, triethylammonium phosphate (TEAP) solution, Streptomycin, HEPES, penicillin and RPMI medium were purchased from Fluka Chemie GmbH and Sigma (Buchs, Switzerland), respectively. FCS was purchased from Gibco BRL (Basel, Switzerland). IL-2 was obtained from EuroCetus B.V. (Amsterdam, The Netherlands). Egg PC was obtained from Lipoid (Cham, Switzerland). PE was obtained from R. Berchtold (Biotechmical Laboratory, University of Bern, Switzerland). SM2 Bio-Beads were from Bio-Rad Laboratories (Glattbrugg, Switzerland). N(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Bodipy 530/550-DHPE), Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (N-Rh-DHPE) and biotin-DHPE (N-biotinyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt) were from Molecule Probes Europe (Leiden, The Netherlands). Sephadex G50 coarse was obtained from Amersham Biosciences (Otelingen, Switzerland). Dynabeads MyOneTM Streptavidin (10 mg ml−1) were purchased from Dynal Biotech (Hamburg, Germany).

The HLA-A2.1-binding HCV peptides Core132 (DLIMGY1PLV, aa 132–140) (32) and Core35 (YLLPRRGPRL, aa 35–44) (32) were obtained from Bachem AG (Bubendorf, Switzerland).

Mice

Immunization experiments were performed in HHD mice transgenic for HLA-A2.1 (A0201) monochrom histocompatibility class I molecule and deficient for both H-2Db and murine
β₂-microglobulin (35). Mice were housed in appropriate animal care facilities and handled according to international guidelines.

**Viruses**

Influenza viruses of the X-31 strain and the A/Sing (A/Singapore/6/86) strain, propagated in the allantoic cavity of embryonated eggs (36), were obtained from Berna Biotech AG (Bern, Switzerland) and purified as described previously (37). The HA : phospholipid ratio was determined by phospholipid determination following the method of Böttcher (38) and HA quantification after SDS-PAGE with the Coomassie-extraction method following the method of Ball (39).

**Preparation of chimeric immunopotentiating reconstituted influenza virosomes**

Chimeric virosomes with HA from the X-31 and the A/Sing Influenza strain, respectively, were prepared by the method described previously (3, 40). Briefly, 32 mg egg PC and 8 mg PE were dissolved in 2 ml of PBS, 100 mM OEG (OEG-PBS). Four micrograms HA of each influenza virus was centrifuged at 100,000 × g for 1 h at 4°C and the pellet was dissolved in 2 ml of OEG-PBS. The detergent-solubilized phospholipids and viruses were mixed and sonicated for 1 min. This mixture was centrifuged at 100,000 × g for 1 h at 20°C and the supernatant was sterile filtered (0.22 μm). Virosomes were then formed by detergent removal using 180 mg of wet SM2 Bio-Beads for 1 h at room temperature with shaking and three times for 30 min with 90 mg of SM2 Bio-Beads each.

**Preparation of liposomes with encapsulated peptide**

A total of 36.4 μmol (28 mg) PC and 15.6 μmol (11 mg) PG (molar ratio 70 : 30) were dissolved in methanol : chloroform (2 : 1). The solvent was removed by a rotary evaporator (Rotavapor R-205, Büchi Labortecnik, Switzerland) at 40°C at a gradual vacuum of 30–10 kPa. The dried lipid film was hydrated with 350 μl PBS containing 2–3.5 mg Core132 peptide to be encapsulated. Before extrusion, the volume was adjusted to 500 μl with PBS. The lipid dispersion was extruded 10 times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 μm; Whatman, Kent, UK) with a 1.5-ml Lipex Extruder (Northern Lipids, Vancouver, Canada). Size determination of extruded liposomes was done by light scattering using a Zetasizer 1000HS instrument (Malvern Instruments, Worcestershire, UK).

**Preparation of Core132-chimeric immunopotentiating reconstituted influenza virosomes**

Chimeric immunopotentiating reconstituted influenza virosomes (CIRIVs; 600 μl in PBS, ~6 mg phospholipid) were incubated with 200 μl (~15 mg phospholipid) of PC/PG-extruded liposomes (0.2 μm diameter) containing the Core132 peptide at 10°C in PBS under constant stirring. To trigger fusion the pH was adjusted to 4.5 with 15 μl of 1 M HCl. After incubation for 30 min, the mixture was neutralized with 15 μl of 1 M NaOH and fusion products were extruded 10 times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 μm) with a 1.5-ml Lipex Extruder (Northern Lipids).

**Fluorescence resonance energy transfer assay**

For in vitro fusion measurements by fluorescence resonance energy transfer (FRET) (41, 42), the following assay was developed: 0.75 mol% of Bodipy 530/550-DHPE and 0.25 mol% of N-Rh-DHPE were incorporated into liposomes consisting of PC : PG (70 : 30). Fluorescence measurements were carried out at discrete temperatures between 4 and 42°C in 5 mM sodium phosphate buffer pH 7.5, 100 mM NaCl, in a final volume of 0.8 ml in 2.5 ml poly(methyl methacrylate) (PMMA) micro-cuvettes (VWR, Dietikon, Switzerland) under continuous stirring. Typically, 1 μl of labelled liposomes (0.3 nmol phospholipid) was mixed with 5–20 μl of virosomes (0.1–0.4 nmol phospholipid) and fusion was triggered by the addition of 3.75–7 μl of 1 M HCl, resulting in a pH of 4.5. The increase in fluorescence was recorded every 5 s at excitation and emission wavelengths of 538 and 558 nm, respectively, with an excitation slit of 2.5 nm and an emission slit of 15.0 nm. Measurements were carried out with an LS 55 Luminescence spectrometer (Perkin Elmer Instruments, Schwerzenbach, Switzerland) equipped with a thermostated cuvette holder and a magnetic stirring device. The maximal fluorescence at infinite probe dilution was reached after addition of Triton X-100 (0.5% w/v final concentration). For calibration of the fluorescence scale the initial residual fluorescence of the liposomes was set to zero and the fluorescence at infinite probe dilution to 100% (maximal fluorescence).

**Affinity precipitation of Core132-CIRIVBio**

Chimeric virosomes with HA from the X-31 and the A/Sing Influenza strain were prepared as described in Preparation of Chimeric Immunopotentiating Reconstituted Influenza Virosomes with addition of 4 mg biotin-DHPE to the phospholipid mixture (CIRIVBio with 1 mg ml⁻¹ biotin-DHPE). Core132-CIRIVBio were prepared as described in Preparation of Core132-Chimeric Immunopotentiating Reconstituted Influenza Virosomes with CIRIVBio instead of regular CIRIVs. For affinity precipitation of Core132-CIRIVBio, 5 μl of Core132-CIRIVBio was added to buffer A (50 mM sodium phosphate, 0.1 M NaCl, pH 7.5) to a final volume of 200 μl. As controls,
Core132-CIRIVBio were supplemented with Triton X-100 to 0.5% (v/v), or Core132-liposomes (equal amount of peptide as Core132-CIRIVBio) were mixed with 5 μl of empty CIRIVBio, in a volume of 200 μl. Hundred microtites of MyOne Streptavidin paramagnetic beads was washed twice in buffer A and incubated with the sample mixtures at 4°C for 1.5 h with continuous shaking. The precipitate was washed twice in buffer A and re-suspended in 25 μl OEG-PBS. After incubation at 4°C for 5 min, the beads were removed and the supernatant was analysed by RP-HPLC as described in Core132 Peptide Quantification.

Immunization and cytotoxicity assay
Where indicated HLA-A2.1 transgenic mice were immunized intramuscularly with inactivated Influenza A/Sing (1 μg HA per dose) 3 weeks prior to immunization with vaccine formulations. Mice were immunized subcutaneously at the base of the tail with 100 μl of virosoome formulation, liposomes with encapsulated peptide, peptide with empty virosoomes, peptide in saline solution, empty virosoomes or saline solution. Mice received Mice were immunized subcutaneously at the base of the tail dose) 3 weeks prior to immunization with vaccine formulations. Where indicated HLA-A2.1 transgenic mice were immunized at 4°C and incubated with the sample mixtures at 4°C for 1.5 h with continuous shaking. The precipitate was washed twice in buffer A and re-suspended in 25 μl OEG-PBS. After incubation at 4°C for 5 min, the beads were removed and the supernatant was analysed by RP-HPLC as described in Core132 Peptide Quantification.

Enzyme-linked immunospot assay
To quantify the frequency of epitope-specific IFNγ-producing cells we used the IFNγ enzyme-linked immunospot (ELISPOT) kit from U-CyTech (Utrecht, The Netherlands). Spleen cells (6 × 10⁶ per well) from immunized mice were re-stimulated in 24-well tissue culture plates with 10 μg ml⁻¹ Core132 peptide or Core35 peptide (negative control) in complete RPMI medium containing 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 5 mM HEPES, 10% FCS and 5 × 10⁻⁵ M 2-mercaptoethanol at 37°C and 5% CO₂. After overnight stimulation 10⁵ and 10⁴ cells from the re-stimulation cultures were transferred in duplicates to a pre-coated and albumin-blocked IFNγ ELISPOT plate (U-CyTech) and incubated for 5 h at 37°C to allow cytokine secretion. Spots were developed following the manufacturer's instructions and were counted by using a microscope. The background level was assessed in wells where effector cells were stimulated with splenocytes pulsed with Core35 peptide. The number of peptide-specific spots was obtained by subtracting the background from the number of spots induced after Core132 peptide stimulation.

Intracellular IFNγ staining
Spleen cells (12 × 10⁶) were incubated with 10 μg ml⁻¹ Core132 peptide or Core35 peptide (negative control) in complete RPMI medium containing 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ Streptomycin, 5 mM HEPES, 5% FCS and 5 × 10⁻⁵ M 2-mercaptoethanol at 37°C and 5% CO₂. In the presence of 5 μg ml⁻¹ Brefeldin A for 4 h. Cells were stained with FITC-conjugated anti-CD8 antibodies, permeabilized and stained with PE-conjugated anti-IFNγ antibodies using the Cytofix/Cytoper kit following the manufacturer’s instructions (BD Pharmingen, San Diego, CA, USA). Data were acquired on a FACScalibur flow cytometer and analysed with WinMDI2.8 software. Frequency of IFNγ-producing cells was calculated as percentage of IFNγ-positive and CD8-positive cells among total CD8-positive cells. The percentage of peptide-specific cells was obtained by subtracting the percentage in samples stimulated with Core35 peptide from the percentage in samples stimulated with Core35 peptide.

Results
The concept of chimeric virosomes
A method was developed that combines the positive properties of virosomes and liposomes with the result of a substantially increased entrapment of peptides or other molecules within functional virosomes (Fig. 1). In a first step, a dried lipid film of PC and phosphatidylglycerol (PG) is prepared, and liposomes are formed by re-hydration of the film with a small volume of a concentrated peptide solution. The formed liposomes efficiently encapsulate the peptide, but are multi-lamellar and heterogeneous. Unilamellar and homo- geneous liposomes are obtained after a defined number of cycles of extrusion through a 200-nm filter. In a second step, CIRIVs with a binary mixture of HA molecules from different influenza virus strains are prepared (hereafter called CIRIVs). The HA molecules of the used strains display different temperature-dependent fusion characteristics: At pH 5 or lower, the X-31 HA triggers fusion efficiently already at low temperatures, whereas at the same pH, the HA from the influenza virus strain A/Sing requires elevated temperatures (>25°C) (13, 16). Thus, CIRIVs containing the HA molecules from both the X-31 and A/Sing virions are able to catalyse two distinct fusion reactions at a pH ~4.5, the first one at a low temperature (<20°C) and the second one at an elevated temperature (>25°C). Finally, peptide-containing CIRIVs result from fusion of liposomes with encapsulated peptides and CIRIVs at a pH ~4.5 and a temperature of 10°C. The fused vesicles are re-sized to a diameter of 200 nm by extrusion. These peptide-containing proteoliposomes are supposed, like intact influenza virus, to be taken up by cells through receptor-mediated endocytosis. In the acidic environment of the endosome, the A/Sing HA molecules are expected to trigger the second fusion reaction between the virosomal and endosomal
membranes, with the consequent release of the peptide into the cytosolic compartment of the cells.

**Generation of Core132-CIRIVs**

CIRIVs containing Core132 peptide were prepared with this novel procedure (Fig. 1). A concentrated peptide solution of 10 mg ml\(^{-1}\) in PBS was used to re-hydrate the lipid film. Determination of peptide concentrations was performed on a RP-HPLC. To remove free peptide, Core132-CIRIVs were purified over Sephadex G50 media with a fractionation range of \(M_r 1.5 \times 10^3 - 3 \times 10^4\) for globular molecules. Therefore, virosomes were not fractionated, whereas peptide was retained by the media. As a control for the efficiency of this method, a defined amount of free Core132 peptide was added to empty liposomes or A/Sing virus and purified over Sephadex G50 (spiking control). No Core132 was found in the purified liposome and virus fractions, (data not shown).

Fifty to seventy five percentage of the added Core132 peptide was encapsulated in the formed liposomes (Table 1). After extrusion, 30–40% of the starting material was still found inside the homogenized Core132-liposomes. To obtain the Core132-CIRIVs, CIRIVs and Core132-liposomes were incubated at a pH ~4.5 at 10°C for 20 min. After neutralization, the fused chimeric vesicles were homogenized by extrusion to a diameter of 200 nm. In contrast to the first extrusion step applied on Core132-liposomes, no encapsulated peptide was lost during the extrusion of the fused chimeric vesicles. Therefore, 30–40% of the starting amount of peptide was finally found entrapped inside the Core132-CIRIVs. Typical concentrations obtained for the encapsulated Core132 peptide were up to 300 \(\mu\)g ml\(^{-1}\). With the conventional detergent-removal method an entrapment efficiency <1% was obtained for the Core132 peptide, as a maximal concentration of 10 \(\mu\)g ml\(^{-1}\) was determined (data not shown). The peptide concentration inside homogenic CIRIVs did not decrease when stored at 4°C for at least 4 weeks (data not shown). The peptide inside CIRIVs is protected against degradation by proteases *in vitro* (e.g. proteinase K; data not shown).

To demonstrate the efficiency of the fusion reaction between the Core132-liposomes and the CIRIVs, an affinity precipitation for Core132-CIRIVs was performed. For this purpose, biotin-DHPE was incorporated into CIRIVs (CIRIV Bio) before the fusion with Core132-liposomes. Precipitation of different samples with and without fusion with magnetic streptavidin beads allowed the isolation of vesicles with biotin (CIRIV before fusion, Core132-CIRIV after fusion), whereas free peptide, and not fused Core132-liposomes, did not precipitate. The precipitate was dissolved in OEG-PBS and Core132 peptide in the precipitate was quantified by RP-HPLC, and compared with the starting amount of peptide (Fig. 2). After precipitation of fused Core132-CIRIV\(_{\text{Bio}}\), ~90% of the peptide was found in the precipitate. The presence of free Core132 peptide did not influence the amount of affinity-precipitated Core132-CIRIV\(_{\text{Bio}}\), indicating the irrelevance of the presence of free peptide on the fusion reaction (data not shown). When fused Core132-CIRIV\(_{\text{Bio}}\) were incubated with streptavidin beads in the presence of detergent [0.5% (v/v) Triton X-100], no Core132 peptide was detected in the precipitate, excluding unspecific binding of Core132 peptide to the magnetic beads. When Core132-liposomes were mixed with empty CIRIV\(_{\text{Bio}}\) without initiating a fusion reaction, no Core132 peptide was detected in the precipitate, excluding stable interactions between liposomes and empty CIRIVs. However, the presence of HA in the precipitate was demonstrated by immunoblotting with an anti-HA antibody (data not shown). These results show that
under the specified conditions at least 90% of the Core132-liposomes fused with CIRIVs.

Core132-CIRIVs are fusogenic

To monitor the pH and temperature-dependent fusion activity of chimeric virosomes with liposomes in vitro, a FRET assay was used (41). This assay makes use of two fluorophores, an energy donor and an energy acceptor, both integrated in the membrane of the liposomes. The fluorescence signal of the energy donor is recorded continuously. Upon fusion of the labelled liposomes with unlabeled virosomes or influenza virus, the two fluorophores move apart and the fluorescence emitted by the donor increases. In this study, Bodipy 530/550-PE was used as the donor and rhodamine-PE as the acceptor. The fusion activity of Core132-CIRIVs was compared with that of CIRIVs and IRIVs (A/Sing) as well as with that of A/Sing and X-31 virus (Fig. 3). As target membranes, unilamellar liposomes prepared of PC : PG (70 : 30) and the two fluorophores (donor : acceptor, 0.75 : 0.25) were used (0.3 nmol of phospholipid) and mixed with virosomes or virus (~0.4 nmol of phospholipid), respectively, in a buffer at neutral pH and pre-incubated at the defined temperature (37 or 10°C). This fluorescence signal constituted the baseline and was set to 0% fluorescence. The fusion process was initiated by addition of HCl to reach a pH of ~4.5, and stopped by the addition of the detergent Triton X-100 to 0.5% (v/v). The latter fluorescence value corresponding to ~4.5, and stopped by the addition of the detergent Triton X-100 to 0.5% (v/v). The latter fluorescence value corresponding to infinite probe dilution was set to 100% fluorescence. As expected, A/Sing virus could fuse to liposomes at 37°C but not at 10°C (Fig. 3A and G). IRIVs with A/Sing HA showed the same properties (Fig. 3B and H), whereas X-31 virus showed fusion activity at both temperatures (Fig. 3C and I). IRIVs with X-31 HA had the same properties as X-31 virus (data not shown). The CIRIVs with A/Sing HA and X-31 HA consequently could fuse both at 37 and at 10°C (Fig. 3D and J). In contrast, Core132-CIRIVs were fusogenic at 37°C, but could no longer fuse at 10°C, indicating that the A/Sing HA remained functional during the first fusion step at 10°C (Fig. 3E and K). As a control, no fusion was observed with Core132-CIRIVs when buffer instead of HCl was added (pH 7.5; Fig. 3F and L), or when unlabeled liposomes or inactivated virosomes were used (data not shown). Homologous Core132-CIRIVs remained fusogenic for at least 4 weeks when stored at 4°C (data not shown).

**CD8⁺ T cell response induced by chimeric virosomes with encapsulated peptide**

To study the immunogenicity of the CIRIVs HLA-A2 transgenic mice were immunized twice with CIRIV formulations containing various concentrations of the Core132 peptide (2, 5.5 and 10 µg per dose) or empty CIRIVs or PBS as negative controls. Two weeks after the last immunization spleen cells of individual mice were isolated and re-stimulated in vitro with peptide. After 5 days of in vitro stimulation, the CTL response for Core132 peptide was investigated by ⁵¹Cr-release assay using HHD-transfected EL4 cells as target cells. In two independent experiments all the Core132 containing CIRIV formulations induced a strong CTL response in all of the immunized mice (Fig. 4A and B). The strength of the induced response was dose dependent with the highest dose giving the highest specific lysis of ~47% lysis at an E : T ratio of 33 : 1. Neither in naive mice nor in mice immunized with empty CIRIVs a substantial lysis of the target cells could be detected.

In addition, IFNγ release was chosen as another indicator of T cell response induced by Core132-CIRIV immunization. IFNγ release was quantified by ELISPOT assay (Fig. 5). The numbers of IFNγ-producing cells correlated well with the peptide-specific cytotoxicity with higher numbers induced by higher amounts of Core132 peptide in the CIRIV formulations. In control experiments with three mice per group using the same immunization schedule and the same methods for analysis neither large unilamellar vesicles (LUVs) with encapsulated peptide (20 µg per dose), nor peptide with empty virosomes (20 µg per dose) nor peptide in saline solution (20 µg per dose) induced a detectable immune response in the ⁵¹Cr-release assay and the ELISPOT assay (data not shown).

To get a better idea about the frequency of specific T cells in the CD8⁺ population, another set of experiments were performed using staining for intracellular IFNγ. In addition, we wanted to test whether pre-existing immunity to influenza, which better reflects the situation in humans would affect the induction of HCV Core132-specific CTL. Therefore, naive and influenza immune mice were vaccinated and boosted 3 weeks later with the same Core132-CIRIV formulations or Core132-LUV and the frequency of specific IFNγ-producing cells was determined in the CD8⁺ population with flow cytomtery.
Compared with the Core132-LUV-vaccinated mice (negative control) in most of the Core132-CIRIV-vaccinated mice an increased frequency of peptide-specific CD8+ T cells could be detected with a maximal frequency of 2.6% (Fig. 6). In addition pre-existing immunity to influenza had no effect on the frequency of peptide-specific CD8+ T cells.

**Discussion**

The experiments described represent an effort to develop an improved viroside-based carrier system for the delivery of macromolecules (e.g., peptides) into cells, especially used as therapeutic vaccines. Since IRIVs are already registered for human use (4) and because of their safety profile and their rapid cellular uptake HA-virosomes represent a promising system. A further advantage of HA-virosomes is their potential targeting to specific cells or tissues by co-reconstituting receptors or antibodies (43) within the virosomal membrane. For this purpose a high concentration of the encapsulated macromolecules is of great importance. In addition, HA-virosomes have been shown to work as an excellent adjuvant in humans (44), a characteristic that is of interest for the use of HA-virosomes in vaccination. We have described a new methodology to improve the encapsulation efficiency by using CIRIVs. CIRIVs combine the advantages of liposomes and virosomes, namely the high encapsulation efficiency of liposomes, the receptor-mediated endocytosis and the membrane fusion properties of virosomes as well as the adjuvant
Fig. 4. Cytotoxic T cell responses in Core132-CIRIV-immunized HHD mice. In two independent experiments (A) and (B) HHD mice (2–3 mice per group) were immunized twice subcutaneously at a 3-week interval with empty IRIVs or Core132-CIRIVs. Spleen cells were isolated 2 weeks after the second immunization. After 5 days of in vitro re-stimulation with Core132 peptide, the stimulated spleen cells were used as effector cells against HHD-transfected EL4 as target cells in a Cr-release assay with an E:T ratio starting at 33:1. (A) Mice were immunized with empty IRIVs (open circles), or CIRIVs containing 2 μg (closed circles) or 5.5 μg (triangles) Core132 per dose. (B) Mice were naive (open circles) or immunized with CIRIVs containing 5.5 μg (triangles) or 10 μg (squares) Core132 per dose. Results show peptide mean specific lysis (lysis of target cells with peptide—lysis of target cells without peptide) of 2–3 individual mice ± standard deviation.

The present method has also been used for the incorporation of other peptides with similar results. Lower entrapment efficiencies <1% were observed. The prepared proteoliposomes were found to undergo efficient fusion with membrane membranes at low pH and elevated temperatures. In contrast to previous reports with other virosomes (45, 46), no leakage was observed with Core132-CIRIVs during the fusion process, in agreement with the situation for alphaviruses (47).

The present method has also been used for the incorporation of other peptides with similar results. Lower entrapment efficiencies were generally obtained with hydrophobic peptides (M. Amacker, unpublished results). Furthermore, CIRIVs can also be used for different applications. Virosomes are, for example, a suitable carrier system for DNA and RNA since these fusogenic particles mimic a virus. The genetic material is enclosed in the viosome and is therefore protected from DNases and RNases. Virosomes could also serve as a drug delivery system to the cytosol. Using mAbs to tumour-associated antigens, virosomes could be targeted to cancer cells and therefore allow specific immunotherapy (48).

As a very important point we were able to show that pre-existing immunity to influenza virus, reflecting the situation in humans, did not affect the induction of IFNγ-producing CD8+ T cells by CIRIV. On the contrary it can be speculated that influenza-specific CD4+ T cells could provide help to rise a CTL response directed against the peptide encapsulated in the virosome. Such a phenomenon has been observed in vitro with human PBMCs where virosomes activated CD4+\textsuperscript{+} CD45RO+ T cells and induced a cytokine profile consistent with Th1 stimulation (51). Alternatively, the lack of adjuvancy...
found in these experiments may also reflect a limitation of the transgenic mouse model.

These data provide evidence that peptide bearing CIRIVs remain fusogenic and are effective at inducing specific, cytotoxic and IFN-γ-producing T cells in HLA-A2.1 transgenic mice. Therefore, such vaccine formulations including combinations of different CTL epitopes could be used to induce not only a strong but also a multi-specific CTL response, making them potential candidates for prophylactic and/or therapeutic vaccines in humans against HCV and other targets, especially as virosome formulation are already accepted for use in humans.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>Bodipy 530/550-DHPE</td>
<td>1,3-diphenyl-1-propynyl-4,4-difluoro-5,6-dihydro-2H-indene-3-carboxylic acid N-ethyl ester</td>
</tr>
<tr>
<td>CIRIV</td>
<td>chimeric immunopotentiating reconstituted influenza virosome</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>enzyme-linked immunospot</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>IRIV</td>
<td>immunopotentiating reconstituted influenza virosome</td>
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References

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immunopotentiating reconstituted influenza virosomes as a non-
live prototype vaccine against hepatitis C virus. *Int. Immunol.* 14:615.


