Vaccination of Rabbits with an Adenovirus Vector Expressing the Papillomavirus E2 Protein Leads to Clearance of Papillomas and Infection

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Received 9 July 2003/Accepted 18 September 2003

Cervical cancer arises from lesions caused by infection with high-risk types of human papillomavirus (HPV). Therefore, vaccination against HPV could prevent carcinogenesis by preventing HPV infection or inducing lesion regression. HPV E2 protein is an attractive candidate for vaccine development because it is required for papilloma formation, is involved in all stages of the virus life cycle, and is expressed in all premalignant lesions as well as some cancers. This study reports vaccination against E2 protein using a rabbit model of papillomavirus infection. A recombinant adenovirus (Ad) vector expressing the E2 protein of cottontail rabbit papillomavirus (CRPV) was tested for therapeutic efficacy in CRPV-infected rabbits. Primary immunization with the Ad-E2 vaccine, compared to immunization with a control Ad vector, reduced the number of papilloma-forming sites from 17 of 45 to 4 of 45. After booster immunization, vaccinated rabbits formed no new papillomas versus an additional 23 papillomas in rabbits that received the control vector. Papillomas in the Ad-E2 vaccinees were significantly smaller than those in the control rabbits, and all four papillomas in the Ad-E2 vaccinated rabbits regressed. No CRPV DNA was detected either in the regression sites or in sites that did not form papillomas, indicating that the vaccination led to clearance of CRPV from all infected sites.

Human papillomaviruses (HPVs) cause cervical cancer, which affects about one-half million women worldwide annually (37, 51, 56). HPV-associated disease includes anal, vulvar (56), oral and other respiratory tract cancers (20), and nearly all skin cancers in patients with epidermodysplasia verruciformis (13). HPV infection is also implicated in nonmelanoma skin cancers in immunocompetent as well as immunodeficient patients (4–6, 13, 26, 29, 40). It has been estimated that 10% of the world's tumor burden is attributable to HPV infection (57).

Cervical carcinogenesis begins with benign epithelial lesions induced by HPV. Because genital HPV infection is highly prevalent, many women are at risk (41). Progression to cervical cancer typically takes more than a decade, so cytological screening can detect high-grade lesions in time for treatment. However, present treatments do not cure all lesions in all patients, and recurrence is a common sequela. Furthermore, cervical cancer has a mortality rate of 33%, clearly indicating the need for better therapy.

Because premalignant lesions caused by HPV can be detected early, vaccination against HPV antigens could provide an effective therapy to induce lesion regression and prevent cancer (9–12, 45). A therapeutic vaccine could also eliminate residual HPV infection after surgical removal of a lesion (9–12, 45). The viral E6 and E7 oncoproteins are presently popular

targets for a therapeutic HPV vaccine. These proteins stimulate cellular proliferation, promote genetic instability, and transform cells, in large part by perturbing the p53 and retinoblastoma tumor suppressor pathways (reviewed in reference 38).

We hypothesized that the E2 protein would make a good candidate for therapeutic vaccination because of its intimate involvement in all stages of the virus life cycle. HPV E2 protein regulates E6 and E7 transcription by reversibly binding to the promoter for both genes (reviewed in references 3 and 36). In infected basal and parabasal cells, low levels of E2 protein activate E6/E7 transcription and thus cellular proliferation. In addition, E2 likely contributes to the partitioning of viral genomes into daughter cells (3, 36), maintaining a stable, low number of viral genomes per cell until, in spinous and granular cells, E2 levels increase sharply, coincident with terminal differentiation. High levels of E2 repress E6/E7 transcription and together with the E1 replication protein drive vegetative HPV DNA replication. Packaging the viral genome into virion particles may also require E2 (36). It has previously been shown, using the cottontail rabbit papillomavirus (CRPV)-rabbit model of HPV-associated cancer, that the E2 protein is required to initiate papilloma formation (53). The CRPV-rabbit model is the only laboratory animal model in which virus-induced papillomas persist despite immunocompetency and evolve under selective host pressure into invasive and metastatic squamous cell carcinomas (7, 8). The CRPV E2 protein has substantial amino acid sequence homology with all HPV E2 proteins (1) and, like HPV E2, transactivates E6/E7 transcription (19). Furthermore, CRPV E2 expression in lesions

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follows the same differentiation-specific pattern as HPV E2 (55).

In this study, we vaccinated rabbits with established CRPV infections by using a recombinant adenovirus (Ad) vector expressing the CRPV E2 protein (Ad-E2). The results show that the Ad-E2 vector, compared to the Ad control vector, significantly reduced papilloma numbers and volumes. Moreover, all papillomas in the Ad-E2-vaccinated rabbits regressed, and CRPV DNA was not detected at either the sites showing regression or those lacking papilloma formation, indicating that the vaccine also led to the clearance of infection.

MATERIALS AND METHODS

Production of recombinant CRPV E1 and E2 proteins. PCR products containing the CRPV E1 and E2 genes were amplified from plasmid CRPV-pLAI1 (52) and purified by the QIAquick PCR protocol (QIAGEN, Valencia, Calif.). The primer set for the E1 gene was E1HisC (5'-GCGCTGACCATGGCTGAA GGTACAGACCC) and E1HisN (5'-GCGCTGACTCGAGTAGAGACTGAG AAGTTC), and for the E2 gene, the primer set was E2HisC (GCCTGATCA TGATCGAGGCTCTCAGCCAGCG) and E1HisN (5'-GCGCTGACTCGAG AAGCCATATAAAATTCCC). The E1 PCR product was digested by *NcoI* and *XhoI*, and the E2 PCR product was digested by *BspHI* and *XhoI*. Each fragment was then ligated to plasmid pET-29a (Novagen, Madison, Wis.) at the corresponding restriction sites and transformed into *Escherichia coli* strain NovaBlue. The correctness of the clones was established by automatic DNA sequencing using an ABI machine (Keck Foundation Biotechnology Resource Laboratory, Yale University). Production of the recombinant E1 and E2 proteins, containing His tag sequences at their carboxyl termini, was induced by using IPTG (isopropyl- β -D-thiogalactopyranoside). Bacterial pellets were resuspended in 10 ml of 6 M guanidine buffer, pH 8.5, and centrifuged at 13,000 rpm in an SS-34 rotor in a Sorvall RC-5 centrifuge for 15 min before the supernatants were applied to 1-ml Ni-NTA agarose columns (QIAGEN) equilibrated with guanidine buffer. The columns were washed, and the proteins were eluted by 8 M urea buffer, pH 8.0, with 50 and 250 mM imidazole. Fractions (1 ml) were collected and dialyzed overnight against Tris-buffered saline at 20°C, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide gel) before transfer to Spectrapor dialysis membranes (molecular weight cutoff = 12 to 14,000) (Spectrum Medical Industries, Los Angeles, Calif.). The recombinant E1 and E2 proteins, containing 30 amino acids from the vector, were 632 and 422 amino acids in length, respectively, with respective estimated molecular masses of 83 and 56 kDa.

Construction of recombinant Ad containing the CRPV E2 gene. We used an Ad5-based vector with a deleted E1 gene, rendering the virus replication incompetent, and a deleted E3 gene, crippling its ability to inhibit the export of major histocompatibility complex (MHC) class I molecules from the endoplasmic reticulum, and previously described methods (27). Briefly, the CRPV E2 gene was isolated from pcDNA3-E2K (31) by digestion with *KpnI* and *XbaI* and inserted at the same restriction sites into the deleted Ad E1 region of pShuttle-CMV. The pShuttle-E2 vector was linearized by *PmeI* digestion and cotransformed with the Ad backbone plasmid pAd Easy1 into *E. coli* strain BJ5183 for homologous recombination. A correct clone of the recombined plasmid, verified by DNA sequencing, was transfected into the E1-complementing 293 cell line for Ad production. Ad titers were determined by endpoint dilution in 293 cells, which were evaluated for cytopathic effects during 10 to 14 days of infection. The same procedure was used to generate an Ad recombinant expressing green fluorescent protein (Ad-GFP).

Demonstration of E2 protein expression from Ad-E2. Northern blots containing lysates of HeLa cells infected 48 h previously with Ad-E2 at a multiplicity of infection of five were hybridized to probes for HPV18 E6/E7 or cellular p21 RNAs, as previously described (22). Negative control cells were infected in parallel with an Ad-GFP or the Ad vector alone (no insert). Total cellular RNA was purified by using Trizol reagent (Invitrogen, Carlsbad, Calif.), and 5 μ g of RNA was subjected to formaldehyde-agarose gel electrophoresis, transferred to a Nytran SuperCharge (Schleicher & Schuell) membrane, and cross-linked to the membrane by UV irradiation. The immobilized RNA was hybridized with the indicated random prime-labeled cDNA, and the signal was detected and quantified with a PhosphorImager (Molecular Dynamics). Sequential hybridizations were performed after stripping the previous probe from the membrane.

Rabbits. Two-kilogram female New Zealand White *Pasteurella multocida*-free rabbits (*Oryctolagus cuniculus*) were purchased from Millbrook Farms (Amherst,

Mass.) and 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.

CRPV infection. After the rabbits were sedated with acepromazine (Henry Schein, Melville, N.Y.) (2 mg/kg of body weight administered intramuscularly), they were infected with CRPV on the right flank by scarification, as described previously (31, 32). Each of three sites received 30 μ l of a 1:50, 1:250, or 1:1,250 dilution of the K216 stock of CRPV, representing high, moderate, and low doses of virus (nine sites per rabbit). This CRPV stock was prepared by growing cottontail rabbit papillomas in immunodeficient mice as previously described (50). Earlier experiments showed that 1:50 to 1:450 dilutions of this stock induced papillomas at 96% to 100% of challenge sites in control rabbits (31, 32, 49, 50).

Delivery of Ad vectors. Prior to being vaccinated, the rabbits were sedated with acepromazine and their fur was clipped. At each vaccination, 5.6×10^{10} particles of recombinant Ad in 0.45 ml of phosphate-buffered saline (PBS) were delivered intradermally with a 27-gauge needle to about 50 sites per rabbit that were adjacent to the CRPV infection sites; i.e., vaccination was not intraslesional. Five rabbits received Ad-E2, and five received Ad-GFP as a negative control 2 and 21 days after CRPV infection.

Collection of clinical data. The rabbits were examined weekly for papillomas, beginning 2 weeks after CRPV infection. At each examination, the number and location of each papilloma was recorded and the size was measured in three dimensions with a digital caliper accurate to 0.1 mm. Papilloma volumes were calculated by using the formula $4/3 \times \pi \times \text{length}/2 \times \text{width}/2 \times \text{height}/2$ (31, 32). The dimensions of the smallest palpable papillomas were estimated to be 0.3 mm by 0.3 mm by 0.3 mm, with a calculated volume of 0.01 mm³. Photographic documentation was performed weekly. The rabbits used in the experiment were held for 133 days. The control rabbits were entered into another treatment regimen after day 56.

Statistical analyses. Statistical analyses were stratified by the dose of the CRPV inoculum used for infection and were performed by using previously established methods (31, 32). The number of infection sites with papillomas was analyzed using repeated, multivariate logistical regression. Because papilloma growth was exponential, the volume data were analyzed using the natural logarithms of the volumes, thereby generating linear relationships between volume and time for statistical analysis. Papilloma volumes were calculated by using data only from the positive sites and ignoring all negative sites. Mean log volumes were analyzed using linear regression and within-animal exchangeable correlations. Differences in mean antibody titers were analyzed using Student's *t* test.

Assessment of CRPV clearance. Epidermal sites that had been infected with the highest dose of CRPV were excised from the Ad-E2-vaccinated rabbits 133 days after CRPV infection and frozen at -70°C. DNAs were extracted from pulverized frozen tissue as described previously (14). Forty nanograms of each sample DNA was used in 20- μ l PCRs. One set of primers amplified a 358-bp product from the CRPV L1 gene, and a second set amplified a 257-bp product from the single-copy rabbit DQ-alpha gene, an MHC class I gene (33). The rabbit DQ-alpha PCR served as a monitor for the quality and integrity of the DNA and as a quantitative reference for calculating CRPV copy number. Reference standards were amplified from a fixed quantity of normal rabbit DNA mixed with various quantities of CRPV-pLAI1 DNA. Densitometric analysis using the National Institutes of Health ImageJ application determined the strength of each PCR band, and the ratio of L1 to DQ-alpha determined the CRPV copy number. The CRPV primers were CR6785C (5'-GCTCAAGGCA TGAACAATGG) and CR7142-N (5'-GGTTCTGGGGGTGGACATTT). The MHC class II DQ-alpha primers were MHC DQ α 5C (5'-TCATCAGCTGACC ACGTTGGC) and MHC DQ α 3N (5'-CTATTGACAGCAGTAGAG).

Ad microneutralization assay. A microneutralization assay was developed to assess the immunogenicity of recombinant Ad vectors in rabbits. Rabbit sera were heat-inactivated at 56°C for 30 min and then diluted in Dulbecco's modified essential medium plus 10% calf serum. Six serial twofold dilutions of each rabbit serum were mixed with 10^5 infectious particles of Ad-GFP, yielding final rabbit serum dilutions of 1:500 to 1:16,000 in Dulbecco's modified essential medium plus 5% calf serum. Meanwhile, 293 cells were plated at a density of 10^5 cells/well in a 48-well tissue culture plate and incubated in 5% CO₂ at 37°C overnight. The culture medium was then replaced by each Ad-GFP-serum mixture (0.3 ml per well). Virus control wells contained Ad-GFP incubated overnight with preimmune rabbit serum, and tissue culture control wells contained medium without virus or serum. Additional control wells contained virus plus preimmune sera from two rabbits. The cultures were further incubated at 37°C for 48 h and then trypsinized with 1 drop of trypsin-EDTA per well for 3 min at 37°C, followed by the addition of 0.2 ml of PBS plus 5% calf serum. Aliquots of cells (25 μ l, containing $\sim 10^4$ cells from the tissue culture control wells) were transferred to

FIG. 1. Recombinant Ad expresses foreign protein in rabbit skin. Rabbit skin was infected intradermally with Ad-GFP, and biopsies were obtained 3 days later. Fluorescence photomicrographs of frozen sections showing one area without GFP expression (A) and another with focal high-level GFP expression (B) are shown.

12-spot glass slides, and the GFP-positive cells were immediately counted under a fluorescence microscope. Virus control spots showed >1,000 GFP-positive cells. The neutralizing antibody titer was defined as the last serum dilution with more than 50 GFP-positive cells per spot.

ELISAs to detect antibodies to CRPV proteins. Enzyme-linked immunosorbent assays (ELISAs) were performed by using as antigen virus-like particles (VLPs) composed of the CRPV L1 protein, generated as previously described (30); recombinant CRPV early proteins E1 and E2, described above; and recombinant E6 and E7 (48, 50). ELISAs were carried out by using 200 ng of recombinant protein per well (Nunc-Immuno Plate; Nalge Nunc International, Roskilde, Denmark) as described previously (32, 48). The positive control sera were from a rabbit vaccinated with the CRPV L1 gene (49), a papilloma-bearing rabbit with naturally occurring antibodies to the E1 and E2 proteins, and rabbits immunized with recombinant protein E6 or E7 (48, 50). The respective positive control sera had antibody titers of 1:12,000, 1:1,280, 1:2,560, 1:12,800 and 1:6,400 to CRPV L1, E1, E2, E6, and E7, respectively. Test sera were collected before CRPV infection and on day 56. Serial twofold dilutions of sera ranging from 1:40 to 1:1,280 (in 0.5% Tween 20–0.5% bovine serum albumin in PBS) were applied to the antigen-coated wells in 96-well microtiter plates. Serum samples with undetectable antibodies at the 1:40 dilution were designated negative. Preimmune sera were universally negative.

RESULTS

Adenoviral infection and immunogenicity in rabbits. Immune responses to foreign proteins expressed from recombinant Ad vectors are readily induced in humans but not in mice (34). To determine the feasibility of evaluating Ad-based vac-

cines in rabbits, we assessed their ability to express a foreign gene in rabbits. Rabbits were inoculated intradermally with 2×10^9 particles per rabbit of Ad-GFP. Microscopic evaluation of tissues taken 48 h later revealed focal areas with high-level GFP expression at the inoculation sites (Fig. 1). This result established that the vector expressed the foreign gene, affirming that rabbits were suitable for the evaluation of Ad-based vaccines.

Ad microneutralization assays were performed to directly assess the immunogenicity of the vaccine vectors. All postvaccination sera had neutralizing antibodies with titers ranging from 1:8,000 to $\geq 1:16,000$ (Table 1). Preimmune sera were uniformly negative. The Ad-E2 and Ad-GFP vectors stimulated equivalent levels of neutralizing antibody production (Fisher's exact test), demonstrating that the vectors were equally immunogenic in rabbits.

Recombinant Ad-E2 expresses E2 protein. The E2 proteins from CRPV, BPV1, HPV1, and HPV18 have similar structures and can interchangeably repress the E6/E7 promoters of homologous and heterologous papillomaviruses, including HPV18, which is endogenous in HeLa cells (21). Transcriptional repression requires high levels of E2 protein (reviewed in reference 36). Fujii et al. previously showed that high (but not low) levels of CRPV E2 protein repressed transcription from the CRPV long control region in an E2-dependent manner (19). Goodwin and DiMaio have shown that bovine papillomavirus (BPV) E2-mediated repression of the endogenous HPV18 E6/E7 promoter in HeLa cells stabilized p53 and increased transcription of the cellular gene encoding the cdk inhibitor p21 (22). To assess the level of E2 protein expressed from Ad-E2 in HeLa cells, we evaluated the effect of Ad-E2 infection on E6/E7 mRNA levels. HeLa cells were infected with Ad-E2, Ad-GFP, or Ad alone (no insert). RNAs were isolated 48 h later. Northern blot analysis showed profound repression of E6/E7 RNA in the Ad-E2-infected cells but not in cells infected with either of the control Ads (Fig. 2A). These results show, indirectly, that Ad-E2 expressed a high level of functional E2 protein. Further analysis showed that Ad-E2 infection strongly activated p21 RNA expression, whereas virtually no expression was detected in either type of control cell (Fig. 2B), another indication of high-level E2 expression.

TABLE 1. Development of humoral immunity to adenovirus and CRPV

Vaccine	Rabbit	Neutralizing antibody to adenovirus (titers)	Rate of seroconversion of CRPV ^a (titers)				
			L1 VLPs	E1	E2	E6	E7
Ad-GFP	1	1:8,000	$\geq 1:1,600$	-	-	-	-
	2	$\geq 1:16,000$	$\geq 1:1,600$	-	-	-	-
	3	1:8,000	$\geq 1:1,600$	-	-	-	-
	4	$\geq 1:16,000$	1:800	1:40	-	1:40	-
	5	$\geq 1:16,000$	$\geq 1:1,600$	1:40	-	-	-
Ad-E2	6	1:8,000	1:200	-	-	-	-
	7	$\geq 1:16,000$	$\geq 1:1,600$	1:80	-	-	-
	8	$\geq 1:16,000$	1:800	-	-	-	-
	9	$\geq 1:16,000$	1:400	-	-	-	-
	10	$\geq 1:16,000$	$\geq 1:1,600$	1:40	-	-	-

^a The highest dilution of serum yielding positive results. Dashes mean bovine serum albumin that negative results were obtained at the lowest dilution (1:40). ELISAs used L1 VLPs, E1, E2, E6, and E7 proteins as antigens. Sera were collected 8 weeks after CRPV infection.

FIG. 2. Repression of HPV18 E6/E7 mRNA. HeLa cells were mock infected, infected with Ad-GFP, or infected with Ad-E2 (encoding CRPV E2 protein). Total RNA was isolated 2 days later. A Northern blot was sequentially probed for HPV18 E6/E7 mRNA and the cdk inhibitor p21.

CRPV-infected rabbits develop antibody to the CRPV capsid protein. Rabbits were infected with CRPV prior to therapeutic vaccination. As a criterion of infection, rabbits were evaluated for serologic reactivity to CRPV VLPs by ELISA, using sera collected 8 weeks after CRPV infection. All rabbits seroconverted, with mean antibody titers of $1:1,180 \pm 1:1,200$ (mean $\pm$ standard error of the mean), and there was no significant difference between the Ad-E2-vaccinated and Ad-GFP-vaccinated control groups (Student's *t* test) (Table 1).

Effects of Ad-E2 vaccination on CRPV-induced disease. The severity of papillomavirus infection is determined by both the virus inoculum and the host genotype. The rabbit experiment tested the therapeutic efficacy of Ad-E2 vaccination against low, moderate, and severe disease while controlling for host genotype. This result was achieved by infecting each rabbit, at different sites, with low, moderate, and high doses of CRPV. Primary vaccination was performed during the subclinical stage of infection (rather than after papillomas formed) to favor the demonstration of therapeutic efficacy. One booster vaccination was given 21 days after the primary vaccination, when many papillomas had already formed. As expected, the numbers and sizes of papillomas correlated directly with the dose of CRPV. This correlation applied to both the experimental and control rabbits (Table 2 and Fig. 3).

When papillomas first appeared, on day 20, their frequencies

in the Ad-GFP-vaccinated control rabbits at sites infected with high, moderate, or low doses of CRPV were 67%, 33%, and 13%, respectively (Fig. 3). At the same time point, the Ad-E2-vaccinated rabbits had formed papillomas at only 9% of sites infected with the high dose of CRPV, and all sites infected with moderate or low doses remained clinically negative. Thus, prior to boosting, the number of papillomas was significantly reduced in the vaccinees ($P = 0.001$). Furthermore, papillomas in the Ad-GFP-vaccinated control rabbits continued to form until 93%, 87%, and 80% of sites infected with CRPV at high, moderate, and low doses, respectively, became positive, whereas no additional papillomas formed in Ad-E2 vaccinees (Table 2 and Fig. 3A) ($P < 0.001$). In addition, all papillomas in vaccinated rabbits regressed after a mean duration of 12 days after onset and all sites remained clinically free of disease until the rabbits were euthanized 110 days after booster vaccination. Only 1 of 40 papillomas in the rabbits given Ad-GFP regressed (Table 2) ($P < 0.001$). The Ad-GFP-vaccinated control rabbits were held for an additional 2 months, but no other papillomas regressed.

Papilloma volumes were also strongly affected by Ad-E2 vaccination, as shown clinically in Fig. 4 and graphically in Fig. 3B. On day 35, for example, when papillomas in the Ad-E2-vaccinated group were 4 mm^3 , their maximum volume, papillomas in the Ad-GFP-vaccinated control group at the corresponding sites were 28 mm^3 ($P < 0.001$). At the end of the experiment, papillomas in the control rabbits had mean volumes of 143 mm^3 , 48 mm^3 , and 14 mm^3 at sites infected with high, moderate and low doses of CRPV, respectively, whereas no disease was detected in the Ad-E2 vaccinees ($P < 0.001$).

Effects of Ad-E2 vaccination on CRPV infection. Vaccines that suppress papilloma outgrowth and/or cause papilloma regression may suppress or clear CRPV infection. To distinguish between these possibilities, we analyzed the only sites where CRPV could have persisted: the infection sites. CRPV could not have persisted elsewhere because CRPV is strictly epitheliotropic and highly localized in domestic rabbits due to the lack of virion replication (7, 8). DNAs were extracted from all sites infected with the highest dose of CRPV: 11 sites where papillomas did not form, and 4 sites where papillomas formed and regressed. PCR analysis showed all 15 sites to be universally CRPV DNA negative (Fig. 5). Since, in contrast, all papillomas but one in the Ad-GFP-vaccinated control rabbits grew larger continually (Table 1; Fig. 3), the data indicate that Ad-E2 vaccination led to CRPV clearance.

TABLE 2. Rates of papilloma appearance, regression, and persistence in CRPV-infected rabbits vaccinated with either Ad-GFP or Ad-E2

Concn of CRPV	Appearance ^a		Regression ^b		Persistence ^c	
	Ad-GFP	Ad-E2	Ad-GFP	Ad-E2	Ad-GFP	Ad-E2
1:50	14/15	4/15 ^e	0/14	4/4 ^e	14/14	0/4 ^e
1:250	14/15	0/15 ^e	1/14	NA ^d	13/14	NA
1:1,250	12/15	0/15 ^e	0/12	NA	12/12	NA
All sites	40/45	4/45 ^e	1/40	4/4 ^e	39/40	0/4 ^e

^a Maximum number of sites that ever formed a papilloma/number of sites.

^b Number of papillomas that completely regressed/maximum number that formed.

^c Number of papillomas that persisted throughout the experiment/number of sites.

^d NA, not applicable.

^e $P \leq 0.001$.

FIG. 3. Papilloma formation and growth in Ad-E2-vaccinated rabbits. The graphs depict the time course of papilloma formation (A) and growth (B), stratified by the CRPV dose used for infection. Vaccination times are marked with an "x." Panel A shows the total number of sites forming papillomas. Panel B shows the mean volumes of papillomas.

Humoral immunity to CRPV early proteins. Antibody responses to CRPV infection might serve as biological markers of clinical response and vaccine efficacy if they differed between the Ad-E2-vaccinated and Ad-GFP-vaccinated control rabbits. Sera from both groups of rabbits were therefore screened by ELISA for antibodies to CRPV E1, E2, E6, and E7 proteins (Table 1). Two Ad-E2-vaccinated rabbits and two Ad-GFP-vaccinated control rabbits developed antibodies against E1, indicating that B-cell epitopes of the E1 protein in papillomas were recognized relatively early during infection by some rabbits. No rabbit developed antibodies to E2 or E7, and only one developed antibody to E6. Thus, antibody responses to CRPV early proteins during the first 6 weeks of infection were unrelated to papilloma frequency or volume and not useful as markers. The lack of antibody to E2 protein in the

vaccinees suggests that non-antibody-mediated mechanisms were responsible for the clinical efficacy of the Ad-E2 vaccine.

DISCUSSION

Prior CRPV E2 DNA vaccine studies tested efficacy in naïve rabbits. Leachman et al. showed that vaccination with CRPV DNAs encoding the E1 plus E2 proteins strongly inhibited papilloma formation and ultimately caused complete papilloma regression (31). Han et al. reported that a similar CRPV E2 DNA vaccine alone induced significant prophylactic immunity (25). Leachman et al. also showed that a CRPV DNA

FIG. 4. Clinical outcomes in Ad-E2-vaccinated rabbits. Photographs show CRPV infection sites in the Ad-GFP-vaccinated rabbit with the median level of disease (A and B) and the Ad-E2-vaccinated rabbit with the most disease (C and D). The arrow in panel B marks the largest papilloma that formed in any Ad-E2-vaccinated rabbit. Low, moderate, and high doses of CRPV were used to infect three sites to the left, middle, and right of each photograph. The infected sites are circled in panels A, B, and D. The photographs in panels A and B were taken on day 35. The photographs in panels C and D were taken on day 56.

FIG. 5. CRPV DNA clearance in Ad-E2-vaccinated rabbits. PCR assays were performed using primer sets for a region of the CRPV L1 gene (A) and a region of the rabbit MHC class II DQ-alpha gene (B). The ethidium-stained agarose gel shows the results of PCR using template DNAs from the three sites infected with the highest dose of CRPV from the three Ad-E2-vaccinated rabbits that formed papillomas (lanes 1 to 3, 4 to 6, and 7 to 9, respectively) and one that did not (lanes 10 to 12), normal rabbit skin (lane 14), CRPV papilloma (lane 15), and plasmid CRPV-pLAI (lane 16). Also shown is a 1-kb DNA ladder (Invitrogen) (lane 13).

vaccine containing ubiquitin-fused versions of the E1 plus E2 plus E7 genes completely prevented papilloma formation (31).

This study tested E2 vaccine efficacy in rabbits with active CRPV infection at the time of vaccination. The results show that an Ad vector expressing E2 protein significantly inhibited papilloma growth. The maximum number of papillomas at sites infected with the highest dose of CRPV was reduced by 90%, and no papillomas were detected at sites infected with moderate or low doses of virus. In addition, papillomas that formed in the Ad-E2 vaccinees were significantly smaller than those in the Ad-GFP-vaccinated control rabbits, persisted for less than 2 weeks, and regressed rapidly. Moreover, 4 months after primary immunization, all vaccinated rabbits remained free of disease and despite seroconversion to CRPV no CRPV DNA was detected at the site of CRPV inoculation, indicating that vaccination had cleared the infection.

Papillomavirus E2 is an attractive candidate for vaccine development because it is a large protein. In addition, E2 proteins have highly conserved regions of amino acid sequence, suggesting that an immune response to E2 may cross-react with E2 epitopes from multiple HPVs. Since more than a dozen HPVs are associated with cancer, a cross-reacting vaccine would be highly advantageous. Additionally, an E2 vaccine might be particularly useful against virus-producing lesions because such lesions contain relatively high levels of E2 protein and lower levels of E6/E7 than advanced lesions. Nevertheless, despite the fact that domestic rabbit papillomas are generally nonproductive (of CRPV), the Ad-E2 vaccine was highly effective.

In an earlier study, Selvakumar et al. showed that a CRPV E2 vaccine, as recombinant protein, induced papilloma regression although it did not suppress papilloma formation even when administered prior to CRPV challenge (43). The apparently greater efficacy of our vaccine is most likely attributable to differences in the vaccine antigens. In their study, E2 was delivered extracellularly as a recombinant bacterial protein, a protocol that usually induces humoral immunity. Antibody would not be expected to affect CRPV-infected cells because E2 is a nuclear protein. The fact that some papillomas ultimately regressed, however, indicates that cell-mediated immune responses also occurred. These responses were probably induced or their efficacy was enhanced by CRPV challenge. In our study, in contrast, the E2 gene was delivered in a recombinant Ad vector. Ad-E2 vaccination resulted in intracellular E2 protein synthesis, presumably followed by E2 degradation through the MHC class I pathway, a protocol that usually induces cytotoxic T-cell immunity.

An E2-based vaccine would have more limited therapeutic value for the treatment of high-grade HPV-associated lesions and cancers because the E2 gene is frequently lost in such lesions through HPV DNA integration (17). However, this feature is not universal, and an estimated 30% of cervical cancers do not contain integrated HPV DNA (18, 35). High-grade lesions and cancers could be screened for E2 expression before a decision were made on whether E2 immunotherapy was appropriate, much as breast cancers are now screened for expression of the HER2/neu protein before Herceptin (trastuzumab; Genentech, San Francisco, Calif.) therapy is chosen (24, 46).

It may be possible to develop a direct CRPV E2-based

therapy with future investigation. This possibility is suggested by our demonstration that CRPV E2, like BPV E2, profoundly repressed the HPV18 E6/E7 promoter and strongly activated p21 expression in HeLa cells. These activities are accompanied by the cessation of cellular proliferation in BPV E2-expressing cells (15, 22, 23). If CRPV E2 similarly caused growth arrest of papillomavirus-infected cells, intralesional injection of CRPV E2, using Ad-E2 or other means, might cause regression. This strategy has potential as a direct treatment for high-grade lesions.

A direct mechanism cannot account for papilloma regression in the present study because it requires the E2 protein to directly bind the E6/E7 promoter, i.e., within CRPV-infected cells. Rabbit skin was infected with CRPV at one set of sites and vaccinated with Ad-E2 at another set of sites. Ad-E2 could not have spread to CRPV-infected cells because the vector lacks the Ad E1 protein and cannot replicate. The E2 protein also could not have entered CRPV-infected cells through cell fusion because Ads do not have this property. The lack of plausible mechanisms for E2 entry into CRPV-infected cells therefore leads us to conclude that the therapeutic efficacy of the Ad-E2 vaccine was due not to a direct effect of E2 on the CRPV E6/E7 promoter but to an indirect mechanism, i.e., immunologic activity.

The therapeutic efficacy of Ad-E2 vaccination was likely due to cell-mediated immunity. Antibody would not be cytotoxic, and vaccinated rabbits did not develop E2 antibodies. Cell-mediated responses might be initiated by Ad-E2 infection of dendritic cells (DCs) or other professional antigen-presenting cells in the skin, causing endogenous E2 expression and resulting in E2 peptide presentation at the plasma membrane. This possibility is supported by reports that recombinant Ad vectors efficiently infect DCs, the most potent antigen-presenting cells in the body (2, 47). Alternatively or additionally, DCs could phagocytose other cell types expressing E2 protein (e.g., following Ad-E2 vaccination), which could lead to E2 cross-presentation. In either case, activated DCs that migrate to draining lymph nodes could activate E2-specific cytotoxic T cells, which, on migrating back to the skin, could kill CRPV-infected cells displaying E2 peptides. Since E2 protein is essential for papilloma formation (53) and is involved in all steps of the virus life cycle (36), it is likely that all CRPV-infected rabbit cells expressed E2 and were susceptible to immunologic attack. When cytotoxic T-cell assays for rabbits become available, the role of cytotoxicity in mediating papilloma regression can be investigated directly.

Ad-E2-vaccination required less than 3 weeks to provide a clinical benefit, since dramatic differences in papilloma outgrowth between the rabbits used in the experiment and the control rabbits were evident by that time. In fact, two Ad-E2 vaccinees never developed papillomas. In the other three, the full effects of primary vaccination may not yet have developed and/or E2 antigens in the established papillomas may have augmented primary responses to effect papilloma regression. Therefore, it is unclear whether booster vaccination contributed to regression. Further, Ad-specific antibodies and/or cellular immune responses induced by primary vaccination may have neutralized the vector upon reinoculation (16).

The clinical efficacy of the Ad-E2 vaccine suggests that it will also cause regression of established papillomas. However, vac-

cination in the present study was performed during the sub-clinical phase of CRPV infection, and regression of established papillomas may require stronger immune responses. If primary and booster vaccination with Ad-E2 is not sufficient, it may be possible to augment the E2-specific response by giving primary vaccinations with a heterologous vector expressing E2 before giving booster vaccinations with the Ad-E2 vector. Recent studies have shown that naked DNA vaccines are particularly effective for primary vaccinations prior to booster vaccinations with an Ad vector (44, 54). Future investigation might determine, therefore, if greater efficacy can be achieved by primary vaccination with either of our E2 DNA vaccines (encoding unfused or ubiquitin-fused E2 protein) and booster vaccination with the Ad-E2 vaccine, or vice-versa. The strategy of giving primary vaccinations with a DNA vaccine and booster vaccinations with a heterologous type of vaccine shows promise in other models (28, 39, 42).

ACKNOWLEDGMENTS

We thank Daniel DiMaio for helpful discussions and suggestions and for critical review of the manuscript, Heather Greenstone for CRPV VLPs, and Daniel Zelterman for assistance with statistical analysis.

This work was supported by grants from the American Cancer Society (to J.L.B., grant 100103 RPG) and the Yale Skin Diseases Research Center (to J.L.B., grant AI401942) and generous gifts from the Ted Mann Foundation (to J.L.B.). E.C.G. was partially supported by National Institutes of Health grant CA16038.

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