Immunization with a tumor-associated CTL epitope plus a tumor-related or unrelated Th1 helper peptide elicits protective CTL immunity

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Immunization with cytotoxic T cell epitope SPSYVYHQF (AH1), derived from MuLV gp70 envelope protein expressed by CT26 tumor cells, does not protect BALB/c mice against challenge with CT26 tumor cells. By contrast, immunization with AH1 plus T helper peptides OVA(323–337) or SWM(106–118) eliciting Th1 and Th0 profiles, protected 83% and 33% of mice, respectively. Interestingly, immunization with AH1 plus both helper peptides reverted the efficacy to 33%. We identified the endogenous T helper peptide p(320–333) from gp70 which elicits a Th1 profile and is naturally processed. As for OVA(323–337), immunization with p(320–333) alone did not protect against tumor challenge. However, p(320–333) plus AH1 protected 89% of mice at day 10 after vaccination. Only 20% of mice vaccinated with AH1 + OVA(323–337) or AH1 + p(320–333) were protected when challenged 80 days after immunization. Treatment with OVA(323–337) or with p(320–333) around established tumors delayed tumor growth. Our results show that tumor-related as well as tumor-unrelated but strong Th1 peptides may be useful for inducing CTL responses in tumor immunotherapy.

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1 Introduction

Cytotoxic T lymphocytes (CTL) play an important role in antitumor immune responses [1, 2]. These cells recognize eight- to ten-amino acid-long peptides derived from tumor antigens presented by major histocompatibility complex (MHC) class I molecules at the surface of tumor cells. It is well documented that induction of CTL usually requires help from $CD4^+$ T helper lymphocytes [3–6], which recognize peptides presented by MHC class II molecules. Thus, it is conceivable that the lack of a competent T helper response induced by tumor antigens might result in tumor growth. Immunization with synthetic peptides containing T helper cell determinants (THd) and T cytotoxic cell determinants (TCd) has allowed the induction of CTL responses against different antigens [3–7]. THd presented by class II molecules may elicit Th0, Th1 or Th2 cytokine profiles [8–10], but, as shown in recent studies, THd eliciting a Th1 cytokine profile induce CTL more efficiently than those eliciting Th2 or Th0 profiles [11, 12]. For the purpose of CTL induction, THd may originate from an exogenous protein not related to the tumor antigens or from an endogenous tumor antigen. However, because immunization with an endogenous tumor-specific THd alone was able to partially protect mice from challenge with MHC class II-negative virus-induced tumor cells [13], it is likely that an endogenous THd might be more suitable than an exogenous THd (from a protein not related to the tumor) for antitumor therapy. This prompted us to evaluate the relative efficacy of endogenous and exogenous THd to induce protective antitumor immune responses. To address this question we used as model TCd the peptide SPSYVYHQF (from now on AH1) derived from MuLV gp70 envelope protein expressed by CT26 cells and presented by H-$2L^d$ MHC class I molecules [14]. Immunization of BALB/c mice with AH1 is unable to induce a protective response against challenge with CT26 tumor cells [14]. To provide T cell help for CTL induction, we tested exogenous Th0 and Th1 peptides as well as an endogenous Th1 peptide from gp70 antigen expressed by CT26 tumor cells, which was identified and characterized in the present work. The relative efficacy of these immunization strategies was tested by challenging vaccinated mice with MHC class II-negative CT26 tumor cells. We report here that immunization with certain combinations of THd and THc induce protective immunity against s.c. or i.v. challenge with CT26 tumor cells.

[I 21182]

Abbreviations: THd: T helper cell determinant / **TCd**: T cytotoxic cell determinant / **MuLV**: Murine leukemia virus

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Table 1. Cytokine profile elicited after immunization of BALB/c mice with exogenous helper peptides OVA(323–337), SWM(106–118) or T cytotoxic determinant AH1 from CT26[a)]

Peptide	IL-2 (mU/ml)[a)]			IFN-γ (pg/ml)[c)]	IL-4 (mU/ml)[c)]	Cytokine profile
	In vitro treatment[b)]					
	None	Anti-CD4	Anti-CD8			
OVA(323–337)	47	21	62	624	< 5	Th1
SWM(106–118)	80	17	72	1,117	398	Th0
AH1	3	NT	NT	< 36	< 5	–

a) Mice were immunized with 50 μg peptide emulsified in IFA. Ten days after lymph node cells were cultured in the presence or absence of peptide and cytokines were measured.
b) Anti-CD4 or anti-CD8 mAb (100 μg) were added to the culture to study their effect in IL-2 production.
c) IL-2, IFN-γ or IL-4 production in the absence of peptide was always below 5 mU/ml, 50 pg/ml and 25 mU/ml, respectively.

2 Results

As mentioned above, immunization of mice with TCd peptide AH1 from gp70 protein of CT26 tumor cells is unable to protect mice from challenge with these cells. For this reason we decided to study whether immunization of mice with AH1 plus different types of THd peptides might be more efficient. In the first part of this work we study exogenous THd peptides. In the second part, we identify an endogenous THd from gp70 and compare its efficacy with the group of exogenous THd peptides.

2.1 Cytokine profiles induced by exogenous T helper determinants following immunization

To study the effect of different cytokine profiles induced by THd in antitumor CTL responses, we measured IL-2, IFN-γ and IL-4 induced by OVA(323–337) from chicken ovalbumin and by SWM(106–118) from sperm whale myoglobin [15, 16]. As a control, we also measured these cytokines after immunization with the immunodominant TCd peptide AH1 from gp70 presented by class I molecules H-2L^d [14]. As shown in Table 1 and reported earlier [12], OVA(323–337) and SWM(106–118) elicit Th1 and Th0 cytokine profiles, respectively, in BALB/c mice. Addition of anti-CD4 but not anti-CD8 monoclonal antibodies strongly reduced IL-2 production, indicating that CD4 cells are responsible for cytokine production. However, AH1 induced very low amounts of IL-2 and was unable to induce significant levels of IFN-γ or IL-4, indicating its inability to function as a THd.

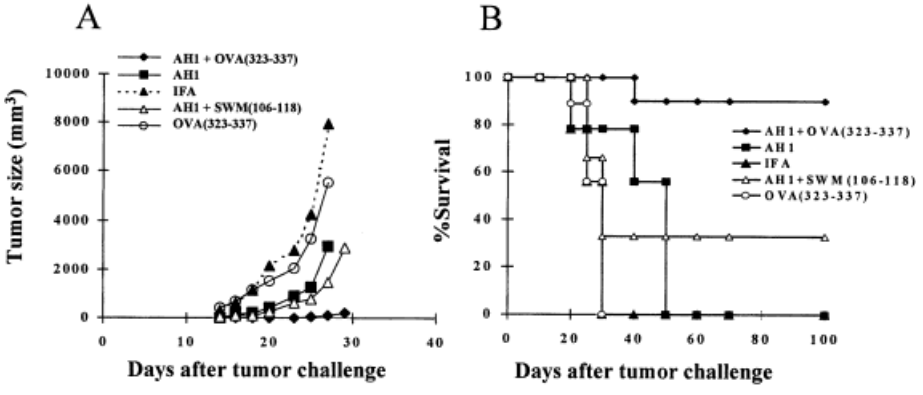

Fig. 1. Evolution of tumor growth after challenge with CT26 tumor cells in groups of BALB/c mice immunized with different peptide combinations. As a control, a group of mice was immunized with IFA alone. Ten days later, animals were challenged by s.c. injection with 5×10^5 CT26 tumor cells. (A) Average tumor size was expressed as the square value of the smallest diameter of the tumor, times the value of its largest diameter. (B) Kaplan-Meier plots of the mice survival after challenge with CT26 tumor cells.

2.2 Efficacy of different peptide combinations to protect mice from CT26 tumor cell challenge

Since from a previous publication [12] we knew that co-immunization of THd OVA(323–337) or SWM(106–118) with a TCd was able to induce a CTL response, we tested whether this strategy could be used to protect animals from challenge with CT26 tumor cells. In a first experiment**,** groups of BALB/c mice were immunized with different THd plus the TCd AH1. These groups were: AH1 + OVA(323–337), AH1 + SWM(106–118), AH1 alone, OVA(323–337) alone, or IFA alone. The dose of THd used was that tested for cytokine production. When lower doses of THd were tested for cytokine production, lower levels of cytokines were elicited but the profiles remained the same as for the higher doses (data not shown).

Animals were then challenged with CT26 tumor cells 10 days after immunization. Co-immunization with AH1 + THd OVA(323–337) prevented tumor growth in eight of nine mice (89%), whereas only three of nine (33%) were protected when using SWM(106–118) as THd ($p<0.05$). By contrast, immunization with AH1 alone only delayed tumor growth, whereas immunization with OVA(323–337) alone, or IFA alone, were without effect (Fig. 1). Indeed, all mice immunized with OVA(323–337) alone, AH1 alone, or IFA alone, developed lethal tumors. Because high levels of IL-4 induced after immunization might be detrimental for CTL induction, we addressed the question of whether the simultaneous immunization with exogenous Th0 and Th1 peptides plus a TCd might be less efficient than the immunization with only the Th1 and the TCd. As indicated in Table 2, we vaccinated mice with AH1 + OVA(323–337), AH1 + SWM(106–118), AH1 + the mixture of SWM(106–118) and OVA(323–337), AH1 alone or IFA alone. This experiment shows that in the first case three of nine (89%) mice were protected, whereas in the second and third cases, only three of nine (33%) of mice were protected, indicating that the presence of a Th0 helper peptide may have a deleterious effect on the efficacy of vaccination. No protection was observed after immunization with AH1 alone or IFA alone. It is interesting to note that cells from mice immunized with AH1 + OVA(323–337) + SWM(106–118), when stimulated with OVA(323–337) alone, SWM(106–118) alone or OVA(323–337) + SWM(106–118) gave 1,800, 400 and 500 pg IFN-γ/ml, respectively, suggesting that the presence of the Th0 peptide lowers the level of IFN-γ production expected for the Th1 peptide.

Table 2. Efficacy of vaccination of BALB/c mice using different peptide combinations

Vaccination mixture[a)]	Percentage of protection[b)]
1. AH1 + OVA(323–337)	8/9 (88.9 %)
2. AH1 + SWM(106–118)	3/9 (33 %)
3. AH1 + OVA(323–337) + SWM(106–118)	3/9 (33 %)
4. AH1	0/9 (0 %)
5. IFA	0/9 (0 %)

a) Mice were vaccinated with the peptide mixtures shown and challenged with CT26 tumor cells 10 days after vaccination. Protection is expressed as the percentage of mice bearing no tumors.

b) $p < 0.0004$ when comparing group 1 versus 5; $p < 0.05$ when comparing group 1 versus 2; non-statistically significant when comparing groups 2 or 3 versus group 5.

2.3 Protective immunity induced by immunization with peptide combinations is mediated by CD8$^+$ CTL

As shown in Fig. 2A, lymph node cells from mice immunized with AH1 + OVA(323–337) but not with AH1 alone, were able to lyse P815 target cells pulsed with AH1. However, some background lysis was observed against unpulsed P815 cells. This background was not detected when using other peptide combinations that were not effective against tumor growth (data not shown). Since it has been reported by Huang et al. [14] that P815 cells express gp70, we believe that this background is related to the expression of this protein. CD8 activity was also measured using an IFN-γ production assay. This experiment showed that only lymphocytes from mice immunized with AH1 + OVA(323–337) but not with AH1 alone or AH1 + SWM(106–118) were able to produce significant amounts of IFN-γ in the presence of AH1 (Fig. 2B). Interestingly, no background noise was observed under these conditions, probably because in this assay APC are spleen cells not expressing gp70. Characterization of the effector cells induced in lymph nodes after immunization with AH1 + OVA(323–337) showed that they were CD8$^+$ (Fig. 2C). To confirm that CD8$^+$ CTL were responsible for *in vivo* protection against tumor challenge, we studied the efficacy of immunizations in CD4$^+$ or CD8$^+$ cell-depleted animals. As expected, all mice depleted of CD8$^+$ cells and immunized with OVA(323–337) + AH1 developed terminal tumors, suggesting that induced CTL are responsible of tumor protection (Table 3). Interestingly, mice depleted of CD4$^+$ cells and immunized with OVA(323–337) + AH1 or with AH1 alone were fully protected from tumor challenge.

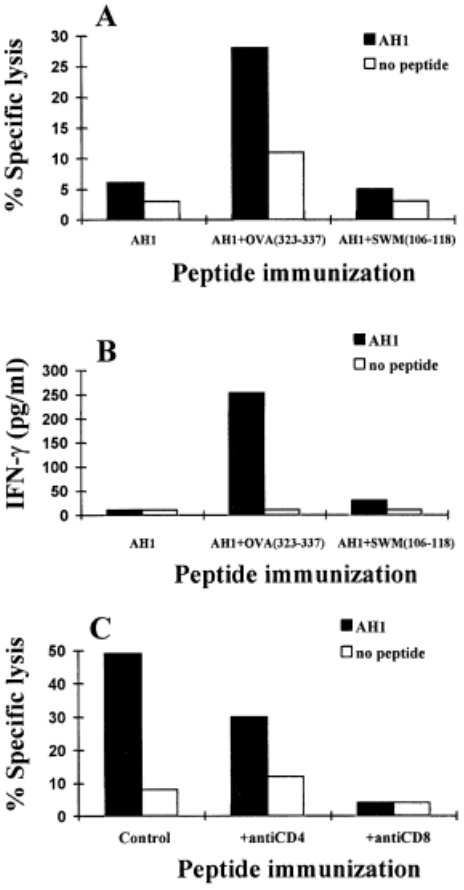

Fig. 2. Characterization of the CTL response induced after peptide immunization. (A) Specific lysis of P815 cells pulsed (filled bars) or unpulsed (empty bars) with peptide AH1 by CTL induced after immunization with AH1 alone or AH1 plus OVA(323–337) or AH1 plus SWM(106–118). (B) IFN-γ production of spleen cells from immunized mice after *in vitro* restimulation in the presence or absence of AH1. (C) Effect of anti-CD4 and anti-CD8 antibodies on the CTL induced after immunization with AH1 plus OVA(323–337). Lymph node cells were incubated with target cells at a ratio of 30:1 with or without 100 μg/ml of anti-CD4 or anti-CD8 monoclonal antibodies.

2.4 Identification and characterization of an endogenous Th1 peptide from gp70

Ossendorp et al. [13] reported that vaccination with a THd from a tumor antigen was able to protect around 40% of mice to challenge with tumor cells. This protection increased to around 90% when a THd and a TCd from the tumor antigen were used. By contrast, immunization with the TCd plus an exogenous THd was less efficient than immunization with the endogenous THd alone [13]

Because our results show that co-immunization with the tumor TCd + OVA(323–337) was able to protect BALB/c mice, we decided to compare the efficacy of this exogenous THd with an endogenous THd from gp70 tumor antigen. Since no sequences with THd character have been previously identified within gp70, we scanned the sequence of gp70 (described in [17]) using the algorithm of Sette et al. [18] to predict I-A^d- and I-E^d-restricted T helper peptides. This afforded the 11 peptides from Table 4, which were synthesized and their helper activity

Table 3. Effect of *in vivo* depletion of $CD4^+$ or $CD8^+$ cells on the induction of antitumor protective immune responses[a)]

Immunized with	*In vivo* treatment	Percentage of protection
AH1 + OVA(323–337)	anti-CD4	6/6 (100 %)
	anti-CD8	0/6 (0 %)
AH1	anti-CD4	6/6 (100 %)
	anti-CD8	0/6 (0 %)
None	anti-CD4	(0/6 (0 %)
	anti-CD8	0/6 (0 %)

a) Mice were depleted of $CD4^+$ or $CD8^+$ cells *in vivo* and immunized with peptide mixtures shown. Ten days after immunization, mice were challenged s. c. with 5×10^5 CT26 tumor cells. Protection is expressed as the percentage of mice bearing no tumors.

Table 4. Cytokines induced after immunization of BALB/c mice with putative binder peptides to I-A^d or I-E^d molecules from gp70 tumor antigen

Peptide	IL-2 (mU/ml)	IFN-γ (pg/ml)	IL-4 (mU/ml)
p (33–46)	91	< 50	< 50
p (109–122)	160	< 50	< 50
p (142–155)	18	< 50	< 50
p (144–157)	5	< 50	< 50
p (153–166)	50	< 50	< 50
p (155–168)	1	< 50	< 50
p (177–190)	70	< 50	< 50
p (260–273)	5	< 50	< 50
p (289–302)	127	< 50	< 50
p (320–333)	183	970	< 50
p (324–337)	32	< 50	< 50

a) Mice were immunized with 50 μg peptide emulsified in IFA. Ten days after lymph node cells were cultured in the presence or absence of peptide and cytokines were measured as described in Sect. 4.

tested in our animal model. For this purpose, BALB/c mice were immunized with 60 μg of these peptides in IFA. Lymph nodes obtained at day 10 were cultured in the presence or absence of peptides and IL-2, IFN-γ and IL-4 production was then measured. Among all the peptides tested, p(109–122), p(289–302) and p(320–333) induced the highest levels of IL-2, but only p(320–333) (LVQFIKDRISVVQA) was able to induce high levels of IFN-γ. It is noteworthy that none of these peptides induced detectable levels of IL-4. Addition of anti-CD4 but not anti-CD8 monoclonal antibodies to the culture *in vitro* of spleen cells from mice immunized with p(320–333), abrogated IFN-γ production, indicating that $CD4^+$ cells are responsible for IFN-γ production (Fig. 3A).

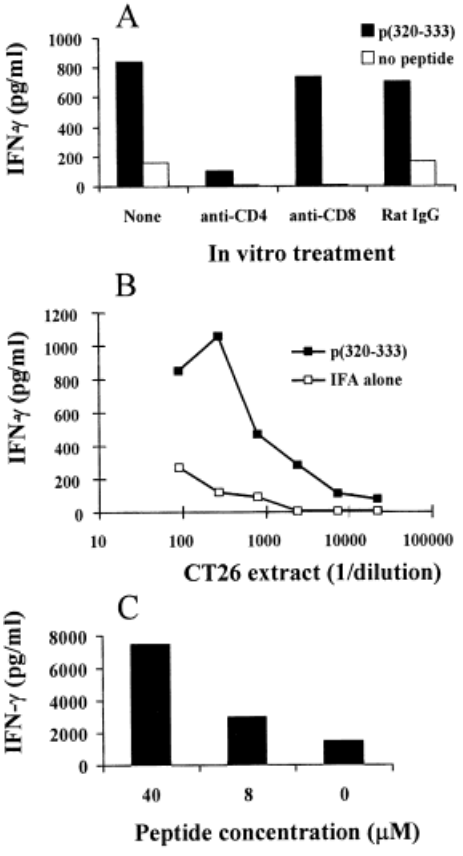

Fig. 3. Characterization of the helper response from p(320–333). Mice were immunized with p(320–333) or with IFA alone. (A) Lymph nodes cells stimulated with or without p(320–333) in the presence of different antibodies. (B) Lymph node cells were stimulated for 48 h with dilutions of CT26 cell lysate and IFN-γ was measured by ELISA. (C) Production of IFN-γ after stimulation with p(320–333) of spleen cells from mice that had rejected CT26 induced tumors due to intratumor administration of dendritic cells engineered to produce IL-12.

To study whether p(320–333) was presented by APC after natural processing of gp70 antigen, we immunized mice with p(320–333) in IFA. After 10 days, lymph node cells were cultured in the presence of different concentrations of a CT26 cell lysate and IFN-γ production was measured after 48 h. Fig. 3B shows that this lysate is able to stimulate IFN-γ production *in vitro* by lymph node cells from p(320–333) immunized animals. By contrast, no IFN-γ production was detected after *in vitro* stimulation of lymph node cells from animals immunized with IFA alone. Moreover, spleen cells from mice that had rejected CT26 induced tumors after intratumor administration of dendritic cells engineered to produce IL-12, as previously described by our laboratory [19] produced IFN-γ when stimulated with p(320–333) (Fig. 3C). Results from Table 4 and Fig. 3 taken together show that p(320–333) is naturally presented by APC and elicits a Th1 cytokine profile. For this reason we tested this peptide in protection experiments, either when used alone or plus AH1. Immunization with AH1 + p(320–333) was able to protect BALB/c mice (9/9 mice) from challenge with CT26 tumor cells, as compared to eight of nine mice after immunization with AH1 + OVA(323–337) (Fig. 4). As expected, *in vivo* depletion of $CD8^+$ cells before challenge, completely abrogated this protection (not shown). As found for OVA(323–337) alone, immunization with p(320–333) alone was unable to protect any of the immunized mice (0/9).

2.5 Effect of peptide vaccination on the protection against lung metastases

The efficacy of vaccination with AH1 plus either OVA(323–337) or p(320–333) to protect against lung metastases after i.v. injection of 1×10^5 CT26 tumor cells is shown in Fig. 5. The degree of protection using either peptide was not significantly different but significantly lower than in the group of non-vaccinated mice ($p<0.001$).

2.6 Comparison of efficacy of exogenous and endogenous Th1 peptides on the induction of long-term antitumor protection

To study if memory induced with endogenous helper peptide p(320–333) was longer than the one induced with exogenous helper peptide OVA(323–337), we immunized 15 mice with AH1 + p(320–333) or with AH1 + OVA(323–337). Five mice from each group were killed at day 80 to measure cytokine production against the peptides used for immunization. No IL-2 nor IFN-γ production could be detected in either group under the stimuli of peptides (not shown). The remaining 10 animals from

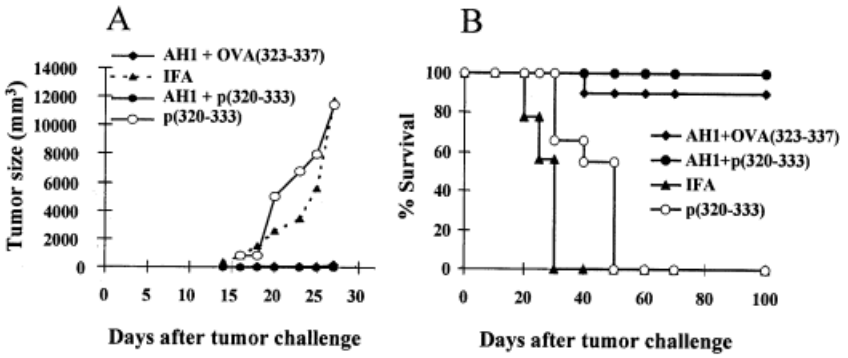

Fig. 4. Evolution of tumor growth after challenge with CT26 cells in groups of nine BALB/c mice immunized with peptide combinations shown. At 10 days after immunizations, animals were challenged by s.c. injection with 5×10^5 CT26 tumor cells. (A) Average tumor size was expressed as the square value of the smallest diameter of the tumor, times the value of its largest diameter. (B) Kaplan-Meier plot of mice survival after challenge with CT26 tumor cells.

each group were challenged with CT26 tumor cells 80 days after immunization. No differences in protection were observed between both groups. Indeed, only 2 animals (20%) from each group were still protected against tumor growth (Table 5).

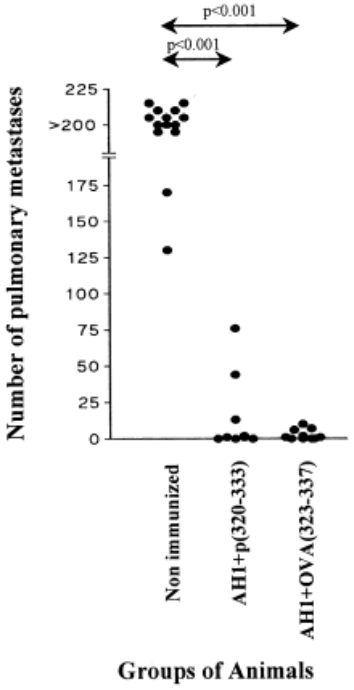

Fig. 5. Protection against lung cancer metastases by vaccination with peptides. Mice were challenged intravenously with 1×10^5 CT26 tumor cells 10 days after vaccination. Metastases were counted 20 days after tumor challenge.

2.7 Treatment of established tumors by immunization with peptides

To test the efficacy of peptides to treat established tumors, we inoculated mice with CT26 tumor cells, and once tumors of around 5 mm were established, peptides were administered in IFA around the tumor. Five groups of mice were treated: (a) with IFA alone, (b) with AH1 +OVA (323–337), (c) with AH1 + p(320–333), (d) with OVA(323–337) alone, and (e) with p(320–333) alone. As shown in Fig. 6, tumor growth at day 14 after treatment, was significantly delayed when administering p(320–333) alone (p=0.0098). A delay in tumor growth, although non-significant (p=0.055), was observed when OVA(323–337) was administered. Tumors continued to develop after day 14, except in one mice per group in those immunized with p(320–333) alone and another with OVA(323–337) alone, where the tumor was rejected.

Table 5. Efficacy of endogenous versus exogenous helper peptides for the protection against challenge with CT26 at different time points

Immunized with[a)]	Day of challenge with CT26 tumor cells	Number of mice Protected/Total
AH1 + OVA(323–337)	10	15/18[b)]
AH1 + p(320–333)	10	16/18[b)]
AH1	10	0/9
AH1 + OVA(323–337)	80	2/10
AH1 + p(320–333)	80	2/10

a) BALB/c mice were immunized at day 0 with peptide combinations shown and were challenged with CT26 tumor cells at days indicated.

b) The number correspond to the sum of two groups of nine mice immunized in two independent experiments.

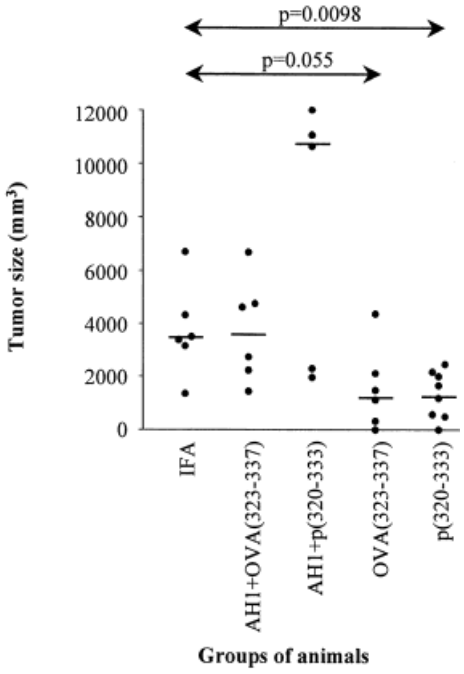

Fig. 6. Treatment of established tumors by immunization with different peptide combinations. Groups of five to eight mice were inoculated with 5×10^5 CT26 tumor cells. When tumor size reached a diameter of around 5 mm, mice were immunized around the tumor area with IFA alone or the combinations of peptides shown. Tumor size was expressed as the square value of the smallest diameter of the tumor, times the value of its largest diameter. Values shown correspond to the size of tumors 14 days after treatment.

3 Discussion

CTL play an important role in the protection against tumor growth, and for this reason, methods aiming at CTL induction are of paramount importance for antitumor therapy. This induction takes place via the help from T helper lymphocytes that are activated when THd peptides are presented by MHC class II molecules. In the present publication we study different types of THd peptides and compare their efficacy in providing T cell help for the induction of protective antitumor CTL responses.

It has been reported that immunization with peptide AH1 emulsified in IFA or with peptide-pulsed dendritic cells is unable to induce CTL [14]. This lack of CTL response was also confirmed in the present study. Moreover, since no production of IL-2, IFN-γ or IL-4 was observed after vaccination with AH1 in IFA, we attributed this lack of response to the absence of T cell help. For this reason, based on previous results from our group [5, 12] as well as from others [3, 4, 6, 7, 20], we speculated that immunization with AH1 plus an efficient THd might induce a protective CTL response. As expected, it was found that co-immunization of this peptide and the exogenous THd OVA(323–337), inducing a Th1 cytokine profile (Table 1), protected 15/18 (83%) mice against challenge with CT26 tumor cells (Table 5).

Interestingly, depletion of $CD8^+$ cells, but not $CD4^+$ cells, abrogated the protective effect of peptide immunizations. At first sight, this data might suggest that $CD4^+$ cells are not relevant for protection. However, as it has been described recently [21] *in vivo* $CD4^+$ cell depletion also depletes $CD4^+$ NKT cells responsible for the repression of immunosurveillance. It is very interesting to note that immunization with AH1 alone in the absence of $CD4^+$ cells efficiently protected mice from tumor challenge (Table 3). We are actively working to characterize this phenomenon.

We have recently reported [12] that peptide SWM(106–118), eliciting a Th0 cytokine profile, is less efficient than Th1 peptides in inducing CTL responses. We show here that vaccination with a TCd in conjunction with a Th1 plus a Th0 helper peptides [OVA(323–337) and SWM(106–118), respectively] is less efficient than vaccination with the TCd plus OVA(323–337) (Table 2). This detrimental effect is probably related to the drop in IFN-γ production observed *in vitro* when both Th1 and Th0 peptides are present. This observation is in line with the recent publication of Song et al. [22] showing that the simultaneous use of a vector expressing carcinoembryonic antigen and a vector expressing IL-4 reduced CTL activity and resistance to tumor challenge. These findings may be important for vaccine design when using combinations of THd and TCd peptides. Using IL-4-deficient mice Schuler at al [23] have shown that IL-4 is required for the generation of Th1-associated CTL-mediated tumor immunity. To reconcile this observation with our data it could be postulated that, although IL-4 may be necessary, high levels of this cytokine may have detrimental effects on CTL induction.

Ossendorp et al. [13] reported that vaccination of C57BL/6 mice with the endogenous env-H19 THd alone protected around 40% of mice against challenge with RMA tumor cells. Enhanced protection (70%) was achieved after immunization with this helper peptide plus the TCd env-K^b8. Moreover, immunization of C57BL/6 mice with env-K^b8 CTL epitope plus OvaH [OvaH is identical to OVA(323–337) used by us, but two amino acids longer] protected only 10% of mice. Since OvaH binds to I-A^b molecules (C57BL/6 mice) four times less efficiently than to I-A^d molecules (BALB/c mice) [24], it is likely that the higher affinity of OVA(323–337) for I-A^d may explain why, in our system, immunization with OVA(323–337) plus AH1 is able to efficiently protect BALB/c mice from CT26 tumor challenge.

In agreement with the work of Ossendorp et al. [13] immunization with the exogenous THd OVA(323–337) alone did not protect mice from tumor challenge. Since immunization with an endogenous THd from a tumor antigen was able to protect against challenge with tumor cells [13, 25], we carried out experiments and identified the endogenous Th1 peptide p(320–333) from gp70. We then immunized mice with p(320–333) plus AH1 and found that 16/18 (89%) mice were protected against challenge with CT26 tumor cells (Table 5), showing that p(320–333) provides efficient T cell help for CTL induction. Also, co-immunization of AH1 with either p(320–333) or OVA(323–337) protected against lung metastases after i.v. injection of tumor cells, demonstrating that these protocols are effective even under more invasive conditions of challenge with tumor cells (Fig. 5).

As opposed to Ossendorp et al. and Miyazawa et al. [13, 25], our endogenous THd alone did not protect mice from tumor challenge, suggesting that our endogenous helper peptide is less efficient than those used by these authors. By contrast, our work shows that efficient protection against challenge with CT26 tumor cells can be achieved by co-immunization with the TCd AH1 plus the endogenous Th1 peptide p(320–333), but also, and most importantly, with exogenous tumor-unrelated Th1 peptide OVA(323–337). Because finding a convenient endogenous Th1 helper peptide from tumor antigens in the context of a given class II molecule might not be straightforward, the use of an exogenous THd, is an alternative to be seriously considered. This THd should elicit a strong Th1 cytokine response in the context of the class II molecule of the vaccinee. Helper and cytotoxic memories might be kept high by repeated immunizations with the exogenous THd plus one or more TCd. This strategy might prevent the onset of metastases after surgical intervention or therapeutic vaccination. An advantage of this strategy would be that only a few Th1 peptides would need to be developed to cover all possible HLA class II restrictions. Alternatively, as suggested by Alexander et al. [24], a promiscuous helper peptide could be used, provided that this peptide was indeed a Th1 peptide in the context of the class II molecule of our interest.

Therapeutic immunizations of mice bearing established tumors with endogenous or exogenous helper peptides, were largely unsuccessful. With the exception of two complete tumor regressions observed after immunization with p(320–333) alone and OVA(323–337) alone, only minor, but statistically significant, delays in tumor progression were observed (Fig. 6). The mechanism by which an exogenous Th peptide can favor the control of established tumor growth it is not clear. However, as described [26], immunization with a good helper peptide may awake the effect of a poorer helper peptide. In our study, the cytokines induced by OVA(323–337) might awake tumor-specific helper and CTL which remained inactive even in the presence of tumor cells. The efficiency of peptide immunization in the treatment of established tumors, might perhaps be improved using Th1 peptides inducing higher levels of cytokines, and concomitantly chemokines capable of attracting activated T lymphocytes to the tumor site.

In conclusion, our results in conjunction with those from others [11, 13, 25] suggest that Th1 peptides provide efficient help for CTL induction against tumors. Also, Th0 peptides when present in immunization mixtures, may diminish the efficient help of Th1 peptides. Moreover, and most important, because after challenge with tumor cells, endogenous but not exogenous Th1 peptides, may be activated to provide T cell help, these peptides should be favored for peptide-based cancer therapy. However, as discussed above, under special circumstances, the T cell help could be provided by an exogenous THd eliciting a Th1 cytokine profile. Last, but not least, it seems advisable that peptide vaccines providing long-lasting immunity should contain several Th1 THd as well as several TCd from one or more antigens from the tumor.

4 Materials and methods

4.1 Peptides

Peptides were synthesized by the solid- phase method of Merrifield [27] using a manual multiple solid-phase peptide synthesizer as described in [12]. At the end of the synthesis, peptides were cleaved, deprotected, and washed six times with diethyl ether. They were lyophilized and analyzed by HPLC. The purity of peptides was above 80% as judged by HPLC.

4.2 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 institutional guidelines.

4.3 Immunization and measurement of CTL response

Mice were immunized by s.c. injection at the base of the tail and footpads with 60 μg peptide emulsified in IFA. For immunizations using a THd and a TCd mixture, 60 μg of each peptide was injected. For CTL measurements, animals were killed 10 days after immunization and their lymph

nodes removed. Cells (8×10^6/well) were restimulated *in vitro* in 24-well plates in 2 ml of culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 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 as described [12].

4.4 Immunization for cytokine measurement

Mice were immunized by s.c. injection at the base of the tail and footpads with 60 μg peptide emulsified in IFA. After 10 days, animals were killed and lymph node cells removed. 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. Supernatants (50 μl) were removed 24 h later to measure IL-2, and 48 h later for IFN-γ and IL-4.

4.5 Measurement of cytokine production

IL-2 was measured using a CTLL bioassay as described [28]. IFN-γ production was measured by ELISA (Genzyme, Cambridge, MA) according to manufacturer's instructions. IL-4 was measured by CT4S bioassay (kindly provided by Drs. W. E. Paul and G. J. Watson, National Institutes of Health, Bethesda, MD) as described [12].

4.6 Tumor challenge experiments

Animals were immunized with different combinations of peptides in adjuvant or adjuvant alone as described above. They were challenged by s.c. injection on the right flank with 5×10^5 CT26 tumor cells at different time points. Tumor size was expressed as the square value of the smallest diameter of the tumor, times the value of its largest diameter. Mice were killed when tumor size reached a volume greater than 10 cm^3. To study the efficacy of peptide immunizations in the development of pulmonary tumor metastases, mice were challenged i.v. with 1×10^5 CT26 tumor cells and killed 20 days after challenge. Metastases were counted under the microscope following injection of 50 μl India ink 5 min before their sacrifice.

In some experiments mice were depleted of CD4$^+$ or CD8$^+$ cells by i.p. injection of 0.3 mg anti-CD4 or anti-CD8 antibodies (obtained from rat anti-mouse hybridomas GK 1.5 and H35.17.2, respectively) on days –1, 0, 1, 6 and 10 as previously described [29], being day 0 the day of peptide immunization. The efficiency of depletions was assessed by flow cytometry.

4.7 Recognition of CT26 tumor cell lysate by lymphocytes from peptide-immunized animals

To study recognition of tumor cells by lymphocytes from peptide-immunized animals, mice were immunized with peptide as described above, lymph node cells extracted at day 10, and stimulated *in vitro* with or without different dilutions of a lysate of CT26 tumor cells. CT26 cells were lysed by six cycles of freezing and thawing, followed by sonication and centrifugation. Culture supernatants were collected after 24–48 h of culture and cytokines measured.

4.8 Statistics

Statistical analysis was carried out to evaluate the therapeutic effect of peptide immunization in established tumors. Non-normally distributed variables were analyzed by the Kruskal-Wallis test, followed by Mann-Whitney's U as a multiple comparison test. The effect of immunization with different peptide combinations was compared with the control group immunized with IFA alone. Comparison of protection between groups was analyzed by Exact test according to Abramson and Gahlinger (Computer Program for epidemiologists, PEPI Version 4.0).

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References

1 **Kast, W. M. and Melief, C. J.,** Fine peptide specificity of cytotoxic T lymphocytes directed against adenovirus-induced tumours and peptide-MHC binding. *Int. J. Cancer Suppl.* 1991. **6:** 90–94.

2 **Greenberg, P. D.,** Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv. Immunol.* 1991. **49:** 281–355.

3 **Fayolle, C., Deriaud, E. and Leclerc, C.,** In vivo induction of cytotoxic T cell response by a free synthetic peptide requires CD4$^+$ T cell help. *J. Immunol.* 1991. **147:** 4069–4073.

4 **Hart, M. K., Weinhold, K. J., Scearce, R. M., Washburn, E. M., Clark, C. A., Palker, T. J. and Haynes, B. F.,** Priming of anti-human immunodeficiency virus (HIV) CD8$^+$ cytotoxic T cells *in vivo* by carrier-free HIV synthetic peptides. *Proc. Natl. Acad. Sci. USA* 1991. **88:** 9448–9452.

5 **Lasarte, J. J., Sarobe, P., Gullon, A., Prieto, J. and Borras Cuesta, F.,** Induction of cytotoxic T lymphocytes in mice against the principal neutralizing domain of HIV-1 by immunization with an engineered T-cytotoxic-T-helper synthetic peptide construct. *Cell. Immunol.* 1992. **141:** 211–218.

6 **Shirai, M., Pendleton, C. D., Ahlers, J., Takeshita, T., Newman, M. and Berzofsky, J. A.,** Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV $CD8^+$ CTL *in vivo* with peptide vaccine constructs. *J. Immunol.* 1994. **152:** 549–556.

7 **Widmann, C., Romero, P., Maryanski, J. L., Corradin, G. and Valmori, D.,** T helper epitopes enhance the cytotoxic response of mice immunized with MHC class I-restricted malaria peptides. *J. Immunol. Methods* 1992. **155:** 95–99.

8 **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L.,** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136:** 2348–2357.

9 **Mosmann, T. R. and Coffman, R. L.,** TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 1989. **7:** 145–173.

10 **Kelso, A.,** Th1 and Th2 subsets: paradigms lost? *Immunol. Today* 1995. **16:** 374–379.

11 **Nishimura, T., Iwakabe, K., Sekimoto, M., Ohmi, Y., Yahata, T., Nakui, M., Sato, T., Habu, S., Tashiro, H., Sato, M. and Ohta, A.,** Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication *in vivo*. *J. Exp. Med.* 1999. **190:** 617–627.

12 **Lopez-Diaz de Cerio, A. L., Casares, N., Lasarte, J. J., Sarobe, P., Perez-Mediavilla, L. A., Ruiz, M., Prieto, J. and Borras-Cuesta, F.,** T(h)1 but not T(h)0 cell help is efficient to induce cytotoxic T lymphocytes by immunization with short synthetic peptides. *Int. Immunol.* 1999. **11:** 2025–2034.

13 **Ossendorp, F., Mengede, E., Camps, M., Filius, R. and Melief, C. J.,** Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* 1998. **187:** 693–702.

14 **Huang, A. Y., Gulden, P. H., Woods, A. S., Thomas, M. C., Tong, C. D., Wang, W., Engelhard, V. H., Pasternack, G., Cotter, R., Hunt, D., Pardoll, D. M. and Jaffee, E. M.,** The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 9730–9735.

15 **Shimonkevitz, R., Colon, S., Kappler, J. W., Marrack, P. and Grey, H. M.,** Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* 1984. **133:** 2067–2074.

16 **Brett, S. J., Cease, K. B. and Berzofsky, J. A.,** Influences of antigen processing on the expression of the T cell repertoire. Evidence for MHC-specific hindering structures on the products of processing. *J. Exp. Med.* 1988. **168:** 357–373.

17 **Timmons, P. M., Brickell, P. M., Latchman, D. S. and Rigby, P. W.,** Activation of endogenous retroviral transcription in SV40-transformed mouse cells. *Nucleic Acids Res.* 1991. **19:** 7215–7218.

18 **Sette, A., Buus, S., Appella, E., Smith, J. A., Chesnut, R., Miles, C., Colon, S. M. and Grey, H. M.,** Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. *Proc. Natl. Acad. Sci. USA* 1989. **86:** 3296–3300.

19 **Melero, I., Duarte, M., Ruiz, J., Sangro, B., Galofre, J., Mazzolini, G., Bustos, M., Qian, C. and Prieto, J.,** Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interleukin-12 induces complete regression of established murine transplantable colon adenocarcinomas. *Gene Ther.* 1999. **6:** 1779–1784.

20 **Grohmann, U., Belladonna, M. L., Bianchi, R., Orabona, C., Silla, S., Squillacioti, G., Fioretti, M. C. and Puccetti, P.,** Immunogenicity of tumor peptides: importance of peptide length and stability of peptide/MHC class II complex. *Cancer Immunol. Immunother.* 1999. **48:** 195–203.

21 **Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D. D., Carbone, D. P., Paul, W. E. and Berzofsky, J. A.,** NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immunol.* 2000. **1:** 515–520.

22 **Song, K., Chang, Y. and Prud'homme, G. J.,** Regulation of T-helper-1 versus T-helper-2 activity and enhancement of tumor immunity by combined DNA-based vaccination and nonviral cytokine gene transfer. *Gene Ther.* 2000. **7:** 481–492.

23 **Schuler, T., Qin, Z., Ibe, S., Noben-Trauth, N. and Blankenstein, T.,** T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J. Exp. Med.* 1999. **189:** 803–810.

24 **Alexander, J., Sidney, J., Southwood, S. et al.,** Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity* 1994. **1:** 751–761.

25 **Miyazawa, M., Fujisawa, R., Ishihara, C., Takei, Y. A., Shimizu, T., Uenishi, H., Yamagishi, H. and Kuribayashi, K.,** Immunization with a single T helper cell epitope abrogates Friend virus-induced early erythroid proliferation and prevents late leukemia development. *J. Immunol.* 1995. **155:** 748–758.

26 **Gerloni, M., Xiong, S., Mukerjee, S., Schoenberger, S. P., Croft, M. and Zanetti, M.,** Functional cooperation between T helper cell determinants. *Proc. Natl. Acad. Sci. USA* 2000. **97:** 13269–13274.

27 **Merrifield, R. B.,** Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963. **85:** 2149–2154.

28 **Lai, M. Z., Ross, D. T., Guillet, J. G., Briner, T. J., Gefter, M. L. and Smith, J. A.,** T lymphocyte response to bacteriophage lambda repressor cI protein. Recognition of the same peptide presented by Ia molecules of different haplotypes. *J. Immunol.* 1987. **139:** 3973–3980.

29 **Lasarte, J. J., Sarobe, P., Prieto, J. and Borras-Cuesta, F.,** *in vivo* cytotoxic T-lymphocyte induction may take place via CD8 T helper lymphocytes. *Res. Immunol.* 1995. **146:** 35–44.

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