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Enhancement of Tumor Outgrowth Through CTL Tolerization After Peptide Vaccination Is Avoided by Peptide Presentation on Dendritic Cells

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Synthetic peptide-based vaccines have been shown to induce potent protective and therapeutic T cell-mediated immunity in preclinical animal models and are now being evaluated in clinical phase I/II studies for their efficacy against tumors or infectious diseases. However, such vaccines might also specifically tolerize T cells causing enhanced tumor outgrowth, as shown by vaccination with two CTL epitopes derived from the adenovirus type 5 early region 1 (Ad5E1) oncogenes. We now report that modification of the Ad5E1 peptide vaccine either through incorporation of the peptides into liposomes or by ligation of the peptides to lipid tails, another vaccine formulation being tested in the clinic, fails to convert immunosuppression into effective antitumor vaccination. Inclusion of a helper T cell epitope into the vaccine likewise induces enhanced tumor outgrowth and thus does not diminish the capacity of the peptides to tolerize Ad5E1-specific CTL. In contrast, the Ad5E1-derived peptides evoke a strong tumor-protective CTL response when presented on dendritic cells (DC), indicating that the in vivo CTL-tolerizing potential of these peptides is converted to specific immunostimulation when presented on DC. These findings have important implications for the development of peptide-based immune intervention strategies and emphasize the superior nature of Ag-pulsed DC over other peptide-based vaccination protocols as well as the crucial importance of the mode of peptide-Ag delivery in setting the balance between T cell stimulation and tolerization. The Journal of Immunology, 1998, 160: 4449–4456.

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ynthetic peptide-based vaccines administered s.c. in adjuvants are able to induce strong protective CTL-mediated immunity against virus infections and malignancies as shown in several preclinical murine model systems (1–4). As a result of these promising results, synthetic peptide-based vaccines are currently being tested in the clinic for their ability to activate virus- and/or tumor-specific CTL responses, as illustrated by the fact that many peptide-based vaccine trials are now enrolling patients with various types of cancer (5, 6).

Peptide Ags can, however, also induce specific peripheral CTL nonresponsiveness (7–9). A peptide derived from lymphocytic choriomeningitis virus (LCMV)* induces protective LCMV-specific CTL when given s.c. in IFA but tolerizes LCMV-specific CTL when given repeatedly i.p. at high peptide doses (10). Recently, we observed that s.c. vaccination with a peptide derived from the human adenovirus type 5 early region 1B (Ad5E1B) oncogene at doses ranging from 100 μg/mouse to as little as 1 μg/mouse evokes specific tolerance of Ad5E1B-directed CTL responses. This peptide-induced tolerization of Ad5E1B-specific CTL responsiveness was strongly associated with the inability of vaccinated mice (in contrast to nonvaccinated animals) to reject a challenge of Ad5E1B-expressing tumors (11). Similar findings were obtained in another tumor system. A single s.c. vaccination with 10 μg of a peptide representing a CTL epitope derived from the Ad5E1 oncogene induced Ad5E1A-specific CTL tolerance, causing enhanced outgrowth of Ad5E1A-transformed tumors (12). In both cases, tolerance induction was associated with rapid systemic diffusion of the peptides from the s.c. IFA depot (11, 12). These observations show that immunization with these particular peptides administered the same way and at the same doses as the aforementioned protective peptide vaccines (1–3) causes systemic peripheral CTL tolerance and indicate that the route and dose of peptide administration causing CTL priming cannot be generalized for all peptide delivered in adjuvants. More importantly, these findings indicate that clinical trials using synthetic peptide-based vaccines for the reinforcement of the host’s immune response against tumors or viral infections should be conducted with caution because they might lead to an effect opposite to that intended, namely, T cell tolerance causing enhanced tumor growth rather than protective T cell immunity.

Since it is of crucial importance to avoid T cell tolerization through peptide vaccination in a clinical setting, we set out to define vaccine formulations that also in the case of potentially tolerogenic peptides lead to protective antitumor immunity. To this end, the effectiveness was determined of different vaccine formulations reported to be capable of priming CTL responses, containing as antigenic component a peptide that readily induces specific
CTL TOLERIZATION AVOIDED BY VACCINATION WITH PEPTIDE-PULSED DC

CTL tolerance when administered s.c. in adjuvants. In this study, we show that peptide-loaded dendritic cells (DC) represent the only formulation tested that is capable of inducing CTL-mediated immunity, leading to tumor protection, whereas all other tested modes of synthetic peptide delivery cause enhanced tumor outgrowth.

Materials and Methods

Mice
C57BL/6 (H-2b) mice were obtained from IFFA Credo (France). Mice were used at 7 to 10 wk of age.

Cell lines and culture conditions
Ad5E1A-transformed mouse embryo cells (MEC) of C57BL/6 origin were generated as previously described (12, 13). Cells expressing Ad5E1A and E1ras (AR cell lines) were generated by transfection of MEC with pAd5E1A, pE1ras, and pTKneo as described previously. (14) The Ad5E1A and E1ras oncogenes are well known to efficiently cooperate in oncogenic transformation (15). In contrast to cells transformed by Ad5E1A alone, Ad5E1A + E1ras-transformed cells are capable of forming tumors in immunocompetent mice (13, 14). All cell lines were tested for expression of the transduced Ad5E1A and Ad5E1B genes by Northern blotting (data not shown). All MEC and Ad5E1A-transformed MEC were maintained in Iscove’s modified Dulbecco’s medium (IMDM) (Biocrom KG, Seromed, Berlin, Germany) supplemented with 4% FCS (HyClone Laboratories, Logan, UT), penicillin (100 IU/ml, Brocades Pharma, Leiderdorp, The Netherlands), and 2-ME (20 μM) at 37°C in a 5% CO2 atmosphere.

Generation of dendritic cells (DC)
Bone-marrow-derived DC were obtained as previously described (16–18). Briefly, bone marrow cells depleted for lymphocytes and MHC class II-positive cells were cultured overnight in IMDM supplemented with 10% FCS, 2-ME (20 μM), penicillin, sodium pyruvate (1 mM), and nonessential amino acids (10 μM) at 37°C in a 5% CO2 atmosphere in 24-well plates (106 cells in 1 ml of culture medium per well). After 24 h, the cells were replated and cultured in the presence of granulocyte-macrophage colony-stimulating factor (100 U/ml), Schering-Plough, Kenilworth, NJ) and murine IL-4 (900 U/ml; R&D Systems, Minneapolis, MN) at 37°C in a 5% CO2 atmosphere for 4 days. The medium was exchanged with fresh medium containing cytokines. On day 8, the DC were pulsed for 2 h at 37°C in 1 ml of IMDM supplemented with 0.5% BSA and 10 μg of peptide. Peptide-pulsed DC were extensively washed before they were used.

Splenic DC were isolated according to the method described by Steinman and Cohn (19). Minor modifications. Splenocytes of B6 mice were injected in vitro with 0.5 ml of collagenase (100 U/ml) (Clostridium histolyticum, type IV, Sigma Chemical, St. Louis, MO), cut into pieces, and incubated at 37°C for 15 min in 1 ml of collagenase. A spleen cell suspension was made in IMDM supplemented with 15% FCS, penicillin (100 IU/ml), kanamycin (100 μg/ml), and 2-ME (2 × 10–5 M). The cells were spun on a discontinuous BSA gradient (Sigma Chemical) of 10, 28, and 35% BSA at 4°C for 30 min at 10,000 × g. The interphase between 10 and 28% BSA was removed, and the cells were cultured for 90 min at 37°C in glass petri dishes. Nonadherent cells were discarded, and the culture medium was replaced. After a further 18 h of culture, the nonadherent cells were harvested and used for peptide loading as described above.

Peptides
Synthetic peptides were constructed on an Abimed 422 multiple peptide synthesizer in Abimed, Langenfeld, Germany. All peptides were synthesized on polymer support as previously described (20). Palmitoyl peptides were prepared by reactions with Palmyr-BOP-NMM (palmitic acid-(benzotriazole-1-yl-oxy-tris-pyrolidinophosphonium hexafluorophosphonato) (–N-methylmorpholine)) as the last step in the synthesis. For the preparation of bis-palmitoyl derivatives, peptides were elongated by coupling with Fmoc-Lys(Fmoc)-OH. After Fmoc removal, coupling was performed with Palmyr-BOP-NMM. Palmitoyl peptides were purified by reversed phase HPLC to ensure the absence of nonpalmitoylated peptide material. In short, a solution of the crude material in 50% H2O/AC was applied to a semipreparative reversed phase HPLC column. Gradient elution was performed in water, acetoni trile, 0.1% trifluoroacetic acid (TFA) with gradients of 1.5 to 2% acetonitrile increase per minute. The following palmitoyl-containing peptides were used in these studies: Pam-TSLTPRCNTAWNR-LKQ4AEFDRAHYVINVT, containing the Friend murine leukemia virus T helper epitope linked to a H-2Dd-restricted CTL epitope derived from the human papilloma virus type 16 (HPV16) E7 protein (3) by the natural sequence of the HPV16 E7 epitope; Pam-STDGSCSAPSNTPEP1, containing the Ad5E1A-encoded CTL epitope and the natural flanking sequences of the Ad5E1A epitope; (Pam)-KSSKOVAAWTLLAKASGPSNTPEP1, where O = cyclohenxylalanine, containing the pan-T helper epitope “PADRE” (that enhances CTL induction in B6 mice) (22), linked to the Ad5E1A epitope by a three-alamine spacer as described elsewhere (23); (Pam)-KSSKOVAAW TLKAAAVBRNCYYI, containing the pan-T helper epitope “PADRE” linked to the H-2Dd-restricted Ad5E1B-derived CTL epitope (14) by a three-alamine spacer.

The purity of the peptides was determined by analytical reversed phase HPLC using a water-acetonitrile gradient containing 0.1% TFA and proved to be at least 90% (UV 214 nm). The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry on a laser mass spectrometer (Finngan MAT, Hemel Hempstead, U.K.). About 5 pmol of the peptide in 0.5 μl of water-acetonitrile containing 0.1% TFA were mixed with 0.5 μl of matrix solution (10 mg/ml in acetonitrile-water, 60:40 (v/v) containing 0.1% TFA) and applied to the instrument. Calibration was performed with peptides of known molecular mass, either as external or as internal references. The amount of free Ad5E1A peptide was less than 1%.

Liposome preparation
Partially hydrogenated egg phosphatidylcholine with an iodine value of 40 was obtained from Asahi Chemical Industry, (Ibarakiken, Japan). Egg phosphatidylglycerol was a gift from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol) was from Sigma Chemical. A lipid mixture of partially hydrogenated egg phosphatidylcholine-egg phosphatidylglycerol-Chol (10:1:4 molar ratio) was prepared in chloroform-methanol (10:1) and dried to a thin film by rotary evaporation. After evaporation for at least 1 h, the lipid film was hydrated with 5% acetic acid (210 μmol of phospholipid per ml) containing the Ad5E1A peptide (5 mg/ml). The resulting peptide-containing liposome dispersion was subsequently diluted 50-fold with HEPES buffer (50 mM HEPES, 73 mM NaCl, pH 7.6). Nonentrapped peptide was removed by ultra centrifugation at 160,000 × g during 20 min. After decantation of the supernatant, the pellet was resuspended and subsequently washed three times with HEPES buffer (10 mM HEPES, 140 mM NaCl, pH 7.4). The liposomes were diluted with 5 mM HEPES buffer to a final phospholipid concentration of 17 μmol/ml. The liposomes were stored at 4°C under nitrogen atmosphere and used to immunize mice the next day.

To determine the amount of peptide encapsulated in liposomes, a mixture of 0.5 ml of liposomes and 0.5 ml of acetonitrile was vortexed for 5 min. The mixture was centrifuged (13,000 × g), and 0.5 ml of supernatant containing the supernatant was lyophilized. The peptide was reconstituted in 1.0 ml of water, and aliquots were applied to reversed phase HPLC. Peptide content was determined by comparison of HPLC peak areas to those of known amounts of peptide. Peptide leakage from liposomes was determined 48 h after construction. Liposomes were washed with PBS, and after concentration of the washings the peptide content of the washings was determined as described above. Peptide leakage was <1%. Estimate the number of peptide encapsulated in liposomes, a mixture of 0.5 ml of liposomes and 0.5 ml of acetonitrile was vortexed for 5 min. The mixture was centrifuged (13,000 × g), and 0.5 ml of supernatant containing the supernatant was lyophilized. The peptide was reconstituted in 1.0 ml of water, and aliquots were applied to reversed phase HPLC. Peptide content was determined by comparison of HPLC peak areas to those of known amounts of peptide. Peptide leakage from liposomes was determined 48 h after construction. Liposomes were washed with PBS, and after concentration of the washings the peptide content of the washings was determined as described above. Peptide leakage was <1%.

Immunizations and tumor cell challenge
Peptide immunizations were performed as described in the legends to the figures. Two weeks after immunization, the mice were challenged s.c. with Ad5E1A + ras-transformed tumor cells in PBS. When a vaccination protocol was studied for its ability to induce Ad5E1A-specific CTL tolerance, the mice were challenged with AR6 cells (106 cells). This tumor cell clone causes growth of small tumors in 80 to 100% of naive B6 mice. After 10 wk, 20 to 30% of the animals have died because of a progressively growing tumors. The other animals that have developed a tumor still carry it or have eradicated the tumor (12). The outgrowth of this tumor can no longer be controlled by the animals after s.c. injection of 10 μg of Ad5E1A peptide inIFA (12). AR5 cells (0.5 × 106 cells/mouse) were used to analyze the ability of peptide-loaded DC to induce protective immunity against an otherwise lethal challenge with this Ad5E1A + ras-transformed tumor cell line. Injection of 0.5 × 106 cells of this tumor cell clone kills 75 to 100% of the animals in 5 to 6 wk.

Generation and analysis of CTL bulk cultures
Ad5E1A-specific CTL in bulk culture were generated as follows: 5 × 106 spleen cells per well derived from B6 mice taken 3 wk or more after the second i.v. immunization with 0.5 × 106 peptide-loaded bone marrow-derived DC were cocultured for 6 days with 10% irradiated (25 Gy) IFN-γ (10 U/ml)-treated Ad5E1A-transformed stimulator cells in 24-well plates.
Next, effector cells were harvested, and dead cells were removed by density centrifugation on Lympholyte M (Cedarlane, Hornby, Canada). These cells were used in a cell-mediated lymphocyte cytotoxicity assay as described previously (3, 14).

Primary in vitro induced Ad5E1-specific CTL bulk cultures were obtained by coculturing $5 \times 10^6$ spleen cells derived from a naive B6 mouse with 10% Ad5E1 peptide-loaded bone marrow-derived DC. After 7 days, the cells were harvested and, after removal of dead cells by density centrifugation, were restimulated with 10% Ad5E1-transformed stimulator cells. Six days thereafter, the bulk CTL cultures were used in a TNF production assay as previously described (24). In short, $5 \times 10^3$ bulk CTL were added to $2 \times 10^3$ stimulator cells in a total volume of 100 µl of medium containing 10 Cetus U rIL-2 (Cetus, Emeryville, CA) in wells of a 96-well U-bottom plate. After 24 h, 60 µl of supernatant were collected, and its TNF content was determined by measuring its cytotoxic effect on WEHI-164 clone 13 cells. The percentage of WEHI cell death was calculated by the formula: $[\text{OD}_{550-560} \text{ in experimental wells} - \text{OD}_{550-560} \text{ in wells containing medium only}] / \text{OD}_{550-560} \text{ in wells containing medium only}] \times 100$

Results
Enhanced tumor outgrowth after immunization with liposome-encapsulated Ad5E1A peptide

Previously, we have shown that immunization with synthetic peptides can lead to enhanced outgrowth of tumors. A single s.c. (local) injection of as little as 10 µg of a peptide derived from the Ad5E1A oncoprotein (sequence, SGPSNTPEP) in IFA led to enhanced outgrowth of Ad5E1A-transformed tumor cells, whereas injection of a peptide derived from the Ad5E1B oncoprotein (sequence, VNIRNCCYI) induced enhanced outgrowth of Ad5E1B-expressing tumor cells (11, 12). In both cases, enhanced tumor outgrowth was accompanied by functional deletion of Ad5E1A- and Ad5E1B-specific CTL, respectively, and associated with rapid systemic distribution of the peptides from the s.c. IFA depot. Since such tolerizing effects of peptide vaccination are unacceptable in a clinical setting, we set out to define vaccine formulations that would not induce CTL tolerance but instead would lead to protective antitumor immunity. We focused on vaccination strategies that 1) have been shown to be able to induce CTL-immunity in other model systems and 2) are likely to retain the peptide locally or are able to deliver the peptide to phagocytes such as macrophages or to DC, because of the possible correlation between systemic distribution of CTL epitope-containing peptides and their ability to induce tolerance (9–12).

FIGURE 1. Vaccination with Ad5E1A peptide encapsulated in liposomes leads to the inability of vaccinated mice to reject a challenge with Ad5E1A + ras-transformed tumor cells. B6 mice were left untreated, were immunized s.c. with $10^7$ irradiated Ad5E1A + ras cells in PBS, were immunized i.v. (A) or s.c. (B) with 10 µg of Ad5E1A peptide encapsulated in liposomes, with empty liposomes only, with 10 µg of Ad5E1A peptide in IFA (B) ($n = 7–8$ animals per group). Two weeks later, the mice were challenged s.c. with $10^7$ live Ad5E1A + ras-transformed tumor cells (tumor cell clone AR6). The percentage of surviving animals is shown. Mice immunized with 10 µg of Ad5E1A peptide entrapped in liposomes die earlier than mice vaccinated with empty liposomes ($p = 0.004$ (A); $p = 0.001$ (B), logrank test).

Enhanced tumor outgrowth after immunization with lipopeptide vaccines

Synthetic lipopeptide-based vaccines can induce peptide-specific CTL responses that cross-react on targets expressing endogenous Ag. Lipopeptide-based vaccines are even considered to be superior in priming CTL responses compared with vaccination with free synthetic peptide in adjuvants, or other vaccination strategies using recombinant viruses and bacteria (23, 29–31). To study the in vivo effects of a lipopeptide containing the Ad5E1A epitope, we immunized mice s.c. with Ad5E1A lipopeptide in PBS. Two weeks later, these mice were challenged with Ad5E1A + ras cells. Lipopeptide-immunized mice are no longer able to control the outgrowth of Ad5E1A-expressing tumor cells (Fig. 2A). Also, addition of a known T helper epitope derived from Friend murine leukemia virus (21) did not prevent the outgrowth of Ad5E1A + ras tumors, but instead led to enhanced tumor outgrowth (Fig. 2A). A similar observation was made when the lipopeptide vaccine was administered together with rIL-2 ($10^5$ Cetus U/mouse), or administered i.v. in PBS (data not shown). Likewise, vaccination with an Ad5E1B-containing lipopeptide vaccine caused an accelerated outgrowth of Ad5E1B-expressing tumors (but not of Ad5E1A + ras-transformed tumors lacking the relevant Ad5E1B-Ag) on challenge with an Ad5E1B-expressing tumor cell (data not provided).

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Mice were immunized s.c. on their left flank with: 10^7 irradiated Ad5E1A (sequence RAHYNIVTF) peptide in IFA; 10 nmol of Ad5E1A peptide linked to a Pam molecule in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of (control) lipopeptide containing the Ad5E1B epitope and the promiscuous T helper epitope “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of lipopeptide containing the Ad5E1A epitope and the promiscuous T helper peptide “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of lipopeptide Pam-TSLTPRCNTAWNRKGGQAEPDRAHYNIVTF (sequence, VNIRNCCY1); or 10 nmol of lipopeptide containing the Ad5E1A epitope and the promiscuous T helper peptide “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of lipopeptide containing the Ad5E1B epitope and the promiscuous T helper epitope “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI) (all shown in A). Likewise, mice were immunized s.c. on their left flank with 10^7 live Ad5E1A + ras cells in PBS; 10 nmol of Ad5E1A peptide in IFA; 10 nmol of Ad5E1B (control) peptide (sequence, VNIRNCCY1) in IFA; 10 nmol of (control) lipopeptide containing the Ad5E1B epitope and the promiscuous T helper epitope “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of lipopeptide containing the Ad5E1A epitope and the promiscuous T helper peptide “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI) (all shown in B) (n = 7–8 animals/group). Two weeks later, the mice were challenged s.c. on their right flank with 10^7 live Ad5E1A + ras-transformed tumor cells (tumor cell clone AR6). The percentage of surviving animals is shown. Mice immunized with Pam-T helper-Ad5E1A peptide die earlier than mice vaccinated with Pam-T helper-HPV16 E7 peptide (p = 0.002, logrank test), whereas mice immunized with Pam2-T helper-Ad5E1A peptide die earlier than mice vaccinated with Pam2-T helper-Ad5E1B peptide (p = 0.005, logrank test).

Peptide-loaded DC induce strong CTL memory responses and protective antitumor immunity

Peptide-pulsed DC have been shown to induce protective and therapeutic antitumor immunity in several model systems, but they have also been reported to present Ag in a tolerogenic fashion (18, 32–36). To analyze whether the CTL induced by immunization with Ad5E1A or Ad5E1B peptides, when pulsed on DC, are able to initiate protective antitumor immunity in vivo, we immunized mice with peptide-loaded DC. Bulk CTL cultures derived from mice immunized with Ad5E1A peptide-pulsed DC lyse Ad5E1A peptide-loaded target cells, as well as Ad5E1-transformed cells (expressing both the Ad5E1A and Ad5E1B onco genes) and Ad5E1A + ras cells (expressing the Ad5E1A oncogene only) (Fig. 4). Bulk CTL cultures obtained from mice immunized with Ad5E1B peptide-pulsed DC kill Ad5E1B-loaded target cells and Ad5E1-transformed cells, but not Ad5E1A + ras tumor cells (not expressing the Ad5E1B oncogene). These data show that immunization with Ad5E1A or Ad5E1B peptide-loaded DC does not induce specific CTL tolerance but leads to a strong peptide- and tumor-specific CTL-mediated immune response in vivo.

To test whether the Ad5E1A or Ad5E1B peptides, when pulsed on DC, are able to initiate Ad5E1-specific CTL-mediated immunity in vivo, we immunized mice with peptide-loaded DC.Bulk CTL cultures derived from mice immunized with Ad5E1A peptide-loaded DC lyse Ad5E1A peptide-loaded target cells, as well as Ad5E1-transformed cells (expressing both the Ad5E1A and Ad5E1B oncogenes) and Ad5E1A + ras cells (expressing the Ad5E1A oncogene only) (Fig. 4). Bulk CTL cultures obtained from mice immunized with Ad5E1B peptide-pulsed DC kill Ad5E1B-loaded target cells and Ad5E1-transformed cells, but not Ad5E1A + ras tumor cells (not expressing the Ad5E1B oncogene). Similar results were obtained when peptide-loaded DC isolated from spleen were used for immunization (data not shown). These data show that immunization with Ad5E1A or Ad5E1B peptide-loaded DC does not induce specific CTL tolerance but leads to a strong peptide- and tumor-specific CTL-mediated immune response in vivo.

FIGURE 2. Mice immunized with an Ad5E1A-containing lipopeptide are unable to control the outgrowth of Ad5E1A + ras-transformed tumor cells. Mice were immunized s.c. on their left flank with: 10^7 irradiated Ad5E1A + ras cells in PBS; 10 nmol of Ad5E1A peptide in IFA; 10 nmol of HPV16 E7 (control) peptide (sequence RAHYNIVTF) peptide in IFA; 10 nmol of Ad5E1A peptide linked to a Pam molecule in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of the control lipopeptide Pam-TSLTPRCNTAWNRKGGQAE PDRAHYNIVTF in PBS (all shown in A). Likewise, mice were immunized s.c. on their left flank with 10^7 irradiated Ad5E1A + ras cells in PBS; 10 nmol of Ad5E1A peptide in IFA; 10 nmol of Ad5E1B (control) peptide (sequence, VNIRNCCY1) in IFA; 10 nmol of (control) lipopeptide containing the Ad5E1B epitope and the promiscuous T helper epitope “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI); or 10 nmol of lipopeptide containing the Ad5E1A epitope and the promiscuous T helper peptide “PADRE” linked to two Pam molecules in PBS (Pam2-KSSKOVAAWTLKAASGPNSNTPPEI) (all shown in B) (n = 7–8 animals/group). Two weeks later, the mice were challenged s.c. on their right flank with 10^7 live Ad5E1A + ras-transformed tumor cells (tumor cell clone AR6). The percentage of surviving animals is shown. Mice immunized with Pam-T helper-Ad5E1A peptide die earlier than mice vaccinated with Pam-T helper-HPV16 E7 peptide (p = 0.002, logrank test), whereas mice immunized with Pam2-T helper-Ad5E1A peptide die earlier than mice vaccinated with Pam2-T helper-Ad5E1B peptide (p = 0.005, logrank test).
ras-transformed tumor cell clone AR5 (Fig. 5). The tumor cell clone AR5 has a much higher growth rate in vivo than AR6. Therefore, an accelerated tumor growth rate after Ad5E1A peptide immunization in IFA is not observed, because the animals in the control group must be euthanized before the differences with the Ad5E1A peptide-immunized group become apparent. Mice immunized with freshly isolated bone marrow cells pulsed with the Ad5E1A peptide are not protected against a challenge with AR5 cells (Fig. 6), illustrating the necessity to use DC for the induction of protective antitumor immunity. A delayed outgrowth of Ad5E1A ras tumors is observed in mice vaccinated with control peptide-loaded DC compared with mice that did not receive DC (Figs. 5 and 7). This is most likely due to “nonspecific” T cell reactivity against FCS components as also observed by others (37) (both tumor cells and DC are cultured in FCS-containing medium), since no delayed outgrowth of tumors is observed when Ad5E1A + ras cells that have been cultured for 3 weeks in serum-free medium are used for tumor challenge (data not shown). DC derived from bone marrow cultures are as potent in eliciting tumor-protective immunity as DC isolated from the spleen (Fig. 7). Both DC populations induce optimal tumor protection when given i.v., whereas only partial protection is induced after s.c. administration of Ad5E1A peptide-loaded DC (Fig. 7). These data indicate that i.v. administration of DC-based vaccines is more effective than s.c. injection of this type of vaccine, as also reported by others (38). Taken together, these results show that the Ad5E1A peptide is able to induce protective antitumor immunity when loaded on DC and point to the superior qualities of DC in the induction of effective antitumor immunity associated with CTL memory responses over several other vaccine vaccine formulations that may lead to specific CTL tolerance.

**Discussion**

Peptide-based vaccines can induce potent antiviral and antitumor immunity as shown in several murine models (1–4). Based on these findings, vaccines using peptide in adjuvants are currently being tested in clinical trials for their ability to prime or boost tumor-reactive CTL in patients with cancer (5). Nonetheless, such
vaccines should be applied with caution, because peptide in adjuvants can also induce specific CTL tolerance (11, 12). The questions why some peptides given locally in adjuvant induce specific CTL immunity whereas others induce specific CTL tolerance are intriguing. One possibility is that tolerance is caused by rapid systemic distribution of the peptides after (local) injection, causing massive peptide presentation on nonprofessional APC or nonactivated professional APC, resulting in insufficient costimulation required for clonal expansion (39). Likewise, systemic distribution of peptide may activate virtually all specific CTL, rather than a fraction of these CTL (as would be the case after local initiation of the immune response), resulting in activation-induced cell death or clonal exhaustion of terminally differentiated effector cells (40).

Peptides that are given systemically or that disperse throughout the body after local s.c. injection have been shown to be able to induce specific CTL tolerance (7, 9, 10, 12). Vaccine approaches capable of controlling the diffusion of Ags would, therefore, have distinct advantages over methods that can lead to systemic distribution of Ags. For this reason, we have focused in this study on vaccine modalities that are likely to retain the peptide locally or that have been shown to deliver the peptides to phagocytes. Immunization of mice with lipopeptide vaccines encoding influenza virus nucleoprotein-derived CTL epitopes leads to the induction of influenza virus-specific CTL (23, 29). In this model, immunization with an influenza epitope-containing lipopeptide was shown to be superior over immunization with free peptide. Moreover, the CTL response was significantly enhanced when a T helper epitope was
incorporated into the vaccine (23, 29, 41, 42). Likewise, in a Plasmodium berghei model, a stronger CTL response was induced after immunization with a lipid-tailed peptide compared with vaccination with free peptide in adjuvant (30). Despite the encouraging results obtained in these models, immunization with an Ad5E1A-containing lipopeptide results in the inability of vaccinated mice to control a challenge with Ad5E1A-expressing tumor cells (Fig. 2A), whereas vaccination with an Ad5E1B-containing lipopeptide leads to the inability of immunized animals to reject a challenge with Ad5E1B-expressing tumor cells (data not shown). Also a lipopeptide mimicking a lipopeptide vaccine designed for use in a clinical setting (23) equipped with two lipid molecules containing the Ad5E1A epitope in addition to a pan class II-binding T helper epitope that has been shown to enhance peptide-induced CTL responses in B6 mice (22) did not induce protective antitumor immunity. Instead, vaccination resulted in the enhanced outgrowth of Ad5E1A-expressing tumor cells (Fig. 2B), indicating that also this vaccine formulation does not prevent tolerization by the Ad5E1A peptide.

Previously, we have shown that s.c. administration of Ad5E1A- or Ad5E1B-peptide in IFA, together with i.v. injection of a CTL clone specifically recognizing the injected peptide, induces a severe respiratory syndrome, most likely due to activation of Ad5E1-specific CTL that are trapped in the vascular bed of the lungs (11, 12). This severe immunopathologic damage induced by peptide Ag treatment in the presence of a substantial amount of memory CTL located in a particular organ can be used as a readout to determine whether the peptides rapidly diffuse out of the s.c. depot into the periphery. Mice given the combination of Ad5E1A lipopeptide and i.v. injection of Ad5E1A-specific CTL (but not of Ad5E1B-specific CTL) suffered from severe respiratory problems. The same was observed when injection of Ad5E1B lipopeptide was combined with i.v. injection of Ad5E1B-specific CTL (data not shown), indicating that s.c. administered Ad5E1-peptides linked to lipid tails are not retained locally but disperse throughout the animals. These findings may be explained by cleavage of the lipopeptides by proteases and/or lipases in the body, liberating free Ad5E1A peptide, which then diffuses throughout the body, instigating Ad5E1-specific CTL tolerance. Moreover, they point to a similar erratic behavior of lipopeptide vaccines in vivo as peptides delivered in adjuvants. In contrast, with Ad5E1A peptide-loaded DC, followed by infusion of Ad5E1-specific CTL, does not induce lung congestion (data not shown).

Soluble Ags entrapped in liposomes induce CTL responses after i.v. injection (27, 28). Depletion of macrophages in vivo abolishes the induction of Ag-specific CTL in this system, indicating that liposomes are able to deliver the encapsulated Ags to cells important for proper CTL induction (28). Nonetheless, i.v. or s.c. immunization with Ad5E1A peptide encapsulated in liposomes leads to enhanced outgrowth of Ad5E1A-transformed tumor cells. Although other types of liposomes may be more effective in eliciting Ad5E1A-specific CTL responses, these observations indicate that also this mode of Ag delivery does not prevent the induction of peptide-specific CTL tolerance, most likely due to release of free Ad5E1A peptide in vivo.

On the basis of these results, we applied the Ad5E1 peptides directly to ex vivo generated DC and used these as vaccine vehicles. Peptide-loaded DC have been shown to induce both protective and therapeutic antitumor immunity when used for vaccination (18, 33, 34, 37, 43). Although DC are unmatched in their ability to activate naive T cells (44), they have also been reported to be able to present Ag in vivo in a tolerogenic fashion (35, 36). The latter is, however, not the case in the Ad5E1-tumor system. DC loaded with Ad5E1A or Ad5E1B peptide are able to induce strong Ad5E1A- and Ad5E1B-specific CTL-responses, respectively, both in vivo and in vitro. More importantly, mice immunized with Ad5E1A peptide-loaded DC are protected against an otherwise lethal challenge with Ad5E1A-transformed tumor cells. These findings indicate that the Ad5E1 peptides do not act as antagonist peptides. Therefore, antagonism of these peptides in vivo is an unlikely explanation for the observed CTL tolerance induction. Also, these peptides can apparently induce protective Ad5E1-specific CTL responses, instead of Ad5E1-specific tolerance, when given in the right context. In this regard, costimulation seems to be particularly important for the induction of protective antitumor immunity, since it has been shown that blockade of CD80 and CD86 by CTLA4-Ig abrogated the tumor-protective effects of peptide-pulsed DC in vivo (34).

Thus, in summary, immunization with Ad5E1A peptide-loaded DC induces protective antitumor immunity against Ad5E1A-positive tumor cells, in contrast to other synthetic peptide vaccination strategies. These vaccination strategies include vaccination with Ad5E1A lipopeptides given s.c. in PBS, with or without known T helper epitopes, with or without IL-2, Ad5E1A lipopeptide given i.v., Ad5E1A peptide given s.c. in adjuvants (like IFA or CFA (12)), Ad5E1A peptide given i.v. in PBS, or Ad5E1A peptide encapsulated in liposomes given s.c. or i.v. Likewise, immunization with Ad5E1B peptide-loaded DC (a CTL epitope presented by different tumor cells and recognized by different CTL) induces Ad5E1B-specific CTL responses both in vitro and in vivo. In contrast, s.c. vaccination of this peptide in adjuvant or Ad5E1B-peptide linked to a lipid tail injected s.c. or i.v. in PBS evokes a specific functional deletion of Ad5E1B-directed CTL (11), and this report). These data show that DC can revert the CTL-tolerizing potential of a synthetic peptide vaccine into a vaccine inducing protective antitumor immunity and provide the rationale for using DC as the primary vehicles of choice for the development of synthetic peptide-based vaccines.

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References


