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T_h1 but not T_h0 cell help is efficient to induce cytotoxic T lymphocytes by immunization with short synthetic peptides

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Abstract

Immunization of BALB/c mice with peptide HVSGHRMAWDMMMNWA, encompassing residues 121–135 from hepatitis C virus E1 protein, induced $CD4^+$ T_h1 cells as well as a long-lasting $CD8^+$ cytotoxic T lymphocyte (CTL) response *in vivo* when the peptide was administered s.c. with or without incomplete Freund's adjuvant. Using truncated peptides from this sequence it was shown that the determinant recognized by cytotoxic T cells was encompassed by residues SGHRMAWDM. Deletion of residues from the N-terminus or the C-terminus of the wild-type peptide abrogated its helper character. When Val122 of the wild peptide was replaced by Ala, the ability to induce a cytotoxic response was lost concomitantly with the loss of the T_h1 pattern of cytokine production. Interestingly, the Ala-modified peptide, when co-immunized with a peptide encompassing residues 323–329 from ovalbumin (OVA), which is able to induce a T_h1 response in BALB/c mice, restored the capacity of the modified peptide to induce CTL. However, co-immunization of the Ala-modified peptide with a peptide encompassing residues 106–118 from sperm whale myoglobin, which induces a T_h0 cytokine profile in BALB/c mice, was much less efficient than the OVA peptide to restore CTL induction. These results demonstrate that CTL induction with a short synthetic peptide requires that this peptide contains domains recognized by T_c cells as well as by T_h1 cells. For those peptides that do not contain this type of T_h domain, competent T cell help can be provided by co-immunization with a distinct peptide that is able to stimulate a T_h1 response.

Introduction

Induction of cytotoxic T lymphocytes (CTL) is an important defensive mechanism of the immune system in response to infection with a virus or other pathogens (1–6). This induction plays a key role in the elimination of infected cells. Similarly, it is also known that tumor growth can be controlled by CTL (7–9). Because of the great potential of CTL in cancer therapy and in the treatment of viral infections, a number of groups have attempted to develop immunization strategies to induce CTL. Thus, the successful use of recombinant viruses (10–12), dendritic cells (DC) pulsed with antigens (13,14), particulate antigens (15,16), lipopeptides (17) and synthetic peptides has been reported. In this last case, several alternative approaches have been tested: short synthetic peptides (18–22), peptide constructs containing a determinant recognized by T_h cells (TD_h) and a determinant recognized by CTL (TD_c) (23–26) or mixtures of TD_h and TD_c peptides not covalently linked (27–29).

Because synthetic peptides do not have any of the potential dangers associated with the induction of an infection, due to recombinant viruses, the use of peptides might be more acceptable for human therapy. Thus, understanding the molecular mechanisms of how synthetic peptides induce CTL is of paramount importance. Experiments carried out by others as well as by our group (19,23–25) demonstrated that CTL induction with synthetic peptides requires T cell help. Also, it has been reported that during infection a T_h1 cytokine profile is required to induce CTL (30,31). All these considerations suggest that short synthetic peptides, that are able to induce a CTL response *in vivo*, should encompass a TD_h (probably of the T_h1 subtype) and a TD_c in their sequence.

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To study the requirements of a short synthetic peptide to induce CTL *in vivo,* we selected a peptide from hepatitis C virus (HCV) E1 protein encompassing residues 121–135 that we had proved was able to induce a CTL response (32). Further characterization in the present study showed that E1(121–135) induced CTL $CD8^+$ and T_h1 $CD4^+$ responses *in vivo* when immunized in incomplete Freund's adjuvant (IFA). Using truncated peptides, we studied the regions within the peptide responsible for T_h and T_c recognition. With the aim of abolishing the ability of the peptide to induce a $CD4^+$ T_h response and concomitantly its ability to induce a CTL response, we replaced Val122 by Ala, affording E1(121–135, Ala122). As expected, replacement of this putative anchor residue of the T_h moiety of peptide E1(121–135) abrogated the induction of T_h response. This opened the possibility of studying the effect of extrinsic T cell help on CTL induction by co-immunization of E1(121–135, Ala122) with two peptides that were able to induce T_h1 and T_h0 cytokine profiles respectively. These experiments shed more light not only in the understanding of how synthetic peptides can induce CTL responses, but also on how these responses can be manipulated.

Methods

Peptide synthesis

Peptides were synthesized by the solid-phase method of Merrifield (33) using the F-moc alternative (34) and a manual multiple solid-phase peptide synthesizer (35). The ninhydrin test of Kaiser was used to monitor every step (36). At the end of the synthesis, peptides were cleaved and deprotected, and washed 6 times with diethyl ether. They were lyophilized and analyzed by HPLC. The purity of peptides was >80% as judged by HPLC.

Mice

Six-week-old female BALB/c mice were purchased from IFFA Credo (Barcelona, Spain). They were hosted in appropriate animal care facilities and handled following international guidelines required for experimentation with animals.

Immunization and measurement of CTL response

Groups of three mice were immunized by s.c. injection at the base of the tail and footpads with 60 μg of peptide emulsified in IFA at days 0 and 15. For immunizations using a TD_h and a TD_c peptide mixture, 60 μg of each peptide was injected. In some experiments, immunizations were carried out in the absence of adjuvant. This was done by i.p. injection of 60 μg of peptide in 0.5 ml of saline at days 0, 7 and 14. In both protocols the animals were killed 10 days after the last immunization and their lymph nodes (LN) and/or spleen removed. Cells (8×10^6/well) were re-stimulated *in vitro* in 24-well plates in 2 ml of culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, penicillin 100 U/ml, streptomycin 100 μg/ml and 5×10^{-5} M 2-mercaptoethanol) with 5 μg/ml of peptide. Cytotoxic activity was measured 5 days after initial culture using the ^{51}Cr-release assay (37). P815 target cells (10^6 cells) were radiolabeled with 50 μCi of $Na_2{}^{51}CrO_4$ at 37°C for 1 h, washed 3 times, and incubated with or without 5 μg/ml of peptide and different numbers of effector cells in triplicate. Spontaneous release was measured from wells without effector cells and maximum release from target cells incubated with 5% Triton X-100. The spontaneous release was in all cases <25% of total release. In selected experiments, 0.1 mg/ml of different antibodies was added during the incubation period. After 4 h of incubation, supernatants were harvested and radioactivity measured in a scintillation counter (Topcount; Packard, Meridan, CT).

In CTL precursor frequency measurement experiments, four different concentrations of LN cells (8×10^5, 4×10^5, 2×10^5 and 1×10^5) were placed in 24 replica cultures (for each dilution) in culture medium in the presence of 10 μg/ml of peptide E1(121–135), complemented with graded numbers of irradiated syngenic spleen cells (3000 rad) to give a total number of 5×10^5 cells/well (96-wells U-bottomed plates) in a final volume of 0.25 ml of medium. After 6 days of culture at 37°C and 5 % CO_2, CTL activity of each individual well was measured using the ^{51}Cr-release assay by transferring 100 μl of each well to a plate containing radiolabeled P815 cells and peptide E1(121–135), and another 100 μl to a plate with P815 radiolabeled cells without peptide. After 5 h of culture, 50 μl of supernatant were removed from each well and the percentage of lysis estimated in a scintillation counter (Topcount). Responses for the individual wells were considered positive when CTL activity in the presence of peptide was 15% higher than in the absence of peptide. Frequencies were calculated using χ^2 analysis according to the method of Taswell (38) on a computer program (kindly provided to us by Dr L. Selin, University of Massachusetts Medical Center, Worcester, MA). CTL precursor frequencies were calculated on the regression curve by interpolating the number of responder cells required to give 37% negative cultures. Only those experiments where data fitted in the single-hit model were considered.

Immunization for cytokine measurement

Groups of three mice were immunized by s.c. injection at the base of the tail and footpads with 60 μg of peptide emulsified in IFA. Ten days after the primary injection, animals were killed and LN cells removed. The lymphocytes were then plated on 96-well plates at 8×10^5 cells/well with culture medium alone or with serial dilutions of peptide in the same medium to a final volume of 0.25 ml. In selected experiments, 0.1 mg/ml of different antibodies was added during the incubation period. Supernatants (50 μl) were removed 24 h later to measure IL-2 content, and 48 h later for IFN-γ and IL-4.

Measurement of cytokine production

IL-2 content in culture supernatants was measured by using a CTLL bioassay as described (39). IFN-γ production was measured by ELISA (Genzyme, Cambridge, MA) according to manufacturer's instructions. IL-4 content was measured by CT4S bioassay (kindly provided by Drs W. E. Paul and G. J. Watson, National Institutes of Health, Bethesda, MD). Briefly, CT4S cells were re-supended in culture medium and were plated (5×10^3 cells/well) in a 96-well flat-bottomed plate with the supernatant to be assayed (50% v/v). After 48 h of culture, cells were pulsed with 0.5 μCi/well of [^{3}H]thymidine for 18 h, harvested and thymidine incorporation was determined in a

scintillation counter (Topcount). Standard curves using known amounts of rIL-4 were also included to determine the amount of IL-4.

Depletion of $CD4^+$ and $CD8^+$ cells in vivo

Mice were depleted of $CD4^+$ or $CD8^+$ cells by i.p. injection of 0.3 mg of anti-CD4 (obtained from rat anti-mouse hybridoma GK1-5) or anti-CD8 (obtained from rat anti-mouse hybridoma H35.17.2) antibodies respectively on days –1, 0, 1, 6, 7, 8, 13, 14 and 15 as previously described (19,40); day 0 being the day of immunization. The efficiency of depletions was assessed by flow cytometry on day 21.

Results

Immunization with peptide E1(121–135) induced a $CD8^+$ CTL response

In a first experiment, BALB/c mice were immunized with peptide E1(121–135) from HCV E1 protein in IFA. As shown in Fig. 1, both LN cells (Fig. 1A) and spleen cells (Fig. 1B) from these animals were able to lyse P815 target cells pulsed with peptide E1(121–135). Thus, peptide E1(121–135) can induce CTL in vivo without external help from a TD_h peptide. Addition of anti-CD8 antibodies plus complement during the ^{51}Cr-release assay, but not of anti-CD4 antibodies plus complement, was able to abrogate the lysis of P815 target cells pulsed with E1(121–135), showing that the phenotype of the induced CTL was $CD8^+$ (Fig. 1C).

In order to identify the region of E1(121–135) recognized by $CD8^+$ cells, LN cells from BALB/c mice immunized with E1(121–135) were stimulated in vitro with E1(121–135) for 5 days and their CTL activity measured against P815 cells pulsed with a number of truncated peptides. Since truncated peptides SGHRMAWDMMMNWA and HVSGHRMAWDM were equally active, and share the region SGHRMAWDM, it was concluded that this region is the one recognized by induced CTL. This was confirmed when using nonapeptide SGHRMAWDM in the CTL assay (Fig. 2).

Immunization with peptide E1(121–135) stimulated a $CD4^+$ T_h1 cytokine profile and concomitantly CTL induction

To characterize the cytokine profile induced after immunization with E1(121–135), LN cells from BALB/c mice immunized with this peptide were stimulated in vitro with 40 μg/ml of peptide. IL-2, IL-4 and IFN-γ were quantified from culture supernatants after 24, 48 and 48 h respectively. As shown in Table 1, the pattern of cytokine production was of the T_h1 subtype, i.e. high IL-2 and IFN-γ production, and no detectable IL-4. Experiments carried out in the presence or absence of anti-CD4 or anti-CD8 antibodies showed that only $CD4^+$ cells were responsible for cytokine production. To characterize the region of E1(121–135) responsible for T_h cell activity, LN cells from BALB/c mice immunized with E1(121–135) were stimulated in vitro with 40 μg/ml of wild-type peptide or with a number of truncated peptides (Table 2). Results show that deletion of residues from either the N-terminus or the C-terminus substantially decreases IL-2 production, showing that the entire sequence is required to provide T cell help.

In another group of experiments, we studied the induction of CTL by immunization with peptide E1(121–135) in the absence of adjuvant. Mice were immunized at days 0, 7 and 14 with 60 μg of E1(121–135) in 0.5 ml of saline. Animals were sacrificed at day 21 and their spleen cells stimulated for 5 days in the presence of E1(121–135). As

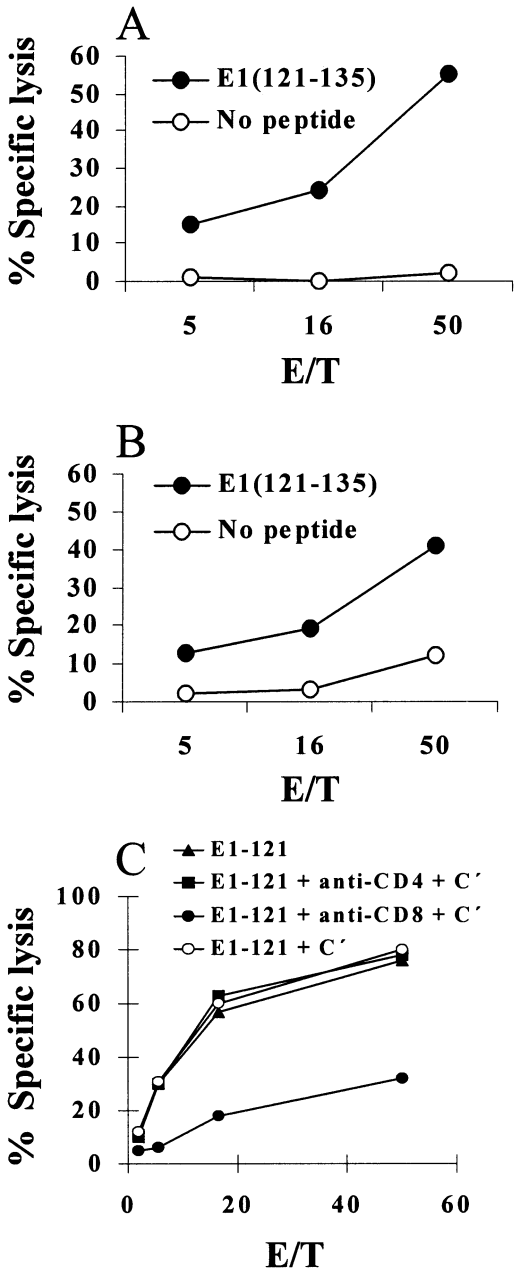

Fig. 1. Induction of CTL in vivo by immunization with peptide E1(121–135). Mice were immunized by s.c. injection with 60 μg of peptide emulsified in IFA at days 0 and 15. Ten days after the last immunization LN (A) and spleen cells (B) were stimulated in vitro for 5 days in the presence of 5 μg/ml of peptide. CTL activity was measured using P815 target cells previously incubated with 5 μg/ml of E1(121–135) or with medium alone. The phenotype of the CTL induced with peptide E1(121–135) was characterized using P815 target cells incubated with E1(121–135) in the presence or in the absence of 0.1 mg/ml of anti-CD4 or anti-CD8 antibodies plus complement (C).

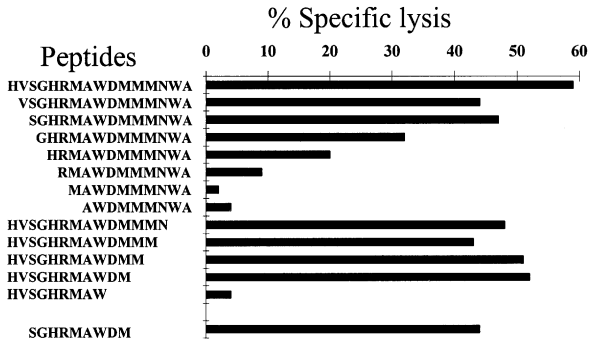

Fig. 2. Region of E1(121–135) recognized by CTL. LN cells from BALB/c mice immunized with peptide E1(121–135) were stimulated in vitro for 5 days. CTL activity was measured against P815 target cells incubated with 5 μg/ml of E1(121–135) truncated peptides shown. An E:T ratio of 25:1 was used in all experiments.

Table 1. Cytokine production after in vitro stimulation with peptide E1(121–135)

Treatment	IL-2 (mU/ml)	IFN-γ (pg/ml)	IL-4 (mU/ml)
None	94.1	333.1	<25
Anti-CD4	2.01	<38	<25
Anti-CD8	88.0	257.4	<25

LN cells from BALB/c mice immunized with E1(121–135) were stimulated with 40 μg/ml of peptide. Supernatants were harvested after 24, 48 and 48 h to quantify IL-2, IL-4 and IFN-γ respectively. mAb were added to the cultures at a concentration of 0.1 mg/ml. IL-2 and IL-4 content was determined using the CTLL and CT4S bioassay respectively, and IFN-γ content was determined by ELISA.

Table 2. Characterization of the region within E1(121–135) responsible for T_h cell activity

Peptide	IL-2 (SI)
HVSGHRMAWDMMMNWA	14.9
VSGHRMAWDMMMNWA	2.0
SGHRMAWDMMMNWA	3.2
GHRMAWDMMMNWA	1.9
HRMAWDMMMNWA	1.6
RMAWDMMMNWA	1.8
MMAWDMMMNWA	1.2
AWDMMMNWA	1.7
HVSGHRMAWDMMMN	3.2
HVSGHRMAWDMMM	1.6
HVSGHRMAWDMM	2.4
HVSGHRMAWDM	2.2
HVSGHRMAW	1.3

LN cells from BALB/c mice immunized with E1(121–135) were stimulated with 40 μg/ml of E1(121–135) truncated peptides. Culture supernatants were harvested 24 h later and production of IL-2 was determined using a CTLL bioassay. IL-2 production is expressed as stimulation index (SI) (ratio between the c.p.m. of CTLL in the presence of peptide and in the absence of peptide).

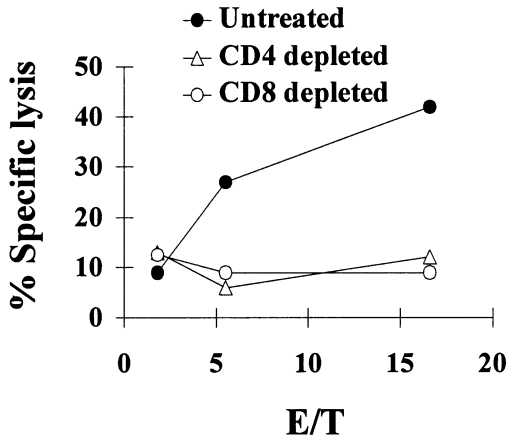

Fig. 3. Effect of in vivo depletion of $CD4^+$ or $CD8^+$ cells on CTL induction. Mice were immunized by i.p. injection with 60 μg of E1(121–135) in 0.5 ml of saline at days 0, 7 and 14. They were depleted of $CD4^+$ or $CD8^+$ cells in vivo or left untreated before and during immunization. Animals were killed 10 days after the last immunization and CTL activity was measured after culturing spleen cells for 5 days in the presence of peptide E1(121–135). CTL activity was measured against P815 target cells pulsed with or without peptide. Results are expressed as the difference between percentage of lysis against peptide pulsed and unpulsed target cells. Specific lysis against unpulsed P815 target cells was always <10%.

shown in Fig. 3, this strategy of immunization was also able to induce a CTL response similar to the one attained when using IFA as adjuvant. Moreover, to study the relevance of $CD4^+$ and $CD8^+$ cells for in vivo CTL induction after immunization with E1(121–135), mice were depleted of either $CD4^+$ or $CD8^+$ cells with mAb. As for non-depleted animals, mice were immunized at days 0, 7 and 14 with 60 μg of E1(121–135) in 0.5 ml of saline. As shown in Fig. 3, depletion of either $CD4^+$ cells or $CD8^+$ cells completely abrogated CTL induction, indicating that both types of cells are needed.

Peptide E1(121–135) induced a long-lasting response

To study the duration of the CTL response induced after two immunizations with peptide E1(121–135) in IFA, animals were killed at days 30, 60 and 90 after the first immunization and CTL activity was measured. As shown in Fig. 4, the activity at day 90 remained stable with respect to that attained at day 60.

Replacement of Val122 by Ala abrogated $CD4^+$ and concomitantly $CD8^+$ responses: restoration of CTL induction by co-immunization with an external T_h1 peptide

Since $CD4^+$ T_h cells were necessary to induce a CTL response with E1(121–135), we decided to modify this peptide outside the CD8 epitope with the aim of abrogating the $CD4^+$ response. Thus, we replaced Val122 by Ala and studied the effect of this replacement on the $CD4^+$ and $CD8^+$ responses. Replacement of Val122 in peptide E1(121–135) by Ala completely abrogated IL-2 production and IFN-γ production (Table 3). Moreover, no CTL were induced after immunization with E1(121–135, Ala122) (Fig. 5). As expected, because position 122 is outside the CTL epitope, replacement of Val 122 by Ala did not change the capacity of the peptide to be

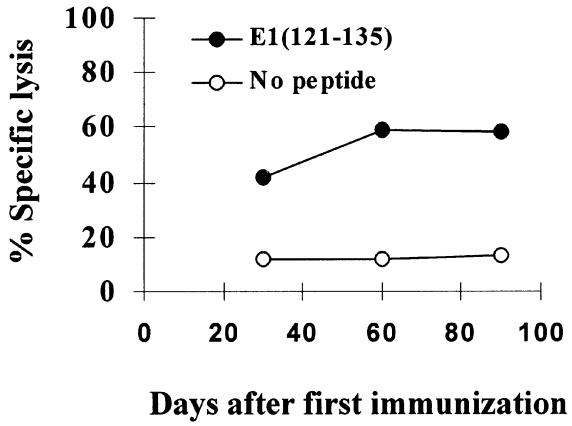

Fig. 4. Duration of the CTL response induced after immunization with peptide E1(121–135). Mice were immunized at days 0 and 15 with peptide E1(121–135) emulsified in IFA. They were sacrificed at days 30, 60 or 90 after the first immunization. Spleen cells were stimulated in vitro for 5 days and CTL activity measured against P815 target cells pulsed with or without E1(121–135). E:T ratio was 50:1 in all cases.

Table 3. Patterns of cytokine production after immunization with different peptides

Peptide	IL-2(mU/ml)	IFN-γ (pg/ml)	IL-4 (mU/ml)
E1(121–135)	25.4	499	<25
E1(121–135, Ala122)	<0.5	<38	<25
OVA(323–339)	33.5	504	<25
FIS[a]	50.2	886	318

LN cells from BALB/c mice immunized with different peptides were stimulated with 40 μg/ml of peptide. Supernatants were harvested after 24, 48 and 48 h to quantify IL-2, IL-4 and IFN-γ respectively. IL-2 and IL-4 content was determined using the CTLL and CT4S bioassay respectively, and IFN-γ content was determined by ELISA.

[a]Peptide FIS corresponds to amino acid sequence 106–118 from sperm whale myoglobin.

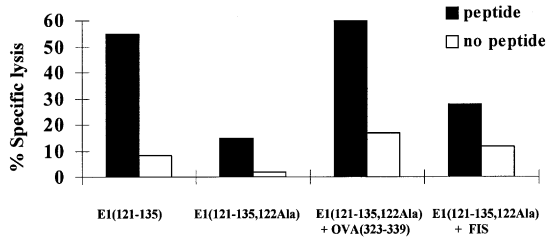

Fig. 5. Effect of internal and external T cell help in CTL induction. Mice were immunized with peptide E1(121–135), with E1(121–135, 122Ala), with E1(121–135, 122Ala) in conjunction with the T_h1 peptide OVA(323–339) or with E1(121–135, 122Ala) in conjunction with the T_h0 peptide FIS emulsified in IFA. LN cells were stimulated in vitro for 5 days in the presence of the corresponding CTL peptide used for immunization and CTL activity measured. In all cases an E:T ratio of 50 was used. Results shown are representative of three different experiments.

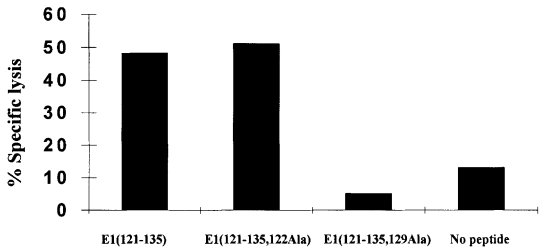

Fig. 6. Effect of single amino acid changes inside and outside the CTL epitope on CTL recognition. Mice were immunized by s.c. injection with 60 μg of peptide emulsified in IFA at days 0 and 15. Ten days after the last immunization LN cells were stimulated in vitro for 5 days in the presence of 5 μg/ml of peptide E1(121–135). CTL activity was measured using P815 target cells previously incubated with 5 μg/ml of E1(121–135), E1(121–135, 122Ala), E1(121–135, 129Ala) or with medium alone. An E:T ratio of 50 was used.

recognized by CTL induced with E1(121–135). However, when Trp129 (which is part of the CTL epitope) was replaced by Ala, the peptide was not recognized (Fig. 6).

We then carried out experiments to restore the CTL activity of this peptide by providing external T cell help and characterize the type of T cell help required. As shown in Table 3, immunization with T_h peptides OVA(323–339) (encompassing residues 323–339 from ovalbumin) and FIS (encompassing residues 106–118 from sperm whale myoglobin) elicited T_h1 and T_h0 cytokine profiles respectively. Thus, we decided to use these peptides to provide external T cell help to E1(121–135, Ala122). Four groups of mice were immunized with single peptides or peptide mixtures emulsified in IFA: (i) E1(121–135), (ii) E1(121–135, Ala122), (iii) E1(121–135, Ala122) in conjunction with OVA(323–339) and (iv) E1(121–135, Ala122) in conjunction with FIS. LN cells were stimulated in vitro for 5 days with either E1(121–135) [group (i) or E1(121–135, Ala122) (groups (ii), (iii) and (iv)] and CTL activity measured against P815 cells pulsed with the same peptide used for in vitro stimulation. As shown in Fig. 5, immunization with peptide E1(121–135, Ala122) by itself is unable to induce CTL in vivo. However, co-immunization of OVA(323–339) and E1(121–135, Ala122) was able to induce a CTL activity of the same level obtained when immunizing with the unmodified E1(121–135). By contrast, co-immunization of FIS and E1(121–135, Ala122) was unable to restore CTL activity to the level attained with E1(121–135). Results from Fig. 5 are representative of experiments carried out 3 times. In order to get a further insight on the magnitude of the CTL responses induced after these immunizations, the T cell precursor frequency was measured by limiting dilution assay. As shown in Table 4, the T cell precursor frequency is in good agreement with the percentage of specific lysis induced (Fig. 5). That is, co-immunization of E1(121–135, Ala122) with OVA(323–339) affords a frequency of CTL similar to the one attained with E1(121–135), whereas co-immunization with FIS affords intermediate CTL precursor levels.

Discussion

It has been reported that CTL induction can be achieved after immunization with short synthetic peptides (18–20,22,41)

Table 4. CTL precursor frequencies induced after immunization of mice with different peptides or peptide mixtures

Immunized with	CTL precursors	95% confidence interval
E1(121–135)	1/356,419	1/282,846–1/481,724
E1(121–135,122A)	1/2,459,329	1/1,536,882–1/6,151,504
E1(121–135, 122A) + OVA(323–339)	1/326,984	1/254,423–1/457,447
E1(121–135, 122A) + FIS	1/1,097,642	1/760,032–1/1,974,902

Mice were immunized with peptide E1(121–135), with E1(121–135, 122Ala), with E1(121–135, 122Ala) in conjunction with the T_h1 peptide OVA(323–339) or with E1(121–135, 122Ala) in conjunction with the T_h0 peptide FIS emulsified in IFA. LN cells were stimulated in vitro in the presence of E1(121–135) or E1(121–135, 122Ala) peptides and the CTL precursor frequency measured after 5 days as described in Methods.

or immunogenic constructs containing combinations of CD4 and CD8 epitopes (23–26). In most of these cases, it was reported that a TD_h should be present in the peptide. Also, as known from in vivo models, when a pathogen enters the body, CTL induction is helped by activation of $CD4^+$ cells of the T_h1 subtype. Thus, we hypothesized that short synthetic peptides that are able to induce a CTL response should contain two types of determinants: those recognized by $CD4^+$ T_h1 cells and those recognized by $CD8^+$ cells. To address this hypothesis we selected peptide E1(121–135) from hepatitis C virus E1 protein which we had found was able to induce a CTL response in BALB/c mice. Characterization of the cytokine profile induced by this peptide showed a T_h1 cytokine profile mediated by $CD4^+$ cells. Also, depletion of $CD4^+$ or $CD8^+$ cells in mice before immunization with peptide E1(121–135) completely abrogated the ability of this peptide to induce CTL in vivo, demonstrating that both types of cells are required for CTL induction. Moreover, using truncated peptides from E1(121–135) we showed that the region recognized by CTL is encompassed by amino acids 123–131 and that the whole peptide E1(121–135) was necessary for providing efficient T cell help for CTL induction.

To study the role of the TD_h encompassed by E1(121–135) we replaced Val122 by Ala to give peptide E1(121–135, Ala122). This was done because Val122 might be an anchor residue for binding to MHC class II molecules, as described for other peptides having TD_h character (42). Replacement of Val122 completely abrogated IL-2 and IFN-γ production (Table 3) as well as CTL induction, showing that Val122 is essential for providing efficient T cell help. Since only the region encompassed by amino acids 123–131 is the one recognized by CTL, replacement of Val122 did not alter the CD8 epitope in peptide E1(121–135, Ala122). Thus, changes in CTL induction can only be attributed to abrogation of the $CD4^+$ T_h response. Moreover, target cells pulsed with peptide E1(121–135, Ala122) were efficiently recognized by E1(121–135)-specific CTL, showing that the modified peptide had a competent CD8 epitope, and retained the ability to be processed and presented by antigen-presenting cell class I molecules (Fig. 6). Since E1(121–135, Ala122) was not immunogenic per se but contained a TD_c, it opened the possibility of studying the effect of an external TD_h peptide to induce CTL in vivo, in a similar manner to the study carried out by Shirai et al. (43) where they tested the use of intrinsic and extrinsic helper epitopes for CTL induction. However, these authors did not study the cytokine profiles induced by the helper peptides. In our work we wished to study the type of cytokines required for efficient CTL induction. Thus, we selected two TD_h peptides, OVA(323–339) and FIS, that are able to induce a T_h1 and a T_h0 cytokine profile respectively. As shown in Fig. 5 and Table 4, the external T cell help provided by the T_h1 peptide was able to efficiently restore CTL induction, whereas the use of T_h0 peptide under our experimental conditions restored CTL activity only partially. This result indicates that T_h peptides that are able to induce T_h1 cytokine profiles are more efficient (if not essential) to induce CTL in vivo than peptides that elicit T_h0 profiles. In vitro studies with $CD4^+$ and $CD8^+$ subpopulations stimulated with synthetic peptides have demonstrated the importance of different cytokines in CTL induction. Thus, IL-2 and IFN-γ are important in early stages of induction, whereas IL-4 is utilized in later stages of CTL maturation (44). It has been reported that IL-12 secretion (one of the most powerful T_h1 skewing cytokines) by DC is dependent upon interaction with $CD4^+$ T_h1 cells, whereas $CD4^+$ T_h2 cells inhibit IL-12 secretion (45). Thus 'good' levels of IL-2 and IFN-γ, and low production of IL-4 might be important for CTL induction. However, as we and others have recently demonstrated (46–48), very high levels of IL-12 and IFN-γ may have a suppressive effect on the induction of T_h1 and CTL responses. Thus, 'adequate' levels of these cytokines are essential for an efficient CTL induction.

It has been recently reported that $CD4^+$ T cell help for CTL induction can be bypassed by activation of DC through CD40, suggesting that the critical role of T_h cells for CTL induction is activation through the CD40–CD40 ligand (49–51). Thus, immunogenic synthetic peptides should contain TD_h and TD_c in order to activate $CD4^+$ T_h cells and $CD8^+$ CTL as well as DC through their cognate interaction with $CD4^+$ cells. Some 9–10mer synthetic peptides (that because of their short length might not have TD_h character) are nevertheless able to induce a CTL response when immunized pulsed onto DC (14,52). In these cases, DC might be either activated in vitro under culture conditions or the peptides might themselves be able to activate DC by an alternative pathway independent of CD40–CD40 ligand, as some viruses do (50). Because in our model depletion of $CD4^+$ cells in vivo abrogated CTL induction we believe that this induction was achieved via a $CD4^+$-dependent pathway.

It is interesting to note that the CTL response induced by peptide E1(121–135) was long-lasting and could also be induced in the absence of adjuvant. These results suggest that the use of combinations of TD_c and T_h1 TD_h regions in immunogenic constructs might be a useful tool in human

vaccination against tumors as well as viruses. External help from a $CD4^+$ TD_h peptide in peptide mixtures has been a very important tool for the characterization of CTL epitopes and their properties (53–56). However, since induction of CTL responses with constructs containing covalently linked epitopes seems to be stronger than the one obtained when using peptide mixtures (24,25,57), these constructs would be more convenient for vaccination as well as for treatment of chronic infections or tumors. Another strategy to induce CTL is the use of naked DNA (58,59). Thus, we carried out preliminary experiments using gene gun-based DNA immunization with a plasmid expressing E1(121–135). After three immunizations with the gene gun, CTL were induced with an efficiency similar to the one attained after immunization with peptide E1(121–135) in IFA (data not shown). We are planning to explore this alternative in more depth in future experiments.

Because of the diversity of MHC class I and class II molecules in humans, peptides used in vaccination or treatment of chronic infections should contain several TD_h and TD_c determinants, to attempt to cover the widest possible proportion of class I and class II restrictions. This implies that the joint effect of several TD_h peptides on the cytokine profile should be studied in great detail, because some TD_h peptides with T_h1 character for a given MHC restriction might have T_h0 or T_h2 character in other restrictions. If this was the case, some combinations of TD_h peptides might be deleterious for efficient CTL induction. Research is being carried out to address these questions.

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Abbreviations

CTL	cytotoxic T lymphocytes
DC	dendritic cells
HCV	hepatitis C virus
IFA	incomplete Freund's adjuvant
LN	lymph node
OVA	ovalbumin
TD_c	T cell determinant recognized by CTL
TD_h	T cell determinant recognized by T_h lymphocytes

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