

A liposomal peptide vaccine inducing CD8⁺ T cells in HLA-A2.1 transgenic mice, which recognise human cells encoding hepatitis C virus (HCV) proteins

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Received 21 October 2003; received in revised form 4 May 2004; accepted 12 May 2004

Available online 19 June 2004

Abstract

Virus specific T cell responses play an important role in resolving acute hepatitis C virus (HCV) infections. Using the HLA-A2.1 transgenic mouse model we investigated the potential of a liposomal peptide vaccine to prime a CD8⁺ T cell response against 10 different HCV epitopes, relevant for human applications. We were able to demonstrate the induction of strong cytotoxic T cell responses and high numbers of IFN- γ -secreting cells, which persisted at high levels for at least 3 months. Co-integrating CpG oligonucleotides into liposomes further increased the number of IFN- γ -secreting cells by 2–10-fold for most epitopes tested. The frequency of specific cells was further analysed with chimeric A2 tetramers bearing the NS31073-1081 epitope and was estimated at 2–23% of the CD8⁺ T cell population. Importantly, mouse effector cells, specific for this epitope, were also capable of lysing a human target cell line expressing HCV proteins. This finding and the specific protection observed in challenge experiments with recombinant vaccinia virus expressing HCV sequences emphasise the biological relevance of the vaccine-induced immune response. In conclusion, such liposome formulations represent a safe and promising strategy to stimulate the CD8⁺ T cell against HCV.

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Keywords: Hepatitis C virus; Liposomes; Peptide immunisation

1. Introduction

The hepatitis C virus (HCV) was identified as the major cause of non-A, non-B hepatitis in 1989 [1] and transmission routes were described to be mainly parenteral [2]. Worldwide an estimated 170 million people are chronically infected [3] and the prevalence of HCV-related long-term complications continues to increase [4]. HCV infection leads to viral persistence in 50–85% of the patients with progression to liver

cirrhosis (20%) and liver cancer (1–5% per year) [4]. The standard therapy with pegylated IFN- α and ribavirin has a limited success rate of 50–60% and can provoke significant side effects [2]. The HCV genotype 1 is particularly poorly susceptible to this treatment [5]. Thus, alternative treatment strategies are urgently required.

The observation that 15–50% of acutely infected individuals can clear the virus without therapeutic intervention has led to extensive studies investigating the parameters of the immune system correlating with viral elimination. One of the major difficulties in combatting HCV is the extremely high mutation rate of this RNA virus [6]. Although antibodies are

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elicited during the infection, they do not seem to prevent progression to chronicity [7]. Viral clearance has been associated with CD4⁺ T cell activation and, importantly, a strong and sustained CD8⁺ T cell response against multiple viral epitopes [8,9]. The chronic state is characterised by a low frequency of specific T cells [10] and reduced responsiveness of the CD8⁺ T cells [11–14]. Based on this, we hypothesize that a therapeutic vaccine should aim at reactivating T cell responsiveness or priming new CD8⁺ T cell responses specific for multiple epitopes to effectively eliminate the virus.

This study combines the knowledge of many HLA-A2-restricted HCV T cell epitopes that have been identified and characterised with the availability of HLA-A2.1 transgenic mice as a preclinical animal model. Our approach uses liposomes as an efficient and immunostimulatory mode to deliver peptides to antigen-presenting cells (APC) [15,16]. Liposomes as well as peptides are produced from natural or synthetic compounds at high purity and liposomal formulations have been safely used in humans in several clinical studies, such as in anti-cancer treatment [17,18].

We investigated the requirement of an extrinsic CD4⁺ T cell epitope or the immune stimulatory molecule CpG for the efficient priming of a CD8⁺ T cell response, concentrating on the capacity of such vaccine formulations to stimulate specific cytotoxicity as well as IFN- γ secretion and to mount a memory response. To demonstrate the biological relevance of the antiviral T cells induced we show effectiveness of our prototype vaccine using a recombinant vaccinia virus challenge model and cytotoxicity elicited against human cells endogenously processing HCV epitopes.

2. Material and methods

2.1. Mice

Six- to eight-week-old HDD mice, transgenic (tg) for HLA-A2.1 (A0201) major histocompatibility complex

(MHC) class I and deficient for both H-2D^b and murine β_2 -microglobulin (β_2m) [19] were used. HLA-2.1 tg mice were bred by IFFA credo (L'Arbresle, France) with the permission of F.A. Lemonnier (Institut Pasteur, Paris, France). During the entire study, mice were kept at the animal care facilities of the Department of Medicine, University of Bern, and experiments were conducted according to the international guidelines for animal experimentation.

2.2. Viruses and cell lines

Recombinant vaccinia virus vv9A, encoding the full-length HCV core protein and part of the E1 protein (amino acids [aa] 1–339 of genotype 1a sequence) was kindly provided by M. Houghton (Chiron, Emeryville, CA). The virus was propagated and titrated in the osteosarcoma cell line 143 TK⁻. As target cells for CTL assays we used EL-4 S3⁻Rob HDD (H-2b) thymoma cells expressing HLA-A2 MHC class I molecules (kindly provided by G. Inchauspé, BioMérieux, Lyon, France). In addition, T1/NS3-4A (clone 2F3), a human HLA-A2⁺B lymphoblastoid \times T cell hybrid cell line constitutively expressing the HCV NS3-4A complex was used. This cell line will be described in detail elsewhere (B. Wölk et al., manuscript in preparation).

2.3. Peptides and oligonucleotides

All peptides were purchased from Neosystem (Strasbourg, France) at a purity of >95%. All peptide sequences were derived from the HCV 1a genotype. For CTL responses, we tested the HCV peptides listed in Table 1. The T helper peptide, derived from the HBV nucleocapsid aa 128–140, (TPPATRPPNAPIL) [20], here after referred to as HBV, was included in some liposomal formulations. The universal T helper epitope PADRE (aKX-VAAWTLKAA, where X is L-cyclohexylalanine and a is D-alanine) [21] was integrated in liposomes or cross-linked to the liposome surface via a linker and a C termi-

Table 1
HLA-A2 restricted HCV epitopes

Protein	Name	aa residue	Sequence	% Conservation in genotype	
				1a	1b
Core	35	35–44	YLLPRRGPRRL	95 (84/88)	96 (453/471)
	132	132–140	DLMGYIPLV	97 (86/88)	95 (452/474)
E2	614	614–622	RLWHYPCTI	82 (19/23)	2 (3/153)
	686	686–694	ALSTGLIHL	90 (20/22)	100 (148/148)
NS3	1073	1073–1082	CINGVCWTV	100 (18/18)	26 (39/150)
	1169	1169–1177	LLCPAGHAV	78 (14/18)	2 (3/141)
	1406	1406–1416	KLVALGINAV	83 (15/18)	0 (0/143)
	1585	1585–1593	YLVAYQATV	100 (18/18)	99 (144/145)
NS4	1769	1769–1777	HMWNFISGI	94 (17/18)	95 (144/151)
	1851	1851–1859	ILAGYGAGV	100 (18/18)	98 (149/151)

Amino acid sequence comparisons within viral genotype 1a and 1b. Sequence comparisons were made using HCV USP library and alignment tool CLUSTALW provided on the public HCV DB database (<http://hcvpub.ibcp.fr/>). Indicated are the calculated percentage of conserved sequences and numbers of identical sequences/total numbers of sequences found.

nal cystein (-AGSGGC). The immunostimulatory oligonucleotide ODN1668 (5'-TCCATGACGTTCCCTGATGCT-3') [22], referred to as CpG was synthesised by Microsynth (Balgach, Switzerland).

2.4. Preparation of liposomes

Small unilamellar liposomes were prepared by freeze-thawing followed by sequential filter extrusion. The basic composition of the liposomes used was 200 mg/ml soy phosphatidylcholine (SPC, L. Meyer GmbH, Hamburg, Germany), 25 mg/ml cholesterol (Fluka, Buchs, Switzerland) and 1.2 mg/ml DL- α -tocopherol (Merck, Darmstadt, Germany). The lipids were dissolved in methanol/methylene chloride (1:1, v:v) and after removal by rotary evaporation, the lipid mixture was solubilised with one of the HCV peptides (4 mg/ml) in phosphate buffer (67 mM, pH 7.4). Some formulations contained, in addition to the CD8⁺ T cell epitope, a CD4⁺ T cell epitope (4 mg/ml) or CpG (250 nmol/ml). The mixture was subjected to 3–5 freeze-thaw cycles, followed by repetitive extrusion through Nuclepore (Sterico AG, Dietikon, Switzerland) filters (0.8, 0.4 and 0.2 μ m pore size) using a LipexTM Extruder (Lipex Biomembranes Inc., Vancouver, Canada). Liposomes were filter sterilized and diluted in BSS (balanced salt solution). Peptide encapsulation was estimated to range between 80 and 90% according to previous determinations [16]. The thiol-modified PADRE peptide was cross-linked to maleimide-functionalised liposomes containing peptides as described by Console et al. [23].

2.5. Immunisation and cytotoxicity assay

HLA-2.1 tg mice were immunised subcutaneously (s.c.) at the base of the tail with 50 μ l of the liposome formulation (~130 μ g peptide) and as negative control with empty liposomes or with a saline solution containing the same peptides (130 μ g peptide). Mice received three injections at a 2-week interval and the response was analysed 2 weeks after the last injection, or 3 months after the last injection for the memory response experiments. Spleen cells (4×10^6 /well) from immunised mice were restimulated for 5 days in 24-well tissue culture plates with 2×10^6 irradiated (1500 rad) spleen cells that had been pulsed with 10 μ g/ml peptide, in complete RPMI medium (Sigma–Aldrich) containing 2 mM L-Glutamine, 100 U penicillin, 100 μ g/ml Streptomycin (Sigma Aldrich), 5 mM HEPES, 10% FCS (Gibco BRL, Basel, Switzerland) and 5×10^{-5} M 2-mercaptoethanol at 37 °C and 5% CO₂. On day 2, 5 U/ml IL-2 (EuroCetus B.V., Amsterdam, The Netherlands) were added. Specific cytolytic activity was tested in a standard ⁵¹Cr release assay against EL-4S3⁻ Rob HHD targets pulsed with 10 μ g/ml of the selected peptides or medium as control. To determine the recognition of endogenously processed epitopes we used the human HLA-A2 expressing cell line T1/NS3-4A. After 4 h incubation ⁵¹Cr release was measured by using a γ -counter. Spontaneous and maximal release

was determined from wells containing medium alone or after lysis with 1N HCl, respectively. Lysis was calculated by the formula: (release in assay – spontaneous release)/(maximum release – spontaneous release) \times 100. Peptide-specific lysis was determined as the percentage of lysis obtained in presence or in absence of the peptide.

2.6. ELISPOT and ELISA assay

To quantify the frequency of epitope-specific IFN- γ -producing cells we used the IFN- γ ELISPOT kit from U-CyTech (Utrecht, Netherlands). Effector cells were restimulated with peptide-pulsed, irradiated and repeatedly washed splenocytes as described for CTL analysis. After overnight stimulation 10^5 or 10^4 cells from the restimulation cultures were transferred in duplicates to a precoated, albumin-blocked IFN- γ ELISPOT plate (U-CyTech) and incubated for 5 h at 37 °C to allow cytokine secretion. Spots were developed following the manufacturers instructions and counted either by using a microscope or the Bioreader 3000 Pro (Bio-Sys GmbH, Karben, Germany). The background level was assessed in wells where effector cells were stimulated with splenocytes pulsed with a control peptide. The number of peptide-specific spots was obtained by subtracting the background from the number of spots induced after HLA-A2-peptide stimulation. ELISA assays were performed according to the manufacturer's protocol (BD Bioscience, Basel, Switzerland) using 100 μ l of supernatants from effector cells restimulated for 2 days as described above.

2.7. Magnetic cell sorting (MACS), tetramer staining and intracellular cytokine staining

The frequency of antigen-specific cells was determined by tetramer staining ex vivo. Spleen cells from immunised mice were sorted on the Vario MACS using CD8 α (Ly-2) MicroBeads and LS columns according to the manufacturer protocol (Miltenyi Biotech, Gladbach Germany). The enriched CD8⁺ T cell population was stained with anti-mouse CD8 α -FITC antibodies (BD Bioscience, Basel, Switzerland) and PE-labelled A-2Kb chimeric tetramer, specific for the epitope 1073 (ProImmune, Oxford, UK), according to manufacturers protocol. As a control, we used PE-labelled A-2Kb chimeric tetramers containing c132 (ProImmune). For the intracellular cytokine staining splenocytes were restimulated over night in 24 well plates (8×10^6 splenocytes in culture medium) with 10 μ g/ml peptide (1073 or control peptide). Cells were subsequently treated for 2.5 h with Brefeldin A (Golgy Stop, BD Bioscience) and permeabilization/fixation was performed with BD Cytoperm/Cytofix (BD Bioscience) according to manufacturer's protocol. Surface CD8 was stained with anti-mouse CD8 α -FITC antibodies (BD Bioscience) and IFN- γ staining was performed with anti-mouse IFN- γ -PE antibodies or isotype control antibodies (BD Bioscience). Fluorescence was analysed on a Coulter Epics XL-MCL flow cytometer (Coulter Corp., Hialeah, FL, USA).

2.8. *In vivo* protection against recombinant vaccinia virus expressing HCV proteins

Mice were immunised twice at an interval of 14 days with liposome formulations containing CpG and the peptides 35 or 132. As negative controls, we used naïve mice or mice immunised with liposomes containing CpG and the peptide 1073, which is not present in the HCV sequence expressed by the recombinant vaccinia virus. Eleven days after immunisation mice were challenged i.p. with 1×10^7 pfu recombinant vaccinia virus vv9A. Five days after vaccinia challenge, mice were sacrificed and ovaries removed. After homogenisation viral load was assessed by plating serial 10-fold dilutions of samples on 24-well plates of confluent 143 TK⁻ cells. After 2 days' culture, cells were stained with crystal violet to detect plaque formation. Groups were compared statistically using the Wilcoxon–Ranks test for non-normally distributed variables.

3. Results

3.1. Liposome-encapsulated CD8⁺ T cell epitopes do not require the help of a CD4⁺ T cell epitope or immune stimulatory molecules for the priming of CTL

The first set of experiments aimed at characterising the efficiency of a liposome based peptide vaccine and the influence of a helper epitope or CpG oligonucleotide on the induction of a specific cytotoxic T cell response in HLA-A2.1 tg mice. As a model peptide in these first experiments we used the well characterised and highly conserved CD8⁺ T cell epitope 132 from the HCV core protein (Table 1), representing one of the most promising candidate epitopes for peptide-based HCV vaccines. Fig. 1A shows that encapsulating the peptide 132 into liposomes was sufficient to induce a specific and strong CTL response against peptide-pulsed target cells. No specific cytotoxicity was detected in the negative control formulations consisting of peptides solubilised in 0.9% NaCl (Fig. 1A) or empty liposomes (data not shown). The addition of the so-called universal T helper epitope PADRE [21] integrated in liposomes or coupled to the surface via a cysteine-maleimide link could not further enhance the response. In contrast, co-administration of immunostimulatory CpG molecules resulted in augmented target cell lysis, however, detectable mainly at low effector to target ratios. In these experiments CpG was co-integrated with the CD8⁺ T cell epitope and liposomes were not dialysed to remove non-integrated CpG, as previous analysis had shown that removal of external CpG by dialysis had no influence on the CTL induction (data not shown). In contrast to other studies [24] we did not observe a CpG-related spleen enlargement or increased lymphocyte numbers in immunised mice.

To determine whether the CD4⁺ T cell epitope PADRE was capable of eliciting a T helper response when co-integrated in liposomes or cross-linked to the surface we

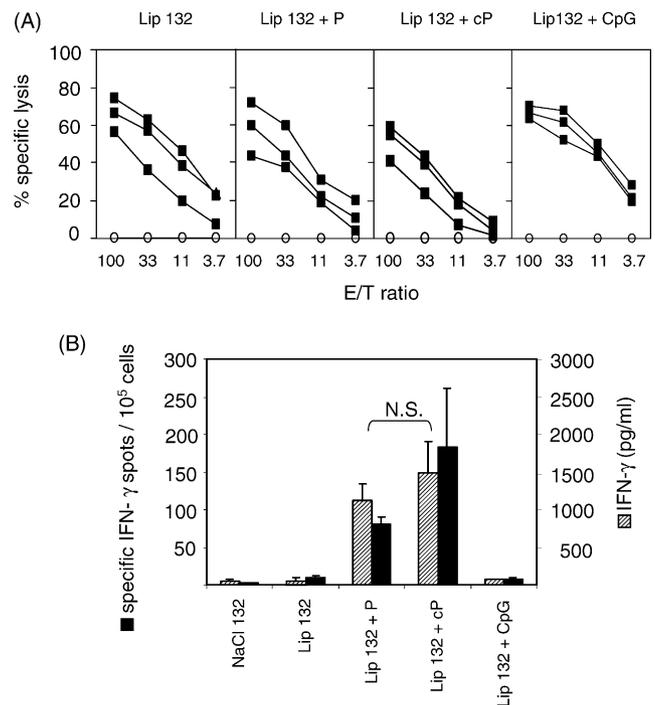


Fig. 1. Efficacy of the liposome formulations and the influence of the T cell helper epitope PADRE and the oligonucleotides CpG on CTL induction. (A) HLA-A2.1 tg mice (three per group; filled squares) were immunised with liposome formulations containing exclusively the CTL epitope core 132 (Lip 132), the CTL epitope and PADRE encapsulated (Lip 132 + P), the CTL epitope encapsulated and PADRE cross-linked to the liposome surface (Lip 132 + cP) or the CTL epitope encapsulated together with CpG (Lip + CpG). A control group was injected with the peptide 132 in saline (open circles representing the mean specific lysis of three control mice). Two weeks after the final immunisation spleen cells were isolated and restimulated for 5 days. Specific lysis was analysed in a standard ⁵¹Cr release assay with peptide-labelled target cells. In (B) the priming of PADRE-specific CD4⁺ T cells is shown. Cell cultures from immunised mice were stimulated over night with the epitope PADRE and IFN-γ production was analysed by ELISPOT (■) or by ELISA (▨).

measured the PADRE-specific IFN-γ secretion by ELISPOT assay and ELISA (Fig. 1B). Mice immunised with formulations containing the helper epitope had a 10–20-fold increased IFN-γ production, suggesting the *in vivo* priming of T helper cells. We found no significant difference between integrated and cross-linked CD4⁺ T cell epitopes. The IL-2 secretion upon *in vitro* stimulation with PADRE was generally high, leading to PADRE-specific proliferation also in cell cultures from naïve mice (stimulation index: 2–9). This may explain the difficulty in demonstrating increased PADRE-specific IL-2 secretion in vaccine-primed cells.

3.2. Induction of high-affinity CTL that recognise endogenously processed epitopes and have the capacity to mount a CD8⁺ T cell memory

The second set of experiments was performed to investigate the recognition of endogenously processed protein by cytotoxic T lymphocytes from liposome-vaccinated mice.

To this end we used as target the human HLA-A2⁺ cell line T1/NS3-4A transfected with the NS3-4A gene sequence. Since in previous experiments we could show that the epitope 1073 was processed and efficiently presented to human CTL (data not shown), the following experiments, investigating the recognition of endogenously processed proteins, were based on this dominant NS3 epitope. Mice were injected three times with liposomal formulations containing either exclusively the epitope 1073 or combinations with the T helper epitope HBV core 128 or with CpG. Two weeks after the immunisation we found strong lysis of peptide pulsed target cells for all formulations tested (Fig. 2A). Interestingly, the mouse effector

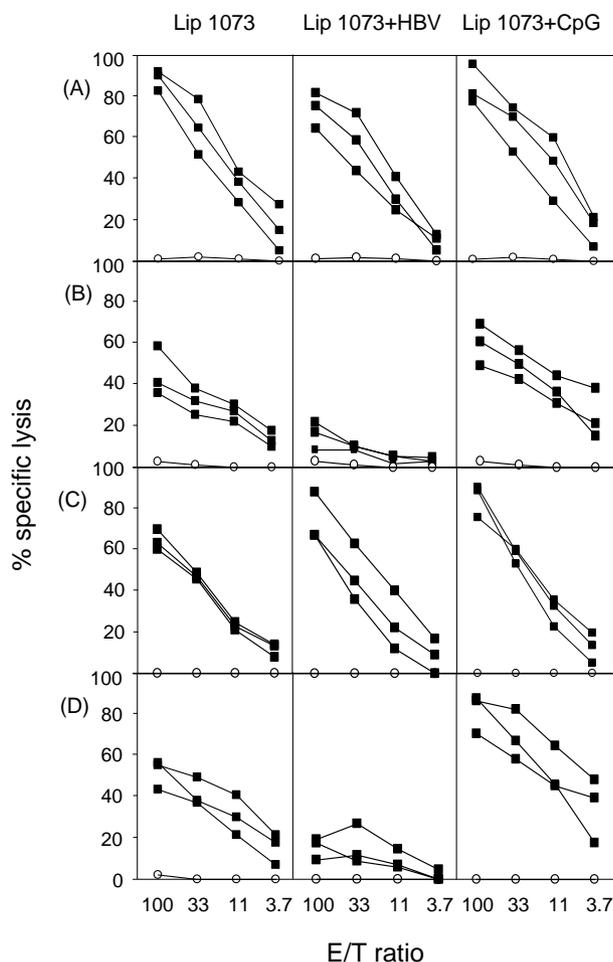


Fig. 2. Recognition of endogenously processed epitope 1073. HLA-A2.1 tg mice (three per group) were immunised with liposomes containing the CTL epitope 1073 (Lip 1073), the CTL epitope plus the T helper epitope HBV core 128 (Lip 1073 + HBV) or the CTL epitope together with CpG (Lip 1073 + CpG). Specific lysis was analysed 14 days after three injections (A) using peptide pulsed EL-4S3^{-/-} Rob HDD target cells. Recognition of endogenously processed epitope 1073 was analysed by using a human HLA-A2.1⁺ target cell line stably transfected with the NS3-4A sequence (T1/NS3-4A) (B). The memory response induced with these liposomal formulations was analysed 3 months after the final immunisation using peptide pulsed target cells (C) or T1/NS3-4A endogenously expressing 1073 (D). The filled squares represent mice vaccinated with liposomal formulations and open circles stand for the mean specific lysis of three control mice immunised with peptide in saline. All experiments were performed 2–3 times.

cells were also able to recognise and lyse the human target cell line T1/NS3-4A, presenting the epitope upon endogenous processing (Fig. 2B). However, the effector cells generated by liposomes with a co-integrated CD4⁺ T cell epitope were very inefficient in lysing these transfected human target cells. This observation was confirmed in three independent experiments and may reflect the difficulty of these effector cells to recognise target cells presenting only low numbers of the epitope, as the addition of exogenous peptides completely restored the lysis of the T1/NS3-4A target cells (data not shown). To investigate the capacity of liposomal formulations to prime memory T cells, the CTL response was also analysed 3 months after the last application (Fig. 2C). The lysis of peptide pulsed target cells remained high and was comparable between the different formulations tested. As already described for the response analysed after 14 days, the cell line T1/NS3-4A was efficiently lysed only by memory CTL induced by formulations containing exclusively the CD8⁺ T cell epitope or, even more efficiently, when CpG was co-integrated in the liposomes (Fig. 2D).

3.3. Liposomal peptide formulations prime and maintain a high number of epitope-specific IFN- γ -secreting T cells

IFN- γ is a potent immunostimulatory and anti-viral cytokine, which is thought to play a significant role in controlling HCV infection by inhibiting viral replication [25]. The frequency of specific IFN- γ -secreting cells stimulated by liposomal formulations exclusively containing the CTL epitope 1073 or combinations with the T helper epitope HBV core 128 and CpG, respectively, was evaluated by ELISPOT assay. Two weeks after three immunisations, we found high numbers of specific IFN- γ -secreting cells (0.2–0.4% of total spleen cells) with comparable results for the different liposomal formulations tested (Fig. 3A). The frequency of specific cells producing IFN- γ persisted at high levels for at least 3 months (0.1–0.2% of total spleen cells; Fig. 3B), demonstrating the efficient priming of memory cells with the capacity to secrete IFN- γ . Interestingly, we repeatedly found that in mice immunised with liposomes containing CpG the IFN- γ -secreting peptide-specific T cells seemed to have further expanded (0.5–0.6% of total spleen cells; Fig. 3B). By separating CD8⁺ and CD8⁻ T cell populations (MACS) we could demonstrate that the specific IFN- γ secretion was restricted to the CD8⁺ T cells (data not shown). The frequency of 1073 specific cells was further determined ex vivo by tetramer staining. Using the enriched CD8⁺ T cell population we stained specific T cells with chimeric A-2Kb tetramers bearing the 1073 epitope (Fig. 3C). We show results from pooled CD8⁺ T cells derived from three mice, immunised either with liposome 1073 or with liposome 1073 plus CpG, or from control immunised mice. The frequency of specific cells determined by tetramer staining 14 days after the last injection was 2% of the CD8⁺ T cells in mice immunised with liposomes containing only the peptide 1073, while mice immunised with liposomes containing CpG had

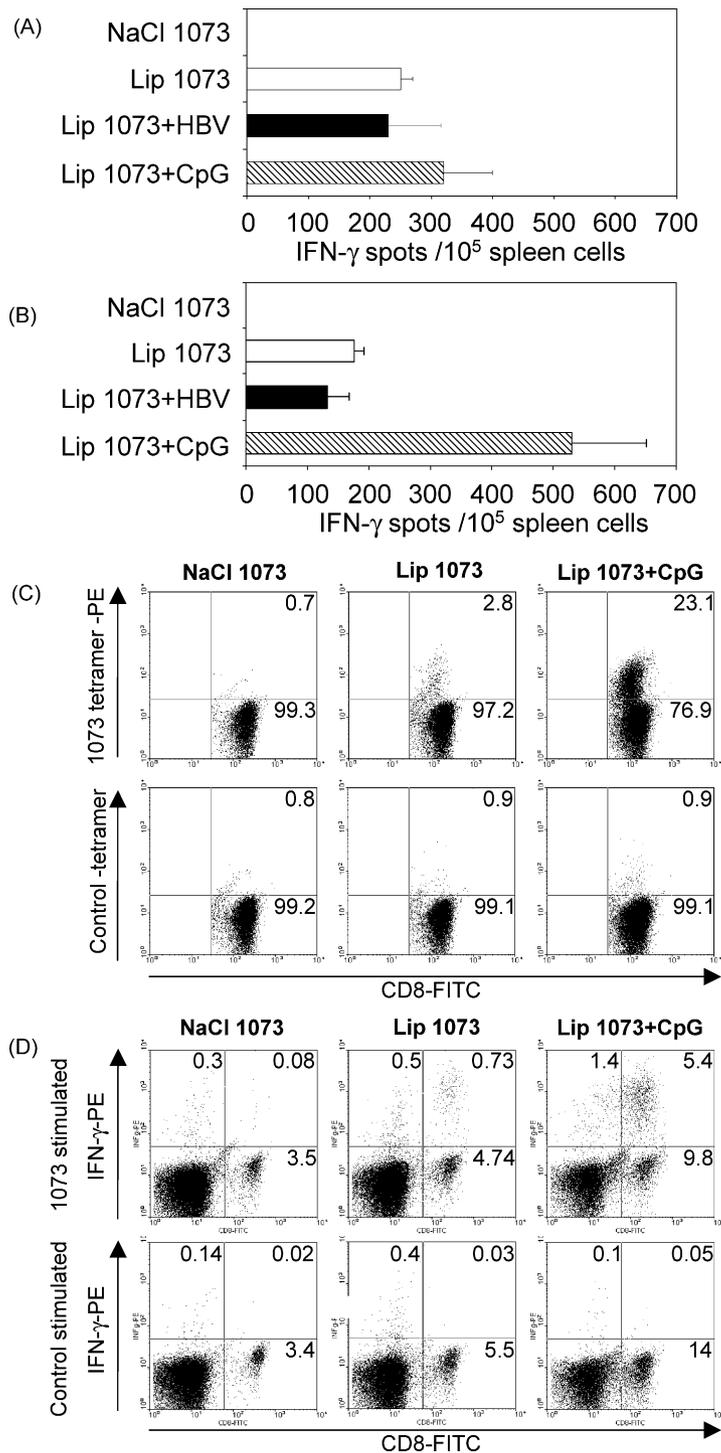


Fig. 3. Priming of IFN- γ -secreting cells. The frequency of IFN- γ -producing cells in mice immunised with liposomes containing only the CTL epitope 1073 (Lip 1073), the CTL epitope plus the T helper epitope HBV (Lip 1073 + HBV) or the CTL epitope together with CpG (Lip 1073 + CpG) was analysed by ELISPOT assay 14 days after three immunisations (A) and 3 months after the last immunisation (B). Spleen cells were restimulated over night with 1073-peptide pulsed and irradiated spleen cells. Cells (10^5 and 10^4) were transferred to pre-coated ELISPOT plates and spot formation analysed after 5 h. The results are presented as specific spots (spots in presence of 1073—spots in presence of a control peptide) per 10^5 spleen cells. The means (\pm S.D.) of three mice are indicated. Part (C) shows tetramer staining of 1073-specific cells from one experiment out of two. Pooled spleen cells from three mice per group, immunised with liposome 1073, liposome 1073 plus CpG or peptide 1073 in saline were enriched for the CD8⁺ T cell population (MACS) immediately after splenocyte isolation and stained with A-2Kb tetramer-PE specific for 1073 (upper panels) or the PE-labelled control tetramer (lower panels) and CD8-FITC. The gated CD8⁺ T cell population was analysed for specific tetramer staining. Part (D) shows intracellular IFN- γ -staining of splenocytes stimulated over night with specific peptide 1073 (upper panel) or a control peptide (lower panel). Typical examples of a liposome 1073, liposome 1073 plus CpG or NaCl 1073 control immunised mice are shown.

22% of tetramer positive CD8⁺ T cells. Furthermore, the frequency of IFN- γ -producing cells was analysed by intracellular cytokine staining upon over night stimulation with peptide (Fig. 3D). The results indicate that in mice immunised with liposomes containing only the peptide approximately 0.6% of total splenocytes (corresponding to 11% of CD8⁺ T cells, data not shown) produced IFN- γ in response to peptide stimulation, while in mice immunised with liposomes containing 1073 plus CpG approximately 5% of total splenocytes (corresponding to 31% of CD8⁺ T cells, data not shown) produced IFN- γ upon specific stimulation. IFN- γ production of CD8⁺ T cells derived from control immunised mice was always around background levels (0.08%).

3.4. The potential of liposomal formulations to stimulate cytotoxicity or IFN- γ production depends on the peptide sequence

Comparative studies of acutely infected and chronic HCV patients indicate that an effective vaccine against HCV should stimulate the response against multiple CD8⁺ T cell target sequences. We thus determined the immunogenicity of potential candidate epitopes. Thereby we could confirm the findings of others [26,27] showing that the extent of IFN- γ secretion strongly depends on the peptide sequence used and does not always correlate with the potential to stimulate cytotoxic T cells. We found that the epitopes 686 and 1169 induced a rather strong cytotoxic T cell response and to a lesser extent IFN- γ secretion, while the peptides 35, 614 and 1406 stimulated only low cytotoxicity but relatively high numbers of IFN- γ -secreting cells (50–150 cells/10⁵ spleen cells). The most immunogenic HCV peptides 132 (Fig. 1), 1073 (Fig. 2), 1585 and 1851 (Fig. 4) were able to stimulate a strong cytotoxic T cell response as well as a high IFN- γ production. In most formulations the co-integration of CpG had a beneficial influence on the IFN- γ production, reflected by a 2–10-fold increased number of IFN- γ secreting cells. In contrast, the CTL induction was only moderately enhanced by CpG. To compensate for the low numbers of CD8⁺ T cells typically found in HLA-A2.1 tg mice (1–6% in the spleen) a relatively high effector to target cell ratio was applied for the CTL assay. This allowed us to compare the responses induced by different epitopes, without the need of an additional *in vitro* restimulation step.

3.5. Reduced viral load in mice immunised by liposomal vaccines upon challenge with recombinant vaccinia virus

No small animal model has been established yet for HCV infection. One possibility to analyse the potency of the CD8⁺ T cells induced by a liposomal vaccine *in vivo* is to challenge immunised mice with recombinant vaccinia virus expressing HCV sequences (vv9a). To this end mice were vaccinated twice with liposomes containing the core epitope 35 or 132 and CpG. A control group was immunised with liposomal

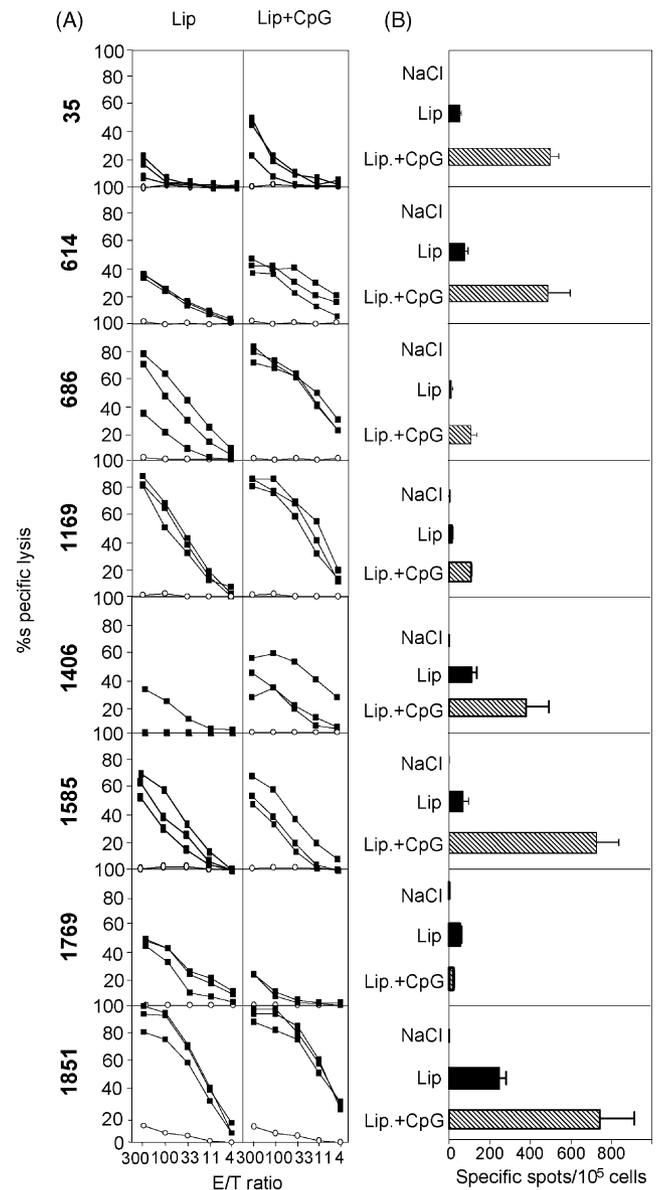


Fig. 4. CTL response and IFN- γ secretion of several different liposome encapsulated CD8⁺ T cell epitopes. Formulations containing the CTL epitope alone or in combination with CpG were tested. Mice (three per group; indicated by filled squares) were immunised three times with liposome formulations or with peptide in saline (open circles, representing the mean specific lysis of three control mice). Spleen cells were isolated 14 days after the last injection and CTL response analysed, after 5 days restimulation against peptide pulsed target cells (A). The frequency of specific IFN- γ producing cells was analysed by ELISPOT assay after over night stimulation of spleen cells with the adequate peptide or a control peptide (B).

formulations containing the NS3 epitope 1073 (not present in vv9a) and CpG. Five days after *i.p.* infection with 10⁷ pfu of recombinant vaccinia virus vv9a expressing the HCV core sequence, ovaries were removed and virus load assessed. Fig. 5 shows a significant reduction of mean viral load (1.5–2 logs) in mice that were immunised with the formulation containing the epitope core 132 and CpG, compared to the control immunised or naïve mice. In contrast, the immunisation with

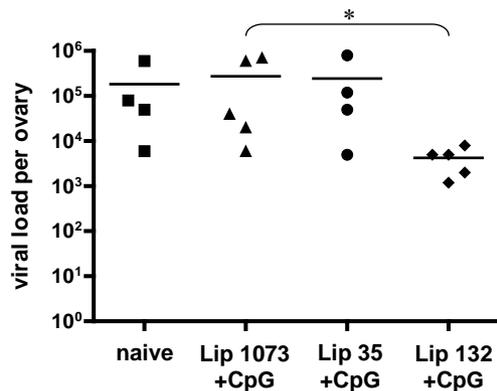


Fig. 5. Protection experiment with recombinant vaccinia virus vv9a expressing HCV core sequence. HLA-A2.1 tg mice (five per group) were immunised two times with liposomes containing CpG and the core epitopes 35 or 132. Naïve mice (5) and mice immunised with liposomes containing CpG and the NS3 epitope 1073 (5) which is not contained within the sequence expressed by vv9a, served as control groups. Eleven days after the immunisations, the mice were infected i.p. with 10^7 pfu of vaccinia virus vv9a. Five days after challenge, ovaries were extracted and the virus titrated in a plaque assay on 143 TK⁻ cells. The log of virus load/ovary is indicated on the y-axis. The significance of the difference between two groups was calculated using the Wilcoxon–Ranks test (* $P < 0.05$).

peptide 35, which was a poor inducer of CTL (Fig. 4), did not lead to protective CTL induction. The 1.5–2 log reduction of viral load in liposome 132 immunised mice was confirmed in three independent experiments.

4. Discussion

This work presents a preclinical analysis of a liposomal peptide vaccine against HCV using the HLA-A2.1 tg mouse model [19]. We analysed the immunogenicity of 10 different HLA-A2 restricted T cell epitopes with potential relevance for a therapeutic vaccine in chronic HLA-A2⁺ HCV patients. We found that the encapsulation of the peptides starting with amino acid 132, 1073, 1585 and 1851 allowed the stimulation of a specific cytotoxic T cell response as well as high frequencies of IFN- γ -secreting cells, while the peptides 35, 614 and 1406 induced preferentially IFN- γ production. Such formulations did not require the help of an extrinsic CD4⁺ T cell epitope for the priming of CD8⁺ T response or the maintenance of a T cell memory. Indeed, the addition of the universal T helper epitope PADRE [21] co-integrated or cross-linked to the liposome surface did not enhance the CTL response, although a PADRE-specific T helper response was induced by these formulations. In contrast the integration of CpG oligonucleotides into liposomes had a significant influence on the CD8⁺ T cell response, increasing the number of IFN- γ -secreting cells approximately 2–10-fold, while the effect on CTL induction was less pronounced.

The biological relevance of the induced CTL response was shown, by demonstrating that immunisation with liposomal peptide vaccines could confer partial protection from

infection with recombinant vaccinia virus expressing HCV sequences. The rather small but significant reduction of viral load (1.5–2 logs in three independent experiments) may be related to reduced numbers of CD8⁺ T cells (1–6% of splenocytes) in this transgenic mouse model and is in line with previously described results showing incomplete protection in a HLA-A2.1 transgenic mouse strain [28]. This is further supported by the finding that identical liposomal formulations containing the dominant epitope gp33 from the lymphocytic choriomeningitis virus (LCMV) lead to complete elimination of LCMV in immunised non-transgenic (C57/BL6) mice [16]. In addition, the cytotoxic T lymphocytes induced in the HLA-A2.1 tg mouse were able to lyse a HLA-A2.1⁺ human cell line endogenously processing the HCV target epitope, which demonstrates the relevance of the CTL response in a situation more closely mimicking the natural infection. Furthermore, the recognition of CTL across species validates HLA-A2.1 tg mice as preclinical model for the analysis of HLA-A2-restricted epitopes with relevance to human applications.

What should a therapeutic HCV vaccine do? The genetic heterogeneity of the virus, the tendency to infect liver cells persistently and the possible suppression of the host immune response may be the major factors responsible for the chronicity of HCV. Viral clearance after acute hepatitis or after treatment with IFN- γ is usually associated with strong and broad peripheral and intrahepatic CD4⁺ and CD8⁺ T cell responses [9,13,29–31]. Since the cell-mediated immunity in patients with chronic HCV infection is generally of low vigour and directed against a small number of epitopes [11,12], the aim of a therapeutic vaccine should be to strengthen and broaden the T cell response. The frequency of specific CTL in the acute phase of a resolving infection was estimated at 1–10% of CD8⁺ T cells for certain CTL epitopes [11,32]. Particular attention should be paid to the selection of the epitopes. While viral proteins, such as E2 and NS5B were not associated with viral clearance, a recent study has correlated the immune response against NS3 with viral resolution [9,33]. The finding that some epitopes might be more relevant for viral clearance than others could be related to their efficient processing and early presentation during viral replication. Alternatively, the immune response to such epitopes may be less prone to viral escape by mutation or depend on other mechanisms reviewed elsewhere [34]. Due to the high mutation rate of HCV, which accounts for its great heterogeneity and for the generation of escape variants, many of the epitopes are not conserved within viral genotypes. Some of the less variable epitopes may be involved in crucial functional structures of the protein and are therefore more sensitive to single amino acid changes. Alternatively, conserved epitopes might represent sequences in protein structures that induce no or only low immune pressure during the course of infection. Importantly, these so-called subdominant epitopes were shown to induce a strong immune response in the context of a peptide vaccine and represent an interesting option to expand the pre-existing immune response. In addition to

the CTL response several studies have underlined the importance of the IFN- γ production as a strategy to inhibit viral replication in absence of cytotoxic T cell-related liver pathology [9,35]. In this respect the identification of epitopes inducing substantial IFN- γ secretion and no cytotoxicity has gained importance and may influence the vaccine development [36]. In particular, vaccines based on subunits, such as peptides, allow the selection of defined epitopes according to certain characteristics, thereby limiting the risk of potential side effects. Studies on peptide-based vaccine strategies for HCV have been recently reviewed by Hunziker et al. [37]. Several other immunization strategies have shown the induction of anti-viral humoral and cellular immune responses in mice. This includes recombinant viral and bacterial vectors or plasmid DNA expressing HCV sequences [26,36,38]. Prime-boost regimens with plasmid DNA and replication-deficient viral vectors or recombinant proteins combine the advantages of different vaccine strategies [36,39]. HCV-derived virus-like particles and recombinant proteins, although difficult to produce at high purity and amounts, have also been shown to induce strong humoral as well as cellular immune responses in several mouse studies [28,40]. In contrast to recombinant proteins or viral-like particles, HCV-derived peptides can be produced synthetically in high amounts. As small peptides consisting only of the CD8⁺ T cell epitope are generally not immunogenic *in vivo*, immunizations were mainly done in combination with adjuvant or lipophilic delivery systems such as lipid tails or lipid vesicles [41,42]. Several studies, mainly conducted in Balb/c mice, have demonstrated the induction of HCV-specific CD8⁺ and CD4⁺ T cell as well as the production of antibodies against peptide mimitopes. An increasing number of vaccine studies are now performed in HLA-A2.1 tg mice. This permits advantage to be taken of the expanding number of CD8⁺ T cell epitopes identified and characterised in HCV patients. A recently performed study has analysed different vaccine strategies (DNA, adenoviral vector and peptides in incomplete Freund's adjuvant) in HLA-A2.1 tg mice for their potential to induce a response against several of the published HCV epitopes [36].

While peptide vaccines seem to induce a rather strong response against single epitopes, this implies the need to expand the immune response to a larger number of different epitopes. In preliminary experiments, we demonstrated priming of T cells against four epitopes by co-injection of different liposomal peptide formulations, although the response per epitope was reduced as compared to immunizations with a single epitope (data not shown).

The chimpanzee model currently provides the only means of studying the protective immunity against HCV by candidate vaccines prior to human trials. Vaccine studies based on E1/E2 (recombinant proteins or plasmid DNA encoding the proteins) were able to confer partial protection against low-level infection with homologous and heterologous virus [43]. Although immunised chimpanzees could be infected even with homologous monoclonal HCV, they often developed only a mild hepatitis [44,45]. In general, immunisation

studies and consecutive experimental infections with HCV in chimpanzees seem to indicate that a vaccine will not be able to confer complete protection, but will rather increase the likelihood of a benign, self-limiting course of infection [46]. Although chimpanzees are infectable with HCV, important differences in infectivity and immunological response as compared to humans have to be considered. The only vaccine trial in humans that entered clinical phase IIa is based on the envelope E1 protein. According to study data released by Innogenetics the induction of high titer anti-E1 antibodies as well as an E1-specific proliferation and IFN- γ production could be demonstrated in healthy probands as well as in chronic HCV patients. In many HCV patients sustained and significant decline of serum alanin-transaminase (ALT) and improvement of liver fibrosis scores were observed [47]. This human trial is of great interest as it demonstrates firstly that the immune response in chronic patients is susceptible to stimulation, secondly that the enhanced immune response does not necessarily lead to exacerbation of immune-mediated liver pathology, and that it can even have beneficial effects on liver fibrosis.

As liposomal peptide vaccines were efficiently tested in the preclinical mouse model we now plan to investigate the immunogenicity of a liposomal or virosomal vaccine in clinical trials. Among the different strategies to deliver peptides through the immunogenic route, liposomes are favourable in many aspects: firstly, they can be produced inexpensively from completely synthetic compounds, secondly they protect peptides from extracellular degradation and thirdly they have been safely used in humans for many years in different clinical applications [16–18]. The immunogenic effect of liposome formulations is probably due to the efficient antigen delivery to dendritic cells (DC) and the activation of antigen-presenting cells in the draining lymph nodes (LN). DC of liposome immunised mice were shown to present the epitope for at least 3 days and the *in vivo* activation of DC in LN was demonstrated by the up-regulation of co-stimulatory molecules [16].

In future experiments we will also investigate the use of HCV protein sequences integrated or cross-linked to the surface of liposomes. This may allow us to simultaneously stimulate the CD4⁺ and CD8⁺ T cell responses against HCV epitopes. Furthermore, we consider prime-boost experiments with plasmid DNA and viral vectors. Thereby the rather broad immune response induced by recombinant viral vectors or plasmid DNA may be combined with the potent and strongly focused CD8⁺ T cell response stimulated by liposomal formulations.

In conclusion, peptide-containing liposomes are an interesting option for a therapeutic HCV vaccine, having the advantage of convenient large-scale production and proven safety in human applications. Using liposomes, we could document the immunogenicity of several HCV epitopes *in vivo*. The stimulated immune response in transgenic mice was strong and focused on selected epitopes, inducing CTL of rather high affinity and elevated numbers of IFN- γ -producing

cells, which persisted for at least 3 months. Thus, this successful immunisation protocol using HCV peptides and liposomes may lead to the development of a phase I clinical trial using combinations of CD8⁺ T cell epitopes and CpG.

Acknowledgments

We like to thank F.A. Lemonnier (Institut Pasteur, Paris, France) for providing us with the HLA-A2.1 tg mouse strain and M. Houghton (Chiron Corp.) for providing us with the recombinant vaccinia virus constructs. This work was supported by the grants QLK2-CT 1999-00356 and QLK2-CT 2002-01329 from the European Commission as well as grant 01 KI 9951 from the Bundesministerium für Bildung und Forschung.

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