

# Eradication of Established Tumors by Vaccination with Venezuelan Equine Encephalitis Virus Replicon Particles Delivering Human Papillomavirus 16 E7 RNA<sup>1</sup>

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## ABSTRACT

The etiological role of human papillomaviruses (HPV) in cervical and other cancers suggests that therapeutic vaccines directed against requisite viral antigens may eradicate tumors or their precursors. A Venezuelan equine encephalitis (VEE) alphavirus vector delivering the HPV16 E7 RNA was evaluated for antitumor efficacy using a murine E7<sup>+</sup> tumor model. Vaccination with VEE replicon particles expressing E7 (E7-VRP) induced class I-restricted CD8<sup>+</sup> T-cell responses as determined by IFN- $\gamma$  enzyme-linked immunospot (ELISPOT), tetramer, and cytotoxicity assays. E7-VRP vaccination prevented tumor development in all of the mice and effectively eliminated 7-day established tumors in 67% of tumor-bearing mice. The induction of protective T-cell responses was dependent on CD8<sup>+</sup>, but not CD4<sup>+</sup> T cells. Long-lasting T-cell memory responses developed in E7-VRP-vaccinated mice as determined by complete protection from tumor challenge 3 months after the final vaccination. These promising results highlight the potent CD8<sup>+</sup> T-cell-mediated antitumor effects elicited by VEE replicon-based vectors and support their further development toward clinical testing against cervical intraepithelial neoplasia or carcinoma.

## INTRODUCTION

Approximately 15% of human cancers worldwide are associated with viruses, the majority of which are attributed to HPV<sup>4</sup> (1). HPV infection and persistence confer risks of developing cervical carcinoma (2, 3), the most prevalent HPV-associated cancer. HPV DNA is detectable in >99% of cervical carcinomas (4) and to varying degrees in other cancers, collectively implicating HPV as the causative agent in ~10% of female cancers (1). The oncogenic potential of the “high risk” HPV genotypes 16, 18, and others is attributed in part to their E6 and E7 genes, which possess transforming and immortalizing activities *in vitro* (5, 6). Sustained E6 and E7 expression is required for maintenance of the malignant phenotype (7, 8) and is evident in cervical carcinomas and their CIN precursors (9, 10). These observations suggest that E6 and E7 may be appropriate targets for eradicating HPV-associated tumors or their precursors by therapeutic vaccination.

Cell-mediated immune responses are important determinants of HPV-associated disease outcome (11). Protective CTL responses against a MHC class I-restricted E7 epitope have been demonstrated

in E7<sup>+</sup> murine tumor models (12, 13). HPV16 E6- and E7-derived peptides capable of binding specific human leukocyte antigen (HLA) class I alleles have been identified and have successfully primed CTL responses in HLA-A2.1 transgenic mice and *in vitro* from normal human donors (14). Memory CTL responses against some of these HLA-restricted peptides from E6 and E7 are detectable in some patients with CIN and cervical carcinoma but not in normal subjects (14). HLA-A2.1-restricted E7 peptides (15) and E6-E7 expressing recombinant vaccinia viruses (16) have been evaluated in Phase I trials with end stage cervical cancer patients and have revealed CTL responses in some cases but poor clinical responses (14). An encouraging recent HPV16 E7 peptide trial in high-grade CIN patients has revealed both increases in CTL activity and lesion regression in many of the vaccinees (17). Whereas vaccine intervention at earlier stages of disease may be one aspect of improving clinical efficacy, more powerful strategies for inducing memory CTLs, preferably against a broader array of HPV epitopes, will be critical for priming or boosting antitumor responses.

Several HPV vaccine candidates have been tested against murine tumors constitutively expressing HPV16 E6 and E7 genes (18). The most practical and efficacious vaccine approaches tested include peptides (12, 13), recombinant fusion proteins (19), chimeric virus-like particles (20), viral vectors (21), and plasmid DNA (22, 23). Limitations currently exist with several vectored approaches such as vaccinia virus and plasmid DNA because of preexisting immunity or vaccine take in humans, whereas peptide vaccines are limited to predetermined target HLA alleles. Although several candidates have not yet been tested clinically, more effective strategies should continue to be investigated in preclinical models. One viral vector under consideration is derived from VEE virus. VEE and other enveloped, positive-stranded RNA AVs like Sindbis and SFV have been engineered as replication-incompetent viral-delivery vectors or replicons (24). Replicon vectors are generated by removing the structural genes of the virus and replacing them with a foreign gene; they contain AV replicase genes, which mediate RNA replication and high-level protein expression but produce no progeny virus. The replicon-recombinant RNA encoding the foreign gene of interest in lieu of the VEE structural genes can be packaged into VRPs on provision of the structural RNAs *in trans* (25). VRPs encoding several viral genes have been shown to be immunogenic and protective in murine, guinea pig, and primate models (25–29).

AVs in general, and VEE in particular, are attractive vaccine vectors for several reasons: (a) there is no widespread preexisting anti-VEE immunity in humans; (b) repeated immunization appears to be possible (25); (c) replicating RNA directs high-level heterologous protein expression (30); (d) double-stranded RNA is produced after AV infection and apoptosis is induced, which may be highly conducive for inducing immune responses (31); and (e) the envelope glycoproteins of VEE, in contrast to those of other AVs, confer dendritic cell tropism (32). These features, as well as the cytoplasmic transcription of VEE RNA and the unlikelihood of RNA integration, suggest

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<sup>4</sup> The abbreviations used are: HPV, human papillomavirus; AV, alphavirus; CIN, cervical intraepithelial neoplasia; GFP, green fluorescent protein; PE, phycoerythrin; SFV, Semliki Forest virus; VEE, Venezuelan equine encephalitis virus; VRP, VEE replicon particle(s); ATCC, American Type Culture Collection; MVA, modified vaccinia virus Ankara; MOI, multiplicity/multiplicities of infection; ELISPOT, enzyme-linked immunospot.

VRPs may be very promising HPV vaccine vectors. In the present study, the antitumor efficacy of VRP vectors was tested using the E7 gene from HPV16, the most prevalent high-risk genotype. The immunogenic, tumor-protective, and therapeutic potential of HPV16 E7-expressing VRPs was evaluated in the E7<sup>+</sup> C3 tumor model.

## MATERIALS AND METHODS

**Mice and Cell Lines.** Specific-pathogen-free 8–12-week-old female C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). Mice were housed in the animal facilities of Loyola University Chicago and Wyeth-Lederle Vaccines under filtertop conditions with water and food *ad libitum*. CD4, CD8, and IFN- $\gamma$  knockout mice (Jackson Laboratories, Bar Harbor, ME) were housed in a specific-pathogen-free barrier facility under sterile conditions. Institutional animal-use guidelines were followed for all experiments.

BHK-21 cells were used for VEE RNA expression, VRP packaging, and titration. MC57G and EL-4 cells were used for cytotoxicity assays. All cell lines were obtained from ATCC (Manassas, VA).

**Preparation of VRPs.** The HPV16 E7 gene (pHPV-16 (no. 45113, ATCC) and the GFP gene (Clontech, Palo Alto, CA) were subcloned into the VEE replicon plasmid, pVR200 (a proprietary vector from Alphavax, Durham, NC). PVR200 was derived from the cDNA of a highly attenuated, nonneurotropic mutant (V3014) of the Trinidad Donkey strain of VEE (33). The VR200 recombinant plasmids and the two other plasmids containing either the VEE capsid or glycoprotein structural genes were linearized by *NotI* digestion. Capped RNA transcripts were synthesized using T7 RNA polymerase (mMessage Transcription kit, Ambion, Austin, TX). VRPs were packaged by electroporation of replicon and helper RNAs into BHK-21 cells by the split helper method (25). VRPs were concentrated from supernatants by centrifugation through a 20% sucrose cushion prepared in PBS. Replication-competent virus was not detectable as determined by an infectious center assay conducted on BHK-21 cells. Titration of VRPs was performed by serial 10-fold dilutions on BHK-21 monolayers in which the number of E7- or GFP-producing cells was determined by staining with an anti-E7 monoclonal antibody (Zymed, San Francisco, CA) or by direct visualization of fluorescing green cells, respectively, and titers were expressed as infectious units per ml. A dose of  $3 \times 10^5$  infectious units per mouse of E7-VRPs or GFP-VRPs was used for all of the experiments.

**Western Blot and Immunofluorescence Staining.** E7-specific protein expression was assessed by Western blot of BHK-21 cell lysates after electroporation of E7- or GFP-VEE replicon RNA and the two helper RNAs. Thirty h after electroporation, cells were lysed with SDS sample buffer (Bio-Rad, Hercules, CA). A His-tagged E7 recombinant protein produced in *Escherichia coli* using the pET-30a expression system (Novagen, Madison, WI) was used as a positive control. The electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA), and the blots were processed with the Western Breeze detection system (Invitrogen, Carlsbad, CA). To detect E7 protein, a primary anti-HPV16 E7 monoclonal antibody was used (Zymed Laboratories, San Francisco, CA) followed by an antimouse alkaline phosphatase-labeled secondary antibody.

**Cytotoxicity Assays.** C57BL/6 mice were immunized s.c. with  $3 \times 10^5$  infectious units of either HPV16 E7-VRPs or GFP-VRPs. Cytotoxicity assays were performed 2 weeks after a single immunization or 3 months after the last of 3 monthly vaccinations. Single-cell splenocyte suspensions were restimulated (20:1) with mitomycin-C-treated MC57G cells infected with recombinant MVA vectors encoding E7 or GFP (E7-MVA or GFP-MVA) at a MOI of 5. CTL activity was measured 5 days later. E7- or GFP- MVA-infected MC57G cells and HPV16 E7<sub>49–57</sub> H-2D<sup>b</sup>-restricted peptide (RAHYNIVTF; Ref. 12)-pulsed EL-4 cells (ATCC) served as targets. MC57G cells were infected for 1 h with either E7- or GFP-expressing MVA at a MOI of 5. EL-4 cells ( $1 \times 10^7$ ) were incubated with peptide (20  $\mu$ g/ml) for 1 h. Target cells were then labeled 3 h later with Europium (Eu<sup>+3</sup>; Sigma Chemical Co., St. Louis, MO) by electroporation. Effector and target cells were incubated at the indicated ratios for 3 h, after which supernatants were harvested and mixed with Enhancer solution (Wallac, Turku, Finland). Eu<sup>+3</sup> release was quantitated by time-resolved fluorescence using a 1234 Delfia fluorometer (Wallac). The percentage of specific lysis was calculated as:

$$\frac{\text{Experimental} - \text{spontaneous release}}{\text{Maximal} - \text{spontaneous release}} \times 100$$

The percentage spontaneous releases ranged from 5 to 10%.

**Tetramer Staining.** H-2D<sup>b</sup> tetramers labeled with PE and containing the HPV16 E7<sub>49–57</sub> peptide were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). CD8<sup>+</sup> T cells were isolated from the spleen using MACS cell sorting (Miltenyi Biotech, Auburn, CA) before tetramer staining. One million CD8<sup>+</sup>-enriched splenocytes were incubated for 1 h with 20  $\mu$ l of 1:100 diluted tetramer and 1:100 diluted anti-CD8<sup>FTTC</sup> antibody (PharMingen, San Diego, CA) in PBS/0.5% BSA. Cells were washed twice in PBS 0.5%/BSA, and the percentage of tetramer<sup>+</sup>/CD8<sup>+</sup> T cells was determined by fluorescence-activated cell sorting analysis.

**ELISPOT Assay.** An ELISPOT assay was used to detect peptide-specific T cells after stimulation with the synthetic HPV16 E7<sub>49–57</sub> peptide. Multi-screen HA plates (Millipore, Bedford, MA) were coated with 5  $\mu$ g/ml anti-IFN- $\gamma$  antibody (PharMingen, San Diego, CA) at 4°C overnight. Plates were washed with PBS/0.5% Tween 20 and blocked with culture medium. Splenocytes were added at  $1 \times 10^6$  and  $2 \times 10^5$  cells per well in medium containing 25 infectious units interleukin 2 and 10  $\mu$ g/ml E7 peptide. After 40-h incubation at 37°C and 5% CO<sub>2</sub>, plates were washed with PBS-Tween and incubated for 2 h at room temperature with 2.5  $\mu$ g/ml biotinylated anti-IFN- $\gamma$  antibodies. After washing with PBS-Tween, 1.25  $\mu$ g/ml Avidin-alkaline phosphatase (Sigma Chemical Co.) were added to the wells in 50  $\mu$ l of PBS and incubated for 2 h at room temperature. The development of the assay was performed using 50  $\mu$ l of alkaline-phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (Promega, Madison, WI) for 15 min. The reaction was stopped by the addition of tap water and the plates were allowed to dry before counting the individual spots with a dissecting scope.

**Immunization and Tumor Challenge.** Groups of eight female C57BL/6 mice were vaccinated with  $3 \times 10^5$  infectious units E7-VRP or GFP-VRP s.c. in the left flank, and a booster dose in the same amount was given 2 weeks later. Two weeks after the booster dose, mice were challenged s.c. in the right flank with  $5 \times 10^5$  C3 tumor cells (12) in 100  $\mu$ l of PBS. Tumor growth was monitored twice a week and quantitated with a spring-loaded caliper in three dimensions. For therapeutic experiments, mice first received a s.c. injection of  $5 \times 10^5$  C3 cells in 100  $\mu$ l of PBS in the right flank. At day 7, all of the mice in the therapy experiments had developed a palpable tumor and received  $3 \times 10^5$  infectious units VRP s.c. in 100  $\mu$ l of PBS in the left flank. The VRP administrations were repeated at 7 and 14 days after the first injection. Tumor sizes in the mice were recorded 2–3 times a week. No vaccine-related toxicities were noted in any of the VRP-vaccinated mice.

## RESULTS

**Construction of Recombinant E7 VEE Replicons.** A recombinant HPV16 E7 VEE replicon vector was constructed, and *in vitro* RNA transcripts were synthesized, electroporated into BHK-21 cells, and assessed for E7 protein expression in total cell lysates by Western blot. Fig. 1A reveals the expected full-length E7 phosphoprotein ( $M_r \sim 20,000$ ) after probing with an anti-E7 monoclonal antibody. The recombinant E7-VEE RNA was packaged using the split-helper method (25) by coelectroporating synthetic RNA encoding the VEE capsid and glycoproteins. Replication-defective E7-VRPs were assessed for infectivity. E7 expression was evident in the cytoplasm and nucleus after immunofluorescence staining with an anti-E7 monoclonal antibody (Fig. 1B) or a rabbit polyclonal anti-E7 peptide antiserum (not shown).

**Cellular Immune Responses Induced by E7-VRP.** To characterize the immune responses induced by E7-VRP vaccinations, C57BL/6 mice were vaccinated s.c. with  $3 \times 10^5$  infectious units of E7-VRP or GFP-VRP. Splenocytes from vaccinated mice were evaluated for CD8<sup>+</sup> class I-restricted cellular immune responses by three assays. An ELISPOT assay demonstrated specific IFN- $\gamma$  release on stimulation of splenocytes with an H-2D<sup>b</sup> binding E7<sub>49–57</sub> peptide RAHYNIVTF (12), but not a control adenovirus 5 E1A H-2D<sup>b</sup> peptide

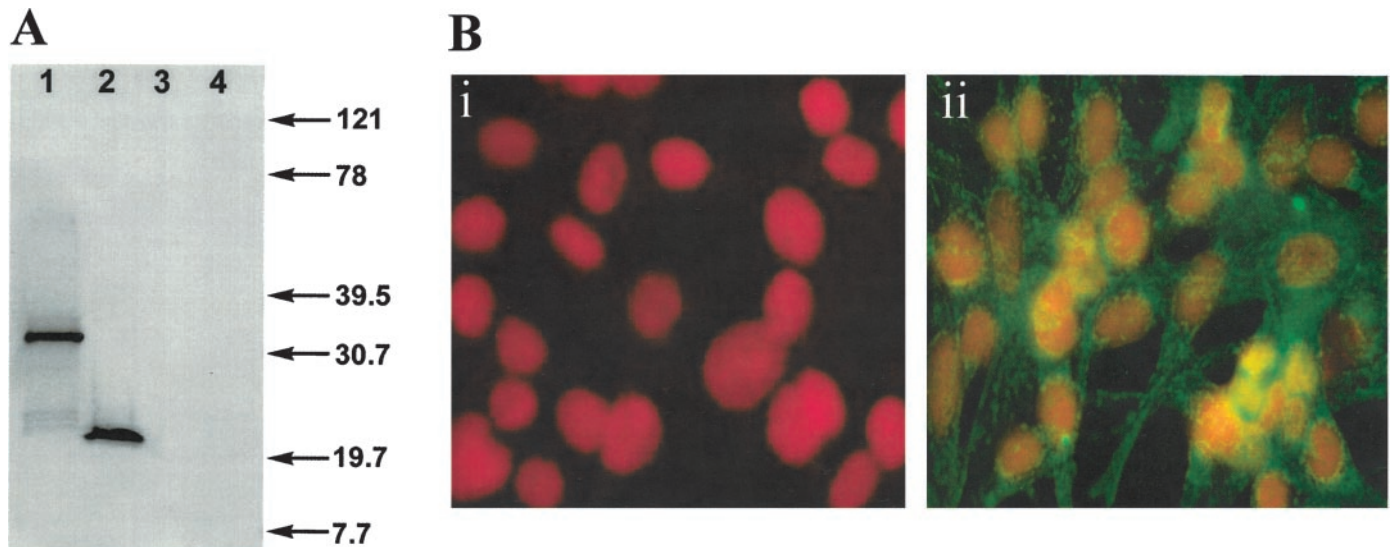


Fig. 1. A, Western blot detection of E7 expression in BHK-21 packaging cell lysate. BHK-21 cells were electroporated with either E7-VEE or GFP-VEE replicon synthetic transcripts together with the helper RNAs encoding VEE capsid and glycoprotein genes. Thirty h after electroporation, total cell lysates were harvested, and the presence of the E7 protein was detected by Western blot with an anti-E7 monoclonal antibody. Lane 1, recombinant E7 fusion protein; Lane 2, E7-VEE RNA-transfected cells; Lane 3, GFP-VEE-transfected cells; Lane 4, mock-transfected BHK-21 cells. B, immunofluorescence staining of E7 after VRP infection. BHK-21 cells were mock infected (panel i) or infected with E7-VRP (MOI, 5; panel ii) and fixed with methanol/acetone (1:1) 18 h later. Fixed cells were incubated with a mouse monoclonal anti-E7 antibody, washed, stained with a goat antimouse immunoglobulin-FITC antibody and mounted in Vectrashield containing propidium iodide. E7 immunoreactive cells were visualized with a fluorescent microscope ( $\times 40$ ).

SGPSNTPPEI (34; Fig. 2A). E7-specific responses were not observed in mice vaccinated with GFP-VRP or in naïve mice. The binding of E7-specific T cells to E7<sub>49-57</sub>/H-2D<sup>b</sup> tetramer complexes was examined by fluorescence-activated cell sorter analysis (Fig. 2B). Double staining of splenocytes from E7-VRP- or GFP-VRP-vaccinated mice with anti-CD8 antibody and E7<sub>49-57</sub>/H-2D<sup>b</sup> tetramers revealed recognition of E7 peptide by CD8<sup>+</sup> T cells, only after E7-VRP vaccination. Approximately 6% of the CD8 population in the spleen was directed against the E7<sub>49-57</sub> peptide after vaccination with E7-VRP. Specific CTL-mediated killing of E7<sup>+</sup> target cells was also tested from VRP-vaccinated mice. E7-VRP vaccination induced lytic activity against both E7<sub>49-57</sub>-pulsed EL-4 cells and E7-MVA-infected MC57G cells 2 weeks after a single immunization (Fig. 2C). In separate experiments, splenocytes from GFP-VRP-vaccinated mice incubated *in vitro* with E7-MVA-infected stimulator cells did not have detectable E7-specific kill against E7<sup>+</sup> target cells even after three immunizations (16 versus 18% and 9 versus 12% specific kill on E7 peptide pulsed or nonpulsed EL-4 targets at 50:1 and 25:1 E:T ratios, respectively).

**E7 VRP Vaccination Protects against Tumor Outgrowth.** C57BL/6 mice ( $n = 8$ /group) were vaccinated s.c. twice with  $3 \times 10^5$  infectious units of E7-VRP or GFP-VRP, and then challenged with  $5 \times 10^5$  HPV16 E7<sup>+</sup> C3 tumor cells (12). Complete protection was observed in E7-VRP-vaccinated mice, whereas control GFP-VRP-vaccinated and naïve mice all developed progressively growing tumors (Fig. 3A). Although all of the mice developed tumors after GFP-VRP vaccination, the individual tumors were consistently smaller than in the naïve mice (Fig. 3B). Tumor-free mice were followed-up until 8 months after tumor challenge, during which time, all of the E7-VRP-vaccinated mice remained tumor free (data not shown). Complete protection was a reproducible finding because two repeat experiments using another E7-VRP preparation gave comparable results (data not shown).

**Role of T-Cell Subsets in Induction of the Immune Response.** The previous data show clear evidence for the induction of E7-specific CD8<sup>+</sup> T cells after the vaccination of mice with E7-VRP. To test the involvement of CD4<sup>+</sup> T cells in the induction of an antitumor

immune response, CD4 and CD8 knockout and wild-type C57BL/6 mice were vaccinated with E7-VRP. Mice were immunized and given booster doses with E7-VRP and challenged with C3 tumor cells as described in Fig. 3. The vaccinated C57BL/6 mice and the CD4 knockout mice were fully protected against a challenge with C3 tumor cells, whereas 100% of both unvaccinated mice and CD8 knockout mice developed progressively growing tumors (Fig. 4). This indicates that CD8 T cells were required, but that CD4 cells were not essential for the induction of a protective immune response by E7-VRP in this model.

**Vaccination Induced IFN- $\gamma$  Suppresses C3 Tumor Growth.** Although 100% of mice developed tumors after vaccination with GFP-VRP, their tumor size was significantly smaller than in naïve mice challenged with the same tumor dosage (Fig. 3). An antigen-nonspecific immune activation may have been induced by vaccination with VRPs that affected the growth of C3 tumor cells. IFN- $\gamma$  was one suspect because *in vitro* cultures of C3 tumor cells grown in the presence of 100 units/ml IFN- $\gamma$  exhibited decreased proliferation (data not shown), and IFN- $\gamma$  is a prominent cytokine produced after AV infection (35). To determine the contributions of IFN- $\gamma$  to both the E7-specific and VRP-nonspecific antitumor effects, IFN- $\gamma$  knockout mice were vaccinated as described in Fig. 3. The differences in tumor growth between naïve and GFP-VRP-vaccinated mice were abolished in IFN- $\gamma$  knockout mice, whereas the outcome of the E7-VRP vaccination initially remained unaffected (Fig. 5).

**Memory Induction by E7-VRP Vaccination.** A very important part of the induction of specific immunity is the formation of a memory response. To determine whether memory was induced by E7-VRP vaccination, groups of eight mice were vaccinated and boosted with E7-VRP, GFP-VRP, or PBS. Three months after the booster dose, all of the mice received a C3 tumor challenge and were monitored for tumor development (Fig. 6). All of the mice vaccinated with GFP-VRP and PBS developed progressively growing tumors, whereas all of the E7-VRP-vaccinated mice were still protected after 3 months. This indicates that two vaccinations with E7-VRP induces long-term memory responses that can prevent tumor outgrowth.

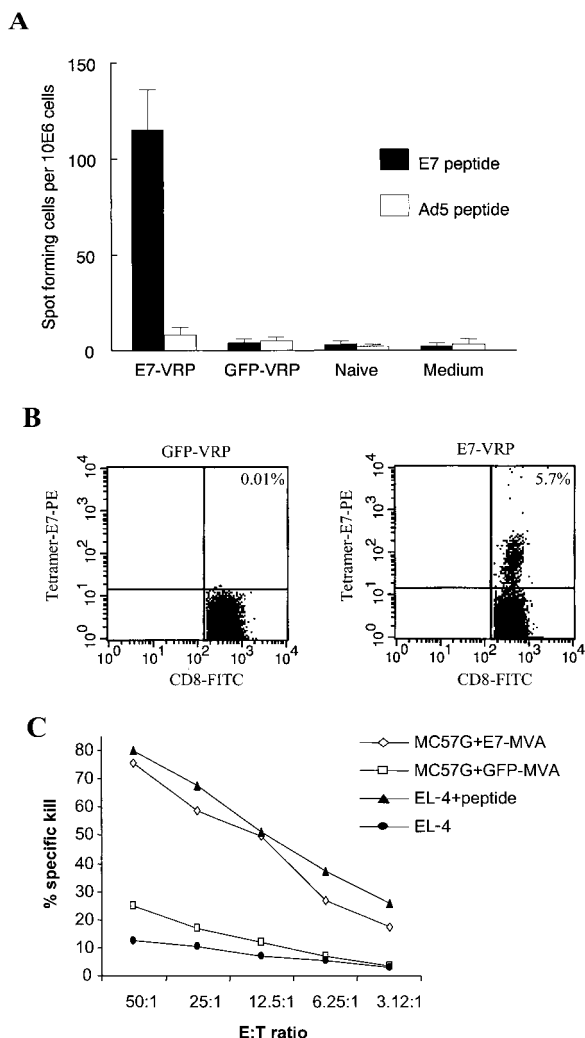


Fig. 2. Determination of cellular immune responses in splenocytes of mice vaccinated with E7-VRP or GFP-VRP or from unvaccinated mice by (A) ELISPOT assay, (B) tetramer staining, and (C) cytotoxicity assays. A, identification of IFN- $\gamma$ -producing cells on stimulation with the HPV16 E7-derived H-2D<sup>b</sup>-restricted CTL peptide RAHYNIIVTF. An adenovirus type 5-derived H-2D<sup>b</sup>-restricted CTL peptide served as a background control. Wells in which the cells were replaced by medium were used as a negative control. Assays were performed in triplicate, and the results were calculated as spot-forming cells per  $10^6$  splenocytes. The graph represents a typical result of multiple similar experiments. B, splenocytes were stained with both FITC-labeled  $\alpha$ CD8 and a PE-labeled E7<sub>49–57</sub>/D<sup>b</sup> tetramer. In the upper right quadrant, the percentages of double-positive cells. This is a representative presentation of five independent experiments with similar results. C, splenocytes from E7-VRP-vaccinated C57BL/6 mice were pooled and restimulated for 5 days with mitomycin C-treated E7-MVA-infected MC57G cells. Cytotoxicity was measured by Eu<sup>3+</sup> release assay on MC57G cells transiently infected with E7-MVA and on E7<sub>49–57</sub>-pulsed EL-4 cells target cells. GFP-MVA-infected MC57G cells and unpulsed EL-4 targets served as negative controls.

#### Eradication of Established Tumors after E7-VRP Treatment.

A therapeutic study was performed to test the ability of E7-VRP to treat existing tumors, a more appropriate measure for an antitumor vaccine. Groups of eight mice received  $5 \times 10^5$  C3 tumor cells. After 7 days, when all of the mice had palpable tumor masses, treatment with E7-VRP, GFP-VRP, or PBS was initiated and repeated on days 14 and 21. A plasmid DNA construct expressing a ubiquitin-E7 minigene fusion protein (S+/Ub) that previously demonstrated therapeutic efficacy (23) was used as a positive control. Fig. 7A reveals that six of eight mice receiving E7-VRP rejected their tumors. The eradication of tumors was reflected in the mean tumor sizes of the groups (Fig. 7B). In contrast, tumors continued to grow in 100% of the mice receiving GFP-VRP and PBS, although there were differences in tumor-growth kinetics between these two groups. Combining the

results of this experiment and a second (data not shown), 11 of 16 mice, or 67%, eradicated established C3 tumors and remained tumor free for a 60-day observation period.

#### DISCUSSION

More than 99% of cervical cancers harbor high-risk HPV DNA (4) and express E6 and E7 oncogenes necessary for the malignant phenotype (7–10). The requirement for sustained E6 and E7 expression by tumor cells implies that tumor eradication may be possible by eliciting strong CTL responses against these viral antigens. The aim of the current study was to evaluate the antitumor efficacy of a VEE replicon vector that has proven highly effective in several viral challenge models (25–29) and may be well suited as an HPV therapeutic vaccine. Because a HPV16 E7<sup>+</sup> tumor model has been established and an immunodominant D<sup>b</sup> E7<sub>49–57</sub> CTL epitope has been identified (12), it was possible to characterize both the immunological and antitumor responses after vaccination with an E7-VRP.

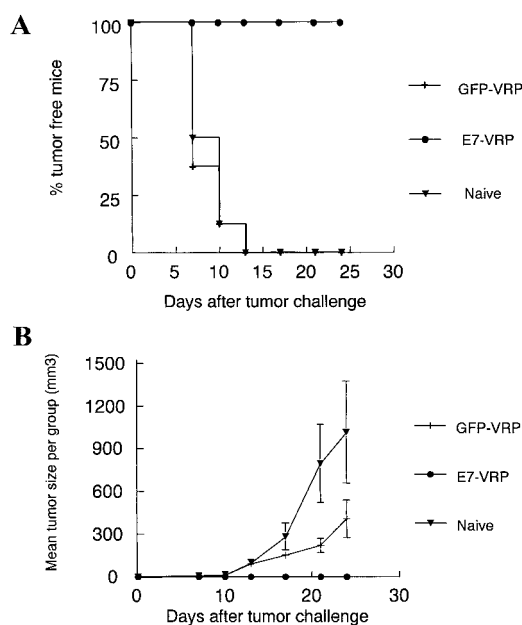


Fig. 3. Three groups of eight C57BL/6 mice were vaccinated s.c. with  $3 \times 10^5$  infectious units of VRPs expressing either HPV16 E7 or GFP in 100  $\mu$ l of sterile PBS. After 2 weeks, booster doses were given, and 2 weeks later, the mice were challenged by a s.c. injection of  $5 \times 10^5$  C3 tumor cells. Naive mice were included to monitor the percentage of tumor take. A, all of the naive and GFP-VRP mice developed a tumor before day 14, and all of the E7-VRP mice remained tumor free. B, the mean tumor sizes in the three groups were significantly different. These results represent one of three separate experiments with similar results.

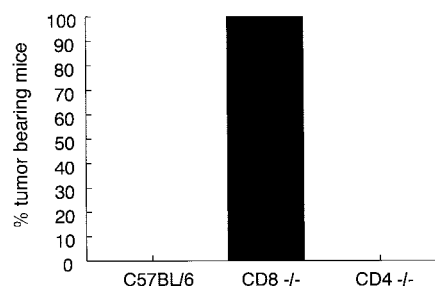


Fig. 4. Wild-type C57BL/6, CD8, and CD4 knockout C57BL/6 mice ( $n = 8$ /group) were vaccinated with E7-VRP and challenged with C3 tumor cells as described in Fig. 3. Four weeks after the tumor challenge, CD8 knockout mice had to be killed as a result of progressively growing large tumor masses in 100% of the mice. The wild-type and CD4 knockout C57BL/6 mice were all protected against the tumor challenge after vaccination with E7-VRP.

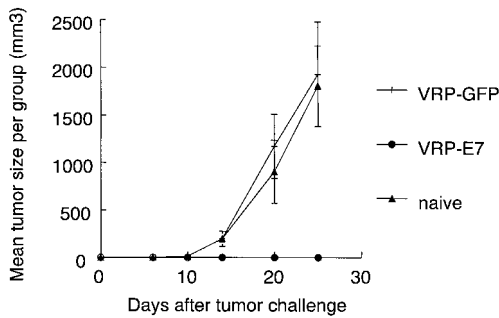


Fig. 5. The role of IFN- $\gamma$  on the tumor protection and tumor growth kinetics after vaccination with E7-VRP and GFP-VRP was studied in IFN- $\gamma$  knockout mice. Vaccinations and tumor challenges were performed as described in Fig. 3. All of the IFN- $\gamma$  knockout mice vaccinated with GFP-VRP and all of the naive mice developed a tumor. The growth kinetics of the tumors in these two groups was similar. All of the IFN- $\gamma$  knockout mice vaccinated with E7-VRP remained tumor free.

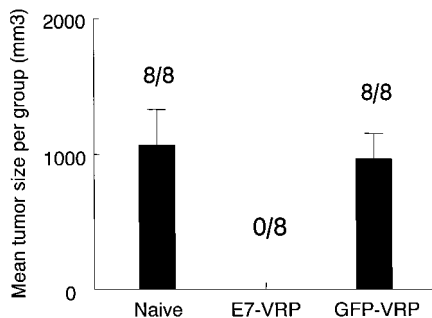


Fig. 6. Three groups of 8 C57BL/6 mice were vaccinated with E7-VRP, GFP-VRP or PBS (naive) as described in Fig. 3. The mice were left untouched for three months at which time all mice were challenged s.c. with  $5 \times 10^5$  C3 tumor cells in 100  $\mu$ l of PBS. The mean tumor size per group is depicted on the Y axis. All of the naive and GFP-VRP-vaccinated mice developed a progressively growing tumor, and all of the E7-VRP-vaccinated mice remained tumor free.

Vaccination of C57BL/6 mice twice with  $3 \times 10^5$  infectious units of E7-VRP elicited MHC class I-restricted CD8<sup>+</sup> T-cell responses against an H-2D<sup>b</sup> E7<sub>49-57</sub> peptide (Fig. 2). Approximately 6% of freshly isolated CD8<sup>+</sup> splenocytes engaged an E7<sub>49-57</sub>/D<sup>b</sup> soluble tetramer (Fig. 2B), indicating that a vigorous E7<sub>49-57</sub> antigen-specific T-cell expansion occurred 2 weeks after E7-VRP vaccination was measurable without *in vitro* restimulation. A proportion of E7<sub>49-57</sub>-specific T cells killed E7<sup>+</sup> target cells (Fig. 2C), which clearly revealed that cytolytic CD8<sup>+</sup> T cells were induced as a result of E7-VRP vaccination. Mice vaccinated by E7-VRP were completely protected against a lethal C3 tumor challenge (Fig. 3). This antitumor response proved to be strictly CD8-T-cell dependent and CD4-T-cell independent (Fig. 4). Collectively, the data presented here demonstrate that E7-VRP vaccination induced robust CD8<sup>+</sup> T cells, which were the effectors responsible for antitumor immunity.

Optimal priming of CD8 CTL activity usually requires CD4 help and is CD40 dependent (36), however examples exist of CD4-independent antiviral responses and antitumor responses, including responses to previous E7 tumor vaccines (19, 20, 23). Similarly, CD4<sup>+</sup> T cells were not required during the priming and initial effector phase (Fig. 4). Nevertheless, a role for helper T cells has been indicated for the rejection of established tumors and induction of protective memory responses (37). Long-lasting T-cell memory was manifest as complete protection from C3 tumor challenge initiated 3 months after the final E7-VRP vaccination (Fig. 6). In separate immunogenicity studies, E7-specific cytotoxicity was readily demonstrable 3 months after E7-VRP boosting (data not shown). It is currently unknown whether any inherent properties of AV vectors favor the elicitation of

long-lived memory CD8<sup>+</sup> T cells with or without contributions by CD4<sup>+</sup> T cells. Nonetheless, for optimal efficacy in humans with diverse HLA alleles, an HPV vaccine should consist of a sufficiently potent vehicle for delivering appropriate viral epitopes in a context highly conducive for eliciting memory cells from both CD4- and CD8-T-cell subsets.

A second important indicator of E7-VRP vaccine efficacy was the ability to control an established tumor load. E7-VRP vaccination 7 days after C3 tumor challenge caused tumor regression against this rapidly growing tumor in 67% of mice after three weekly injections (Fig. 7). The eradication of tumors that were established in all of the mice before day 10 is likely dependent on a rapid CTL induction and mobilization to the progressively growing tumor and may be further improved by increasing VRP dosage or routes of administration. Other E7-specific vaccines exhibiting this level of therapeutic efficacy required modification of E7 by fusing it to a heat-shock protein (19), by altering its intracellular trafficking (21, 22) or facilitating its degradation (23). Routing E7 through the endoplasmic reticulum to a class II loading compartment by incorporating a LAMP-1 sequence has been shown to be important for both vaccinia virus- and plasmid DNA-vectored vaccines against E7<sup>+</sup> TC-1 tumor cells (21, 22). A plasmid DNA that encodes E7 minigenes required ubiquitination and administration by gene gun to observe a high therapeutic efficacy against C3 tumor challenge (23). E7 modification was unnecessary in the E7-VRP vaccine; however, as already noted, there may be strategies to improve E7-VRP therapeutic efficacy by intra- or intercellular targeting (21–23) or coadministration with VRPs that contain cytokines or costimulatory molecules.

Other AV vectors derived from SFV and Sindbis virus have shown efficacy as tumor vaccines in formats including replicon particles (38), naked RNA (39), and plasmid DNA encoding a viral replicase (40). Therapeutic efficacy has been observed against day-2-established CT26.CL25 tumors expressing the *Lac Z* gene when alphaviral

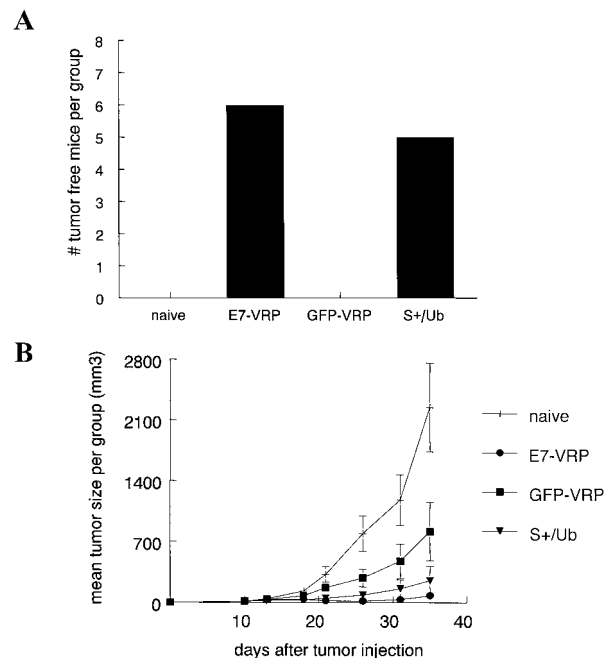


Fig. 7. Mice received  $5 \times 10^5$  C3 tumor cells on day 0, and by day 7, all of the mice had a palpable tumor. Therapeutic vaccination was then initiated with  $3 \times 10^5$  infectious units of E7-VRP or GFP-VRP or with 2  $\mu$ g of S+/Ub DNA (23) and repeated on days 14 and 21. A, all of the naive mice and the mice vaccinated with GFP-VRP developed a tumor. Six of eight mice vaccinated with E7-VRP, as well as five of the mice that received the DNA vaccine, remained tumor free. B, the mean tumor sizes of the groups reflect the differences in the therapeutic potential of the treatments.

replicon RNA (39) or pDNA (40) were injected at low doses. These studies attributed antitumor efficacy to qualitative rather than quantitative properties of the AV vector, most notably the induction of apoptosis (39, 40). Gene gun vaccination with E7-VEE replicon RNA protected 6 (75%) of 8 mice against C3 tumor challenge (data not shown), which revealed that E7-VEE replicon RNA, like other AV RNA-based tumor vaccines, elicits protection against tumor challenge that is not directly dependent on packaged replicon particles. Although E7-VEE RNA immunization was not as protective as VRP (Fig. 3), additional studies are required to define the optimal delivery method for VEE RNA. Although VEE replicons have not been tested clinically, the use of packaged replicons to deliver RNA should be highly efficacious because they mimic a natural infection pathway.

During the preparation of this report, results were published (41) assessing SFV replicons encoding both the E6 and E7 genes packaged in a manner analogous to the one described here. Using the TC-1 tumor model, Daemen *et al.* observed 40% tumor protection after administering  $5 \times 10^6$  infectious units of E6 and E7 containing SFV replicons three times. The notable vector and insert differences between this and the present studies include any relevant tropism differences that may exist between SFV *versus* VEE and the use of E6-E7 inserts (41), in which E7 translation by leaky scanning from an ATG of E6 may not be as optimal as the single E7 insert used herein. SFV vaccination appeared to prime E7-specific effector cells, although the CTL restimulations were considerably longer (11–18 days) and included interleukin-2 (41); these were far greater *in vitro* restimulation conditions than what was required to measure CTLs after E7-VRP vaccination (Fig. 2C). Some unknown differences may exist between the tumors used, although TC-1 does not appear to be an inferior CTL target compared with C3 because it is more readily lysed *in vitro* than C3 by an anti-E7-specific T-cell clone, which may relate to its higher E7 expression levels.<sup>5</sup> Direct comparisons of E7-VRP in both models may clarify these discrepancies.

Several tumor challenge experiments (Fig. 3B and 7B) indicated activation of an immune response by GFP-VRPs, because tumor sizes, after GFP-VRP administration, were found significantly smaller than in naïve mice. Viral infections and double-stranded RNA are known to induce IFN- $\alpha/\beta$  responses, which induce the production of IFN- $\gamma$  by T cells and natural killer cells. Our experiments in IFN- $\gamma$  knockout mice indicate that IFN- $\gamma$  is involved in the tumor growth delay of C3 cells in mice. The ELISPOT and tetramer analyses, however, do not reveal an increased CTL induction against E7, which indicates that the IFN- $\gamma$  is not produced by E7-specific CTLs. Activation of helper T cells and NK cells by viral particles is a plausible explanation for IFN- $\gamma$  production, but these cells are not detected by the assays mentioned above. The tumor growth inhibition might be a direct effect of IFN- $\gamma$  on the C3 cells, which we have previously observed *in vitro*. Vaccination with E7-VRP in IFN- $\gamma$  knockout mice did not affect the tumor protection. This indicates that the cytolysis of C3 cells *in vivo* is IFN- $\gamma$  independent and most likely induced by perforin release of E7-specific CTLs.

The safety concerns for the clinical advancement of VEE replicon-based vaccines expressing HPV genes can be addressed through a number of precautions. Several safety features are inherent in the VEE vector itself, including its derivation from an attenuated vaccine strain (33) and the split helper packaging system, which minimizes the likelihood of replication-competent recombinant viruses (25). Preclinical primate studies to date portray very favorable safety profiles with no indications of VRP-related toxicities, even when doses of  $5 \times 10^8$  were administered *i.v.* (26, 28). The data presented herein support the

continued investigation of the safest and most efficacious strategies for bringing these RNA vaccines to the clinic as therapeutics against cervical carcinoma and CIN.

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