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Multiepitope Trojan Antigen Peptide Vaccines for the Induction of Antitumor CTL and Th Immune Responses¹

Jun Lu,* Yuichiro Higashimoto,[†] Ettore Appella,[†] and Esteban Celis^{2*}

We describe in this study a strategy to produce synthetic vaccines based on a single polypeptide capable of eliciting strong immune responses to a combination CTL and Th epitopes with the purpose of treating malignancies or preventing infectious diseases. This strategy is based on the capacity of Trojan Ags to deliver exogenous Ags into the intracellular compartments, where processing into MHC-binding peptides takes place. Our previous work demonstrated that Trojan Ags containing a CTL epitope localized to intracellular compartments, where MHC class I-binding peptides were generated in a TAP-independent fashion by the action of various exopeptidases and the endopeptidase furin. In this study, we report that Trojan Ags containing several CTL epitopes joined via furin-sensitive linkers generated all of the corresponding MHC class I-binding peptides, which were recognized by CTL. However, Trojan Ags prepared with furin-resistant linkers failed to produce the MHC class I-binding peptides. We also present data indicating that Trojan Ags bearing both CTL and Th epitopes can generate the corresponding MHC class I- and II-binding peptides, which are capable of stimulating T cell responses. Most significantly, in vivo vaccination of mice with a single injection of multiepitope Trojan Ags resulted in strong CTL and Th responses that translated into significant antitumor responses in a model of malignant melanoma. The overall results indicate that Trojan Ags prepared with furin-sensitive linkers are ideal candidates for producing synthetic multiepitope vaccines for the induction of CTL and Th responses that could be used against a variety of diseases, including cancer. *The Journal of Immunology*, 2004, 172: 4575–4582.

Antigens recognized by T lymphocytes, which are expressed exclusively or preferentially by tumor cells, have been intensively investigated as potential immunogens for antitumor immunotherapy (1–3). These Ags represent the source of short peptides that bind to MHC class I and II molecules and are presented to the TCR of tumor-reactive CTLs or Th cells, respectively. Peptide immunization using the epitopes derived from the processing of proteins that are preferentially expressed on tumor cells appears to be a reasonable approach to induce antitumor immunity aimed at achieving therapeutic benefit (4, 5). However, the usefulness of each peptide is limited because only a fraction of the patient population will express the appropriate MHC allele that restricts the corresponding T cell response. Furthermore, it would be beneficial to use T cell epitopes from more than one tumor Ag to increase disease coverage and prevent the emergence of tumor-escape variants. Although the use of peptide mixtures in a vaccine formulation could potentially address these issues, such mixtures would also create logistical problems and manufacturing challenges that may hinder the development of these vaccines (5).

Our laboratory has recently developed an approach to introduce large peptides containing T cell epitopes directly into the cytoplasm of APC (6). Our results showed that linking a Trojan peptide sequence derived from HIV-I Tat (carrier) with a CTL epitope

(cargo) leads to the effective generation of MHC class I/peptide complexes in a TAP-independent fashion. These Trojan Ag (TA)³ constructs were capable of delivering the peptide epitopes directly into the endoplasmic reticulum (ER) and *trans*-Golgi network (TGN), where resident peptidases efficiently generate MHC class I-binding peptides. Furthermore, we observed that furin, a TGN-resident endopeptidase that recognizes RXRR or RXKR motifs (7), played an important role in releasing the CTL epitope cargo portion from the Trojan peptide carrier (6). These observations opened the possibility of using TA constructs composed of multiple T cell epitopes joined via furin-sensitive linkers to deliver large Ags into intracellular compartments, where the appropriate generation of the individual MHC class I-binding peptides can take place. The present results show that, indeed, this approach is successful for the induction of CTL and Th responses to several well-characterized murine epitopes. Moreover, the T cell responses induced by a single vaccination with TAs were sufficiently robust to provide a significant survival benefit against a tumor challenge or existing tumors. These findings are discussed with respect to their value in the design of cost-effective, safe, and clinically useful multi-T cell epitope peptide-based vaccines for cancer.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 mice, DO11.10 TCR-transgenic mice (8), and OT-I TCR-transgenic mice (9) were purchased from The Jackson Laboratory (Bar Harbor, ME). The 2C TCR-transgenic mice (10) and TcrLCMV (lymphocytic choriomeningitis virus) (B6.D2-TgN[TrcLCMV]327Sdz) TCR-transgenic mice (11) were kindly provided by L. Pease (Mayo Clinic).

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³ Abbreviations used in this paper: TA, Trojan Ag; CpG, unmethylated cytosine-guanine; DC, dendritic cell; ER, endoplasmic reticulum; LCMV, lymphocytic choriomeningitis virus; ODN, oligodeoxynucleotide; TGN, *trans*-Golgi network; TRP2, tyrosinase-related protein 2.

Cell lines

The TAP-competent EL-4, EG7 (EL-4 transfected with the OVA gene) mouse cells, and the TAP-deficient RMA/S cell line were used as APC in cytotoxicity and lymphokine release T cell assays. The P815 mastocytoma (H-2^d) cell line was used as APC in ELISPOT assays to measure CTL response against the P1A tumor Ag (12). The mouse melanoma cell line B16 transfected with the OVA gene (B16-OVA) was provided by E. Lord (University of Rochester Medical Center, Rochester, NY) and used for tumor challenges. All cell lines were maintained in tissue culture in IMDM supplemented with 10% FBS, L-glutamine, and gentamicin.

Peptide synthesis

Synthetic TAs were prepared by producing synthetic peptides containing the multiple minimal CTL or Th epitopes linked together through either furin-sensitive linker RVKR or furin-resistant linker VRVV, then joined to the HIV-1 Tat protein membrane-translocating domain (RKKRRQRRR) at either the C- or N-terminal end. The amino acid sequences of minimal T cell epitopes (12–18) and multiepitope TAs used in this study are presented in Table I. All synthetic peptides were synthesized according to standard solid-phase synthesis methods using an Applied Biosystems (Foster City, CA) apparatus and were purified by reverse-phase HPLC. The purity (>95%) and identity of peptides were determined by analytical reverse-phase HPLC and mass spectrometry analysis. Peptides were dissolved at 10 mg/ml in DMSO containing 0.1% trifluoroacetic acid and were stored in small aliquots at –20°C until further use.

Cytotoxicity assays

The processing and presentation of TAs were determined in a standard 4-h ⁵¹Cr release assay. Effector cells were obtained by harvesting splenocytes from OT-I, 2C, or TcrLCMV TCR-transgenic mice and stimulating the CTL in vitro with 3 μM the appropriate peptide for 7 days in complete IMDM medium containing IL-2 (25 IU/ml). Target cells were prepared by pulsing EL-4, EG7, or RMA/S cells with the appropriate concentration of minimal peptide epitope or TAs. Later, target cells were labeled with 300 μCi ⁵¹Cr sodium chromate (Amersham Pharmacia Biotech, Piscataway, NJ) for 1–2 h at 37°C. The labeled targets were then mixed with effector CTLs at various E:T ratios in 96-well round-bottom plates in a final volume of 0.2 ml of complete medium. After a 4-h incubation period at 37°C, 30 μl of supernatant was collected from each well and the amount of radioactivity was determined in a scintillation counter. The percentage of specific lysis was determined according to the formula: ((cpm of the test sample – cpm of spontaneous release)/(cpm of the maximal release – cpm of spontaneous release)) × 100. All assays were done in triplicate samples.

ELISPOT assays

CTL responses were also evaluated in ELISPOT assays. Responder cells were prepared by purifying CD8⁺ T cells from draining lymph nodes 7 days after vaccination using Ab-coated magnetic beads (Miltenyi Biotec, Auburn, CA). APC were prepared by overnight pulsing of EL-4 (H-2^b) or P815 (H-2^d) cells with 10 μM the appropriate peptides. Serial dilutions of purified CD8⁺ T cells were cocultured with 1 × 10⁵ irradiated (3000 rad) target cells at 37°C for 48 h in 96-well ELISPOT plates. The assay was

performed and developed according to the kit's manufacturer (BD Pharmingen, San Diego, CA).

Flow cytometry

The role of furin on processing and presentation of TAs was also evaluated by fluorescent flow cytometry using mAb 25D1.16 specific for the Kb/OVA₂₅₇ complexes (kindly provided by R. Germain, National Institutes of Health, Bethesda, MD) (19). In brief, APC were washed twice with serum-free medium and pretreated with 60 μM furin inhibitor decRVKR-CMK (Bachem, King of Prussia, PA) for 30 min, and subsequently pulsed with equimolar concentrations of various TAs at 37°C overnight with the continuous presence of the decRVKR-CMK. To exclude the involvement of protease component in serum, we used serum-free AIM-V medium (Life Sciences, St. Petersburg, FL) during peptide-pulsing process. After washing the cells twice with flow buffer (PBS supplemented with 2% FCS and 0.2% sodium azide), peptide-loaded APC were stained with 25D1.16 mAb, followed by FITC-labeled goat anti-mouse IgG for flow analysis. Untreated APC were pulsed with peptides and stained simultaneously as controls.

Dendritic cell (DC) preparation and peptide pulse-chase kinetic analysis

Mouse DCs were derived from bone marrow-derived macrophages of (C57BL/6 × BALB/c)F₁ mice and were generated in tissue culture in the presence of rGM-CSF (5 ng/ml) and IL-4 (10 ng/ml). On day 7, DCs were harvested and pulsed with 3 μM either minimal T cell peptide or TA6 for 2 h at 37°C. Following three washes with medium, peptide-pulsed DCs were kept in a 37°C incubator for various time periods. At different time points (0, 12, 24, 36, 48 h after peptide pulsing), 3 × 10⁴ peptide-loaded DCs were cocultured with 1 × 10⁵ previously activated (7 days before) T cells derived from TCR transgenic mice OT-1 and DO.11.10. Supernatants were harvested after 24-h incubation and measured for mouse IFN-γ release by ELISA, according to the kit manufacturer's instructions (BD Pharmingen).

Mice immunization and tumor challenge

To vaccinate mice, peptide mixture (100 μmol each peptide per mouse) or TAs (100 μmol/mouse) were administered s.c. emulsified in IFA at the nape of neck. Mice received three daily s.c. injections of 100 μg each of 1826 unmethylated cytosine-guanine (CpG)-oligodeoxynucleotide (ODN) (TCCATGACGTTCCCTGACGTT) as adjuvant, administered on days –1, 0, and +1 of the peptide vaccine (20). Spleen and draining lymph nodes were harvested 7–10 days after immunization to study Ag-specific CTL and Th responses, as described above. The antitumor effects of vaccination with either TA or peptide mixture were evaluated in both the prophylactic and therapeutic settings. In the prophylactic model, the mice were first immunized with vaccine formulations, and 7 days later they were challenged with 5 × 10⁴ live B16-OVA melanoma cells given s.c. in the lower flank. In the therapeutic protocol, the mice were first challenged with tumor cells (as described above), and 7 days later they received the corresponding peptide vaccines. Mice were observed daily or every other day to monitor tumor growth and survival. Tumor-bearing mice either died on their own or were euthanized when tumors became ulcerated or surpassed 2 cm in diameter. Statistical analyses for evaluating the survival advantages were

Table I. Amino acid sequences for minimal epitopes and TAs used in these studies^a

Name	Sequence	MHC Restriction	Reference
OVA ₂₅₇	SIINFEKL	Kb	13
SIYR	SIYRYGL	Kb	14
LCMV ₃₂	IKAVYNATCG	Kb	15
TRP2	SVYDFVWL	Kb	16
Ad5E1A	SGPSNTPPEI	Db	17, 21
P1A	LPYLGWLVF	Ld	12
OVA ₃₂₃	ISQAVHAAHAEINEAGR	Ab/Ad	18
TA1	IKAVYNFATCGRV <u>RVKRSI</u> YRYGYL <u>RVKRSI</u> INFEKLAARKRRQRRR	Kb-Kb-Kb	
TA2	IKAVYNFATCGV <u>RVVSI</u> YRYGYL <u>RVVSI</u> INFEKLAARKRRQRRR	Kb-Kb-Kb	
TA3	SIINFEKL <u>RVKRI</u> KAVYNFATCGRV <u>RVKRSI</u> YRYGLAAARKRRQRRR	Kb-Kb-Kb	
TA4	RKKRRQRRRAAASVYDFVWL <u>RVKRL</u> PYLGWLVF <u>RVKRS</u> SGPSNTPPEI	Kb-Ld-Db	
TA5	RKKRRQRRRAAASVYDFVWL <u>RVVLP</u> PYLGWLVF <u>RVVSGP</u> SGPSNTPPEI	Kb-Ld-Db	
TA6	SIINFEKL <u>RVKRSI</u> QAVHAAHAEINEAARKRRQRRR	Kb-Ab/Ad	

^a Linkers for the multiepitope TA constructs are shown underlined.

performed using log-rank analysis. All the experiments were performed at 5–10 mice/group, and Mayo Clinic institutional animal care and use committee approved all the experimental protocols.

Results

TAs containing multiple CTL epitopes and furin-sensitive linkers sensitize APC for CTL recognition

We first tested the hypothesis that TAs containing multiple CTL epitopes connected via furin-sensitive linkers would render target cells sensitive to lysis by CTL specific to each individual epitope. In addition, this hypothesis predicted that TA constructs containing linkers not sensitive to furin cleavage would not be as effective for target sensitization as the TAs bearing furin-sensitive linkers. Thus, we prepared three synthetic TAs (TA1, TA2, and TA3) that contained three CTL epitopes derived from OVA₂₅₇, 2C, and LCMVgp₃₂ (Table I) linked through either the furin-sensitive linker RVKR (TA1 and TA3) or via the furin-resistant linker VRVV (TA2). All three TAs contained the HIV-I Tat membrane-translocating sequence (RKKRRQRRRR) at their C-terminal end (Table I). The antigenicity of multiepitope TA constructs was then tested by measuring their capacity to sensitize target cells for CTL lysis. As shown in Fig. 1, the two TAs bearing furin-sensitive linkers (TA1 and TA3) were effective in sensitizing both TAP-deficient RMA/S and TAP-competent EL-4 target cells for lysis by Ag-specific CTL derived from TCR-transgenic mice OT-I, 2C, and TcrLCMV (Fig. 1). The extent of specific lysis of targets pulsed with either TA1 or TA3 was comparable to that observed using the corresponding minimal peptide epitopes. In addition, the order that the CTL epitope sequences occupied within the TA construct did not seem to bear a significant effect in the capacity to generate the corresponding MHC/peptide complexes recognized by the CTL (Table I, compare TA1 vs TA3). Notably, TA2, which contained furin-insensitive linkers (VRVV), failed to sensitize target cells for CTL lysis for both TAP-deficient and TAP-competent target cells. This data indicate that TAs containing multiple CTL epitopes can produce individual MHC class I-binding peptides in a TAP-independent fashion, which are then effectively presented on the surface of APC to CTL. Furthermore, these results suggest that the TGN-resident protease furin plays a crucial role in the generation of the separate CTL epitopes, independently both of the order that the epitopes occupy within the construct and of the presence of TAP on the APC.

To further examine the processing of TAs and the importance of the furin-sensitive linker, we measured the expression of specific peptide/MHC class I complexes on Ag-pulsed APC using mAb 25D1.16, which specifically binds to Kb/OVA₂₅₇ complexes (19). At the same time, we studied the effect of furin inhibitor decRVKR-CMK on the processing of TAs leading to the generation

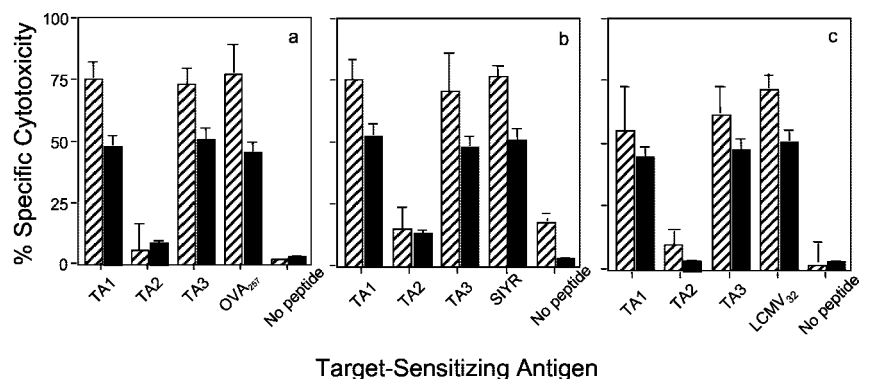
of surface Kb/OVA₂₅₇ complexes. As shown in Fig. 2, only those APC pulsed with TA1 (Fig. 2a) and TA3 (Fig. 2c), which bear furin-sensitive linkers, expressed significant amounts of Kb/OVA₂₅₇ complexes on the cell surface. In contrast, APC pulsed with TA2 (Fig. 2b), which contained furin-insensitive linkers, displayed very low levels of Kb/OVA₂₅₇ complexes. Most significantly, the production of peptide/MHC class I surface complexes by the TA1- and TA3-sensitized APC was abolished by the furin inhibitor decRVKR-CMK. Together these results emphasize the importance that the TGN-resident endopeptidase furin plays in the processing of multiepitope TAs into individual MHC class I-binding peptides, indicating that the use of furin-sensitive linkers in TA will assure that each CTL epitope will be generated.

Multiepitope TAs generate strong Ag-specific CTL responses in vivo

To evaluate the feasibility of using multiepitope TAs for vaccination, we compared the in vivo immunogenicity of TA1 against a mixture of the three minimal peptide epitopes (OVA₂₅₇, SIYR, and LCMVgp₃₂) after a single s.c. injection into naive mice. The data show that TA1 was as effective as the minimal epitope peptide mix in generating in vivo CTL responses to all three epitopes (Fig. 3). These results demonstrate that a single entity corresponding to a TA construct can indeed be used to adequately immunize against all its constituent CTL epitopes.

Because the CTL epitopes present in TA1 (OVA₂₅₇, SIYR, and LCMVgp₃₂) are all known to be highly immunogenic, we decided to evaluate a TA composed of the less immunogenic tumor-related CTL epitopes, tyrosinase-related protein 2 (TRP2) (16), Ad5E1A (17, 21), and P1A (12) (Table I). We thus compared TA4 (containing furin-sensitive linkers), TA5 (with furin-insensitive linkers), and the corresponding peptide mixture for their immunogenicity after a single vaccination. As shown in Fig. 4, only TA4, which contains furin-sensitive linkers, was able to generate significant CTL responses toward all three components. In contrast, both TA5 (bearing control linkers) and the peptide mixture failed to elicit Ag-specific CTL responses (except for some level of background lysis). Simultaneously to the cytotoxicity assay, we also measured the CTL responses to vaccination using a lymphokine release ELISPOT assay. The results presented in Fig. 4b show that mice immunized with TA4 had higher T cell responses against all three epitopes as compared with those mice immunized with the minimal epitope peptide mix. In contrast to the results obtained in the cytotoxicity assay (Fig. 4a), TA5 (containing furin-insensitive linkers) was capable of generating lymphokine secretion T cell responses to all three MHC class I epitopes. However, the magnitude of the responses of TA5 to the Ad5E1A and P1A epitopes

FIGURE 1. Evaluation of the antigenicity of multiepitope TAs. TAP-deficient RMA/S cells (▨) and TAP-competent EL-4 cells (■) were pulsed with various peptide constructs (for sequences, see Table I) at a final concentration of 1 μM for 12 h at 37°C. After extensively washing the cells, they were labeled with ⁵¹Cr, as described in *Materials and Methods*. The capacity of the peptide-loaded target cells to serve as targets for CTL lysis was evaluated using previously activated CTL from the following TCR transgenic mice: OT-I (a), 2C (b), and TcrLCMV (c) at an E:T ratio of 10:1 in a conventional 4-h ⁵¹Cr release assay. Results represent the average percentage of specific cytotoxicity of triplicate samples with SD of the means (error bars).



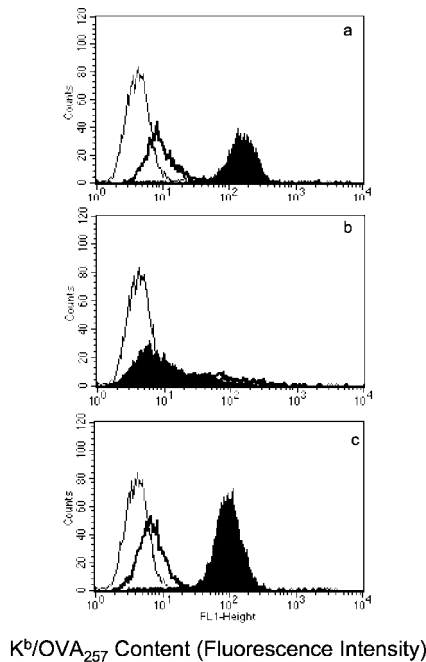


FIGURE 2. Role of furin in the generation of cell surface Kb/OVA₂₅₇ complexes on APC. RMA/S cells were incubated with 3 μ M TA1 (a), TA2 (b), or TA3 (c) for 6 h at 37°C both in the presence and absence of 60 μ M the furin inhibitor decRVKR-CMK. After extensively washing the APC, the levels of surface Kb/OVA₂₅₇ complexes were measured by flow cytometry using mAb 25D1.16, which specifically reacts with these complexes. Experimental conditions: Kb/OVA₂₅₇ complexes in the absence of decRVKR-CMK (shaded area); Kb/OVA₂₅₇ complexes in the presence of decRVKR-CMK (thick lines); isotype control (thin line).

was lower than those obtained using TA4. Surprisingly, the lymphokine release response to the TRP2 epitope induced by TA5 was higher than that of TA4 (Fig. 4b), which did not correlate with the cytotoxicity assay (Fig. 4a, right bottom panel).

Induction of immune responses with TAs containing both CTL and Th epitopes

Because both CTL and Th cells are required for effective and long-lasting antitumor immune responses, we decided to evaluate a TA construct containing both a CTL and a Th epitope for OVA, joined via a furin-sensitive linker (TA6, Table I). We first examined the capacity of TA6 to sensitize APC for CTL and Th recognition in a pulse-chase experiment. Purified DC from C57BL/6 mice were pulsed with equimolar amounts of either TA6 or a mixture of OVA₂₅₇ and OVA₃₂₃, and after washing the cells extensively to remove the unbound peptides, the APC were resuspended in fresh medium and studied at various time points for their ability to stimulate MHC class I-restricted, OVA₂₅₇-specific, OT-I CTLs or MHC class II-restricted, OVA₃₂₃-reactive, DO11.10 Th cells. The results presented in Fig. 5 show that the TA6-pulsed DC were as effective as the DC pulsed with the peptide mixture in stimulating both OT-I CTLs and DO11.10 Th cells at time zero (immediately after pulsing and washing the DC). Moreover, the DC that were pulsed with TA6 maintained their full stimulatory activity for OT-I CTLs during the entire chase period (50 h), while the stimulatory activity of the peptide mix-pulsed DC decreased with time, losing one-half of the original activity at \sim 30 h of the chase period (Fig. 5a). With respect to the stimulatory activity of the Ag-pulsed DC for DO11.10 Th cells, both the TA6-pulsed and the peptide mix-pulsed APC decreased their antigenicity at the same rate, where \sim 50% of the original activity remained after 12 h into the chase

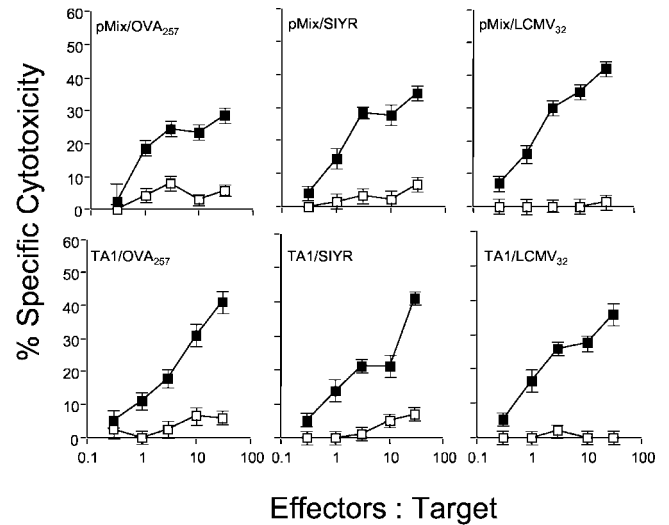


FIGURE 3. Evaluation of the immunogenicity of multiepitope TAs containing highly immunogenic CTL epitopes. Naive C57BL/6 mice (3 per group) were immunized s.c. with 100 μ mol of either TA1 or minimal epitope peptide mixture. Both vaccines were emulsified in IFA and administered with CpG-ODN as adjuvant. Spleens and draining lymph nodes harvested 7 days after vaccination were pooled in each group, and the effector cell populations were kept in culture with IL-2 (25 IU/ml) for 3 days. T cell responses were measured in a 4-h cytotoxicity assay against EL4 (\square) and peptide-pulsed EL4 (\blacksquare) target cells at various E:T ratios. Upper three panels, Represent the CTL responses elicited by minimal epitope peptide mixture (pMix); lower three panels, responses induced by the TA1 vaccine. Left panels, Display the responses against the OVA₂₅₇ epitope; middle panels, the responses to the SIYR epitope; and right panels, the responses to the LCMV_{gp32} CTL epitope. Each data point represents the average of triplicate samples.

period. These results indicate that TA constructs containing mixtures of CTL and Th epitopes are capable of producing both MHC class I- and II-binding peptides that can be presented to CTL and Th cells, respectively.

Next, we evaluated the immunogenicity of TA6 after a single s.c. injection into naive C57BL/6 mice. Groups of three mice each were vaccinated with equimolar amounts of either TA6 or the OVA₂₅₇ plus OVA₃₂₃ peptide mix, and 7 days later the Ag-specific responses of purified CD8⁺ and CD4⁺ T cells were measured by cytotoxicity and lymphokine release assays, respectively (Fig. 6). The cytotoxic activity of the CD8⁺ T cells was evaluated with or without an in vitro Ag restimulation step. Interestingly, when the CD8⁺ T cells were simply kept in culture without the addition of Ag in medium containing IL-2, the cells derived from the TA6-vaccinated mice displayed \sim 100-fold higher levels of cytotoxicity as compared with the cells from the peptide mix-vaccinated mice (Fig. 6a, compare number of effectors from each group required to obtain 25% lysis). Moreover, the cytolytic activity was high against both peptide-pulsed EL-4 and EG7 (OVA-transfected) targets, demonstrating the high quality of the response. Alternatively, when the CD8⁺ T cells were restimulated in vitro with Ag, the effector cell populations from both the TA6- and peptide mix-immunized mice had the same level of activity (Fig. 6b). The Ag reactivity of purified CD4⁺ Th cells from the vaccinated mice was evaluated by stimulating the cells with various concentrations of OVA₃₂₃ peptide in the presence of MHC class II-expressing APC. The data shown in Fig. 6c indicate that both the cells derived from the TA6-vaccinated mice and the cells derived from the peptide mix-immunized animals had relatively similar levels of activity. The overall results indicate that a TA containing linked MHC class

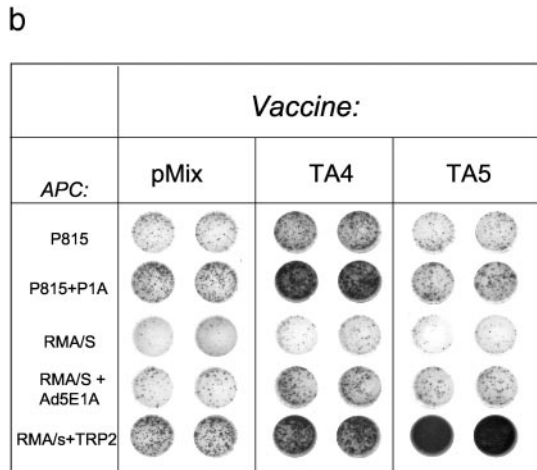
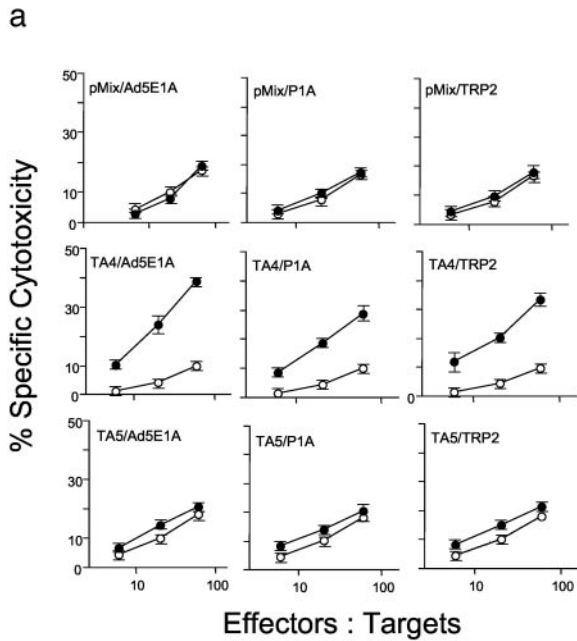


FIGURE 4. Evaluation of the immunogenicity of multipitope TAs containing poorly immunogenic CTL epitopes. Naive C57BL/6 mice (3 per group) were immunized s.c. with 100 μ mol of TA4, TA5, or minimal epitope peptide mixture (pMix) using IFA and CpG-ODN as adjuvant. Seven days later, the spleens and draining lymph nodes were harvested and pooled for each group, and the Ag-specific CTL responses were analyzed in a 4-h cytotoxicity assay or a 48-h IFN- γ release ELISPOT. *a*, Before the cytotoxicity assay, the effector cells were kept in tissue culture with IL-2 (25 IU/ml) for 3 days. T cell responses were detected in a 4-h chromium assay for peptides TRP2 and Ad5E1A against EL4 (○) and peptide-pulsed EL4 (●) and for peptide P1A against P815 (○) and P1A-pulsed P815 (●). Each data point represents the average of triplicate samples. *b*, IFN- γ release ELISPOT assays of the effector cells. A total of 1×10^5 purified CD8⁺ cells from each group was incubated with an equal number of peptide-pulsed or unpulsed APC in each well of an Ab-coated ELISPOT 96-well plate for 48 h under tissue culture conditions. After this time period, the plates were processed, as recommended by the kit manufacturer (BD PharMingen), to develop spots generated by the lymphokine-producing cells.

I and II epitopes is capable of inducing strong immune responses for both CTL and Th cells. Moreover, the CTL responses induced by TAs are superior to those elicited by an equivalent peptide mix in conditions when the responding cells are not restimulated with Ag.

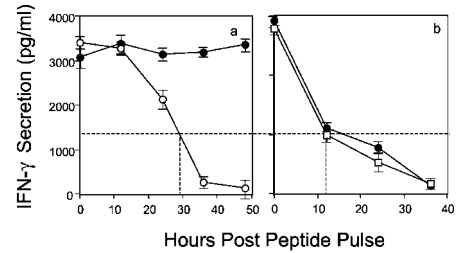


FIGURE 5. Evaluation of antigenicity of TA containing MHC class I and II T cell epitopes. Bone marrow-derived DC from (C57BL/6 \times BALB/c)_{F1} mice were pulsed with 3 μ M OVA₂₅₇ (○), OVA₃₂₃ (□), or TA6 (●) for 2 h at 37°C. After this time period, the APC were washed three times and irradiated (3000 rad). The ability of peptide-pulsed DC to induce present Ag to CTL and Th cells at various time points during the Ag chase was determined by adding 1×10^5 T cells (previously activated) from TCR transgenic OT-I or DO11.10 mice to 3×10^4 DC and removing the supernatants 48 h later for measuring the amount of IFN- γ produced by an ELISA. Each data point represents the average of triplicate samples.

Antitumor effects of multipitope TAs

Demonstration that a vaccine is capable of inducing T cell responses does not necessarily imply that such responses will be effective against a disease-causing agent. Thus, we proceeded to test whether TA immunization generated a sufficiently robust T cell response that would be translated into antitumor effectiveness (Fig. 7). For these experiments, we compared as vaccines the TA6 construct with a mixture of OVA₂₅₇ plus OVA₃₂₃, or the OVA₂₅₇ CTL epitope alone for their ability to increase the survival of C57BL/6 mice either before (prophylactic protocol, Fig. 6*a*) or after (therapeutic protocol, Fig. 6*b*) a challenge with OVA-transfected B16 melanoma cells (B16-OVA). In the prophylactic model, immunization with OVA₂₅₇ alone prolonged the median survival by ~12 days compared with nontreatment group, but all mice eventually developed tumors and died or had to be euthanized (Fig. 7*a*). The inclusion of the Th cell epitope OVA₃₂₃ into the vaccine as a peptide mixture slightly increased the median survival by ~20 days, with 2 of 10 mice surviving for at least 3 mo. TA6 vaccination provided a significantly improved protection of the mice against tumor challenge as compared with either the untreated or OVA₂₅₇-alone groups, with 4 of 10 mice remaining tumor free for 3 mo after the tumor challenge. More importantly, the antitumor effect provided by TA6 vaccination was superior to that of vaccination with the OVA₂₅₇ and OVA₃₂₃ mixture.

In the therapeutic protocol (Fig. 7*b*), vaccination with OVA₂₅₇ alone failed to offer any significant survival advantage over the nontreatment group ($p > 0.05$). In this model, both TA6 and the OVA₂₅₇ plus OVA₃₂₃ peptide mix significantly enhanced the median survival by ~21 days ($p < 0.01$), but ultimately all animals developed tumors and succumbed to their disease. Overall, these results demonstrate that vaccination with a TA construct provides a survival advantage in both prophylactic and therapeutic models, and that the inclusion of a Th cell epitope into the peptide vaccine is important for achieving the antitumor effects.

Discussion

Although peptide-based vaccines appear to be an attractive way to treat malignant diseases because of their ease and low cost to manufacture, there are numerous barriers that have to date prevented their success in the clinic (5). Among these obstacles, the inherent lack of immunogenicity of peptides is being addressed through the use of strong adjuvants and immunopotentiating strategies. We have recently shown that peptides representing CTL and Th cell

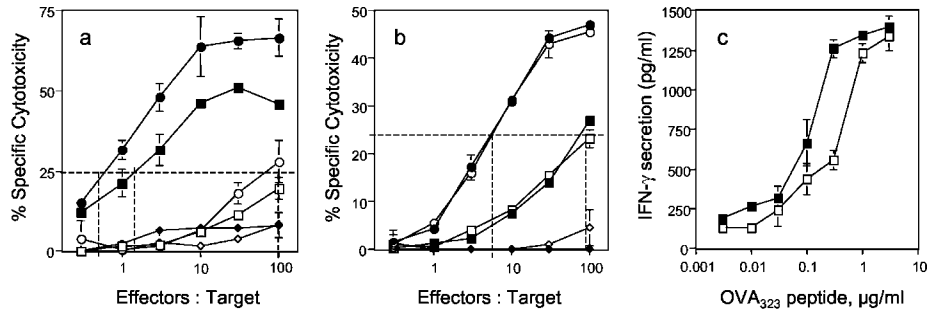


FIGURE 6. Evaluation of immunogenicity of TA containing MHC class I and II T cell epitopes. Naive C57BL/6 mice (three per group) were vaccinated s.c. with 100 μ mol of TA6 (filled symbols) or the minimal peptide mixture (open symbols) containing the of OVA₂₅₇ and OVA₃₂₃ T cell epitopes. Seven days after, spleens and draining lymph nodes were harvested and pooled for each group, and the responding T cell populations were evaluated for their capacity to recognize Ag in the context of MHC class I and MHC class II. *a*, Four-hour cytotoxicity assay of responding cells maintained in culture for 5 days without Ag restimulation. Dotted lines represent number of effector cells required to attain 25% specific lysis. *b*, Four-hour cytotoxicity assay of responding cells maintained in culture for 5 days with Ag restimulation (3 μ M OVA₂₅₇). The following target cells were evaluated at various E:T ratios: OVA₂₅₇-pulsed EL-4 (circles); unpulsed EL-4 (diamonds); EG7 (squares). Dotted lines represent number of effector cells required to attain 25% specific lysis. *c*, Purified CD4⁺ T cells (1×10^5 per well) were mixed with 2×10^5 irradiated (3000 rad) splenocytes from normal C57BL/6 mice as APC in each well of a 96-well round-bottom plate with various concentrations of OVA₃₂₃ peptide. The culture supernatants were removed 48 h later and evaluated for the content of IFN- γ using a commercial ELISA kit (BD PharMingen). TA6-immunized mice (\square); peptide mix-immunized mice (\blacksquare). Each data point represents the average of triplicate samples.

epitopes administered in combination with synthetic CpG-ODNs, which activate DC through Toll-like receptor 9, induce strong and effective antitumor immune responses (20). Moreover, in those circumstances in which the targeted tumor Ag is also expressed by normal cells, such as the melanosomal product, TRP2, the use of CTLA-4 blockade together with peptide vaccination and CpG-ODN adjuvant was shown to elicit a significant therapeutic antitumor outcome in a mouse model of malignant melanoma (22).

Another major obstacle for the development of peptide-based vaccines stems from the necessity to use more than a single T cell epitope in each vaccine preparation as a result of the stringency imposed by MHC restriction and the prerequisite to include epitopes derived from diverse tumor Ags to increase disease coverage and prevent the emergence of escape mutants. Moreover, substantial evidence has been gathered to indicate that to be clinically effective, vaccines must stimulate not only tumor-reactive

CTLs, but should also trigger antitumor Th cell responses (23–26), necessitating the inclusion of additional peptide sequences representing MHC class II-restricted epitopes. A simple solution to this dilemma could be the use of peptide mixtures representing mixtures of various CTL and Th cell epitopes restricted by common MHC alleles and derived from more than one tumor Ag. Nevertheless, this approach creates a multitude of problems when developing vaccines for human use. Not only each separate component of a peptide mixture has to be manufactured separately under tight regulations (e.g., following Good Manufacturing Practices), but also extensive and costly quality control and safety testing must be performed individually for each of the components as well as for the final peptide mixture. This cumbersome scenario extends to the stability of peptide mixtures, in which if only one of the peptides was to degrade rapidly, even if the remaining components were highly stable, the vaccine would become inadequate for

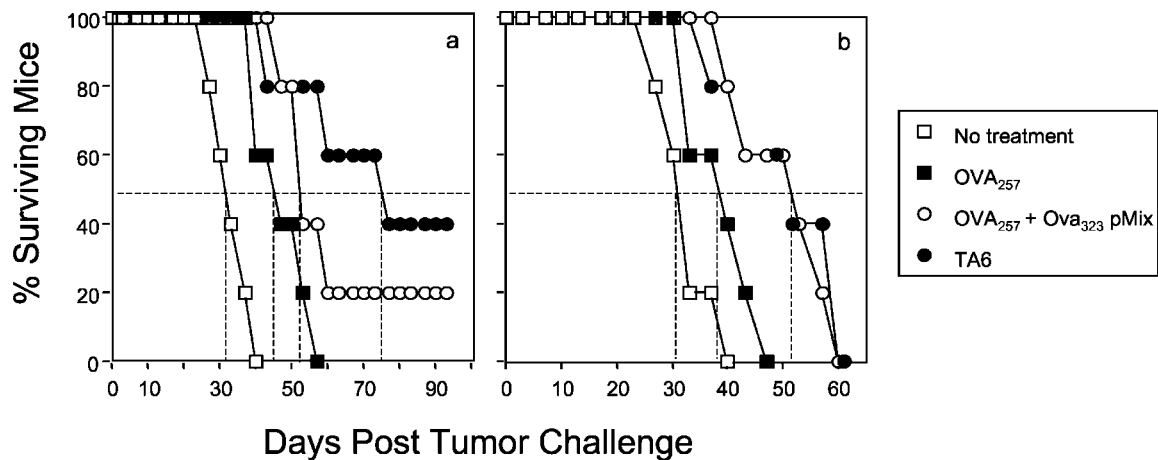


FIGURE 7. Antitumor effects resulting from peptide vaccination. C57BL/6 mice (10 per group) were vaccinated s.c. once with peptides OVA₂₅₇ (\blacksquare), OVA₂₅₇, OVA₃₂₃ peptide mixture (\circ), or TA6 (\bullet) either 7 days before tumor challenge (*a*) or 7 days after tumor challenge (*b*). Dotted lines represent the medial survival (days) for each group. In all cases, tumor challenge consisted of an s.c. injection (in the lower flank) of 5×10^4 B16-OVA melanoma cells. Mouse survival was monitored every other day. Animals with ulcerated tumors or tumor masses larger than 2 cm in diameter were sacrificed according to our institution's animal care procedures. Statistical analyses were done by log rank Mantel-Haenzel tests and demonstrated significant differences in *a* between each vaccine group compared with the unvaccinated group (all, $p < 0.01$). In addition, the TA6 group was found to be statistically significantly different from the OVA₂₅₇ + OVA₃₂₃ mix ($p < 0.05$). In *b*, only the TA6 and the OVA₂₅₇ + OVA₃₂₃ mix were found statistically significantly different from the unvaccinated group ($p < 0.05$). However, TA6 and OVA₂₅₇ + OVA₃₂₃ mix groups were not different from each other.

clinical use. At first sight, the most immediate solution to this problem would be to simply join together in tandem all of the epitopes into a single polypeptide chain. However, there are no clear rules that can follow to guarantee that proteasomal cleavage and peptide trimming will produce all the epitopes correctly, avoiding the possible destruction of an epitope by unpredictable protease cleavage. The studies presented in this work were planned to address a possible strategy to produce single synthetic peptide constructs containing multiple T cell epitopes that would be capable of inducing effective antitumor immune responses.

We previously demonstrated that TA containing a single CTL epitope linked to a Trojan peptide carrier were effective in generating MHC class I/peptide complexes in a TAP-independent fashion, and that furin played an important role in the processing of these Ags in the ER and TGN compartments (6). In this study, we have shown that, indeed, the use of furin-sensitive linkers to join the CTL epitopes was critical for the effective production of the corresponding MHC class I/peptide complexes (Figs. 1 and 2). Interestingly, the importance of furin-sensitive linkers was observed not only for the TAP-deficient target cells, but also for the TAP-competent APC (Fig. 1). The antigenic TAs (TA1 and TA3) as well as the minimal CTL epitopes appeared to be more effective in sensitizing the TAP-deficient target cells than the TAP-competent APC (Fig. 1). This effect is probably due to the fact that there are more peptide-receptive MHC class I molecules in the ER and TGN, and on the surface of TAP-deficient APC as compared with TAP-competent cells. Nevertheless, TAs were shown to be effective in producing the necessary amounts of peptide/MHC class I complexes in TAP-competent cells for CTL recognition (Fig. 1) and for inducing CTL responses in TAP-competent mice (Fig. 6). Notably, TA2, which lacked furin-sensitive linkers, failed to yield CTL epitopes in the TAP-competent APC, suggesting that proteasomes are not capable of producing the MHC class I-binding peptide precursors. These results indicate that even in circumstances in which proteasomal cleavage and TAP transport are operational, multiepitope TAs require processing in the TGN and ER, and that furin-sensitive linkers are needed to separate the CTL epitopes from each other.

With respect to immunogenicity, the multiepitope TA constructs performed as well, if not better, than the corresponding minimal epitope mixtures (Figs. 3 and 4). When the CTL epitopes tested represented highly immunogenic Ags, the CTL responses from mice vaccinated with TA constructs were almost identical with those obtained from mice immunized with peptide mixture (Fig. 3). In contrast, when less immunogenic CTL epitopes were studied, the TA constructs outperformed the peptide mixture (Fig. 4a). Although the exact reason for these differences is not known, it is possible that the TAs function better than the minimal epitopes from poorly immunogenic Ags because APC from the TA-immunized animals are qualitatively superior than the APC generated in the peptide mix vaccine recipients. For example, the APCs processing TAs were able to present Ag for a more extended period of time than the APCs pulsed with minimal epitopes (Fig. 5) (27). It is important to note that the results derived from the cytotoxicity assays (Fig. 4a) did not entirely correlate with the lymphokine release assays (Fig. 4b), in which the CTL derived from mice immunized with either the peptide mixture or the TA with furin-resistant linkers (TA5) exhibited some level of response. This discrepancy could be due to the differences in incubation time existing between cytotoxicity assays (4 h) and ELISPOTs (48 h), which would allow low affinity CTL the opportunity to react to Ag in conditions of prolonged stimulation.

Although APC pulsed with TA were capable of efficiently presenting MHC class I epitopes for an extended time period (up to

50 h), this effect was not mirrored with regard to the presentation of Ag via MHC class II molecules (Fig. 5). Thus, it appears that the TA intracellular pools are able to last for a long period of time, but can only supply material to the MHC class I pathway. In contrast, because the kinetics of antigenicity decay for MHC class II presentation of the TA-pulsed APC was identical with that of the peptide-pulsed APC (Fig. 5b), we assume that the MHC class II-processing path of the TA follows a similar route to that of the minimal peptide epitope. Thus, it is possible that the TA could directly bind to cell surface MHC class II molecules as the minimal epitope does, because there is no major restriction on the length of peptides for these interactions to occur. It is also possible that the TA reaching the intracellular endocytic compartments where peptides usually bind to MHC class II molecules are degraded at the same speed as proteins/peptides that are normally ingested by the APC. Regardless of the mechanism leading to peptide/MHC class II complex formation by TAs, our results demonstrate that TAs composed of CTL and Th epitopes are capable of generating both MHC class I and II T cell epitopes. More importantly, the CTL-Th TA constructs were shown to be effective at inducing *in vivo* CTL and Th responses after a single injection (Fig. 6). The CTL responses derived from the TA-vaccinated mice appeared to be significantly higher than the responses obtained from peptide mix-immune mice, when the responding cells were maintained in culture without the addition of Ag (Fig. 6a). However, these differences disappeared if the T cell cultures were restimulated with synthetic peptide (Fig. 6b). Interestingly, while the potency of the CTL response of the cells from peptide mix-immunized mice increased when the cells were restimulated with peptide, the opposite occurred with the responding cells derived from the TA-vaccinated animals. One possible explanation for these results could be differences in the amounts of effector killer cells vs memory CD8⁺ T cells that are induced by the two types of vaccines. Specifically, if the responder cells from TA-immunized mice contain larger numbers of activated effector killer cells than the responder cells from peptide mix-vaccinated mice, one would expect to obtain, without Ag restimulation, higher CTL responses in the former than the latter. In addition, Ag restimulation of the effector killer T cells present in the cultures derived from the TA-vaccinated mice could result in significant loss of activity due to activation-induced cell death, which is known to readily take place when recently activated CTL re-encounter Ag in the presence of IL-2 (28–31). In contrast, memory CTLs require Ag restimulation to become activated and display their cytotoxic function. Thus, our results suggest that both the TA and the peptide mix vaccines elicit similar levels of memory CTL responses, but that the TA vaccine is more effective at maintaining effector CTL levels for a longer period of time (~6 days) than the peptide mix vaccine. Additional experiments are required to explore these possibilities.

It is clear that the induction of CTL and Th responses by any type of vaccine without clear demonstration of some type of benefit against disease is meaningless for the translation of basic research into the clinical setting. The results presented in this work demonstrate that the immune responses induced by multiepitope TA vaccines were effective in increasing, to some extent, the survival of tumor-bearing mice both in the prophylactic and therapeutic settings (Fig. 7). Thus, the use of TA-based vaccines in cancer patients is an attractive possibility for inducing immunity against multiple T cell epitopes using a single compound. Nevertheless, we realize that to obtain an outstanding antitumor effect, additional immunological manipulations, such as CTLA-4 blockade or the removal of T regulatory cells, may be necessary.

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