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Pharmacokinetic Differences Between a T Cell-Tolerizing and a T Cell-Activating Peptide

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Vaccination with a peptide representing a CTL epitope from the human papillomavirus (HPV) 16 E7 protein induces a specific CTL response that prevents the outgrowth of HPV16 E7-expressing tumors. In contrast, vaccination with a peptide encoding an adenovirus type 5 (Ad5) E1A CTL epitope results in CTL tolerance and enhanced growth of an Ad5 E1A-expressing tumor. It is unclear why these peptides induce such opposite effects. To determine whether a difference in pharmacokinetics can explain the functional contrasts, tritiated Ad5 E1A and HPV16 E7 peptides were injected into mice. Results show that the tolerizing peptide spread through the body 16 times faster than the activating peptide and was cleared at least 2 times faster. The HPV16 E7 peptide kinetics correlated with the kinetics of HPV16 E7-specific CTL induction. In contrast, Ad5 E1A peptide injection resulted in physical deletion of preexisting Ad5 E1A-specific CTLs within 24 h after injection. This tolerization occurred at the time when the peptide reached its maximum peptide concentration in the organs. These data suggest that ubiquitous expression of the tolerizing Ad5 E1A peptide within a short period of time causes activation-induced cell death of Ad5 E1A-specific CTLs. Therefore, information on the pharmacokinetics of peptides is vital for the safety and efficacy of peptide-based vaccines. *The Journal of Immunology, 2001, 166: 7151–7157.

Peptide-based vaccines have been successful in inducing protective immunity against a variety of infections and tumor challenges (1, 2). For example, vaccination with a CTL epitope from the human papillomavirus (HPV) type 16 E7 protein protected mice against the outgrowth of tumor cells expressing the HPV16 genome (3). However, in some tumor models, peptide-based vaccines promote tumor growth. Examples of such peptides are CTL epitopes derived from adenovirus type 5 (Ad5) E1A (4) and E1B (5) and the CTL epitope derived from the P815 mastocytoma tumor Ag (6). Mice that were immunized s.c. with these peptides in IFA and then challenged with tumor cells expressing the Ags developed tumors more rapidly than nonimmunized mice. Furthermore, the functional activity of Ad5 E1A-specific CTLs that were adoptively transferred into nude mice was no longer detectable after the mice were immunized with the Ad5 E1A peptide (4). Additionally, when mice received Ad5 E1A-specific CTLs i.v. and the Ad5 E1A peptide s.c. simultaneously, they died of extensive lung destruction within 16 h after injection (4). However, when HPV16 E7-specific CTLs and HPV16 E7 peptide were injected simultaneously, lung destruction did not occur and CTLs remained functionally active. This suggests that there is an important difference between the HPV16 E7 and Ad5 E1A peptides, which could be related to a difference in diffusion rates throughout the body.

In the human setting, peptide-based vaccines have also induced diverse effects. Several clinical trials applying peptide-based vaccines in cancer patients have demonstrated peptide-specific immune responses in the majority of the patients (7–10). However, many of the clinical trials testing peptide-based vaccines have shown limited or no peptide-specific immune response (11, 12) with undetectable clinical effects (13–15). Although many explanations for the absence of immune induction have been offered, the deletion of specific CTLs after peptide vaccination has not been studied. Moreover, vaccination-induced T cell tolerance would be deleterious to the desired objective of establishing immunity and to the immediate health of the patients. Therefore, it is important to determine why peptide-based vaccines can give such contrasting results. To our knowledge, there is no published study that has explored a possible difference in pharmacokinetics as an important property that determines immunogenicity of peptides in vivo. Information on peptide pharmacokinetics could greatly influence the design of peptide-based vaccine strategies.

In this study, we compared the pharmacokinetics of a T cell-activating peptide derived from the HPV16 E7 protein and a T cell-tolerizing peptide of the Ad5 E1A protein. The results show that the Ad5 E1A peptide egressed from the vaccine site at a higher rate than the HPV16 E7 peptide. As a consequence, the Ad5 E1A peptide reached its maximum concentration in all organs much faster than the HPV16 E7 peptide; maximum concentrations were reached at ~10 and 160 h in all organs for the Ad5 E1A and the HPV16 E7 peptide, respectively. Furthermore, the HPV16 E7 peptide remained in all organs significantly longer than the Ad5 E1A peptide. These differences in pharmacokinetics could contribute to the differential effects of HPV16 E7 and Ad5 E1A peptide injection on peptide-specific CTLs in vivo.
Materials and Methods

Mice, cell lines, and peptides
Six-week-old female C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and were housed in a specified pathogen-free environment. RMA-S cells were cultured in IMDM (BioWhittaker, Walkersville, MD) supplemented with 2 mM l-glutamine (Mediatech, Herndon, VA), 10% FCS (HyClone Laboratories, Logan, UT), and 1 mg/ml kanamycin (Sigma, St. Louis, MO). Peptides were dissolved in PBS and emulsified in IFA (Difco, Detroit, MI) at a ratio of 1:1 using the Polytron homogenizer PT-3100 (Brinkmann Instruments, Westbury, NY). This method of emulsification of peptides is superior to the classical method of emulsification (i.e., vortexing or repeatedly passing through a syringe). This method guaranteed that both peptides were equally well emulsified. A total of 300 µg of H-labeled peptide, equivalent to 0.51 µCi H, was injected s.c. in a volume of 250 µl on the right flank of the mouse.

Peptide synthesis
Ad5 E1A234-243 (NH2-GSGSNTPEPPEO-COOH) (16) and HPV16 E734-57 (NH2-RAHYNIVTF-COOH) (3) were synthesized and purified as reported previously for 35S-labeled peptides (17). Briefly, uniformly labeled H-Ile (New England Nuclear, Billerica, MA) with a specific radioactivity of 108 Ci/mmol, was used for BOC or F-moc groups as described (16). Peptides were dissolved in 0.1% trifluoroacetic acid (TFA) and purified by preparative reverse phase HPLC using a Rainin Dynamax SD-200 HPLC (Rainin Instruments, Emeryville, CA) and a Zorbax 300-SB C4 column (MAC-MOD Analytical, Chaddsford, PA). Purity was assessed by analytical HPLC using a Rainin 25 × 0.5 cm C18 column (Rainin Instruments) and a linear gradient over 1 h of 0.1% TFA in water to 0.1% TFA in 70/30 acetonitrile/water. The identity was confirmed by matrix-assisted laser desorption ionization/time-of-flight or electron spray mass spectrometry. The peptides were determined to be >99% pure. The specific activity of the Ad5 E1A and HPV16 E7 peptides was 1899 cpn/nmol and 2062 cpn/nmol, respectively.

RMA-S-peptide binding assay
The MHC-peptide binding assay, which measures the ability of the peptide to bind H2-Dd, was assessed using the RMA-S cell line as described previously (3). Briefly, RMA-S cells were incubated for 36 h at 26°C followed by a 4-h incubation with 100 µg/ml tritiated and nontritiated HPV16 E7 or Ad5 E1A1 peptides. Cells were washed with a biotin-conjugated H2-Dd-specific Ab (BD PharMingen, San Diego, CA), followed by avidin-FITC (BD Biosciences, San Jose, CA). The level of H2-Dd expression, as determined by FACS analysis, correlates with the peptide binding affinity.

Organ collection
At the following time points after injection, mice were sacrificed by cervical dislocation: 0, 0.5, 1, 2, 4, 8, 24, 48, 72, 120, 168, and 336 h. At these times, the following organs were collected: vaccine site, lymph nodes, spleen, thymus, heart, liver, lungs, kidney, intestines, brain, and blood. Each organ was frozen at −20°C until analysis. Whole blood was heparinized and processed as described below.

Organ processing
All organs were homogenized in deionized water at 3 ml/g wet weight using the Polytron PT-3100. A total of 0.3 µl homogenate was digested in 1 ml Soluene-350 (Packard, Meriden, CT). To the vaccine site and organs weighing <100 mg, 0.3 ml deionized water and 1 ml Soluene-350 was added. The mixture was incubated at 50°C overnight, after which 10 µl Hionic Fluor scintillation mixture (Packard) was added. To whole blood, 1 ml Soluene-350/isopropanol (1:1) was added. After a 2-h incubation at 50°C, 0.2 ml of 30% H2O2 was added. Following a 30-min incubation at 50°C, 10 µl Hionic Fluor scintillation mixture was added. 3H content of all samples was measured by a liquid scintillation counter (Beckman Coulter, Fullerton, CA). The cpm were converted to dpm by standard quench curves. Furthermore, the measured dpm were corrected for the percentage of dpm due to chemiluminescence.

HPLC of homogenates
Homogenates were centrifuged at 17,000 × g, filtered using a 0.22-µm filter, and subjected to reverse phase HPLC as described above but with the gradient shortened to 0.5 h. Fractions were collected, and the radioactivity was measured by liquid scintillation counting.

Analysis of kinetic data
The data were analyzed as the mean dpm for each data point (i.e., three mice at each time point and each organ). Multiple kinetic schemes were tested; nonlinear fit of the data to the appropriate equation was performed using the Kaleidagraph program (Abelback software).

Enzyme-linked immunospot (ELISPOT)
Spleens were isolated at defined times after peptide injection. Splenocytes were frozen as a single-cell suspension. Multiscreen 96-well filtration plates (Millipore, Bedford, MA) were coated with 5 µg/ml anti-IFN-γ Ab (BD PharMingen) overnight. Splenocytes were thawed and plated in triplicate at 5 × 10⁴ and 2 × 10⁴ cells/well in the presence of 30 U/ml IL-2 and 5 µg/ml Ad5 E1A, HPV16 E7 peptide, or no peptide. After a 24-h incubation, ELISPOT plates were washed with PBS containing 0.1% Tween 20, and 2.5 µg/ml anti-IFN-γ-biotin (BD PharMingen) was added to each well. Plates were incubated for 2 h at room temperature, and 1.25 µg/ml avidin-alkaline phosphatase (Sigma) was added to each well for 2 h. Plates were washed with PBS containing 0.1% Tween 20 followed by PBS. A solution of 5-hromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Promega, Madison, WI) in alkaline phosphatase buffer was added to each well and, after a 15-min incubation, plates were washed with deionized water and air-dried. Spots in the lower dilution wells were counted, averaged, and subtracted from the background spots counted in the wells stimulated without peptide. This number was converted to the average number of CTL precursor cells per 1 × 10⁵ cells present in the immunized mice.

Tetramer analysis
C57BL/6 splenocytes isolated for the ELISPOT were used for this assay. CD8+ T cells were positively selected using anti-CD8 conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). These cells were stained for anti-CD8-FITC (BD PharMingen) overnight. Splenocytes were thawed and plated in triplicate at 5 × 10⁵ and 2 × 10⁵ cells/well in the presence of 30 U/ml IL-2 and 5 µg/ml Ad5 E1A and HPV16 E7 tetramer or Ad5 E1A (SGPSNTPPEI)-H2-Dd tetramer, which were both conjugated with PE (National Institute of Allergy and Infectious Diseases tetramer facility, Atlanta, GA), for 1 h on ice. Expression levels of CD8 and specific TCRs on 4 × 10⁵ gated viable cells were analyzed by FACS.

Results

Peptide-MHC binding is not affected after intrinsic labeling with 3H
To test whether the HPV16 E7 and Ad5 E1A peptide binding capacity to H2-Dd was altered by tritiation, a standard RMA-S binding assay was performed. The level of H2-Dd expression on the cell surface when RMA-S cells were loaded with regular peptide or tritiated peptide was compared by FACS analysis. No difference in MHC expression could be detected (Fig. 1), indicating that the peptide-MHC binding capacity was not affected by intrinsic tritiation. Based on these data, the tritiated peptides emulsified in IFA were compared for in vivo biodistribution after s.c. injection in mice.

The tolerizing peptide diffuses out of the vaccine site more rapidly than the T cell-activating peptide
The tritium content of the vaccine site was assessed at different times after injection with 300 µg 3H-labeled Ad5 E1A or HPV16 E7 peptide emulsified in IFA. Fig. 2A shows that, 3 h after injection, the tolerizing Ad5 E1A peptide started to egress from the vaccine site, whereas increased egression of the activating HPV16 E7 peptide started 10 h after injection. Furthermore, egression of the Ad5 E1A peptide halted after 72 h, and egression of the HPV16 E7 peptide stopped after 200 h (Fig. 2B). Comparison of the egression rate constants (Ko) of the two peptides (Table I) showed that the Ad5 E1A peptide egressed from the Ag depot 2.7 times faster than the HPV16 E7 peptide.

The tolerizing Ad5 E1A peptide reaches its maximum concentration in all organs earlier and declines in concentration faster than the activating HPV 16 E7 peptide.

To establish the pharmacokinetic behavior of the two peptides with respect to organ distribution, several organs were analyzed for...
their tritium content at different points in time, and influx and egression parameters were calculated. In four independent experiments with three mice per time point, the pharmacokinetic behavior of each individual peptide in all organs was found to be very consistent (Table I). To determine the pharmacokinetics in lymphoid organs, in which induction of the CTL response should take place, spleen, thymus, and lymph nodes were analyzed for their tritium content. Fig. 3 shows the pharmacokinetics of both peptides in the spleen. The maximum Ad5 E1A peptide concentration in the spleen was similar to that of the HPV16 E7 peptide concentration; however, the maximum Ad5 E1A peptide concentration reached after ~10 h, and it declined to an equilibrium after ~100 h. The HPV16 E7 peptide reached the maximum concentration in the spleen ~140 h after injection, and its concentration had not equilibrated to baseline levels as late as 350 h (Fig. 3B). Identical peptide concentration and kinetics were detected in the thymus as indicated in Table I. Tritium concentration in pooled lymph nodes was also measured but never attained levels above background. This is most likely due to the extremely small size of these organs, resulting in an undetectable tritium concentration.

Simultaneous adoptive transfer of Ad5 E1A-specific CTLs and Ad5 E1A peptide injection into nude mice resulted in severe lung destruction 16 h after injection (4). The suggested hypothesis was that this could be attributed to a rapid influx of Ad5 E1A peptide in the lungs. To test this hypothesis, pharmacokinetics of both peptides were measured in the lungs and are shown in Fig. 4. The kinetics of both HPV16 E7 and Ad5 E1A peptides in the lungs are very similar to those detected in the spleen in that maximum peptide concentrations are reached at 10 and 170 h after Ad5 E1A or HPV16 E7 peptide injection, respectively (Fig. 4A), due to the large difference (~36-fold) in influx between both peptides into the lungs as represented by the slope of the curves (Fig. 4B). Moreover, the HPV16 E7 peptide persists in the lungs at least 100 h longer then the Ad5 E1A peptide (Fig. 4B). These results indicate that, while both peptides spread to and egress from all organs with similar kinetics, each individual peptide does so with distinct kinetics. This difference in kinetic behavior between these two peptides depends on the nature of the peptide rather than that of the organ.

**Intact peptides can be detected 24 h after injection**

To determine whether the tritium measured in the different organs originated from intact peptide, free amino acids, or other degradation products, homogenates of most organs obtained 24 h after injection were analyzed by reversed phase HPLC analysis and compared with pure synthetic tritiated peptide (Fig. 5). With the exception of the intestines, the tritium label eluted from all organs in the same fraction as intact synthetic peptide (fraction 17 and 18 for the Ad5 E1A peptide and fraction 23 for the HPV16 E7 peptide), demonstrating that the label was still contained within intact peptide. In the intestines, the label eluted in the void volume, indicating that the peptide had been degraded to amino acids or other polar products.

**The kinetics of Ad5 E1A- and HPV16 E7-specific CTL induction corresponds to the peptide kinetics**

The previous data indicate a clear difference in the kinetics of the tolerizing (Ad5 E1A) and the activating peptides (HPV16 E7). However, these differences may not be the cause of the observed functional

![FIGURE 1. Tritiation of peptides does not alter binding to H2-D\(^b\). RMA-S cells were loaded with tritiated or nontritiated CTL peptides, and the MHC class I (H2-D\(^b\)) expression was compared by FACS analysis for the HPV16 E7 peptide (A) and the Ad5 E1A peptide (B). The thin line represents RMA-S cells without peptide, the bold line represents RMA-S cells loaded with tritiated peptide, and the dotted line represents RMA-S cells loaded with nontritiated peptide.](image1)

![FIGURE 2. The tolerizing Ad5 E1A peptide egresses from the vaccine site faster than the activating HPV16 E7 peptide. Intact vaccine sites were collected at different times after peptide injection, and \(^3\)H was measured. Curve fit was performed according to the following equation: \(A - A_{eq} = (A_{eq} - A_0)e^{-kt}\), where \(A\) is the radioactivity in the vaccine site, and \(A_0\) and \(A_{eq}\) are parameters representing the initial radioactivity and the final ("equilibrium") radioactivity. Curve fits are shown on a logarithmic (A) and a linear (B) scale to enable data analysis at different times after peptide injection. The SEM has been included for each time point. This is one representative experiment of four independent experiments performed. Squares represent the HPV16 E7 peptide, and circles represent the Ad5 E1A peptide.](image2)
differences in the induction of a CTL response. Therefore, we assessed the kinetics of the Ad5 E1A- and HPV16 E7-specific CTL responses after peptide injection. Mice were immunized s.c. with 100 μg Ad5 E1A or HPV16 E7 peptide in IFA. At defined times after peptide injection, splenocytes were isolated and the number of Ad5 E1A- and HPV16 E7-specific CTLs was measured by ELISPOT. Fig. 6A shows that an increase in HPV16 E7-specific CTL precursors was observed 72 h after peptide injection. The CTL response peaked at 120–168 h after peptide injection and returned to the original CTL level after 2 wk. A similar pattern was observed in the HPV16 E7-H2-Db tetramer analysis performed at the same time points after peptide injection (Fig. 6B). As observed in the ELISPOT assay, the number of HPV16 E7-specific CTLs increased 120 h after peptide injection and declined 2 wk after injection. The E7-specific CTL kinetics corresponds strongly with E7 peptide kinetics, which also showed a peak in E7 peptide concentration between 120 and 168 h after injection (Fig. 3). In contrast, a significant Ad5 E1A-specific CTL response was not detected by ELISPOT (Fig. 6C) or by Ad5 E1A-H2-Db tetramer staining (Fig. 6D). The positive control (spleenocytes isolated from mice immunized with irradiated tumor cells expressing the Ad5 E1A protein) showed a significant induction of Ad5 E1A-specific CTLs by tetramer staining (Fig. 6D). This proves that Ad5 E1A-specific CTLs can be induced, but Ad5 E1A peptide in IFA did not induce Ad5 E1A-specific CTLs.

FIGURE 3. The tolerizing Ad5 E1A peptide appears in and disappears from the spleen significantly earlier than the activating HPV16 E7 peptide. The spleen was collected at different times after injection, and 3H was measured at different times after injection. For the consecutive first order reactions A→B→C, the concentration of B is given by the equation: B = A0k1 / k1 – k2 (e⁻kt1 – e⁻kt2), where k1 and k2 are rate constants for the two reactions. The radioactivity deviated from this equation at later time points, because it returned to slightly above the original baseline. Accordingly, the scheme was modified to take this into account, resulting in the following equation: radioactivity = aex⁻kt1 – bex⁻kt2 + c, in which a ≠ b, and a, b, and c are aggregated kinetic parameters including terms for rate constants and apparent pool sizes. Curve fits are shown on a logarithmic (A) and a linear (B) scale to enable data analysis at different times after peptide injection. This is one representative experiment of four independent experiments performed. The SEM has been included for each time point. Squares represent the HPV16 E7 peptide, and circles represent the Ad5 E1A peptide.

Table I. Peptide pharmacokinetics and organ distribution

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<th>Peptide</th>
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<th>Tmax</th>
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<th>Rate Constant (K2)</th>
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* This is a representative experiment (n = 4) in which three mice per time point per peptide were sacrificed to obtain tritium content of different organs.
* Cmax refers to the maximal peptide concentration reached as a percentage of total injected peptide.
* Tmax refers to the time (h) at which the Cmax is reached.
* Rate constant (h⁻¹) into the organ. This is indicated by the slope of the curve in the linear area; a steep curve and therefore a high rate constant indicates a high influx of the peptide into the organ.
* Rate constant (h⁻¹) out of the organ.
* The rate constant into the organ is not applicable (NA) for the vaccine site.
Ad5 E1A-specific CTLs are deleted within 24 h after Ad5 E1A peptide injection

The absence of an Ad5 E1A-specific CTL response after Ad5 E1A peptide injection could be due to a functional or physical deletion of Ad5 E1A-specific CTLs. To test this, C57BL/6 mice were preimmunized twice with irradiated Ad5 E1A-positive tumor cells to induce a significant number of Ad5 E1A-specific CTLs. Two weeks after the last cellular immunization, mice were injected s.c. with Ad5 E1A peptide in IFA. At defined times after peptide injection, splenocytes were isolated and the number of Ad5 E1A-specific CTLs was determined by Ad5 E1A-H2-D10 tetramer analysis. As shown in Fig. 7, before peptide injection (0 h after peptide injection), a significant Ad5 E1A-specific CTL response was present: 1.58% of the CD8+ splenocytes was Ad5 E1A specific. Within 24 h after Ad5 E1A peptide injection, the number of Ad5 E1A-specific CTLs gradually reduced to levels detected in a naive mouse and remained unchanged up to 2 wk after peptide injection. These data indicate that Ad5 E1A peptide injection results in physical deletion of Ad5 E1A-specific CTLs. Furthermore, the observed physical deletion is very rapid, as is the appearance of the Ad5 E1A peptide in the spleen. The rapid increase of Ad5 E1A peptide concentration within the first 10 h of peptide injection results in deletion of Ad5 E1A-specific CTLs in that same time period.

Discussion

Ag localization, dose, and persistence determine whether an Ag will immunize or tolerize (19). Local administration of CTL peptides in adjuvant can result in an optimal induction of a specific CTL response. Other routes of delivery (i.v. and i.p.) that result in a rapid, systemic distribution and high Ag concentrations induce CTL tolerance in animals (20, 21). However, the route of delivery does not always predict the outcome of peptide-based vaccination, because s.c. peptide-based vaccination in IFA can also lead to tolerization of peptide-specific CTLs (4–6). In this study, we have compared the pharmacokinetics of a T cell-tolerizing and a T cell-activating peptide with the kinetics of CTL induction in vivo. The results provide strong evidence for the hypothesis that T cell activation or tolerization is determined by Ag concentration over time and Ag persistence. The ability of adjuvants to prevent tolerance is believed to reflect a slow Ag release from the adjuvant that results in an extended stable and low level of Ag presentation (20). Furthermore, adjuvants provide an Ag depot, allowing professional APCs to enter the vaccine site and collect the peptide. These Ag-loaded APCs then migrate to the lymph nodes and initiate T cell activation (22). This study presents evidence that the activating HPV16 E7 peptide persists in all organs longer than the tolerizing Ad5 E1A peptide as a result of a slow release of the HPV16 E7 peptide from the vaccine site (Fig. 2). As a consequence of the rapid diffusion of the Ad5 E1A peptide from the vaccine site, the Ad5 E1A peptide concentration reaches its maximum concentration in a relatively short time in all organs.
but it also declines more rapidly than the HPV16 E7 peptide concentration (Figs. 3 and 4).

The peptide kinetics offers an explanation for the observation that mice injected with Ad5 E1A-specific CTLs i.v. and the Ad5 E1A peptide s.c. die of extensive lung destruction within 16 h after injection (4). When the Ad5 E1A peptide reaches the lungs within 16 h (Fig. 4A), it activates Ad5 E1A-specific CTLs that are entrapped in the microvascular beds of the lungs after adoptive transfer. Peptides will bind to the MHC class I molecules of the endothelial cells, and the activated CTLs will cause destruction of the endothelial cells presenting the peptide. In contrast, mice injected simultaneously with HPV16 E7-specific CTLs and HPV16 E7 peptide remained alive, because the HPV16 E7 peptide slowly egresses and does not reach its maximum concentration until 160 h after injection (Fig. 4B). At that time, the HPV16 E7-specific CTLs will have migrated from the lungs and will not cause the lung destruction as observed in the Ad5 E1A peptide-injected mice.

Interestingly, we observed that the peptides detected after 24 h in most organs were intact (Fig. 5). These findings are in contrast to a report that studied the stability of a MART-1 peptide in human plasma in vitro in which the peptide half-life was calculated to be 22 s (23). Our data indicate that both Ad5 E1A and HPV16 E7 peptides remained in circulation for a much longer period. Peptides may be protected from degradation by binding to MHC class I molecules. This could explain the difference between these two studies, because MHC-expressing cells are absent in plasma. One exception to this generalization is the intestines, in which the radioactivity was recovered in a different fraction from the intact peptide after HPLC analysis, indicating peptide degradation. This should not be surprising, because the intestines contain both proteases and bacteria, which may have degraded the peptides.

The mechanism by which the peptide-specific CTLs are tolerized is not fully understood. However, based on the kinetics of the Ad5 E1A peptide, we infer that tolerance is likely induced by activation-induced cell death (AICD). AICD occurs as the consequence of repeated antigenic stimulation of T cells and is mediated by Fas-Fas ligand interactions in CD4+ T cells (24) and engagement of TNFRs in CD8+ T cells (25). We show in the spleen that Ad5 E1A peptide-specific CTLs are confronted with a rapid influx of a high peptide concentration within 24 h after peptide injection (Fig. 3). The analysis of the Ad5 E1A-specific CTL precursor frequency obtained from preimmunized mice after Ad5 E1A peptide injection demonstrated that, within 24 h after peptide injection, the number of preexisting Ad5 E1A-specific CTL (1.6% of the CD8+ T cell population) was significantly reduced. We propose that this is the result of AICD, even though we did not actually measure apoptosis. This is in agreement with a study showing that i.p. injection of high peptide doses, resulting in systemic distribution, readily deleted their specific CTLs in wild-type mice. However, in TNFR/Fas double-knockout mice, i.p. injection of peptide did not induce AICD, suggesting that peptide-induced tolerance is dependent on the presence of these two death receptors (26). In contrast, a slow buildup of the HPV16 E7 peptide and persistence of the peptide in lymphoid organs seems optimal for immunization. This is supported by our finding that the increased HPV16 E7 peptide concentration is closely followed by an increase in HPV16 E7 CTLs (Fig. 7).
6, A and B). Another possible explanation for the reduction of Ad5 E1A-specific CTLs could be retargeting of these CTLs. However, because the Ad5 E1A peptide spreads to all organs with similar kinetics, this seems less likely.

It remains to be answered why the Ad5 E1A peptide displays such different kinetics compared with the HPV16 E7 peptide. Differences in kinetics may be the consequence of a combination of intrinsic peptide characteristics, i.e., hydrophobicity, charge, structure, and MHC class I binding affinities of the peptide. Comparison of these two peptide sequences revealed that the Ad5 E1A peptide is more hydrophilic than the HPV16 E7 peptide. However, tolerance could not be prevented by using an Ad5 E1A peptide that was engineered to increase hydrophobicity (27). Furthermore, the Ad5 E1A peptide is negatively charged as compared with a positively charged HPV16 E7. Other structural features, such as the presence of aromatic amino acids in HPV16 E7 and not in Ad5 E1A, may contribute to their functional differences. Another important difference is that the fact the Ad5 E1A peptide has a 100-fold higher binding affinity for the H2-D^d molecule than for the HPV16 E7 peptide (28). However, this feature by itself does not explain the functional difference between the two peptides, because MHC-peptide binding analysis of a number of T cell-tolerizing and -activating peptides showed that tolerizing peptides do not have a consistently higher binding affinity than activating peptides (29). The pharmacokinetic properties of other T cell-tolerizing and -activating peptides should be analyzed to determine whether differences in pharmacokinetics could indeed predict the tolerizing or activating property of peptides in general. Ultimately, prediction of the effects that peptides may have on the immune system using pharmacokinetic profiles may increase the safety and efficacy of peptide-based vaccinations in humans.

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References