DNA vaccines against the human papillomavirus type 16 E6 or E7 oncoproteins

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DNA vaccines expressing the E6 or E7 oncoproteins of human papilloma virus type 16 (HPV-16) in either their wild-type form or fused to sequences that affect intracellular trafficking were tested for induction of protective immunity against tumor cell challenge in two models based on BALB/c and C57Bl/6 mice. The DNA vaccines to E7 gave uniformly disappointing results, while the DNA vaccine that expressed E6 linked to a viral leader sequence protected BALB/c mice against tumor cell challenge given before or after vaccination. The efficacy of this vaccine could be enhanced by a DNA vector prime/viral vector boost regimen. In contrast, priming of mice with the DNA vaccines to E7 reduced the efficacy of a viral vector expressing the same antigen.

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Cervical cancer is most commonly a consequence of sexually transmitted, persistent infections with oncogenic types of HPVs, most notably HPV-16.¹ Cervical cancer is the second most common cause of cancer death in women worldwide, claiming approximately 300,000–500,000 lives each year. Upon sexual transmission, HPV-16 (and other oncogenic types of sexually transmitted HPVs) infects the basal and parabasal cells of the cervical mucosa where transcription and translation of the early antigens of HPV-16 leads to cellular atypia followed by preinvasive neoplasia.² Cellular transformation is initiated by the three viral oncoproteins, E5, E6 and E7, which cause dysregulation of cell cycle control.^{2,3} The oncoproteins of HPV-16 being causative for transformation of HPV-16-infected cells are thereby natural targets for active immunotherapy of HPV-16-associated cervical cancer.

A number of vaccine modalities to HPV-16 oncoprotein-expressing tumor cell lines have been tested in preclinical animal models focusing mainly on vaccines to E7,^{4–10} although studies with vaccines to E6^{4,7,10} and E5¹¹ have also been reported. Our vaccine effort initially focused on viral recombinant vaccines expressing the E6 or E7 protein. They showed that E1-deleted adenoviral vectors to E7 induce potent and tumor-protective CD8⁺

T-cell responses in C57Bl/6 mice. In contrast, BALB/c mice displayed partial CD4⁺ T-cell-mediated protection induced by vaccinia or adenoviral recombinant vaccines expressing the E6 of HPV-16 but could not be protected by the vaccines to E7.⁷

In this report, we extended our studies to DNA vaccines expressing the E7 or E6 proteins of HPV-16. In addition to those expressing wild-type versions of the oncoproteins, we also tested DNA constructs that express the oncoproteins linked to various polypeptides that affect intracellular trafficking. In one construct, the oncoproteins were fused to a viral leader sequence that prevents nuclear localization and instead affects secretion. A second modification involved addition of a leader sequence and the lysosome-associated membrane protein (LAMP)-1, which channels proteins upon secretion into lysosomes resulting in improved association with MHC class II determinants. This modification, used previously for HPV-16 E7 vaccines, was shown to also improve the induction of MHC class I-restricted CD8⁺ T-cell responses.^{5,12} Lastly, we expressed E6 and E7 linked to ubiquitin that promotes proteolytic degradation of proteins, thus increasing the pool of peptides available for transport into the endoplasmic reticulum where binding to MHC class I occurs.¹³ Our results showed poor efficacy of DNA vaccines expressing E7 in a C57Bl/6 subcutaneous tumor cell challenge model. In contrast, DNA vaccines expressing E6 linked to a leader sequence provided protective immunity in the BALB/c tumor cell challenge model both if given prophylactically or therapeutically. In BALB/c mice, but not in C57Bl/6 mice, vaccine efficacy could be improved by DNA vaccine priming followed by a booster immunization with a low dose of an E1-deleted viral recombinant vector expressing the corresponding antigen.

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Materials and methods

Animals

Female 6–8-week-old BALB/c mice (H-2d) and C57Bl/6 mice (H-2b) (Taconic, Germantown, NY) were used for all experiments. Mice were housed at The Animal Facility of The Wistar Institute. Rats were purchased from Harlane Sprague-Dawley (Indianapolis, IN) and used at 3 months of age.

Cell lines

The following cell lines were used throughout the experiments: 293 cells were used for propagation of adenoviruses. COS-7 cells were utilized for transfection studies. The t(10)1rasE7E6 cell line is derived from a primary embryonal BALB/c fibroblast cell line spontaneously immortalized due to the loss of both p53 alleles and transformed by transfection with a triple promoter retroviral vector expressing v-Ha-ras and E6 and E7 of HPV-16.⁷ The TC-1 cell line (provided by TC Wu, John Hopkins, Baltimore, MD) originated from lung epithelia cells immortalized by transfection with the v-Ha-ras, E6 and E7 genes of HPV-16.⁵ All of the above-described cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), HEPES buffer and antibiotics. HT-2 cells, which are dependent on IL-2 or IL-4 for survival and proliferation were grown in DMEM supplemented with 10% FBS, 10^{-6} M 2-mercaptoethanol and 10% rat concanavalin A supernatant.

Expression vectors

DNA vaccine constructs. All DNA vaccines are based on a modified version of the expression vector pVR1012.2. containing the cytomegalovirus (CMV) promoter (generously provided by Vical, Inc., San Diego, CA). The kanamycin-resistance gene was removed from the vector and replaced with the ampicillin-resistance gene. The ampicillin-resistance gene has two strong CpG motifs that are active in mice.¹⁴ Constructs pVRE6, pVRE7, pVRLDE6 and pVRLDE7 have been previously described.¹⁵ The plasmids pVRLDE6 and pVRLDE7 express the E19 leader sequence of the human adenovirus (AdHu) serotype 2 fused to the amino terminus of the HPV-16 oncoproteins.

Constructs pVRE6LAMP-1 and pVRE7LAMP-1 were generated from pre-existing constructs (pCVMSigE6-LAMP-1 and pCVMSigE7LAMP-1). Specific oligonucleotides (CyberSyn, Lenni, PA) were generated based on the sequence for the signal peptide and the LAMP-1 cytoplasmic domain¹² containing the *Xba*I and *Bam*HI restriction sites (5' and 3' ends respectively). The pre-existing constructs were used as templates for a standard polymerase chain reaction (PCR). The PCR fragment was digested with *Xba*I and *Bam*HI and ligated into similarly digested pVR1012.2. These constructs express the E6 and E7 oncoproteins fused to the signal sequence and the integral membrane sequence of the LAMP-1 protein.

To generate constructs pVRE6UBI and pVRE7UBI, the pVRE6 and pVRE7 vectors were digested with *Xba*I and *Bam*HI enzymes. The liberated oncogenes were ligated into pVRp53UBI from which the p53 insert had been removed using the same restriction enzymes.¹⁶ The final constructs express the ubiquitin protein fused to the amino terminus of the HPV-16 oncoproteins.

Correct insertion of the fragments was verified by sequencing through the Genomics Facility of The Wistar Institute.

Adenoviral expression constructs. E1-deleted replication-defective human serotype 5 adenovirus recombinants expressing either the E6 or E7 genes of HPV-16⁷ or the L1 of HPV-16¹⁷ have been described previously.

Recombinant baculovirus expression constructs. Baculovirus recombinants to HPV-16 E7 and E6 were used to generate oncoproteins as described previously.¹⁵

Expression of baculovirus recombinant proteins and their purification. Recombinant baculovirus was generated by the Vector Core Facility of The Wistar Institute by cotransfection of the transfer vector with BaculoGold DNA (BD, Pharmingen, Torre Pines, CA) into Sf9 insect cells as described.¹⁸ For protein production, Sf9 cells (500 ml: 5×10^6 cells/ml) were infected at a multiplicity of infection of 10 with the various baculovirus recombinants for 72 hours. Cells were then harvested and freeze/thawed $3 \times$ in PBS. Following low-speed clarification, cell debris-free supernatants were precipitated with ammonium sulfate. Precipitated proteins were aliquoted and protein content was determined by the Bradford method using a Bio Rad protein assay (Bio Rad, Hercules, CA).

Propagation and purification of viruses

Adenovirus recombinants were propagated on the E1-transfected 293 cell line. Semiconfluent monolayers of cells were infected with 0.1 plaque forming units (PFU)/cell of virus. Cells were harvested after 3 days and freeze/thawed three times. Cell debris was removed by centrifugation at 2000 r.p.m for 30 minutes. Virus was titrated on 293 cell monolayers. Cells plated in 6-well plates were infected with serial dilutions of virus-containing medium. The following day cells were overlaid with agarose. Viral plaques were counted 4–8 days later.

Immunization of mice

DNA vaccines (50–100 μ g) were injected into mice intramuscularly (i.m.) in both quadriceps muscles, dividing the dose equally to reduce variability. Mice were boosted once with the same DNA vaccine (50–100 μ g) 3 weeks after the initial injection. Some mice were further boosted subcutaneously (s.c.) 2–3 weeks later with 10^6 PFU of replication-defective adenovirus expressing the E6, the E7 or the L1 of HPV-16. At 3 weeks after the DNA boost or 2 weeks after the viral boost, mice were challenged s.c. with 3×10^4 t(10)1rasE7E6 cells or with 2×10^5 TC-1 cells. After tumor cell challenge, mice were

checked three times/week. They were scored as tumor bearing when the s.c. growth exceeded 2 mm in diameter. Mice were euthanized once tumors reached a size of ~1 cm in diameter. Mice were depleted of NK cells with a rabbit serum against asialo-GM1 ganglioside (Wako Inc., Wako, TX) as described.¹⁹

In vitro expansion of splenocytes

Splenocytes were harvested 2 weeks after immunization and cultured at 6×10^6 cells in 1.6 ml. of DMEM (with 2% FCS, HEPES buffer, 10 mM 2-mercaptoethanol, nonessential amino acids, glutamine, sodium pyruvate and antibiotics). Adenoviral recombinants (0.1 PFU/cell) or baculovirus produced protein (1 g/ml) were added to each well.

Intracellular cytokine staining

Freshly harvested splenocytes were incubated with 1 μ g/ml of the peptide carrying the immunodominant H-2b MHC class I epitopes of E7 (RAHYNIVTF) or a p53 control peptide for 5 hours in 96-well U-bottom plates (Costar, Cambridge, MA) at a concentration of 10^6 cells/well in a volume of 200 μ l complete DMEM supplemented with 50 U/ml of human recombinant IL-2 and 1 μ l/ml of Brefeldin A (GolgiStop, Pharmingen, La Jolla, CA²⁰). After 5 hours, the cells were washed and surface-stained with an FITC-conjugated rat anti-mouse CD8 antibody (Pharmingen, La Jolla, CA) followed by fixation/permeabilization (Cytofix/Cytoperm, Pharmingen) and intracellular staining for IFN- γ (PE-conjugated rat anti-mouse IFN- γ , Pharmingen) according to the manufacturer's instructions. After washing, cells were examined by two-color flow cytometry using an EPICS Elite XL (Beckman-Coulter, Inc., Miami, FL) and data were analyzed using the WinMDi software.

Cytokine-release assay

IL-2 secretion was tested for by culturing 6×10^6 splenocytes of immune or sham-vaccinated mice in 1.6 ml. DMEM supplemented with 2% FCS and 10^{-6} M 2-mercaptoethanol. Baculovirus-derived E6 or E7 protein was added to a final concentration of 1 μ g/ml. Supernatants were harvested 24 hours later and tested for induction of proliferation of the IL-2-dependent HT-2 indicator cell line as described.²¹

Results

Validation of DNA constructs

Construction of pVRE6, pVRE6LD, pVRE6LAMP-1, pVRE6UBI, pVRE7, pVRE7LD, pVRE7LAMP-1 and pVRE7UBI were derived as described in Material and methods. The inserts were sequenced and transcript expression was confirmed by a reverse transcription PCR. The different vectors including the viral vectors used for vaccination are listed in Table 1.

Table 1 List of vectors used for vaccination

Vaccine constructs	Inserts (derived from HPV-16)
<i>DNA vectors</i>	
pVR1012.2 ^a	None
pVRE6	Wild-type E6 sequence
pVRE7	Wild-type E7 sequence
pVRE6LD	E6 sequence fused to viral leader sequence
pVRE7LD	E7 sequence fused to a viral leader sequence
pVRE6LAMP-1	E6 sequence fused to a leader sequence and the lysosome-associated membrane protein
pVRE7LAMP-1	E7 sequence fused to a leader sequence and the lysosome-associated membrane protein
pVRE6UBI	E6 sequence fused to ubiquitin
pVRE7UBI	E7 sequence fused to ubiquitin
<i>E1-deleted adenoviral vectors</i>	
AdHu5E6	Wild-type E6 sequence
AdHu5E7	Wild-type E7 sequence
AdHu5L1	Wild-type L1 sequence

^aIn the pVR1012.2 vector used for all of the constructs, the original kanamycin-resistance gene was replaced with an ampicillin-resistance gene.

Preventative DNA vaccination

Vaccines were tested for efficacy in two transplantable tumor models in C57Bl/6 mice and in BALB/c mice using two transplantable tumor models based on TC-1 and t(10)1rasE6E7 cells originated from C57Bl/6 and BALB/c mice, respectively. Both tumor cell lines express E6 and E7 of HPV-16 together with v-Ha-ras. After two immunizations with DNA vaccines expressing the different forms of the E6 or E7 oncoproteins, mice were challenged s.c. with tumor cells, that is, t(10)1rasE7E6 (BALB/c) or TC-1 (C57Bl/6) cells, and monitored for tumor formation. Mice were scored as tumor bearing when tumors reached ~2 mm in diameter. Greater than 90% of mice receiving an empty vector alone developed tumors after challenge (Fig 1). The DNA vaccines expressing wild-type E6, or E6 fused to LAMP-1 or ubiquitin protected a small percentage (~20%) of BALB/c mice (Fig 1a). The vaccine expressing E6 linked to a viral leader sequence (pVRE6LD) protected ~70% of the animals against tumor development and delayed onset of tumors in those mice that remained susceptible to tumor challenge. In C57Bl/6 mice, none of the E6-expressing vaccines induced significant protection against challenge with the syngeneic TC-1 tumor cell line (Fig 1c). The DNA vaccines expressing E7 were also largely ineffective in BALB/c (Fig 1b) or C57Bl/6 (Fig 1d) mice. Only the construct expressing E7 linked to ubiquitin provided some protection (~20%) to BALB/c mice, while the pVRE7-LAMP-1 vaccine provided protection to ~50% of C57Bl/6 mice. Nevertheless, neither of these results reached statistical significance.

Figure 1 Efficacy of preventative DNA vaccination. BALB/c or C57Bl/6 mice were vaccinated i.m. twice with 100 μ g of E6 and E7-expressing vectors. At 3 weeks after the final vaccination, mice were challenged s.c. with either 3×10^4 t(10)1rasE7E6 cells (BALB/c mice) or 2×10^5 TC-1 cells (C57Bl/6 mice). (a) BALB/c mice vaccinated with DNA vaccines to E6, (b) BALB/c mice vaccinated with DNA vaccines to E7, (c) C57Bl/6 mice vaccinated with DNA vaccines to E6, (d) C57Bl/6 mice vaccinated with DNA vaccines to E7. X—mice vaccinated with the insert-free pVR1012.2 vector. Number of animals: (a) 29, (b) 5, (c) 26, (d) 17. Open squares—mice vaccinated with DNA vaccines expressing wild-type E6 or E7: (a) 28, (b) 5, (c) 28, (d) 17. Closed squares—mice vaccinated with DNA vaccines expressing E6 or E7 linked to a viral leader sequence (a) 18, (b) 5, (c) 29, (d) 15. Squares with cross—mice immunized with DNA vaccines modified to express E6 or E7 linked to a signal sequence and LAMP: (a) 8, (b) 5, (c) 5, (d) 10. Half-open squares—mice immunized with DNA vaccines expressing E6 or E7 linked to ubiquitin: (a) 8, (b) 5, (c) 5, (d) 10. The graph shows the combined results of several experiments using the number of mice indicated above. Statistical significance of % tumor-free survival and of the kinetics of onset of tumors was calculated by Student's *t*-test. Data with *P*-values < .05 were considered significant. *Significant increase in % tumor-free survival. **Significant delay in onset of visible tumors.

DNA prime/viral recombinant boost vaccination

The immune response to DNA vaccines can be augmented by booster immunization with a heterologous vaccine carrier such as a viral vector expressing the same antigen.^{22,23} As neither of the DNA vaccines induced complete protection against tumor cell challenge in BALB/c or C57Bl/6 mice, we tested whether boosting of DNA vaccine primed mice with a low dose of an E1-deleted adenoviral recombinant expressing the corresponding antigen augmented resistance to tumor cell challenge. In these experiments, BALB/c mice were tested using DNA vaccines expressing wild-type E6 or E6 linked to a leader sequence (Fig 2a), while C57Bl/6 mice were tested with the entire set of DNA vaccines to E6 (Fig 2b)

Figure 2 Efficacy of a DNA vaccine prime-adenoviral recombinant boost regimen. BALB/c or C57Bl/6 mice were vaccinated i.m. twice with 100 μ g of the DNA vaccines. At 3 weeks after the second dose of DNA, mice were boosted s.c. with either 10^6 PFU of AdHu5E7 or AdHu5E6 virus. At 7 days following viral recombinant boost, mice were challenged with either 3×10^4 t(10)1rasE7E6 cells (BALB/c) or 2×10^5 TC-1 cells (C57Bl/6) s.c. (a) BALB/c mice vaccinated with vaccines to E6: open squares—mice primed with the pVRE6 vector and boosted with the AdHu5E6 virus; *n* (number of animals)=15. Closed squares—mice primed with the pVRE6LD vector and boosted with the AdHu5E6 virus; *n*=15. Open crosses—mice primed with the insert-free pVR1012.2 control vector and boosted with the AdHu5E6 virus; *n*=15. Open circles—mice primed with the pVRE6 vaccine and boosted with the AdHu5L1 vaccine; *n*=5. Closed circles—mice primed with the pVRE6LD vaccine and boosted with the AdHu5L1 vaccine; *n*=5. Closed crosses—mice primed with the insert-free pVR1012.2 vector and boosted with the AdHu5L1 vaccine; *n*=5. (b) C57Bl/6 mice immunized with vaccines to E6 and (c) C57Bl/6 mice immunized with vaccines to E7: open crosses—mice immunized with the insert-free pVR1012.2 vector and boosted with the AdHu virus expressing E6 or E7; number of animals: *n*=5 (b) and 10 (c). Open squares—mice immunized with pVRE6 or pVRE7 and boosted with the corresponding AdHu5 vector, *n*=5 (b) and 8 (c). Closed squares—mice immunized with pVRE6LD or pVRE7LD and boosted with the corresponding AdHu5 vector, *n*=5 (b) and 9 (c). Squares with cross—mice immunized with pVRE6LAMP or pVRE7LAMP and boosted with the corresponding AdHu5 vector *n*=5 (b) and 9 (c). Half-open squares—mice immunized with pVRE6UBI or pVRE7UBI and boosted with the corresponding AdHu5 vector, *n*=5 (b) and 10 (c). Data were collected as described in the legend to Figure 1.

or E7 (Fig 2c). Priming of BALB/c mice with the pVRE6LD vaccine followed by booster immunization with the AdHu5E6 vaccine provided a statistically significant increase in protection compared to priming with an empty DNA vector prior to immunization with the AdHu5E6 vaccine. None of the C57Bl/6 mice immunized with DNA vaccines to E6 followed by a booster immunization with the AdHu5E6 vaccine were protected. The E7 vaccines had a paradoxical effect in C57Bl/6 mice. In this model, priming with any of the DNA vaccines to E7 impaired the efficacy of the AdHu5E7 vaccine that upon immunization of control vector-primed mice protected most, that is, 80%, of the animals while priming with E7-expressing DNA vaccines reduced the level of protection to $\leq 50\%$.

Therapeutic immunization

Considering the nature of HPV-16 infections and the sequence of expression of the different viral antigens, vaccines to the viral oncoprotein should be used therapeutically rather than for prevention of an infection, which can best be achieved by vaccines inducing neutralizing antibodies to the viral surface antigen L1.²⁴ We tested if the most effective of our DNA vaccines, that is, the pVRE6LD vaccine could prevent tumor progression if given after challenge in BALB/c mice. Naïve BALB/c mice were injected with 10^4 t(10)1rasE7E6 cells s.c. After 3 days, the same mice were immunized i.m. with 100 μ g/mouse of the pVRE6LD vaccine or the insert-free vector. Mice were boosted with 100 μ g of DNA/mouse 7 days after the initial DNA vaccination. The pVRE6LD vaccine caused tumor regression in $\sim 80\%$ of the animals (Fig 3a). The empty vector achieved protection in $\sim 30\%$ of the animals. To test if innate immune responses such as NK cells activated by the bacterial components of the vector²⁵ rather than just E6-specific adaptive immune responses contributed to the therapeutic effects of the vaccine, mice were depleted of NK cells by treatment with an antiserum to asialo-GM1 ganglioside and then tested in a postchallenge vaccination experiment. As shown in Figure 3b, depletion of NK cells did not influence tumor development suggesting that this lymphocyte subset did not contribute to the postchallenge efficacy of the pVRE6LD vaccine.

Mechanisms of protection

Protection against HPV-16-associated tumors induced by vaccines to the viral oncoproteins is linked to the induction of cellular rather than humoral immunity.⁷ To test if the DNA vaccines induced detectable -cel T-cell responses to the transgene product, BALB/c mice were vaccinated with the pVRE6, the pVRE6LD or the insert-free vector or C57Bl/6 mice were vaccinated with the entire set of DNA vaccines to E7. Splenocytes were harvested 3 weeks after the second vaccination and cultured with an irrelevant protein or with the viral oncoprotein expressed by the DNA vaccine, a procedure that activates primarily antigen-primed CD4⁺ T-cells. Supernatants were harvested 24 hours later and tested for

Figure 3 Efficacy of DNA vaccines given after tumor cell challenge. (a) BALB/c mice were challenged s.c. with 10^4 t(10)1rasE7E6 cells. At 3 and 10 days postchallenge, the mice were immunized i.m. with 100 μ g of insert-free pVR1012.2 vector (X, $n=13$), or pVRE6LD (closed squares, $n=8$). (b) mice treated with a depleting dose of a serum to NK cells as well as untreated control animals were challenged and vaccinated as described in Figure 4a. X—insert-free pVR1012.2; vector; open crosses—anti-NK treatment and + pVR1012.2; closed squares—pVRE6LD; squares with cross—anti-NK treatment and + pVRE6LD.

IL-2 on the HT-2 indicator cells and for IL-4 and IFN- γ by an ELISA. We were unable to demonstrate in any of the experiments significant release of either IFN- γ or IL-4 in response to the vaccine antigen (data not shown). Secretion of low levels of IL-2 could be detected from splenocytes of mice vaccinated with the pVRE6 or the pVRE6LD vector (Fig 4a). Levels of secretion were comparable and thus not linked to the superior efficacy of the pVRE6LD vaccine. In C57Bl/6 mice, none of the E7 expressing vaccines induced secretion of detectable amounts of cytokines (i.e., IFN- γ , IL-2 or IL-4) as shown in Figure 4b for IL-2.

In addition, splenocytes were tested for CD8⁺ T-cell activity by a number of different techniques. We were unable to demonstrate significant lysis of HPV-16 oncoprotein expressing target cells by a traditional ⁵¹Cr-release assay using *in vitro* expanded splenocytes as effector cells. Stimulation of splenocytes for 5 hours with a peptide carrying the immunodominant epitope of E7 in presence of a drug that inhibits secretion of proteins revealed low frequencies (i.e., $<0.4\%$) of E7-specific IFN- γ -producing CD8⁺ T-cells in mice immunized with the pVRE7LD or pVRE7LAMP-1 vaccines (Table 2). Frequencies were below those previously reported for AdHu5E7-vaccinated C57Bl/6 mice (0.8–1%⁷). They failed to increase upon challenge with TC-1 cells (data not shown), which was also in contrast to our previous observation with mice vaccinated with a protective dose of the AdHu5E7 vaccine.²⁰

Figure 4 IL-2 release by splenocytes from DNA vaccinated mice. (a) BALB/c or (b) C57Bl/6 mice were immunized twice with the DNA vaccines. At 3 weeks after the second immunization, splenocytes were cultured for 24 hours with E7 peptide (black bars) or a control peptide (striped bars). Cell-free supernatants were tested for induction of proliferation of the HT-2 cell line. Data show means of triplicate samples $\pm$ standard deviations. TdR—thymidine.

Table 2 Frequency of IFN- γ ⁺ CD8⁺ cells^a E7 peptide

	+	–	Δ Frequency ^b
pVR1012.2	0.07	0.07	0
PVRE7	0.07	0.12	–0.05
PVRE7LD	0.52	0.15	0.37
PVRE7LAMP-1	0.33	0.13	0.18
PVRE7UBI	0.08	0.12	–0.04

Groups of BALB/c mice were immunized three times in 2-week intervals with 100 μ g of DNA. At 3 weeks after the last injection, splenocytes were harvested and cultured with the control peptide (–) or the E7 peptide (+) for 5 hours in the presence of Brefeldin. Cells were then stained for CD8 and intracellular IFN- γ .

^aData show the frequency of IFN- γ ⁺CD8⁺ cells over all CD8⁺ cells.

^bFrequency of IFN- γ ⁺CD8⁺ cells in cultures incubated with the E7 peptide minus frequencies of IFN- γ ⁺CD8⁺ cells in cultures incubated with the control peptide.

Discussion

We tested a set of DNA vaccines expressing either the E6 or E7 antigens of HPV-16 for induction of protection to

challenge with HPV-16 E6/E7-expressing tumor cells in two different mouse models. We had shown previously, using viral recombinant vaccines, that is, E1-deleted adenoviral vectors derived from the human serotype 5 and vaccinia virus recombinants from the Copenhagen strain, that E1-deleted adenoviral vectors expressing E7 provide complete protection to TC-1 cell challenge in C57Bl/6 mice, while both types of viral vectors expressing E6 provide partial protection (30–40%) to BALB/c mice against challenge with the t(10)1rasE7E6 cell line.⁷ This discrepancy in protection was at least in part linked to genetic restriction of T-cell-mediated immune responses to the short oncoproteins of HPV-16.²⁶ E7 carries an immunodominant epitope able to associate with MHC class I determinants of the H-2b haplotype²⁷ but not of the H-2d haplotype.⁷ The E7 protein lacks a functional epitope with high affinity to either H-2d or H-2b MHC class II determinant.⁷ Protection in C57Bl/6 mice was consequently mediated by CD8⁺ T-cells, which are best induced by E1-deleted adenoviral vectors. The E6 antigen lacks a CD8⁺ T-cell epitope for either mouse haplotype but apparently carries an immunogenic MHC class II epitope for BALB/c mice.⁷ In BALB/c mice, protection was mediated by CD4⁺ T-cells, which could be induced by vaccinia virus vectors as well as by E1-deleted adenoviral recombinants. Ir-gene control of T-cell responses thereby limited the usefulness of E6 and E7 viral recombinant vaccines in inbred strains of mice and one would expect genetically restricted efficacy in an outbred human population as well.

DNA vaccines, through yet to be defined mechanisms, have been shown to overcome Ir-gene control and to induce responses to proteins that are otherwise poorly immunogenic in certain MHC haplotypes if given by other vaccine carriers.²⁸ To assess if we could expand the efficacy of vaccines to HPV-16 oncoproteins, we developed a set of DNA vaccines expressing either E6 or E7 in wild-type form or fused to sequences known to affect antigen trafficking, processing and presentation by MHC class I or class II determinants and tested these vaccines in both BALB/c and C57Bl/6 mice.

Our results with DNA vaccines to E6 yielded promising results in BALB/c mice. The DNA vaccines expressing E6 linked to a viral leader sequence induced protection in the majority of mice if given before or even after tumor cell challenge. In the BALB/c model, protection is mediated by CD4⁺ T-cells and DNA vaccines expressing a secreted form of E6 outperformed viral recombinant vaccines, which as reported previously induce protective immunity in ~30–40% of vaccinated BALB/c mice.⁷ We showed previously that a DNA vaccine expressing a biologically inactive form of mouse p53 linked to a viral leader sequence induced better protection against a tumor cell line expressing high levels of p53 compared to DNA vaccines expressing other modifications of p53.¹⁶ In this system, both CD4⁺ and CD8⁺ T-cells mediated protection. Addition of a leader sequence to proteins with nuclear localization domains such as p53 or E6 ensures cytoplasmic retention of the protein and in case of E6 channeling into the secretion pathway. Secretion of the

protein may, through reprocessing, increase antigen presentation in association with MHC class II determinants and thus increase activation of antigen-specific CD4⁺ T-cells. This could not be confirmed experimentally as splenocytes from mice immunized either with the pVRE6 or the pVRE6LD vectors secreted negligible amounts of IL-4 and comparable levels of IL-2. Considering the intricacies of T-cell trafficking, lack of an *in vitro* T-cell-mediated recall response does not necessarily reflect lack of such a response *in vivo*. In both the p53 and the E6 tumor models, vaccines expressing the tumor-associated antigens linked to a leader sequence outperformed E1-deleted adenoviral vectors as well as poxvirus vectors.¹⁶ The result is surprising considering that viral vectors induce in general far more potent transgene product-specific immune responses compared with DNA vaccines.²⁹ It is conceivable that epitopes with comparatively low affinity to MHC determinants may be more immunogenic if delivered by a DNA vaccine than by a viral recombinant vector. DNA vectors, unlike viral vaccine carriers, do not express any competing epitopes, which may help the induction of T-cells to low-affinity epitopes, which are displayed in limiting amounts by MHC-molecules on the cell surface. In the same vein, DNA vaccines are thought to provide long-lasting production of antigen that may also optimize the chances of low levels of MHC-epitope complexes to attract the attention of T-cells with corresponding receptors.

The DNA vaccines expressing E7 yielded uniformly disappointing results upon i.m. administration of two or more doses (protection results are only shown for the two-dose regimen) by providing limited protection to TC-1 cell challenge and by inducing only marginal CD8⁺ T-cell responses to the immunodominant epitope of E7. Previous reports have shown that protective immunity to TC-1 cell challenge could be induced by a DNA vaccine expressing E7 fused to LAMP-1.³⁰ In these studies, in contrast to ours, the DNA vaccine was delivered by gene gun immunization that generally favors a Th2 response rather than a Th1 response that is best suited to drive activation of CD8⁺ T-cells.³¹ Accordingly, frequencies reported in these studies were even lower than those achieved with our immunization protocol with the pVRE7LAMP-1 construct. Nevertheless, protection against TC-1 cell challenge upon immunization of mice with a variety of DNA vaccines expressing modified E7 given alone or in conjunction with genetic adjuvant has been established.^{8,9,30} These contrasting results can best be explained by differences in cell numbers used for tumor cell challenge; we used a dose of 2×10^5 TC-1 given s.c. This dose leads to rapid development of tumors; within 10 days tumors commonly reach a size that requires euthanasia of the animals. Previously published results were based on a challenge model in which mice are injected with 10^4 tumor cells intravenously.³⁰ In this type of challenge, only a fraction of the tumor cells become established in the lung and proliferate into nodules.³² The number of tumor cells that has to be eliminated by tumor antigen-specific CD8⁺ T-cells is thus fairly low, which obviously influences vaccine efficacy.

Others have shown that gene gun delivery of DNA vaccines to E7 resulted in enhanced tumor growth of an E6/E7-transformed mouse cell line given s.c. to C57Bl/6 mice.³³ The DNA vaccination was shown to direct the immune response towards the Th2 pathway resulting in an immune response that was incompatible with tumor cell eradication. We obtained comparable results when we inoculated the DNA vaccines to E7 through the i.m. route and then boosted mice with a low dose of the E1-deleted AdHu5E7 vector. This type of prime boost regimen, previously described to enhance the efficacy of vaccines to other viral antigens,²⁹ reduced the level of protection achieved with the AdHu5E7 vaccine against tumor cell challenge in the C57Bl/6 mouse model presumably by impairing activation of a protective CD8⁺ T-cell response.

Other vaccine modalities expressing E7, such as E1-deleted adenoviral vectors,⁷ viral replicons¹⁰ or bacterial vaccine vehicles³⁴ have been shown to induce more potent CD8⁺ T-cell responses and reliable protection to experimental tumor cell challenge than DNA vaccines to E7. Especially, the findings that DNA vaccines to E7 can increase tumor progression or subvert the efficacy of other vaccine modalities if used for priming, raise concern for the validity of such vaccines for use in humans.

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