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In vitro studies of core peptide-bearing immunopotentiating reconstituted influenza virosomes as a non-live prototype vaccine against hepatitis C virus

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Abstract

Evidence from both animal and human viral diseases indicate that cytotoxic T lymphocytes (CTL) are crucial in antiviral defense. However, a major problem to generate cytotoxic immunity is that *in vivo* exogenous antigens are usually presented via MHC class II pathway and normally fail to induce CTL. The aim of this study is to describe a novel non-live prototype vaccine based on immunopotentiating reconstituted influenza virosomes (IRIV) as vehicles to deliver HLA-A*0201-restricted hepatitis C virus (HCV) peptides (core 35–44 and 131–140) into the cytoplasm of at least three different target cell types [including T2, a transporter associated with antigen processing (TAP)-deficient cell line] resulting in MHC class I peptide presentation and lysis by peptide-specific CTL lines. Comparison of kinetics and analysis of the influence of peptide-stripping and Brefeldin A (BFA) reveal that there exists an endogenous, TAP-independent and BFA-sensitive pathway for virosomally delivered peptides. Moreover, virosomes containing influenza matrix peptide 58–66 can efficiently re-stimulate *in vivo* primed CTL and, importantly, IRIV containing HCV core peptides can even prime CTL from peripheral blood mononuclear cells of HCV$^-$ healthy blood donors *in vitro*. The fact that *in vitro* primed CTL are also able to specifically lyse target cells infected with recombinant vaccinia virus encoding the HCV core protein is of great importance for future studies based on *in vivo* mouse models. One of the most evident advantages of the virosomes *in vivo* will be their capability to protect the incorporated peptide from a large variety of degrading proteases.

Introduction

The interest to analyze the adjuvant effect of liposomes, on the one hand, and in parallel, morphological and immunological aspects of influenza virus, on the other hand, led to the creation of the first so-called virosomes in 1975 (1); a name selected to reflect the structural similarities between the viral liposomes and the actual influenza virus particles. While liposomes have been proposed for a long time as vehicles to deliver peptides or proteins to antigen-presenting cells and some more-sophisticated variants are still very popular for CD8$^+$ T cell responses *in vivo* (2), nowadays many different protocols concerning the preparation of virosomes exist, partly referring to the growing diversity of different types, e.g. rabies immunosomes (3), virosomes reconstituted from HIV (4), Epstein–Barr virus (EBV)-based virosomes (5) and, finally, the classical immunopotentiating reconstituted influenza virosomes (IRIV) (6–10).

IRIV are spherical, unilamellar vesicles with a mean diameter of ~150 nm. They can serve as carriers for multiple antigens which will have good immunogenicity and are well tolerated (11), and in contrast to classical liposomes they are fusogenic due to the influenza-derived hemagglutinin (HA) (12,13), which makes them an attractive tool for vaccine studies. HA has several important functions: (i) it binds sialic acid-containing receptors on the surface of macrophages,

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lymphocytes or other immunocompetent cells (14), (ii) it mediates the membrane fusion between virosomes and endosomal membranes of target cells, thereby facilitating antigen delivery (15), and (iii) it serves as a 'recognition antigen', since most humans are primed to HA due to prior exposure to either influenza disease or vaccination (16,17). Finally, the protective role of the virosomes is by far the most important one: as stated by Widmann *et al*. (18), soluble synthetic peptides have differential stabilities due to the activity of proteases which may have important consequences for attempts to manipulate the development of an immune response *in vivo*. In addition, using virosomes also avoids the induction of tolerance—a phenomenon that has been observed under certain conditions by immunizing mice i.p. with soluble peptide (19).

Several different vaccine trials based on virosomes have been performed so far, mainly focusing on influenza or hepatitis A virus (HAV) with the aim to induce protective, neutralizing antibodies (20–24).

In contrast to HAV and HBV viruse where protective antibody titers can be obtained after immunization, the role of immunoglobulins against HCV remains to be defined. Envelope antibodies would be the prime candidates for virus neutralization, but their presence in chronically infected patients as well as the observation that chimpanzees can be infected repeatedly by the identical HCV strains argue against an efficient humoral virus neutralization *in vivo* (25,26). On the other hand, antibody-mediated immune pressure seems to be directly correlated with an evolution of viral escape mutants during the course of chronic infection (27,28).

As cytotoxic T lymphocytes (CTL) seem to be of greater importance in HCV elimination and since the core protein is the most highly conserved structural protein in the HCV genome (29), we are focusing on vaccine development based on virosomes containing either core 35–44 (IRIV-core 35) or core 131–140 (IRIV-core 131) inside. The function of the virosome is, on the one hand, to protect its contents from early protease degradation and, on the other hand, to deliver the core peptides into the MHC class I pathway. In our study we were able to show that it is possible to lyse EBV-transformed B cells (JY) as well as T1 and T2 cells preincubated with HCV core peptide-containing virosomes in a standard Cr-release assay using core peptide-specific effector cell lines. We analyzed the method of peptide presentation in the virosome model in more detail based on peptide-stripping experiments and on the post-Golgi inhibitor Brefeldin A (BFA), and using the EBV-transformed B cell line JY, the transporter associated with antigen processing (TAP)-competent cell line T1 and its TAP-deficient counterpart T2.

Furthermore, using virosomes as vehicles to channel peptides into the cytoplasm of peripheral blood mononuclear cells (PBMC) from HCV$^-$ healthy human blood donors, we showed that correct epitopes can be presented on the cell surface, efficiently enough to prime naive CTL against HCV epitopes *in vitro*. Although primary CTL induction *in vitro* is also possible based on soluble peptide, the protective effect of the virosomal carrier will be of great importance for the performance of *in vivo* vaccine trials.

Methods

Synthetic peptides

The following synthetic peptides were used: two HCV core-derived peptides, core 131–140 (ADLMGYIPLV) (30,31) and core 35–44 (YLLPRRGPRL) (30,31), and one control peptide, influenza matrix 58–66 (GILGFVFTL) (32), used for re-stimulation of memory CTL, all obtained by Neosystem (Strasbourg, France) or the Institute for Biochemistry (Epalinges, Switzerland). Peptides were dissolved in DMSO (20 mg/ml) and diluted in PBS to a stock solution (1 mg/ml).

IRIV

Preparation of peptide-IRIV was performed according to the following procedure (Swiss Serum and Vaccine Institute, Bern, Switzerland): 32 mg of phosphatidylcholine (Lipoid, Ludwigshafen, Germany) and 8 mg of phosphatidylethanolamine (R. Berchtold, Biochemical Laboratory, Bern, Switzerland) were dissolved in 2 ml of PBS containing 100 mM of octaethyleneglycol (PBS/OEG) (Fluka Chemicals, Buchs, Switzerland). Influenza A/Singapore HA was purified as described previously (33). A solution containing HA (4 mg) was centrifuged at 100,000 *g* for 30 min and the pellet was dissolved in 1 ml of PBS/OEG. Then 5 mg of the hydrophobic peptides was dissolved in 100 μl of DMSO and 900 μl of PBS/OEG were added. The phospholipids, the HA solution and the peptide solution were mixed and sonicated for 1 min. This mixture was then centrifuged at 100,000 g for 1 h and the supernatant sterile filtered (0.22 μm). Virosomes were then formed by detergent removal using BioRad (Glottbrugge, Switzerland) SM Bio-beads. Quantification of the peptide incorporation rate was performed using radioactively labeled ^{14}C-tracer core 131 peptide. The actual peptide concentration incorporated was measured as 166 μg peptide/ml and all experiments performed with peptide-bearing virosomes were based on that quantification. If proceeded before having performed the quantification, experiments were then adapted to that standard concentration retrospectively, thereby explaining the unusual concentrations of virosome-derived peptide in some cases.

To make sure that there was no 'contamination' of soluble peptide outside of the virosomes, some batches of peptide-containing virosomes were purified by means of a G10 column in addition. Whether or not this procedure was performed, there was no difference in the results obtained in our experiments. Via HPLC we obtained another proof for virosomal purity: in the virosome formulations there was no peak seen corresponding to free peptide outside of the constructs (data not shown).

Recombinant vaccinia virus (rVV)

rVV virus encoding the full-length HCV core protein and part of the E1 protein vv9A (C/5′ end of E1; Met1–Arg 39) was kindly provided by Dr Michael Houghton (Chiron, Emeryville, CA).

For rVV infection 1×10^6 JY EBV-transformed B cells were put into a 15 ml tube and centrifuged. Then, the virus was added to the cell pellet in 1 ml with a concentration of 10 m.o.i. (corresponding to 1×10^7 p.f.u.) with 1 h incubation on a shaker at room temperature. Cells were centrifuged again and

resuspended in 1 ml of fresh medium and then incubated at 37°C for later use in a standard Cr-release assay.

Cell lines

JY, an EBV-transformed B cell line (HLA-A2.1, -B7, -Cw7, -DR4, -Drw6, -Dpw2; kindly provided by Dr F. V. Chisari), T1 cells and their TAP-deficient counterpart T2 cells—both originally produced by fusing the T leukemic line CEM with the TAP^- mutant LCL 721.174 (34,35)—were cultured in RPMI 1640 medium (Sigma, St Louis, MO) enriched with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and HEPES (5 mM), and containing 10% (v/v) heat-inactivated FCS (FCS medium).

The T1 hybrid has normal antigen-presenting function, and expresses the HLA-A1, -A30 and -B8 alleles of the CEM parent and the HLA-A2.1 and -B51 alleles of the 721.174 parent; the T2 hybrid was derived from T1 by selecting for loss of the CEM HLA locus and resembles its 721.174 parent in being TAP-deficient and defective in antigen presentation, with a 70–80% reduction in surface HLA-A2.1 expression compared to T1 and virtually no detectable surface HLA-B51 (36).

Induction of primary and secondary (recall stimulation) CTL

Buffy coats or lymphaphereses of $HLA\text{-}A2^+$ healthy human blood donors (negative for HIV, HBV and HCV serology) were separated on Ficoll-Paque density gradients (Pharmacia Biotech, Uppsala, Sweden) and washed at least 3 times in 50 ml of PBS containing 10% FCS medium.

Then, as described previously (37), 4×10^6 PBMC were incubated either with synthetic peptide i.e. HCV core 131, core 35 or influenza matrix 58 or with virosomes IRIV-core 131, IRIV-core 35 and IRIV-flu containing core 131, core 35 and influenza matrix peptide respectively in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and HEPES (5 mM), and containing 10% (v/v) of heat-inactivated human AB serum (AB medium) in 24-well plates for 3 days.

On day 3 and weekly thereafter, 1 ml of complete AB medium supplemented with rIL-2 (20 U/ml; EuroCetus, Amsterdam, The Netherlands) was added to each well. On day 7 and weekly thereafter, cultures were re-stimulated with 10^6 irradiated (7400 rad) autologous feeder cells in 1 ml of AB medium containing rIL-2 (20 U/ml) and the corresponding peptide or virosomes.

Cytotoxicity (CTL activity) of the *in vitro* stimulated PBMC was then determined in a standard 4-h Cr-release assay between 2 and 7 weeks of *in vitro* stimulation as indicated above.

Generation of peptide-specific cell lines

CTL bulk cultures with a peptide specificity of >80% at an E:T ratio of ~20:1 obtained in our primary *in vitro* inductions as described above were depleted of $CD4^+$ cells by negative selection according to the manufacturers instructions (Dynabeads; Dynal, Oslo, Norway) and plated at 100 effector cells/well in 96-well plates in FCS medium enriched with phytoHA (1 μg/ml) and rIL-2 (30 U/ml) containing irradiated (10400 rad) allogeneic PBMC (10^6/ml) and irradiated (22000 rad), peptide-pulsed (10 μg/ml; 1 h) JY EBV-B cells (10^5 cells/ml).

Peptide-specific wells were expanded in 48-, 24- or 12-well plates and the resulting peptide-specific cell lines could be kept in culture over several months.

Cr-release assay (cytotoxicity assay)

Target cells (JY, T1 or T2; 10^6 per condition) were incubated with peptide (10 μg/ml) or virosomes (IRIV) containing peptide (varying concentrations) in FCS medium at 37°C and 5% CO_2 overnight—or in case of rVV-infected target cells the protocol was followed as indicated above under 'rVV'.

Preincubated target cells were then labeled with 100 μCi of ^{51}Cr (Amersham, Little Chalfont, UK) for 1 h at 37°C and washed 4 times with cold PBS containing 10% FCS medium to remove non-incorporated Cr. Cytolytic activity was determined in a standard 4-h Cr-release assay using U-bottom 96-well plates containing 2500 target cells. Depending on the number of effector cells available, E:T ratios between 60:1 and 1:1 were selected. Percent cytotoxicity was determined using the formula: $100 \times$ [(experimental release – spontaneous release)/(maximum release – spontaneous release)]. Maximum release was determined by lysis of targets in 2 N HCl, spontaneous release by lysis of targets in FCS medium. Spontaneous release was usually ~12%, but always <25% of maximum release. Specific lysis was calculated as difference between lysis of targets with peptide (or peptide-bearing virosomes) and targets without peptide (or naked virosomes). All results were based on duplicates with the exception of the analysis of primary induction cultures, where we only performed unicates due to lack of cells.

For the peptide preincubation time curves, targets cells (JY or T2) were only incubated for the times indicated, i.e. a few hours or even fractions of an hour instead of the overnight incubation.

Peptide 'stripping' by mild acid elution

After overnight incubation of T2 or JY target cells with peptide or virosomes at 37°C and 5% CO_2 they were washed once with cold PBS containing 10% FCS medium and then put on ice in 1 ml of an ice-cold citric acid–Na_2HPO_4 buffer at pH 3.0, the mixture of equal volumes of 0.263 M citric acid and 0.123 M Na_2HPO_4 containing 1% BSA (290 mosmol/kg H_2O), for 2 min (38). Immediately after the acid treatment, cells were washed with an excess of FCS medium to neutralize the acid buffer.

Cytolytic activity against peptide-stripped targets was either measured directly after the mild acid treatment or after another 5-h incubation of the targets at 37°C and 5% CO_2. In the case of virosome-preincubated targets this 5-h incubation was either performed with or without the addition of new peptide-bearing virosomes.

BFA treatment of JY and T2 target cells

Target cells (JY and T2) were preincubated with various BFA concentrations ranging from 3 μg/ml to 10 ng/ml at 37°C for 4 h, before adding either soluble core 131 or virosomes containing core 131 for overnight incubation.

The next morning, cells were collected and prepared for Cr-release assay.

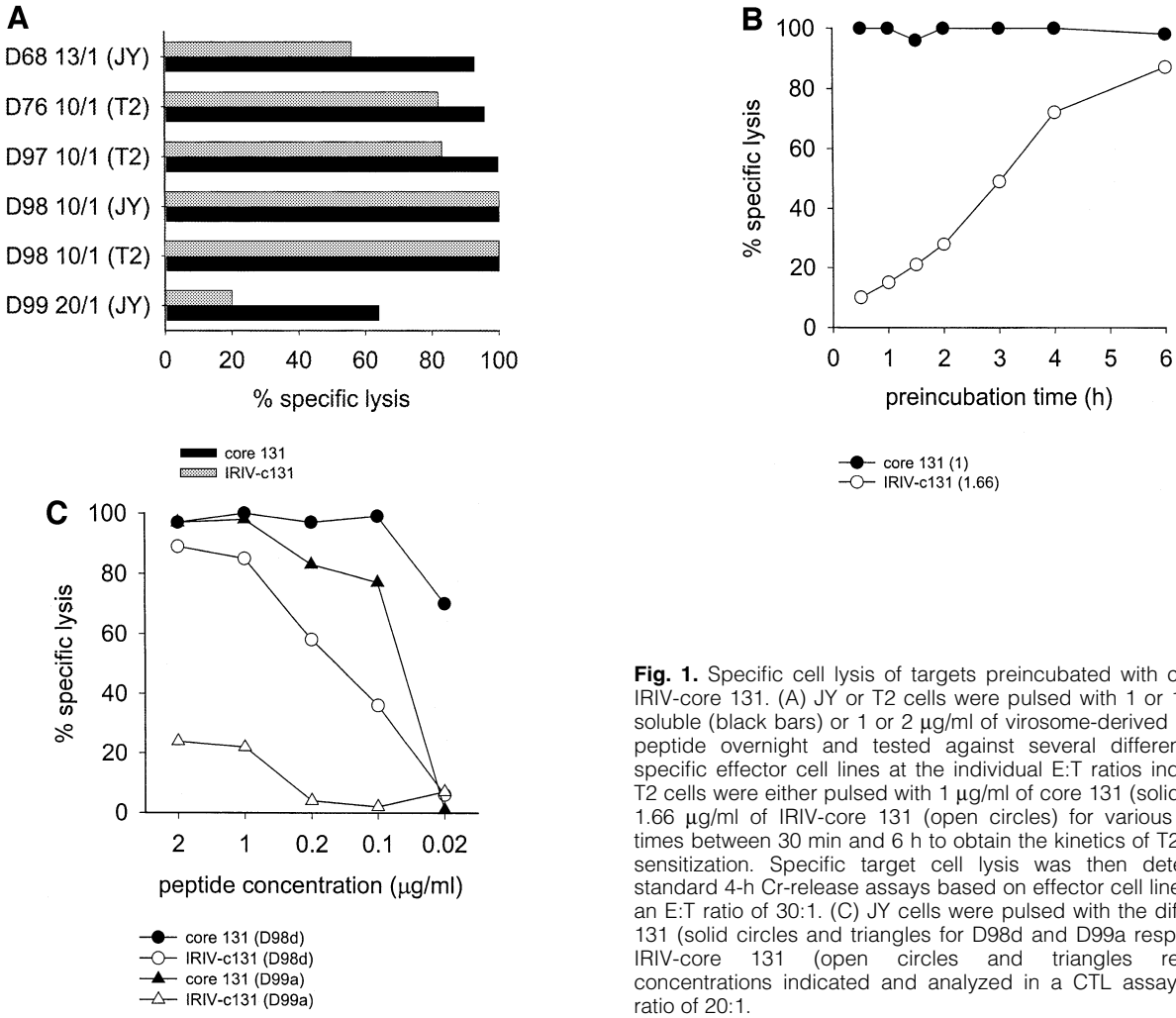


Fig. 1. Specific cell lysis of targets preincubated with core 131 or IRIV-core 131. (A) JY or T2 cells were pulsed with 1 or 10 μg/ml of soluble (black bars) or 1 or 2 μg/ml of virosome-derived (gray bars) peptide overnight and tested against several different peptide-specific effector cell lines at the individual E:T ratios indicated. (B) T2 cells were either pulsed with 1 μg/ml of core 131 (solid circles) or 1.66 μg/ml of IRIV-core 131 (open circles) for various incubation times between 30 min and 6 h to obtain the kinetics of T2 target cell sensitization. Specific target cell lysis was then determined in standard 4-h Cr-release assays based on effector cell line D97a and an E:T ratio of 30:1. (C) JY cells were pulsed with the different core 131 (solid circles and triangles for D98d and D99a respectively) or IRIV-core 131 (open circles and triangles respectively) concentrations indicated and analyzed in a CTL assay at an E:T ratio of 20:1.

Results

Target cell sensitization of JY, T1 and T2 cells

First, two different target cell lines, i.e. JY, an EBV-transformed B cell line, and T2, a TAP-deficient cell line, were pulsed with either soluble core 131 (1 or 10 μg/ml) or IRIV-core 131 (1 or 2 μg/ml) overnight. Several peptide-specific effector cell lines were then used at various E:T ratios indicated to test the efficiency of the target cell sensitization in standard 4-h Cr-release assays (Fig. 1a). All five cell lines (D68, D76, D97, D98 and D99) tested against JY and/or T2 could lyse targets incubated with soluble and virosome-derived peptide, although varying in their absolute amounts of specific cell lysis. Both targets were able to present virosome-derived peptide on MHC class I via a TAP-independent internal pathway that was analyzed in further experiments. There was a general tendency towards a higher lysis in the case of soluble peptide.

Secondly, a time curve based on T2 target cell sensitization with either core 131 (1 μg/ml) or IRIV-core 131 (1.66 μg/ml) was obtained to analyze the kinetics of soluble versus virosome-derived peptide based on CTL line D97a at an E:T ratio of 30:1 (Fig. 1b). After 30 min of incubation there was maximum target cell lysis in the case of soluble peptide, whereas for IRIV-core 131 preincubated targets half of the maximum cell lysis was obtained after ~3 h and a lysis nearly comparable to the one obtained with soluble peptide was obtained after 6 h. Between 6 and 12 h of target sensitization there was a constant cell lysis as analyzed in an additional experiment (data not shown). The two different time curves obtained for an efficient peptide presentation on the target cell surface of both T2 and JY targets (data not shown) are the first hint that two independent pathways of exogenous (soluble peptide) versus endogenous (IRIV-core 131) target cell sensitization are used.

Third, we obtained a concentration curve using soluble and virosome-derived peptide ranging between 2 μg/ml and 20 ng/ml (Fig. 1c) to give a direct comparison of the efficiency of these two different forms of peptide on target cell lysis based on an E:T ratio of 20:1. There is a remarkable difference between the resulting cell lysis obtained for core 131 and IRIV-core 131 preincubated targets (Fig. 1c): compared to the lysis obtained based on soluble peptide, virosome-derived peptide at a similar concentration of 0.2 μg/ml led to a target cell killing

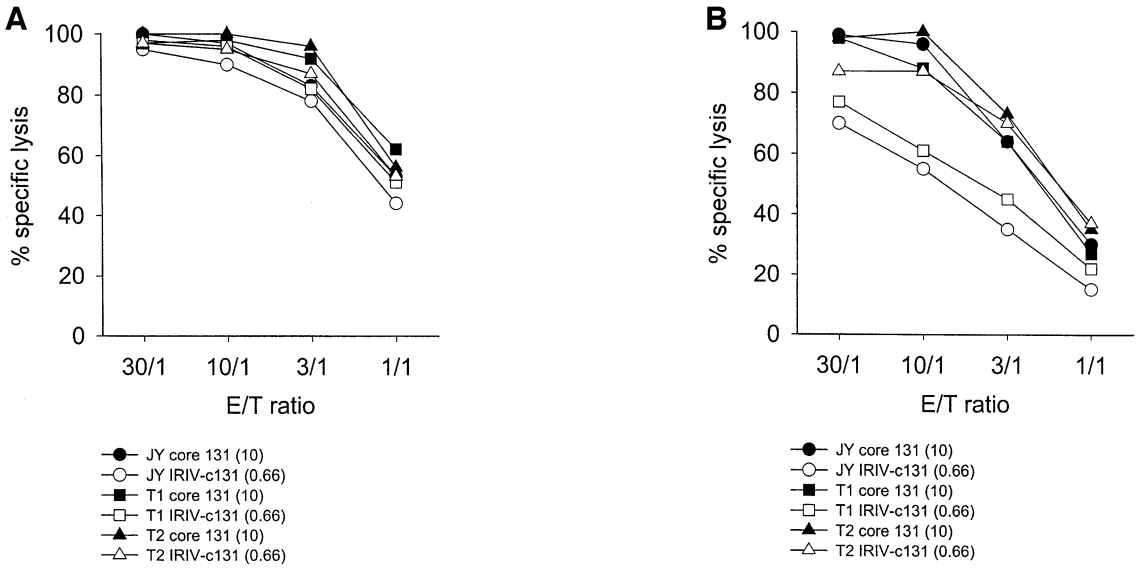


Fig. 2. JY, T1 and T2 target cell sensitization in comparison. The three different targets were either pulsed with 10 μg/ml of core 131 or 0.66 μg/ml of IRIV-core 131 overnight. Then, a 4-h Cr-release assay was performed based (A) on effector cell line D98b or (B) on cell line D99a at various E:T ratios ranging from 30:1 to 1:1.

reduction of 39% for D98d and 79% for D99a respectively. The varying peptide concentrations necessary to obtain similar target cell lysis with both effector cell lines might be partly explained due to differences in the TCR–peptide–MHC class I affinity.

Finally, to focus on TAP-bearing and -deficient targets more directly, we analyzed JY, T2 and its TAP-bearing parental cell line T1 in parallel (Fig. 2a and b). Targets pulsed with core 131 were generally slightly more efficiently killed than those incubated with IRIV-core 131. The CTL line of donor D98 (Fig. 2a) again showed a higher peptide affinity than that of donor D99 (Fig. 2b), as demonstrated in the higher killing of core 131-pulsed targets at low E:T ratios. Interestingly, the D99a CTL line was not able to lyse IRIV-core 131-sensitized TAP-competent T1 and JY targets as efficiently as TAP-deficient T2 cells. In fact, it seems that the lower the peptide affinity of the effector cells is, the bigger the discrepancy between the killing potential of TAP-deficient versus TAP-bearing IRIV-core 131 preincubated targets, also observed in other experiments not shown.

Peptide stripping of T2 and JY cells pulsed with soluble core 131 versus IRIV-core 131

T2 cells were incubated in the presence of either core 131 peptide (1 μg/ml) or IRIV-core 131 (1.66 μg/ml) overnight. Then, cells were treated with a mild acid solution as described in Methods and incubated for another 5 h with or without the addition of new virosomes.

Based on the effector cell line D97a, there was a high lysis of peptide-pulsed targets at all E:T ratios indicated, which could only be reduced to about three-quarters of the original lysis after the acid treatment. In the case of IRIV-core 131, however, the original cell lysis was only about half the amount obtained with soluble peptide and it could be reduced to zero after peptide stripping (Fig. 3a). It appears that the whole internal peptide pool derived from the virosomes was presented at once, since there was no efficient reloading of the HLA-A2 molecules during the ongoing 5-h incubation after having washed the present peptide from the surface of the T2 cells, except after the addition of new peptide-bearing virosomes which led to a recovery of target cell lysis to even a slightly higher amount than before the acid treatment, comparable to the killing obtained with core 131 after peptide stripping.

In parallel, we also performed another peptide stripping experiment based on JY target cells and three different effector cell lines (D68, D98d and D99) specific for core 131 using very low peptide concentrations (0.1 μg/ml) for target cell pulsing (Fig. 3b). Based on all three cell lines, there was a reduction of specific cell lysis after the mild acid elution by 50–100% depending on the particular effector cells used. There seems to be a clear correlation between the peptide amount for target cell pulsing and the efficiency of the peptide stripping, which explains the data obtained in Fig. 3(a) with soluble peptide after the mild acid treatment.

BFA and its influence on peptide-presentation

To make sure that the virosome-derived core 131 was actually presented via an endogenous pathway and not directly binding to surface class I molecules after leakage of the virosomal peptide carrier, in addition to the time curves performed in parallel with core 131 and IRIV-core 131 (Fig. 1b) we performed a post-Golgi blocking study with different BFA concentrations ranging from 10 ng/ml to 3 μg/ml. Targets were therefore first incubated with varying amounts of BFA for 4 h and then either soluble core 131 (1 μg/ml) or IRIV-core 131 (1 μg/ml) was added in addition.

BFA did not alter the percentage of specific lysis of targets pulsed with soluble peptide, neither in JY and based on CTL

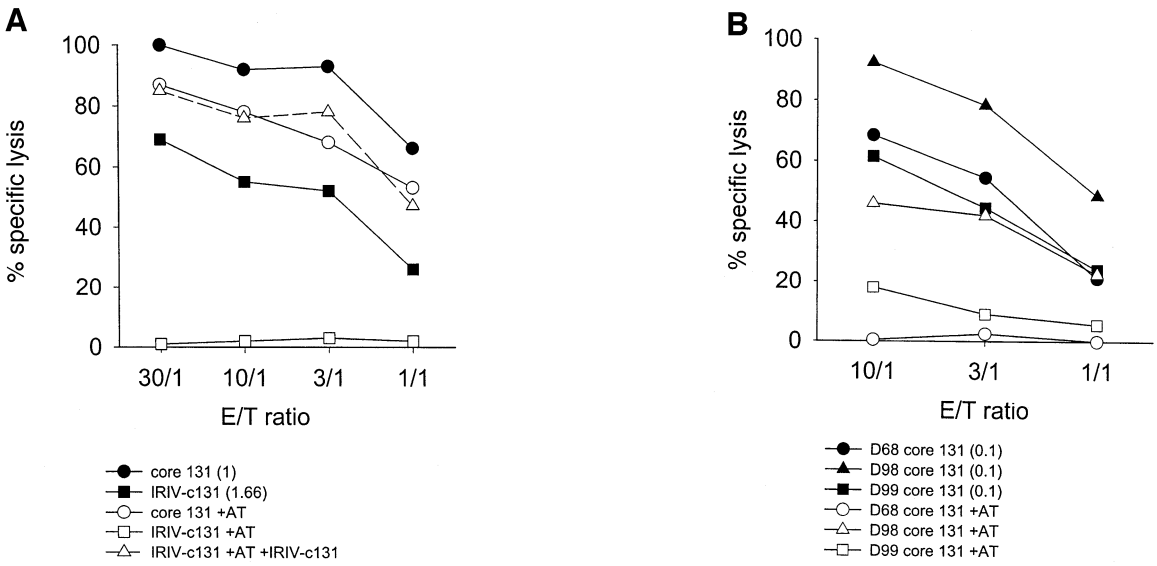


Fig. 3. Influence of mild acid treatment on target cell lysis. A peptide-stripping based on a mild acid treatment followed by another 5 h of incubation was performed with T2 cells either preincubated with 1 μg/ml of soluble core 131 or 1.66 μg/ml of IRIV-core 131 (A) or JY cells preincubated with 0.1 μg/ml of core 131 (B) overnight. (A) The Cr-release assay performed was based on CTL line D97a and E:T ratios ranging between 30:1 and 1:1 as indicated. Control cells pretreated with core 131 (solid circles) or IRIV-core 131 (solid squares) were compared to acid-treated T2 cells preincubated with core 131 (open circles) or IRIV-core 131 (open squares) and to acid-treated target cells that were incubated with new virosomes after the peptide stripping (open triangles) for another 5 h. (B) The assay was based on CTL lines D68, D98d and D99 using E:T ratios of 10:1, 3:1, and 1:1. Solid symbols represent control cells and open symbols acid-treated cells.

line D98d (Fig. 4a) nor in T2 cells and based on CTL line D68c (Fig. 4b). In the case of IRIV-core 131 preincubated targets both JY and T2 cells were less efficiently lysed in the presence of 0.1 μg/ml of BFA. As for JY targets (Fig. 4a) the cell lysis in the presence of 0.1 μg/ml of BFA decreased to half of the lysis in the absence of BFA and, with regard to T2 cells, this decrease was even more relevant (Fig. 4b). Higher BFA concentrations did not increase the negative effect on cell lysis. Importantly, even at very low peptide concentrations (0.05 μg/ml), BFA did not have any negative effect on cell killing based on soluble peptide (Fig. 4c).

In conclusion, this is the proof that virosome-derived peptide is presented in part in a BFA-sensitive internal pathway, whereas exogenous soluble peptide is presented in a BFA-independent pathway. This, together with the results obtained from the time curve, indicates that the two pathways of peptide presentation for core 131 and IRIV-core 131 are independent.

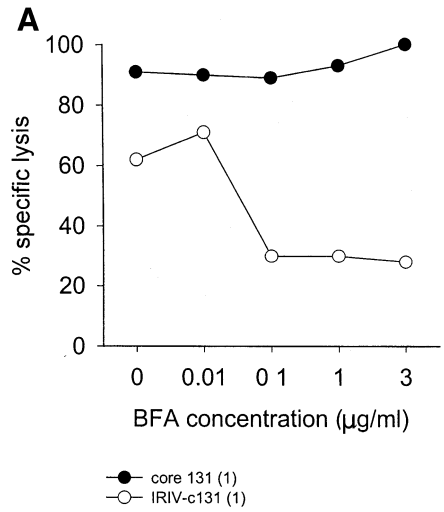
Primary induction based on core 131, core 35 and their virosome-derived counterparts

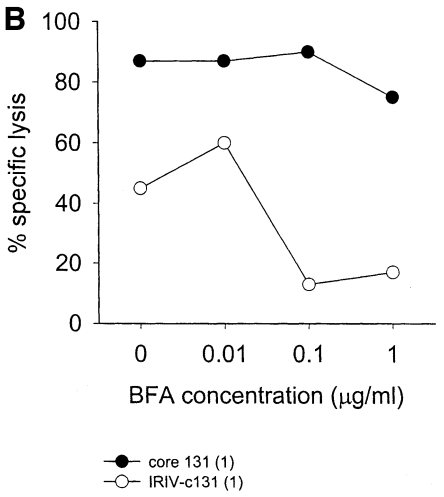
PBMC obtained by four HCV^- healthy blood donors (D96, D97, D98 and D99) were stimulated with soluble peptide (core 131 or core 35) or peptide-bearing virosomes (IRIV-core 131 or IRIV-core 35) in induction cultures of 4×10^6 cells for several weeks according to the protocol indicated.

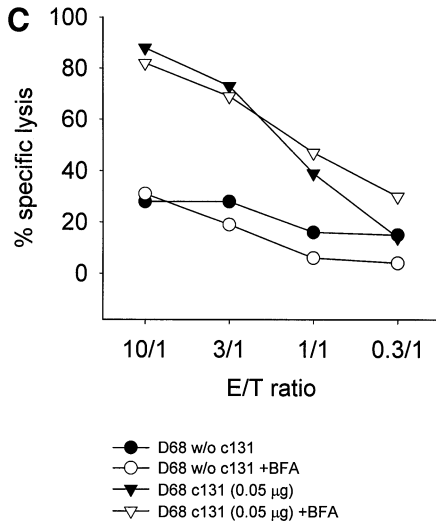
In a first step, we compared the efficiency of soluble peptide (10 μg/ml) versus virosome-derived peptide (3.3 and 0.8 μg/ml in experiments 1 and 2 respectively) based stimulation for primary induction *in vitro*. In Table 1 two experiments are summarized as an overview. In experiment 1, four out of six cultures stimulated with core 131 were positive, whereas no positive culture could be obtained with IRIV-core 131 stimulation as determined in CTL assays. In the case of core 35, three out of six cultures were positive using the soluble peptide and one well was positive after IRIV-core 35 stimulation, shown in more detail in Fig. 5(a).

In experiment 2, seven out of 12 cultures stimulated with core 131 were positive and eight out of 12 cultures were positive after IRIV-core 131 stimulation, whereas one single well was positive after core 35 stimulation and six of them were positive after IRIV-core 35 stimulus. The considerable variation in individual experiments and the differing numbers of positive cultures of various blood donors obtained is due to a very low precursor frequency of CTL specific for HCV epitopes in seronegative healthy human blood donors—a phenomenon that was already observed previously (37). Nevertheless, it was discovered that virosome-derived peptide can be as efficient as soluble peptide to prime CTL *in vitro*. Indeed, the importance of these results will be more obvious with respect to the situation *in vivo* focusing primarily on humans where the use of a suitable adjuvant and delivery system for a peptide-based vaccine is crucial. The effect of priming was dose dependent, since no positive cultures were obtained using 10 times lower IRIV-core 131 or IRIV-core 35 concentrations (data not shown).

The kinetics of primary versus secondary stimulation is shown in Fig. 5(a and b). *In vitro* generation of CTL to influenza peptide (secondary response) as well as to HCV core 131 or IRIV-core 131 and core 35 or IRIV-core 35 respectively are







presented in parallel (Fig. 5a and b). The data shown represent typical examples out of the results obtained in soluble peptide- or IRIV-peptide-induced cultures. As indicated, there is not a big difference between the time needed for a successful priming of an induction culture for a primary CTL response based on soluble and virosome-derived peptide *in vitro*. However, there is a big difference between a primary and a secondary *in vitro* stimulation, as shown in the curves obtained for ourinfluenza control peptide that had its peak around week 3 or 4, which was 2–3 weeks earlier compared to the peak response obtained with core 35 or core 131.

Primary induction compared to secondary stimulation in vitro based on virosomes

In a second step, we compared the responses of primary induction cultures induced with IRIV-core 131 or IRIV-core 35 to the secondary responses induced with IRIV-flu based on PBMC obtained by three different HCV$^-$ blood donors D84, D85 and D98 (Table 2). As for memory responses induced with IRIV-flu stimulation, we measured CTL activities between 2 and 4 weeks of stimulation, whereas in the case of primary *in vitro* induction, we checked for lysis of targets between 4 and 6 weeks of stimulation. Concerning IRIV-flu stimulation, all four replicates of all three donors were positive at the first time point checked as expected for a secondary stimulation based on an influenza virus-derived peptide that is well known by the immune system of most individuals. The following week, most of them had an even higher lysis potential which decreased after 1 month of *in vitro* stimulation. In the case of IRIV-core 131, all replicates of donor D98 showed peptide-specific lysis after 4 weeks of stimulation, reaching a maximum after 5 or 6 weeks and progressively loosing their specificity again (data not indicated). Unfortunately, no positive cultures were obtained against IRIV-core 35 in the three donors evaluated in this assay, which has again to be attributed to a very low precursor frequency, thereby reducing the chances of bearing peptide-specific precursors in each individual induction culture well. After IRIV-core 131 and IRIV-flu stimulation targets preincubated with soluble and virosome-derived peptide could be lysed efficiently, thereby pointing to the fact that the effector cells were really peptide specific.

Then, we wanted to analyze if there was any correlation between different IRIV-flu concentrations and the actual time of stimulation needed to obtain positive induction cultures based on PBMC from donor D96. As shown in Table 3, a higher IRIV-flu amount led to positive cultures faster: stimulation of cultures with 1.66 μg/ml of IRIV-flu led to three positive

Fig. 4. Influence of BFA on peptide presentation in (A) JY and (B) T2 target cells. Targets were preincubated either in the absence or the presence of various BFA concentrations ranging between 10 ng/ml and 3 μg/ml for 4 h and then either 1 μg/ml of core 131 (solid circles) or 1 μg/ml of IRIV-core 131 (open circles) was added for an overnight incubation. In another experiment (C), JY cells were pulsed with a very low peptide concentration of 0.05 μg/ml either in the presence of absence of 0.1 μg/ml of BFA. Percentage of cytotoxicity was determined in a standard Cr-release assay based on (A) CTL line D98d at an E:T ratio of 20:1, (B) CTL line D68c at an E:T ratio of 10:1 and (C) CTL line D68 at various E:T ratios indicated.

Table 1. Primary *in vitro* induction of HCV peptide specific CTL with soluble versus virosome-derived peptide

Stimulus	Target	Experiment 1 (D96, D97a)		Experiment 2 (D96, D98, D99a)		Total
		Peptide concentration (μg/ml)	Positive/total wells[b]	Peptide concentration (μg/ml)	Positive/total wells[b]	
Core 131	core 131	10	4/6	10	7/12	11/18
IRIV-c131[c]	IRIV-c131[e]	3.3	0/6	0.8	8/12	8/18
Core 35	core 35	10	3/6	10	1/12	4/18
IRIV-c35[d]	IRIV-c35[e]	3.3	1/6	0.8	6/12	7/18

[a]Different blood donors tested.
[b]Positive was determined as a specific target cell lysis >15%.
[c]IRIV-c131 = virosomes containing core 131 (ADLMGYIPLV).
[d]IRIV-c35 = virosomes containing core 35 (YLLPRRGPRL).
[e]Positive responses were also obtained against targets pulsed with core 131 or core 35 respectively.

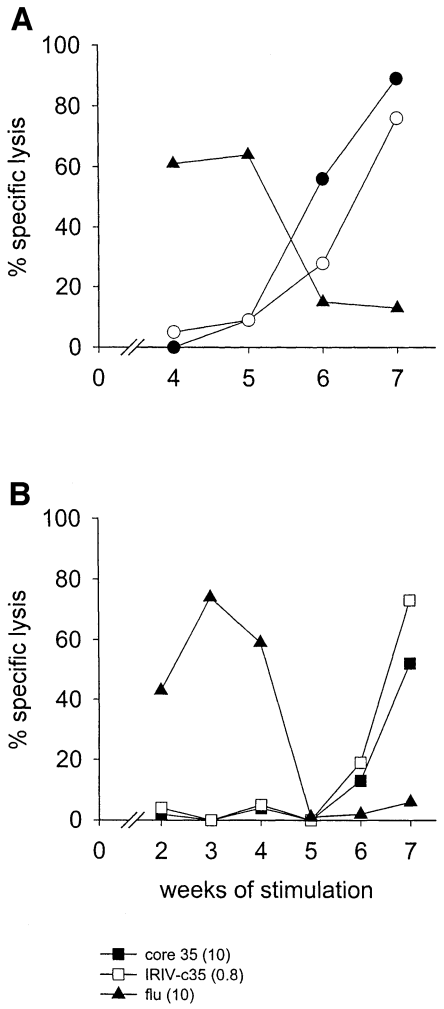


Fig. 5. Primary *in vitro* induction using (A) core 131 or IRIV-core 131 and (B) core 35 or IRIV-core 35 respectively against secondary *in vitro* stimulation. PBMC (4×10^6) were stimulated with either the corresponding soluble or virosome-derived peptide. Percentage of cytotoxicity was then determined after various weeks of stimulation against JY targets. Both, (A) and (B) represent typical examples out of the results shown in Table 1.

out of three cultures after 2 weeks, whereas the lower amounts such as 0.166 and 0.0166 μg/ml led to one and zero positive out of three cultures respectively. Finally, after 4 weeks of stimulation, there were two positive cultures obtained after both of the lower stimuli.

Recognition of endogenously processed core 131 peptide by epitope-specific CTL

To determine whether *in vitro* primed CTL were not only able to lyse target cells preincubated with soluble core 131 or peptide-bearing virosomes, but could also recognize endogenously processed peptide, we performed Cr-release assays using rVV9A encoding the full-length HCV core protein.

The experiment was based on both donor D76 and D98 effector cell lines and JY targets infected with rVV9A leading to specific target cell killing of nearly 40% at an E:T ratio of 40:1 based on donor D98 effector cell line (Fig. 6).

Discussion

The aim of this study was to analyze HCV core peptide-bearing IRIV *in vitro* as a novel non-live vaccine approach to induce primary CTL against two well-characterized HLA-A*0201 binding epitopes of core, the best conserved HCV protein representing also the main target of several other vaccine studies published so far (29,39–41). One central point was the characterization of the intracellular pathway with respect to TAP dependence, BFA sensitivity and mild acid treatment, and to check the remaining lysis after peptide stripping.

Overnight incubation of JY and the TAP-deficient cell line T2 in the presence of IRIV-core 131 resulted in the lysis of both targets by peptide-specific CTL effector cell lines (Fig. 1a). This led to two main questions: (i) how to rule out the possibility that virosomes are leaky and peptide is simply released into solution after a certain time of incubation (thereby binding from outside to MHC class I), and (ii) more generally, what are the possible different mechanisms involved in such a supposed TAP-independent pathway?

Target cell sensitization time curves using core 131 and IRIV-core 131 in parallel showed an obvious difference in kinetics of a successful pulsing between soluble and

virosome-derived peptide (Fig. 1b). In addition, the virosomal pathway was BFA-sensitive (Fig. 4a and b), as the egress of MHC class I peptide-loaded molecules to the cell surface was partly inhibited (42), whereas BFA did not have any negative effect on external soluble peptide sensitization of MHC class I on the surface of target cells, even at a very low peptide concentration of 0.05 μg/ml (Fig. 4c). These findings led to the assumption that leakiness of the virosomes could be definitely ruled out, especially as the virosome preparations had been purified on a G10 column to eliminate any possible free peptide.

In a dose curve based on various core 131 and IRIV-core 131 concentrations ranging between 2 μg/ml and 20 ng/ml there was a clear dose–response relationship for soluble and virosome-derived peptide (Fig. 1c). However, the two effector cell lines used required targets presenting different soluble and virosome-derived peptide concentrations for a comparable cell lysis, probably due to differences in the TCR–peptide–MHC class I affinity.

In a following study focusing in parallel on IRIV-core 131 sensitization of JY, T1 and T2 we compared the influence of the presence or absence of TAP on virosome-derived peptide presentation (Fig. 2). Although both effectors cell lines, D98b (Fig. 2a) and D99a (Fig. 2b), were able to kill virosome-preincubated targets, there was a difference between TAP-bearing and TAP-deficient cell lines: especially in the case of the weaker effector cell line D99a, JY and T1 cells incubated with IRIV-core 131 were less efficiently lysed than T2 cells (Fig. 2b). A finding confirmed in other experiments not shown was that there is a positive correlation between a strong effector cell line and an efficient lysis of TAP-bearing targets sensitized with virosome-derived peptide. TAP deficiency even seemed to be an advantage to obtain higher lysis especially if dealing with effector cells of lower killing potential. An explanation for this phenomenon could be that in T2 cells there is no real competition between endogenous TAP-transported peptide and core 131 as in TAP-competent cell lines.

There are some well-known classical types of peptides which are TAP independent: those that are naturally derived from signal sequences (43,44) and that have direct access to the endoplasmic reticulum where they are liberated by signal peptidase or those artificially linked to N-terminal signal sequences (45–47), peptides of membrane-bound proteins, i.e. transmembrane proteins (36,48), or finally those from within secretory proteins (49). It should be mentioned here that our HCV core peptides are lipophilic, therefore possibly behaving like normal transmembrane proteins, which might be one reasonable explanation in favor of a TAP independence. However, we rather support the hypothesis that the TAP independence is somehow related to the virosomal pathway *per se* and not limited to particular epitopes as HCV core 131 or core 35.

Table 2. Primary *in vitro* induction versus secondary stimulation of peptide-specific CTL based on virosome-derived peptide (IRIV-core 131 and IRIV-flu respectively)

Stimulus	Donor	Replicate	Percent specific lysis of targets presenting soluble peptide (P) or virosome-derived peptide (V)[c]									
			2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
			P	V	P	V	P	V	P	V	P	V
IRIV-flu[a]	D84	1	24	23	17	12	0	5	ND[d]	ND	ND	ND
		2	22	32	11	9	1	1	ND	ND	ND	ND
		3	24	25	65	51	65	38	ND	ND	ND	ND
		4	26	22	34	26	42	29	ND	ND	ND	ND
	D85	1	89	71	84	60	69	40	ND	ND	ND	ND
		2	82	71	66	53	54	41	ND	ND	ND	ND
		3	77	70	56	46	19	23	ND	ND	ND	ND
		4	85	62	79	48	38	26	ND	ND	ND	ND
	D98	1	100	80	91	84	75	72	ND	ND	ND	ND
		2	100	87	87	79	28	34	ND	ND	ND	ND
		3	92	93	89	91	73	73	ND	ND	ND	ND
		4	87	93	89	90	60	83	ND	ND	ND	ND
IRIV-c131[b]	D84	1	ND	ND	ND	ND	1	7	0	0	0	0
		2	ND	ND	ND	ND	0	0	0	0	0	0
		3	ND	ND	ND	ND	1	0	0	0	0	0
		4	ND	ND	ND	ND	0	0	0	0	0	0
	D85	1	ND	ND	ND	ND	1	3	0	0	0	2
		2	ND	ND	ND	ND	0	0	0	0	0	3
		3	ND	ND	ND	ND	0	0	0	0	0	0
		4	ND	ND	ND	ND	3	0	0	0	2	0
	D98	1	ND	ND	ND	ND	47	57	50	54	1	3
		2	ND	ND	ND	ND	30	41	67	60	80	72
		3	ND	ND	ND	ND	29	20	35	49	9	4
		4	ND	ND	ND	ND	46	44	69	69	23	27

[a]IRIV-flu = virosomes containing influenza matrix peptide (GILGFVFTL).
[b]IRIV-c131 = virosomes containing core 131 (ADLMGYIPLV).
[c] After weeks (w) of *in vitro* stimulation.
[d]Not determined.
No positive results were obtained using IRIV-c35 (YLLPRRGPRL) in this experiment.

Table 3. Comparison of secondary *in vitro* stimulation of influenza-specific CTL at various IRIV-flu concentrations

Stimulus	Peptide concentration (μg/ml)	Positive/total wells analyzed[a]		
		2 weeks[b]	3 weeks	4 weeks
Flu	10	3/3	3/3	2/3
IRIV-flu	1.66	3/3	3/3	3/3
IRIV-flu	0.166	1/3	1/3	2/3
IRIV-flu	0.0166	0/3	0/3	2/3

[a]Positive was determined as a specific target cell lysis >15%.
[b]After weeks of *in vitro* stimulation.

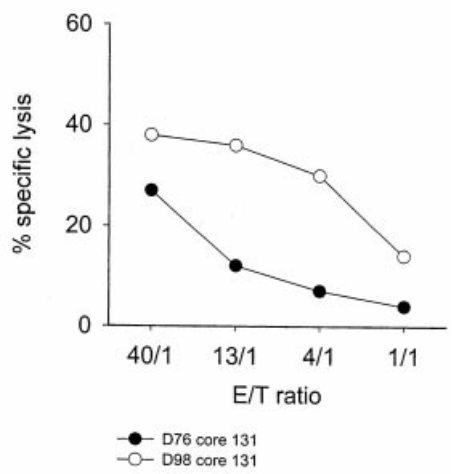


Fig. 6. Killing of endogenously processed peptide based on targets infected with rVV. JY target cells were incubated with rVV (at 10 m.o.i. or 1×10^7 p.f.u.) expressing the core protein for 3 h and then a Cr-release assay was performed based on donor D76 (solid circles) or D98 effector cell line (open circles) at E:T ratios of 40:1, 13:1, 4:1, and 1:1.

To obtain more information about the TAP-independent pathway, we focused on the influence of peptide stripping based on a mild acid treatment (38,50,51) of T2 targets (Fig. 3a). Cells pulsed with virosomes and incubated for another 5 h after the acid treatment were supposed to be able to present new peptide on their surfaces derived from an internal pool in the cytoplasm. However, it became evident that IRIV-core 131-pulsed cells could be efficiently peptide stripped, whereas core 131-pulsed T2 cells could not. Obviously, no MHC class I molecules could be reloaded with new peptide, except after the addition of new virosomes, leading to the assumption that IRIV-derived peptide was presented at once, not leaving any internal pool left. Evidently, in the case of soluble core 131, many more MHC molecules were presenting peptide than in the case of IRIV-core 131, which means that the acid treatment failed, as not enough peptide could be removed from the cell surface, explaining the high remaining lysis after mild acid treatment. This was confirmed in additional peptide-stripping experiments performed at lower core 131 concentrations of 0.1 μg/ml and based on JY target cells (Fig. 3b), where the mild acid treatment could reduce target cell killing by 50–100%, again depending on the killing potential of the corresponding effector cells. Alternatively, exogenously added peptides may be transported by recirculating MHC class I complexes to an intracellular compartment, thus forming a protected intracellular pool. On the other hand, the loss of target cell killing in IRIV-core 131-incubated cells might be partly due to the fact that a certain percentage of the original peptide was degraded despite the protecting virosomal vehicle after the release of core 131 into the cytoplasm or else it could be that the general efficiency of the TAP-independent transport is limited. Although only ~200 MHC class I molecules need to present a certain epitope to induce target cell lysis by specific effector cells (52) due to the possibility of serial triggering of many TCR by only a few peptide–MHC complexes (53), there was obviously still not enough core 131 left on the cell surface. Interestingly, addition of new virosomes together with further incubation led to sensitivity and resulting target cell lysis again, even with a slightly higher efficiency than before the acid elution, which might be partly due to the accumulation of new virosome-derived core 131 with remaining peptide. It may also be that acid treatment led to the availability of increased amounts of recycled empty class I molecules available for IRIV-derived peptides.

Importantly, it was possible to induce a secondary stimulation *in vitro* based on IRIV-flu which was dose dependent (Table 3), and even to prime CTL with IRIV-core 131 and IRIV-core 35 (Table 1) as successfully as based on the respective soluble peptides. According to the same protocol we were also able to prime CTL from spleen cells of BALB/c mice *in vitro* based on either core 133–142 (LMGYIPLVGA), IRIV-core 133 or influenza NP peptide 147–155 (TYQRTRALV) (data not included). Although *in vitro* there is no real difference in the number of positive primary induction cultures obtained between soluble and IRIV-derived peptide, focusing on the importance of an *in vivo* model, the most striking fact becomes evident: while it is impossible to inject soluble peptide not protected from extracellular proteases into a human body, thereby obtaining CTL specific against the desired epitope, virosomes might be an important peptide carrier for the induction of a cellular immune response as based on our studies performed so far. It remains to be determined if the efficacy of such a proposed vaccine approach based on HCV core peptide-bearing IRIV will be successful in the HLA-A*0201 transgenic mouse model, too. However, so far we could already show that *in vitro* primed CTL are able to specifically kill targets infected with rVV expressing HCV core

protein, demonstrating their capacity to lyse endogenously processed peptide—which is of great value for the planned *in vivo* vaccine trials.

Actually, there already exist different vaccines on the market based on virosomes and aimed at inducing protective antibodies in vaccinees. One of them, Epaxal Berna, is directed against HAV (9,11,20); a second one, Inflexal V Berna, against influenza virus (21,24). The difference of our vaccine project is to focus primarily on CTL induction by incorporating HCV-derived peptides into the vehicles instead of linking them onto the virosomal surface. A possible combination of both, extra-virosomal and incorporated epitopes would be one solution to induce both humoral and cellular immunity in parallel—the optimal combination for a vaccine against viral infections.

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Abbreviations

BFA	Brefeldin A
CTL	cytotoxic T lymphocyte
EBV	Epstein–Barr virus
HA	hemagglutinin
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
IRIV	immunopotentiating reconstituted influenza virosome
OEG	octaethyleneglycol
PBMC	peripheral blood mononuclear cell
rVV	recombinant vaccinia virus
TAP	transporter associated with antigen processing

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