Published in final edited form as:

Vaccine. 2010 December 6; 28(52): 8345–8351. doi:10.1016/j.vaccine.2009.04.082.

Reversal of papilloma growth in rabbits therapeutically vaccinated against E6 with naked DNA and/or vesicular stomatitis virus vectors

Janet L. Brandsma^{1,2,3,4}, Mark Shlyankevich¹, Yuhua Su⁵, Daniel Zelterman^{3,5}, John K. Rose^{2,3}, and Linda Buonocore²

¹ Section of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520

² Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520

³ Yale Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520

⁴ Yale Skin Disease Research Center, Yale University School of Medicine, New Haven, Connecticut 06520

⁵ Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06520

Abstract

Persistent infection with high-risk human papillomaviruses (HPVs) is the greatest risk factor for the development of HPV-associated cancers. In this study rabbits bearing persistent and potentially malignant papillomas were used to test the efficacy of vaccination with a recombinant DNA and/or vesicular stomatitis virus (VSV) targeting the cottontail rabbit papillomavirus (CRPV) E6 protein. Immune responses were primed with either vector and boosted twice with the homologous or heterologous E6 vector. Over the course of 18 weeks, E6 vaccination reduced papilloma volumes to one third the volume in the controls, and the rabbits boosted with an heterologous vector tended to mount stronger responses. Small and medium-sized papillomas responded significantly but only slightly better than large papillomas. Finally the initial papilloma burden per rabbit, ranging from $<100 \text{ mm}^3$ to $>1000 \text{ mm}^3$, was not prognostic of antitumor efficacy. In summary both E6 vaccines elicited significant therapeutic immunity, and their sequential use tended to be advantageous.

1. Introduction

Human papillomavirus (HPV) infections induce a variety of premalignant epithelial tumors. Such tumors normally regress spontaneously following the development of HPV antigen-specific cell-mediated immunity [1,2], but tumors that persist can progress to carcinoma. High-risk HPV-associated lesions precede the development of some skin cancers, many head and neck cancers and most anogenital cancers [3–5] and HPV infection is a necessary cause of cervical cancer, the most common and best studied HPV-associated malignancy [5]. Patients with high-grade cervical neoplasia or carcinoma generally lack evidence of HPV-specific cellular immunity [6–9] suggesting that vaccination against HPV tumor antigens has the potential to benefit such patients.

The standard treatment for HPV-associated high grade neoplasia is surgical excision or laser ablation. Unfortunately HPV infection often persists in normal-appearing tissue, and lesions recur in about 20% of treated patients [10,11]. Patients with recurrent lesions may be

excellent candidates for HPV-specific therapeutic vaccination because after treatment, the majority of recurrence-free patients exhibit proliferative T-cell responses to HPV16 E6 and E7 antigens and expression of T-helper type 1(Th1) cytokines in contrast to patients with recurrent disease [12]. Therapeutic vaccination may be particularly valuable for such patients, especially if combined with standard treatment.

The most attractive targets for therapeutic vaccination against HPV-associated lesions are the viral E6 and E7 oncoproteins, which are expressed in all HPV-induced lesions. In cervical cancer cell lines moreover the deliberate inhibition of E6/E7 transcription reactivates dormant tumor suppressor pathways and causes senescence [13]. Antigens in the HPV E6 protein appear to be particularly important for the stimulation of therapeutically effective immune responses because healthy subjects previously infected with HPV16 or HPV18 exhibit E6-specific CD4+ Th1 responses and memory T-cells, whereas E6-specific responses in cervical cancer patients are negligible [14–16].

The induction of cell-mediated immune responses sufficient for antitumor efficacy will almost certainly require repeated vaccination [17]. Naked DNA vectors have a distinct advantage in this regard because the absence of vector proteins precludes the induction of neutralizing antibodies, which often limit the efficacy of boosting with virus-based vectors [17,18]. This feature could be especially beneficial to the development of anti-cancer vaccines, and some DNA-based anti-cancer vaccines currently in clinical trials [19–23].

Virus-based vaccine vectors attenuated for pathogenicity have complementary attributes. Viral mechanisms of cell entry are highly evolved [24], and in vivo viral vectors express greatly elevated levels of recombinant antigens compared to DNA vectors. Simultaneously, they express vector proteins which, being naturally immunogenic, enhance the strength of both humoral and cellular immune responses. Additionally, if the viral vector is replication-competent, expression of the recombinant antigens (vaccine targets) is amplified and prolonged, further contributing to the induction of effective immunity. In this study we used an attenuated, replication-competent vesicular stomatitis virus (VSV)-based vector. VSV-based vaccines induce strong cellular and humoral immunity as well as long-term memory responses [25,26].

The only small animal model of high-risk HPV infection and spontaneous malignant progression is the cottontail rabbit papillomavirus (CRPV)/rabbit model [27]. CRPV infection predictably induces papillomas that persist for long periods of time in the virtual absence of regression, until one or more regions spontaneously progresses to invasive carcinoma in most rabbits. Beyond infection, the greatest risk factor for the development of HPV-associated carcinoma is HPV persistence [7]. Because persistence involves the development of mechanisms of immune evasion, it makes the development of antitumor vaccines particularly challenging. Therefore the persistence and malignant potential of CRPV-induced papillomas make them physiologically relevant targets for preclinical therapeutic vaccine development.

The level of immunologic resistance to viral infections and malignant progression can frequently be augmented by boosting. An especially effective immunization strategy is to prime an immune response with one vector and boost it with a heterologous vector encoding the same antigen [28–30]. The current study evaluated the antitumor efficacy of prime-boost protocols using a DNA-based vector encoding a ubiquitin-fused CRPV E6 protein (UbE6 DNA) and a vesicular stomatitis virus based vector encoding the wild-type E6 protein (VSV-E6). Previous studies showed that a single immunization with each vector individually induced significantly prophylactic and/or therapeutic immunity against CRPV-

induced disease. The results show that all E6 prime/boost protocols significantly reduced papilloma volumes in rabbits with established lesions at the time of therapeutic intervention.

2. Methods

2.1 CRPV infection of rabbits

Two-kilogram female New Zealand white Pasteurella-free rabbits (Millbrook Farms, Amherst, MA) were maintained in the animal facilities at the Yale University School of Medicine. All experiments were performed in accordance with procedures approved by the Yale Institutional Animal Care and Use Committee. Each rabbit was infected with CRPV at nine cutaneous sites, each having an area of approximately 1 cm². Briefly, three sites per rabbit were infected by scarification with a 1:50, 1:250 or 1:1250 dilution of our K216 stock of CRPV.

2.2 VSV neutralizing antibody titers

Serial two-fold dilutions of sera were assayed for neutralizing antibodies to the G protein of rVSV Indiana as previously described [31]. Differences in antibody titers were analyzed using t-Tests.

2.3 Vaccines and vaccination

The VSV-E6 vaccine (VSV-E6.5) and the negative control VSV vector encoding green fluorescent protein (VSV-GFP) were described previously [32]. The UbE6 DNA vaccine, based on pcDNA3, was also described previously [33]. Prior to vaccination, the rabbits were sedated with intramuscular Acepromazine (J.A. Webster Inc., Sterling, MA, USA) (35 mg/kg). For VSV vaccination, each rabbit was inoculated intradermally with 5×10^7 pfu of VSV on the shoulder after clipping the fur, as previously described [32]. For DNA vaccination, the left flank was clipped and remaining fur was depilated using Nair (vondor). The DNAs were then delivered with the aid of a helium-driven PowderJectR XR-1 research device (gift of Powderject® Vaccines, Madison, WI, USA) to nine cutaneous sites on the left flank using 1 µg DNA/0.5 mg gold per site, as described previously [33].

2.4 Collection of clinical data

Rabbits were examined for papilloma formation 17, 21, 31 (baseline period), 43, 52, 63, 72, 84, 93 (first boost period), 99, 112, 134, 143 and 154 (second boost period) days after CRPV infection. Data entries on day 17 and 21 were ignored because they preceded vaccination. The data were used to calculate the frequency of papilloma formation (number of positive sites/all sites) and the volumes of the papillomas ($\frac{4}{3} \times \pi \times \text{mean length}/2 \times \text{mean width}/2 \times \text{mean height}/2$). At each examination, the number, location and dimensions (length, width, height) of each papilloma were recorded.

2.5 Statistical analysis

The statistical analysis of vaccine effects on papilloma volumes were based on cumulative volumes due to the heterogeneity of the volume data as in a previous study [34]. The cumulative papilloma volumes were calculated by using the trapezoidal rule for the area under the curve generated by plotting the volume at each site over time. For the sites without any palpable papilloma at all during the study, data entries were treated as missing. The missing data were mainly at the sites infected with the low doses of CRPV. Data entries of zero volume (prior papilloma formation) were replaced by 0.00065 mm³ to enable analysis of log volumes. The efficacy of each treatment was summarized using cumulative papilloma volumes at each CRPV infection site for different time periods. The cumulative volumes were further divided by the length of the duration of the underlying time period to generate

standardized cumulative volumes. The standardized cumulative volumes were used to describe the papilloma experience over time. ANCOVA models were used to compare the effects of vaccines between two vaccinated groups and/or between the vaccinated and the control. The PROC MIXED procedure of SAS version 9.1 was used in computation.

3. Results

3.1 Experimental Design

The experimental design is illustrated in Figure 1. Twenty-five rabbits were infected at a total of nine sites with CRPV dilutions of 1:50, 1:250 and 1:1250. Papillomas grew for one month prior to assigning the rabbits to five groups to achieve equivalent papilloma volumes in each group (Table 2). Two groups were then primed with the UbE6 DNA vaccine and two with the VSV-E6 vaccine. Three and nine weeks later, the rabbits were boosted, with the homologous vector or heterologous E6 vector. The control group was primed with the empty pcDNA3 vector and boosted with pcDNA3 (n=3) or VSV-GFP (n=2). The rabbits were followed for 154 days after CRPV infection.

3.2 Immunization with the VSV vectors elicits immunity to the vector

Recombinant viral vectors elicit immune responses to the vector proteins as well as the recombinant protein. To determine whether the VSV-E6 and VSV-GFP vectors elicited immune responses, we measured VSV-specific neutralizing antibodies in the sera of all rabbits. All rabbits vaccinated with either VSV-E6 or VSV-GFP developed high-titered VSV-specific neutralizing antibody titers, confirming that the VSV vectors were immunogenic (Table 1). In contrast, all sera from the rabbits inoculated with DNA alone were negative, confirming the specificity of the antibody reactivity. Further analysis showed that VSV-specific neutralizing antibody titers were not significantly different in rabbits that received VSV-E6 two or three times (groups 3 and 5) compared to rabbits that received VSV-E6 once (group 4). ($P=0.14$).

3.3 Clinical status of the rabbits prior to vaccination

The severity of disease at the time of therapeutic intervention may influence the therapeutic responses to vaccination. For this reason, the individual rabbits were assigned to groups to achieved equivalent papilloma numbers and volumes in each group just before priming. At that time, papillomas had formed at all sites infected with the high dose of virus, all but one site infected with the moderate dose, and at 83% of sites infected with the low dose (Table 2). They had reached a mean volume of 97 mm³ at the CRPV high dose sites, 18 mm³ at the moderate dose sites, and 3 mm³ at the low dose sites, or a total mean burden per rabbit of 354 mm³. The five groups showed no significant differences in either papilloma frequency or volume at any CRPV dilution (Table 2).

3.4 Time course of responses to UbE6 DNA and/or VSV-E6 vaccination

Initial analysis showed no difference in papilloma volumes between the control rabbits boosted with the VSV-GFP vs. empty DNA vector (groups 1a and 1b, Fig. 1), so the data from the five rabbits were pooled for further analysis. The time course of papilloma growth in the control and five E6 vaccinated groups at each set of CRPV infection sites is shown in Fig. 2. Within about two weeks after priming, papillomas in the vaccinated groups began to grow more slowly than in the controls. About three weeks after the first boost, papillomas in the vaccinees actually diminished in volume relative to earlier time points. This period of growth reversal lasted about two weeks before the rabbits were re-boosted, six weeks after the first boost. About three weeks after the second boost, papilloma growth was again reversed in the vaccinees relative to the controls. As immune responses require about two

weeks to develop following vaccination, the timing of the clinical responses strongly suggests that they were immunologically based. All four E6 prime-boost protocol reduced cumulative papilloma volumes. As cumulative volume equals the total papilloma burden carried by the rabbits throughout the experiment, it is a stringent measure of efficacy. Overall (all sites in all rabbits), papilloma volumes were reduced by $55.4\% \pm 13.7\%$ in group 2, $71.1\% \pm 9.2\%$ in E6 group 3, $61.8\% \pm 7.4\%$ in group E 6 5 and $77.9\% \pm 4.3\%$ in E6 group 4 (see Fig. 1 for group designations). Additionally, there were no differences in efficacy between any of the E6 vaccination protocols. Thus, both E6 vaccines were immunotherapeutic, confirming earlier work [35].

3.5 Contrasts of interest

We next assessed whether the UbE6 DNA vs. VSV-E6 vaccine was more effective for priming or boosting and whether boosting with homologous vs. heterologous vectors was more effective. For this purpose we calculated for each infection site the cumulative papilloma volume, i.e. the sum of the papilloma volume at each measurement interval. The cumulative volumes at the nine sites per rabbit were then totaled, yielding a single value per rabbit. Four orthogonal contrasts were then analyzed using the logarithm of the standardized cumulative papilloma volumes from day 43 after priming to day 154, the end of the experiment, and the logarithm of the volumes on day 31, the day of priming, as the baseline. The analyses showed that vaccination against E6 antigens (all protocols) reduced papilloma volumes by 66.5% relative to the controls overall, and this effect was significant even after adjusting the p-value for multiple comparisons (Table 3). Additional analyses showed that VSV-E6 vaccine was slightly more effective than the UbE6 DNA vaccine for priming (18.1%) and boosting (23.5%), but neither difference was statistically significant. Finally, the heterologous combination of E6 vectors was 38.5% more effective than the homologous combination and this effect was marginally significant ($P=0.0724$). Thus, vaccination with the UbE6 DNA and/or VSV-E6 vectors was highly effective, and the use of both vaccines in sequence may have augmented therapeutic efficacy.

3.6 Relative efficacy of therapeutic vaccination against small, medium-sized and large papillomas

Each rabbit had three size-classes of papillomas induced by the low, moderate or high dose of CRPV, respectively, and the difference in volume between each pair of sites was highly significant. The experimental design therefore allowed us to compare the antitumor responses of the small, medium and large-sized papillomas. For this analysis, the mean cumulative volume at each set of papillomas was calculated and the values in the vaccinated rabbits were then normalized to the mean value of the control group at the corresponding CRPV dose. The results showed that relative to the controls, treatment with UbE6 DNA and/or VSV-E6 vaccines significantly reduced papilloma volumes, by $28.4\% \pm 7.1\%$, $29.1\% \pm 4.8\%$, and $37.7\% \pm 4.5\%$ at the low, moderate and high dose CRPV sites respectively. Further analysis showed that the small and medium-sized papillomas responded significantly better than the large papillomas ($P=0.009$), although the magnitude of the difference was small, just 8.9%. No difference however was detected between the responses of the small and medium-sized papillomas ($P=0.847$).

3.7 Relative efficacy of therapeutic vaccination in rabbits with different papilloma volumes

Rabbits are genetically diverse and vary considerably in their natural responses to CRPV infection. On the day of priming, the papilloma burden per rabbit ranged from a low of 77 mm^3 to a high of 1114 mm^3 in different individuals. To formally assess the relationship between the individuals' initial burden and vaccine efficacy, we ordered each rabbit according to its total papilloma burden on day 31 before priming and plotted the data (Fig. 4A, top panel). The same order was then used to plot the cumulative papilloma volume (Fig.

4A, bottom panel). The results showed that the antitumor responses of the vaccinees with initially small papilloma burdens were variable, *e.g.* compare rabbit # 8 and #9, as were those of vaccinees with initially large papilloma burdens, *e.g.* compare rabbit #19 and #20. Statistical analysis showed that the papilloma burden before therapeutic intervention was not a good prognosticator of the antitumor response to vaccination with the UbE6 DNA and/or VSV-E6 vectors (correlation coefficient = 0.260).

As the above result was somewhat unexpected, we approached the question in a different way, by intentionally maximizing the volume differences. Thus, the five rabbits with the smallest papilloma burden were assigned to volume group 1 (V1), the next five to group V2 and so forth, ending with the five rabbits with the largest papilloma burden in group V4. In the new groups, the mean papilloma burden per rabbit ranged from a low of 92 mm³ in group V1 to a high of 845 mm³ in group V4. Now the initial papilloma burden in each group was significantly different from the next group ($P \leq 0.003$ for V1 vs. V2 (V1/V2), V2/V3, and V3/V4). Nonetheless, at the end of the experiment, the differences between any two groups were astonishingly small and lacked statistical significance ($P=0.191$ for V1/V2, $P=0.873$ for V2/V3 and $P=0.915$ for V3/V4). Even the initial 9.2-fold difference in papilloma burden between groups 1 and 4 ($P=0.00002$) was reduced to a 1.8-fold difference after vaccination ($P=0.105$). Thus the rabbits' initial responses to CRPV infection had little to do with their responses to therapeutic intervention.

4. Discussion

In earlier work we developed naked DNA vaccine vectors encoding the CRPV wild-type (E6) or ubiquitin-fused E6 (UbE6) protein. Each vector induced prophylactic immunity after a single immunization, and the UbE6 DNA vaccine was significantly more effective than the E6 DNA vaccine [36,37]. In a therapeutic study, a single immunization the UbE6 DNA vector, combined with DNA vectors encoding UbE1, UbE2 and UbE7 proteins, greatly reduced growth in rabbits with papillomas at the time of vaccination [35]. The UbE6 DNA vaccine alone had not been previously tested for therapeutic efficacy or in a prime/boost protocol. In other studies we developed recombinant VSV-based vaccines encoding the E6 or UbE6 protein. A single immunization with either VSV vector significantly suppressed papilloma growth, and the individual vectors yielded nearly identical results, showing that ubiquitin-fusion did not have the same potency in the VSV vector as the DNA vector, at least in the CRPV/rabbit model [32].

Previous studies have shown that humoral, cellular and protective immune responses elicited by vaccination with a VSV vector can be significantly elevated by priming [38] or boosting [25,39,40] with another virus vector encoding the same antigen. One study used DNA vectors to prime VSV-induced protective immunity [41], but unlike our study, one of the DNAs encoded a powerful vaccine adjuvant [42].

Our study evaluated the therapeutic efficacy of sequential vaccination with the UbE6 DNA and/or VSV-E6 vector in rabbits with a mean papilloma burden of 354 mm³ at the time of priming. The vaccinees were primed with the UbE6 DNA vector or the VSV-E6 vector and boosted twice, three and nine weeks later, with the homologous or heterologous E6 vector. The control group was inoculated at the same times with the VSV vector encoding an irrelevant protein (GFP) and/or the empty DNA vector. Overall, the E6 prime-boost protocols reduced papilloma volumes to just one-third the volume in the controls ($P=0.02$), a very significant effect considering that the analysis used just one value per rabbit. Additionally, boosting with a heterologous E6 vector was 15.6% more effective than boosting with an homologous E6 vector, and the difference was marginally significant. Complete regression in some rabbits therapeutically vaccinated with the VSV-E6 vector

occurred in a previous study [32] but not in this study. In the previous study however rabbits were vaccinated four weeks earlier after CRPV infection. While the different times of therapeutic intervention were not directly compared, the results suggest that stronger immune responses will be required to eliminate established papillomas (this study) relative to subclinically infected cells [32]. Since in the current study the equivalent pre-vaccination tumor burden in a 70 kg woman would be 12 cm³, the efficacy of the UbE6 DNA and/or VSV-E6 vaccination protocols can probably be improved by tumor debulking at the time of vaccination.

The antitumor effects exhibited by the vaccinated rabbits were almost certainly mediated by cellular immune responses. This conclusion is based first on the time course of clinical efficacy (reduction in papilloma volume) which generally began about three weeks after vaccination, a reasonable amount of time for the development of cell-mediated immunity and subsequent killing of tumor cells, and second by previous studies in which therapeutic vaccination of rabbits with either the UbE6 DNA or VSV-E6 vector elicited antitumor efficacy in the absence of detectable E6-specific antibodies [32,37] and data not shown). Moreover, numerous other studies in animal models as well as patients have documented the importance of cell-mediated immunity for the clearance of papillomavirus-associated lesions [43–45].

The VSV-E6 vaccine was slightly more effective than the UbE6 DNA vaccine, but the difference was not statistically significant. As DNA vectors are generally significantly weaker than viral vectors encoding the same protein, potential reasons for the high level efficacy of the UbE6 DNA vector relative to the VSV-E6 vector were considered. One factor enhancing the efficacy of the UbE6 DNA vector was almost certainly the ubiquitin fusion, compared to what might have been achieved using the wild-type E6 DNA vector, based on direct comparison in a prophylactic study [36]. On the other hand, the lack of ubiquitin fusion in the VSV-E6 vector is unlikely to have any effect, based on direct comparison of VSV vectors expressing E6 vs. UbE6 vectors in an earlier therapeutic vaccination study [32]. Another factor perhaps explaining why the VSV-E6 vector did not appear to be more effective is that only half the VSV-E6-primed rabbits benefited from boosting, as shown previously by direct comparison [32]. The lack of efficacy of boosting was probably due to the presence of VSV-specific neutralizing antibodies induced by priming [32]. Both groups of UbE6 DNA-primed rabbits on the other hand most likely benefited from homologous as well as heterologous E6 boosting. This interpretation is supported by the similar titers of VSV-specific neutralizing antibody in all VSV-E6-vaccinated rabbits, regardless of whether they received VSV-E6 once, twice or three times.

The study also showed that small and medium-sized papillomas responded significantly better to therapeutic vaccination than large papillomas. However the differences were small (about 9%) especially when compared to the actual volumes which were 4.3-fold and 1.8-fold smaller respectively at sites infected with the low or moderate dose of CRPV relative to the high dose. No difference was found between the responses of the small vs. medium-sized papillomas. Thus, the therapeutic efficacy of UbE6 DNA and/or VSV-E6 vaccines was similar among all papillomas, regardless of the starting volume.

The genetic diversity of rabbits and the physiologic similarities between persistent lesions caused by CRPV or HPV prompted us to address another issue, namely whether the papilloma burden per rabbit at the time of immunization had prognostic value regarding vaccine efficacy. If so, then tumor volume could be a useful parameter for the eventual selection of human subjects most likely to benefit from vaccination, *e.g.* with HPV homologues of the CRPV vectors. Instead, the results showed that the therapeutic responses to vaccination correlated poorly with the natural responses to early CRPV infection,

suggesting that therapeutic vaccination may have stimulated different components of the immune system and/or stimulated the same components in different ways.

In summary, sequential vaccination of rabbits with a DNA and/or VSV-based vector encoding the CRPV E6 protein in homologous and heterologous prime/boost protocols reduced the volumes of persistent premalignant tumors by 67% relative to the controls, regardless of the specific protocol. Additionally, heterologous prime/boost protocols tended to induce stronger responses than homologous protocols. The results suggest that analogous vectors targeting the HPV E6 protein could be of therapeutic value in patients with persistent premalignant tumors associated with high-risk types of HPV. They might be especially useful for preventing the recurrence of premalignant tumors following, or in conjunction with, removal of the visible tumor.

Acknowledgments

This work was supported by grants from the American Cancer Society (#TURPG MBC-100103) and the National Cancer Institute (5R01CA98355).

References

1. Baseman JG, Koutsky LA. The epidemiology of human papillomavirus infections. *Journal of Clinical Virology*. 2005; 32 (Suppl 1):S16–24. [PubMed: 15753008]
2. Schiffman M, Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. *Journal of the National Cancer Institute*. 2003; (31):14–9. Monographs. [PubMed: 12807940]
3. Akgul B, Cooke JC, Storey A. HPV-associated skin disease. *Journal of Pathology*. 2006; 208(2): 165–75. [PubMed: 16362995]
4. Hobbs CGL, Sterne JAC, Bailey M, Heyderman RS, Birchall MA, Thomas SJ. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. *Clinical Otolaryngology*. 2006; 31(4):259–66. [PubMed: 16911640]
5. zur Hausen H. Papillomavirus infections--a major cause of human cancers. *Biochimica et Biophysica Acta*. 1996; 1288(2):F55–78. [PubMed: 8876633]
6. Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. *Cancer Research*. 1997; 57(14):2943–50. [PubMed: 9230206]
7. Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. *Current Cancer Drug Targets*. 2007; 7(1):79–89. [PubMed: 17305480]
8. Rensing ME, van Driel WJ, Celis E, Sette A, Brandt MP, Hartman M, et al. Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type 16-positive cervical lesions against a human leukocyte antigen-A *0201-restricted E7-encoded epitope. *Cancer Research*. 1996; 56(3):582–8. [PubMed: 8564975]
9. Tindle RW. Immune evasion in human papillomavirus-associated cervical cancer. *Nature Reviews Cancer*. 2002; 2(1):59–65.
10. Gok M, Coupe VMH, Berkhof J, Verheijen RHM, Helmerhorst TJM, Hogewoning CJA, et al. HPV16 and increased risk of recurrence after treatment for CIN. [see comment]. *Gynecologic Oncology*. 2007; 104(2):273–5. [PubMed: 17157365]
11. Paraskevaidis E, Arbyn M, Sotiriadis A, Diakomanolis E, Martin-Hirsch P, Koliopoulos G, et al. The role of HPV DNA testing in the follow-up period after treatment for CIN: a systematic review of the literature. *Cancer Treatment Reviews*. 2004; 30(2):205–11. [PubMed: 15023438]
12. Sarkar AK, Tortolero-Luna G, Follen M, Sastry KJ. Inverse correlation of cellular immune responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 with recurrence of cervical intraepithelial neoplasia in a cross-sectional study. *Gynecologic Oncology*. 2005; 99(3 Suppl 1):S251–61. [PubMed: 16188303]

13. Goodwin EC, DiMaio D. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97(23): 12513–8. [PubMed: 11070078]
14. Welters MJP, de Jong A, van den Eeden SJF, van der Hulst JM, Kwappenberg KMC, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. *Cancer Research*. 2003; 63(3):636–41. [PubMed: 12566307]
15. Welters MJP, van der Logt P, van den Eeden SJF, Kwappenberg KMC, Drijfhout JW, Fleuren GJ, et al. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. *International Journal of Cancer*. 2006; 118(4):950–6.
16. de Jong A, van Poelgeest MIE, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. *Cancer Research*. 2004; 64(15):5449–55. [PubMed: 15289354]
17. Schlom J, Gulley JL, Arlen PM. Paradigm shifts in cancer vaccine therapy. *Experimental Biology & Medicine*. 2008; 233(5):522–34. [PubMed: 18375829]
18. Anderson RJ, Schneider J. Plasmid DNA and viral vector-based vaccines for the treatment of cancer. *Vaccine*. 2007; 25 (Suppl 2):B24–34. [PubMed: 17698262]
19. Wu TC. Therapeutic human papillomavirus DNA vaccination strategies to control cervical cancer. [comment]. *European Journal of Immunology*. 2007; 37(2):310–4. [PubMed: 17273998]
20. Cheng W-F, Hung C-F, Pai SI, Hsu K-F, He L, Ling M, et al. Repeated DNA vaccinations elicited qualitatively different cytotoxic T lymphocytes and improved protective antitumor effects. *Journal of Biomedical Science*. 2002; 9(6 Pt 2):675–87. [PubMed: 12432234]
21. Haupt K, Roggendorf M, Mann K. The potential of DNA vaccination against tumor-associated antigens for antitumor therapy. *Experimental Biology & Medicine*. 2002; 227(4):227–37. [PubMed: 11910045]
22. Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. *Advances in Genetics*. 2005; 55:25–40. [PubMed: 16291211]
23. Stevenson FK, Rice J, Ottensmeier CH, Thirdborough SM, Zhu D. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunological Reviews*. 2004; 199:156–80. [PubMed: 15233733]
24. Brave A, Ljungberg K, Wahren B, Liu MA. Vaccine delivery methods using viral vectors. *Molecular Pharmaceutics*. 2007; 4(1):18–32. [PubMed: 17274663]
25. Haglund K, Leiner I, Kerkisiek K, Buonocore L, Pamer E, Rose JK. Robust recall and long-term memory T-cell responses induced by prime-boost regimens with heterologous live viral vectors expressing human immunodeficiency virus type 1 Gag and Env proteins. *Journal of Virology*. 2002; 76(15):7506–17. [PubMed: 12097563]
26. Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses from DNA. [erratum appears in *Proc Natl Acad Sci U S A* 1995 Sep 12;92(19):9009]. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92(10):4477–81. [PubMed: 7753828]
27. Kreider JW, Bartlett GL. The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression. *Advances in Cancer Research*. 1981; 35:81–110. [PubMed: 6280459]
28. Dale CJ, Thomson S, De Rose R, Ranasinghe C, Medveczky CJ, Pamungkas J, et al. Prime-boost strategies in DNA vaccines. *Methods in Molecular Medicine*. 2006; 127:171–97. [PubMed: 16988455]
29. Woodland DL. Jump-starting the immune system: prime-boosting comes of age. *Trends in Immunology*. 2004; 25(2):98–104. [PubMed: 15102369]
30. Ramsay AJ, Kent SJ, Strugnell RA, Suhrbier A, Thomson SA, Ramshaw IA. Genetic vaccination strategies for enhanced cellular, humoral and mucosal immunity. *Immunological Reviews*. 1999; 171:27–44. [PubMed: 10582164]

31. Rose NF, Roberts A, Buonocore L, Rose JK. Glycoprotein exchange vectors based on vesicular stomatitis virus allow effective boosting and generation of neutralizing antibodies to a primary isolate of human immunodeficiency virus type 1. *Journal of Virology*. 2000; 74(23):10903–10. [PubMed: 11069984]
32. Brandsma JL, Shlyankevich M, Buonocore L, Roberts A, Becker SM, Rose JK. Therapeutic efficacy of vesicular stomatitis virus-based E6 vaccination in rabbits. *Vaccine*. 2007; 25(4):751–62. [PubMed: 16962690]
33. Brandsma JL. The cottontail rabbit papillomavirus model of high-risk HPV-induced disease. *Methods in Molecular Medicine*. 2005; 119:217–35. [PubMed: 16350405]
34. Brandsma JL, Shlyankevich M, Su Y, Roberts A, Rose JK, Zeltermann D, et al. Vesicular stomatitis virus-based therapeutic vaccination targeted to the E1, E2, E6, and E7 proteins of cottontail rabbit papillomavirus. *Journal of Virology*. 2007; 81(11):5749–58. [PubMed: 17392369]
35. Brandsma JL, Shlyankevich M, Zeltermann D, Su Y. Therapeutic vaccination of rabbits with a ubiquitin-fused papillomavirus E1, E2, E6 and E7 DNA vaccine. *Vaccine*. 2007; 25(33):6158–63. [PubMed: 17630050]
36. Leachman SA, Shlyankevich M, Slade MD, Levine D, Sundaram RK, Xiao W, et al. Ubiquitin-fused and/or multiple early genes from cottontail rabbit papillomavirus as DNA vaccines. *Journal of Virology*. 2002; 76(15):7616–24. [PubMed: 12097575]
37. Sundaram P, Tigelaar RE, Xiao W, Brandsma JL. Intracutaneous vaccination of rabbits with the E6 gene of cottontail rabbit papillomavirus provides partial protection against virus challenge. *Vaccine*. 1998; 16(6):613–23. [PubMed: 9569473]
38. Roediger EK, Kugathasan K, Zhang X, Lichty BD, Xing Z. Heterologous boosting of recombinant adenoviral prime immunization with a novel vesicular stomatitis virus-vectored tuberculosis vaccine. *Molecular Therapy: the Journal of the American Society of Gene Therapy*. 2008; 16(6):1161–9. [PubMed: 18388911]
39. Tan GS, McKenna PM, Koser ML, McLinden R, Kim JH, McGettigan JP, et al. Strong cellular and humoral anti-HIV Env immune responses induced by a heterologous rhabdoviral prime-boost approach. *Virology*. 2005; 331(1):82–93. [PubMed: 15582655]
40. Ramsburg E, Rose NF, Marx PA, Mefford M, Nixon DF, Moretto WJ, et al. Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. *Journal of Virology*. 2004; 78(8):3930–40. [PubMed: 15047809]
41. Egan MA, Chong SY, Megati S, Montefiori DC, Rose NF, Boyer JD, et al. Priming with plasmid DNAs expressing interleukin-12 and simian immunodeficiency virus gag enhances the immunogenicity and efficacy of an experimental AIDS vaccine based on recombinant vesicular stomatitis virus. *AIDS Research & Human Retroviruses*. 2005; 21(7):629–43. [PubMed: 16060834]
42. Schadeck EB, Sidhu M, Egan MA, Chong S-Y, Piacente P, Masood A, et al. A dose sparing effect by plasmid encoded IL-12 adjuvant on a SIVgag-plasmid DNA vaccine in rhesus macaques. *Vaccine*. 2006; 24(21):4677–87. [PubMed: 16288822]
43. Nicholls PK, Stanley MA. The immunology of animal papillomaviruses. *Veterinary Immunology & Immunopathology*. 2000; 73(2):101–27. [PubMed: 10690928]
44. Stanley MA. Immunobiology of papillomavirus infections. *Journal of Reproductive Immunology*. 2001; 52(1–2):45–59. [PubMed: 11600177]
45. Stern PL. Immune control of human papillomavirus (HPV) associated anogenital disease and potential for vaccination. *Journal of Clinical Virology*. 2005; 32 (Suppl 1):S72–81. [PubMed: 15753015]

(A)

Boost	Prime		
	pcDNA3 ^a	UbE6 DNA	VSV-E6
pcDNA3	Group 1a	--	--
VSV-GFP ^b	Group 1b	--	--
UbE6 DNA	--	Group 2	Group 4
VSV-E6	--	Group 3	Group 5

^a The empty DNA vector.

^b VSV expressing green fluorescent protein.

Figure 1. Schematic illustration of the experimental design

Panel A shows the priming and boosting vector administered to each rabbit group. Panel B shows the experimental schedule for CRPV infection, papilloma formation and immunization. The experiment ended 154 days after CRPV infection.

Figure 2. Time course of papilloma growth in the vaccinated and control groups

Panels A, B and C show the time course of changes in papilloma volume at sites infected with the 1:50 (A), 1:250 (B) or 1:1250 (C) dilutions of CRPV (mean plus S.E.M. of the natural logarithm of the volume). The markers identify the treatments: control (triangles), primed with U6E6 DNA (circles) or VSV-E6 (squares), and boosted with the homologous (shaded markers) or heterologous (filled markers) vector. Panel D shows the overall efficacy of each vaccination protocol as measured by the difference between the control group and each E6 vaccinated group (mean $\pm$ S.E.M. of the natural logarithm of the volumes).

Figure 3. Clinical effects of vaccination

The panels show the flanks of representative rabbits from each treatment group. Papillomas induced with the 1:1250, 1:250 and 1:50 dilutions of CRPV (low, moderate and high doses) are located on the left, middle and right of each photograph. Photographs were taken during week 20 of the experiment.

Figure 4. Antitumor responses of rabbits with varying papilloma volumes at the time of priming
 In Panel A, the control and vaccinated rabbits were ordered according to total papilloma volume prior to vaccination, *i.e.* the sum of the volumes at all nine infection sites. Listed on the x-axis are the rabbit number (top row) and the vector used for priming (middle row) and boosting (bottom row). The stacked bars on the y-axis show the total papilloma burden broken into the contributions from each set of CRPV infection sites, after normalization to the mean total burden of all rabbits in each chart (means plus S.E.M.s). The charts in panel A show papilloma volumes prior to vaccination (upper chart) and the corresponding cumulative volumes (lower chart). In panel B, the vaccinated rabbits were re-grouped to maximize the volume differences prior to priming. The bars indicate the normalized mean papilloma volume per rabbit before priming (lightly hatched) and thereafter (heavily hatched) (mean plus S.E.M.). The groups in panel B contain rabbits #6 to 10 (Vol 1), #11 to 15 (Vol 2), #16 to 20 (Vol 3) and #21 to 25 (Vol 4) (numbers from panel A).

Table 1

VSV-specific neutralization titers

Group # ^a	VSV vector	VSV-E6 Inoculation		VSV neutralizing antibody titer ^b
		Day 31	Day 52	Day 94
1a	N/A	-	-	Negative ^c
1b	VSV-GFP	-	+	10,240 ± 0
2	N/A	-	-	Negative
3	VSV-E6	-	+	4,096 ± 1,536
4	VSV-E6	+	-	2,432 ± 768
5	VSV-E6	+	+	4,608 ± 512

^aSee Fig. 1 for full description of the rabbit groups.

^bThe highest dilution ⁻¹ of serum that neutralized VSV (mean plus standard error of the mean (S.E.M.)).

^cNegative at the lowest dilution tested (1:40).

Table 2

Clinical status of the rabbits on the day of vaccination

CRPV ^a	Group ^b	Papilloma Frequency		Time to appearance ^d		Papilloma Volume ^e
		Number ^c	Percent	Days	per Site	
1:50	1	15 ± 15	100	17.3 ± 0.3	80.2 ± 5.0	
	2	15 ± 15	100	18.1 ± 0.8	135.6 ± 49.7	
	3	15 ± 15	100	18.3 ± 0.6	77.6 ± 34.6	
	4	15 ± 15	100	17.8 ± 0.8	109.2 ± 37.5	
	5	15 ± 15	100	17.3 ± 0.3	80.5 ± 38.5	
1:250	1	15 ± 15	100	25.8 ± 1.6	7.4 ± 0.8	
	2	15 ± 15	100	22.7 ± 2.8	24.5 ± 9.9	
	3	14 ± 15	93.3	23.0 ± 2.4	20.1 ± 10.5	
	4	15 ± 15	100	24.3 ± 2.0	20.6 ± 8.6	
	5	15 ± 15	100	22.1 ± 1.7	19.2 ± 10.1	
1:1250	1	12 ± 15	80	30.0 ± 1.0	1.4 ± 0.4	
	2	10 ± 15	66.7	26.0 ± 2.6	3.7 ± 1.3	
	3	13 ± 15	86.7	27.8 ± 2.5	2.9 ± 1.4	
	4	14 ± 15	93.3	28.3 ± 1.3	2.7 ± 0.8	
	5	13 ± 15	86.7	28.3 ± 1.2	3.8 ± 1.7	

^aDilution of the CRPV inoculum.

^bThe groups were subsequently primed/boosted as shown in Figure 1.

^cNumber of papilloma-forming sites/total number of sites.

^dMean number of days after CRPV infection (± S.E.M.)

^eMean papilloma volume per site ± S.E.M. in mm³.

Table 3

Papilloma Volumes in the Contrasts of Interest

Comparison	Groups ^a	CRPV Dilution			ANCOVA	
		1:50	1:250	1:1250	All sites	Raw ^b Adj. ^c
All rabbits						
Controls	CC	52.0 ± 6.6 ^d	41.4 ± 6.6	30.1 ± 9.2	100.0 ± 15.2	
Vaccinees	DD+DV+VD+VV	29.8 ± 3.6	20.0 ± 3.3	16.0 ± 4.0	33.5 ± 4.7	0.018
Priming vector						
UbE6 DNA	DD+DV	32.8 ± 6.0	21.2 ± 5.8	15.9 ± 6.8	36.8 ± 8.2	
VSV-E6	VV+VD	26.6 ± 3.8	18.8 ± 3.2	16.0 ± 4.3	30.1 ± 4.9	1.000
Boosting vector						
UbE6 DNA	DD+VD	28.7 ± 5.4	24.4 ± 4.6	19.5 ± 6.5	37.9 ± 7.0	
VSV-E6	VV+DV	30.9 ± 4.8	15.1 ± 4.1	12.0 ± 4.1	29.0 ± 3.9	1.000
Prime-boost combination						
Homologous	DD+VV	33.8 ± 5.1	23.9 ± 4.5	18.7 ± 6.2	41.4 ± 7.4	
Heterologous	DV+VD	25.2 ± 4.2	15.5 ± 4.2	12.6 ± 4.3	25.5 ± 4.9	0.302

^aControl (C), UbE6 DNA vector (D), VSV-E6 vector (V).^bAnalyzed as described in Materials and Methods^cAdjusted for multiple comparisons.^dPapilloma volume per CRPV dilution of CRPV, normalized to the mean volume of the controls at all sites.