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Recombinant *Listeria monocytogenes* Vaccination Eliminates Papillomavirus-Induced Tumors and Prevents Papilloma Formation from Viral DNA

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***Listeria monocytogenes* is a gram-positive, facultative intracellular bacterium that enters the cytoplasm of infected cells and spreads directly into neighboring cells without encountering the extracellular environment. Cytoplasmic *L. monocytogenes* efficiently presents secreted proteins to the major histocompatibility complex class I pathway which can stimulate protective T-cell-mediated immune responses. We have used a cottontail rabbit papillomavirus (CRPV) rabbit model to test the ability of recombinant *L. monocytogenes* strains secreting the viral E1 protein (E1-rLm) to protect outbred rabbits against CRPV- and CRPV DNA-induced tumors. CRPV infection of outbred rabbits serves as a model for oncogenic papillomaviruses since CRPV-induced papillomas progress with high frequency to malignant carcinoma. Rabbits were vaccinated with wild-type *L. monocytogenes* or E1-rLm and then challenged with CRPV or viral DNA. In contrast to 0% papilloma regression in control animals, 77% of E1-rLm-vaccinated rabbits generated protective immunity that controlled and induced complete regression of tumors induced by CRPV. Latent viral DNA was not detected at 71% of the papilloma regression sites examined 4.5 months postregression. E1-rLm responder rabbits were completely resistant to papilloma formation from viral DNA. In contrast to controls, peripheral blood mononuclear cells from E1-rLm responder rabbits were able to proliferate in response to in vitro E1 stimulation. These results indicate that E1-rLm immunization generated a systemic anti-CRPV E1 cell-mediated immune response which protected outbred rabbits from tumors induced by CRPV or CRPV DNA challenge.**

Many human cancers have been linked to viral infections, including Epstein-Barr virus, human T-cell leukemia virus, hepatitis B virus, and several human papillomaviruses (PVs). Immunological control of neoplastic progression is suggested by the higher incidence of virus-associated cancers in immunocompromised individuals (32, 41). Vaccine strategies which target viral gene products to the immune system may be able to prevent or induce regression of virus-induced cancers. Cottontail rabbit papillomavirus (CRPV) infection of domestic rabbits provides a well-established animal model for high-risk PVs associated with the development of cancer (4, 26, 57). This model has been used for studies of virus-host interactions and for the development of immune-based strategies aimed at controlling PV infection and carcinogenesis.

PVs are small, nonenveloped, double-stranded DNA viruses which are highly species and tissue specific (42, 57). They are responsible for the localized development of benign epithelial neoplasms (papillomas or warts), which can progress to malignant carcinomas. PVs infect cutaneous and mucosal tissues, and viral protein expression and DNA replication are tightly linked to epithelial cell differentiation (8, 17, 27, 59). Although infection with PV induces both humoral and cellular anti-PV immune responses, the effect of antibody- and cell-mediated immunity (CMI) on the virus-host interaction is variable (2, 30, 47, 54). PV infection can be latent or result in the formation of papillomas which persist for the lifetime of the host, completely regress, or progress to malignant tumors (3, 10, 26, 57). Papilloma regression is associated with a cellular immune response characterized by tumor-infiltrating T lymphocytes and macrophages (2, 10, 35, 49). The observation that PV infection may be influenced by immunological mechanisms has prompted the development of anti-PV vaccines.

Early studies of CRPV infection demonstrated the induction of both humoral and cellular immune responses which are thought to prevent superinfection with virus and mediate spontaneous regression of tumors, respectively (15, 16). Progression of papillomas to invasive carcinomas further stimulates both humoral and cellular immunity as the tumor disseminates throughout the host (30, 47). Several CRPV proteins have been used to generate protective anti-CRPV immunity; these include the early proteins, E1 and E2, as well as the late proteins, L1 and L2 (29, 48). E1 and E2 are required for viral DNA replication, maintenance, and papilloma formation (7, 8, 12, 56, 58). L1 and L2 are the structural proteins of the capsid (42). Rabbits immunized with plasmid DNA encoding L1, full-length nondenatured L1, or virus-like particles generate neutralizing antibodies which recognize conformational epitopes (6, 14, 24, 28). Such animals are protected against viral challenge but not against CRPV DNA challenge (28). Immunization with the nonstructural protein E1 or E2 stimulates regression of papillomas, and in some animals, regression occurs in the absence of antigen-specific antibodies, suggesting that CMI was responsible for papilloma regression (48).

Listeria monocytogenes is a gram-positive, facultative intra-

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cellular bacterium that we and others have developed as a live vaccine vector for the generation of cell-mediated immune responses against heterologous antigens (19, 23, 44, 51, 52). It is able to enter phagocytic and nonphagocytic cells, escape from endosomes, multiply within the host cell cytoplasm, and spread directly to adjacent cells without exposure to the extracellular environment (50, 53). *L. monocytogenes* proteins are processed and presented by both major histocompatibility complex (MHC) class I and II pathways, stimulating a variety of cell-mediated immune responses (36, 37, 43). *L. monocytogenes* antigen-specific, MHC-class-I-restricted, cytotoxic $CD8^+$ T cells generated during primary infection of mice are able to protect naive recipients against lethal *L. monocytogenes* challenge following adoptive transfer (11). Protective $CD8^+$ T cells primarily recognize epitopes from secreted *L. monocytogenes* proteins (20, 21). Viral antigens, such as the lymphocytic choriomeningitis virus and influenza virus nucleoproteins, secreted by intracellular, recombinant *L. monocytogenes* are also processed and presented by MHC class I molecules (19, 23, 51, 52). Viral antigen-specific $CD8^+$ T cells primed by recombinant *L. monocytogenes* vaccination provide protection against viral challenge (19, 51, 52).

This study describes the ability of recombinant *L. monocytogenes* vaccine strains encoding CRPV E1 sequences (E1-rLm) to protect outbred domestic rabbits against CRPV- or viral DNA-induced tumor development. The majority of E1-rLm-vaccinated rabbits controlled virus-induced papilloma formation and growth and eventually eliminated all papillomas. Latent viral DNA could not be detected in the majority of papilloma regression sites several months postregression. In contrast to the susceptibility of control rabbits, E1-rLm responder rabbits were completely protected against CRPV DNA-induced tumor formation. Papilloma regression induced by E1-rLm vaccination correlated with an in vitro E1-specific proliferative response by peripheral blood mononuclear cells (PBMC). These results indicate that E1-rLm immunization of an outbred population stimulated protective immunity through a systemic, anti-E1 cell-mediated mechanism which controlled and eliminated developing papillomas, preventing their progression to carcinoma.

MATERIALS AND METHODS

Plasmid and recombinant *L. monocytogenes* construction. The antigen expression cassette of pEJ140P was constructed by cloning a 290-bp fragment of *hly*, containing the promoter and signal sequence amplified by PCR, into pBR322 (34). The *hly* promoter fragment was translationally fused with a *Bam*HI-*Asc*I-*Pac*I-*Hin*dIII linker and the *Escherichia coli phoA*, which lacks its own promoter and signal sequence (18). The antigen expression cassette contains *aphA-3*, which confers kanamycin resistance and is flanked by *Not*I sites. PCR was used to amplify the entire open reading frame of CRPV E1, encoding amino acids 2 to 602, as well as three fragments encoding amino acids 2 to 190, 172 to 416, and 384 to 602. All PCR products were amplified with *Bam*HI and *Asc*I sites at their 5′ and 3′ ends, respectively. *Bam*HI-*Asc*I-digested PCR products were translationally fused at their 5′ end to the third codon after the signal peptidase cleavage site of *hly* and at their 3′ end to *phoA*. All constructs were sequenced through the fusion junctions and then cloned into pHS-LV as *Not*I fragments (51). pHS-LV derivatives were introduced into the wild-type *L. monocytogenes* 10403S by electroporation (40). Genomic integration of antigen expression cassettes and selection for and identification of desired recombinant strains were performed as previously described (51).

Western blot analysis. Cultures of *L. monocytogenes* were grown to logarithmic phase (optical density at 600 nm, 0.8) at 37°C in brain heart infusion broth buffered to pH 7.5 with 100 mM MOPS [3-(*N*-morpholino)propanesulfonic acid]. Supernatant proteins were concentrated by trichloroacetic acid precipitation. Total cellular proteins were obtained by lysing washed cell pellets with *L. monocytogenes* bacteriophage endolysin, PLY118, for 5 min at 37°C (31). Protein extracts were normalized and electrophoresed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis for Coomassie brilliant blue staining or anti-PhoA immunoblot analysis performed with enhanced chemiluminescence as previously described (51). Coomassie blue staining demonstrated minimal contamination of cellular proteins in supernatant preparations and equivalent lane loading among total cellular proteins and among supernatant proteins (data not shown).

Rabbit vaccination and challenge. Groups of 3- to 4-month-old, female New Zealand White (NZW) rabbits obtained from Irish Farms (Norco, Calif.) were immunized intravenously with three doses of 10^5, 10^6, and 10^7 CFU (naive rabbit 50% lethal dose, $\approx 10^6$ CFU/kg of body weight) of 10403S or the E1-rLm cocktail per kg of body weight given at 2-week intervals. The E1-rLm cocktail contains equal amounts of the recombinant *L. monocytogenes* strains EJL244NP, MP, and CP, each expressing fusion proteins containing overlapping portions of CRPV E1. Three weeks after the final immunization, rabbits were challenged at four sites/rabbit with CRPV (Washington B strain) applied to 2 cm^2/site of lightly scarified skin. The amount of CRPV required to induce papillomas on NZW rabbits was determined previously. Some immunized rabbits were simultaneously challenged at 30 to 38 sites/rabbit each with 50 μg of recombinant CRPV DNA per site inoculated subcutaneously with a Ped-o-jet injector (28). Papillomas were monitored and quantitated up to 193 days postchallenge. Individual papilloma areas were calculated from the smallest and largest diameters.

PCR analysis of skin biopsies. Skin punch biopsies (5-mm diameter) were aseptically collected from regressed CRPV challenge sites of E1-rLm-vaccinated rabbits and the normal skin and a papilloma from a 10403S-vaccinated rabbit. The skin samples were immediately frozen in liquid nitrogen and stored at −70°C. Total DNA was prepared from the tissue samples and subjected to PCR as previously described (28). Briefly, minced tissue was digested with proteinase K for 1 h at 55°C and then boiled for 10 min. Debris was removed, and 5 μl of supernatant was used in a 50-μl PCR containing two sets of primers. One set of primers amplified a 344-bp fragment from the CRPV E7 gene (28). The second primer pair produced an internal control, 159-bp fragment from rabbit cytochrome P-450 (form 3a, exon 2) (28). Five microliters of the PCR mixtures was electrophoresed on 1.8% agarose gels for ethidium bromide staining and photography and then transferred to nylon membranes for Southern blot analysis using probes for CRPV E7 or cytochrome P-450 labeled by random priming. The detection limit of the assay has been estimated to be approximately one copy of viral DNA per 100 cells (28).

Isolation of PBMC and in vitro proliferation assay. PBMC were isolated as previously described (47). Briefly, arterial blood from the ear was collected into heparinized tubes and diluted with phosphate-buffered saline. Diluted blood was layered over Histopaque-1077 (Sigma) and centrifuged. PBMC at the interphase were collected and washed three times in phosphate-buffered saline and once in minimal essential medium and resuspended to a final concentration of 5×10^6 viable cells/ml of culture medium. Proliferation assays were carried out in 96-well flat-bottom microtiter plates. One hundred microliters of PBMC (5×10^5 cells) was added to triplicate wells containing 100 μl of medium with no mitogen-antigen, 5 μg of concanavalin A (ConA) per ml as a positive control for T-cell proliferation, 1 μg of TrpE per ml, or 1 μg of TrpE-E1 per ml. Vectors containing *trpE* and *trpE-E1* were constructed, and proteins were expressed and purified as previously described (30, 48). Cultures were incubated for 3 to 6 days at 37°C in humidified 5% CO_2. Twenty hours prior to harvest, 1 μCi of [^{3}H]thymidine (New England Nuclear) was added to the wells to be sampled. DNA was harvested from wells onto filter discs with a semiautomatic cell harvester, and mean [^{3}H]thymidine incorporation was determined by a liquid scintillation counter. Because of variability in the background response to TrpE, which ranged from 4.4 ± 0.9 (mean kilocounts per minute [kcpm] ± standard error of the mean [SEM]) on day 3 to 17.9 ± 5.1 on day 6, the data are expressed as an E1-specific stimulation index (SI) (47). The SI represents the ratio of mean [^{3}H]thymidine incorporation from cells stimulated with TrpE-E1 to that from cells stimulated with TrpE on the same day of culture. SIs greater than 2 are considered a positive, antigen-specific proliferative response (47).

Statistical analysis. Data were analyzed with StatView 4.5 for the Macintosh (Abacus Concepts) and are represented as the means ± SEMs. Statistical significance was determined by unpaired *t* test comparison.

RESULTS

Expression of CRPV E1 by recombinant *L. monocytogenes* strains. We have previously shown that transcription, translation, and secretion directed by the *L. monocytogenes hly* promoter and signal sequence allow foreign antigens to be exported from *L. monocytogenes* and introduced into the MHC class I processing pathway for presentation to $CD8^+$ T cells (51). We initially constructed a recombinant *L. monocytogenes* strain, EJL244WP, which expresses the entire CRPV E1 protein as a translational fusion between the *L. monocytogenes hly* signal sequence and *E. coli* PhoA (Fig. 1A). Fusion with PhoA allows an assessment of secretion in *E. coli* during plasmid construction and serves as an immunological marker for monitoring fusion protein localization in recombinant *L. monocytogenes*. Export of the E1-PhoA fusion protein did not occur in *E. coli*, and this fusion protein was inefficiently secreted from

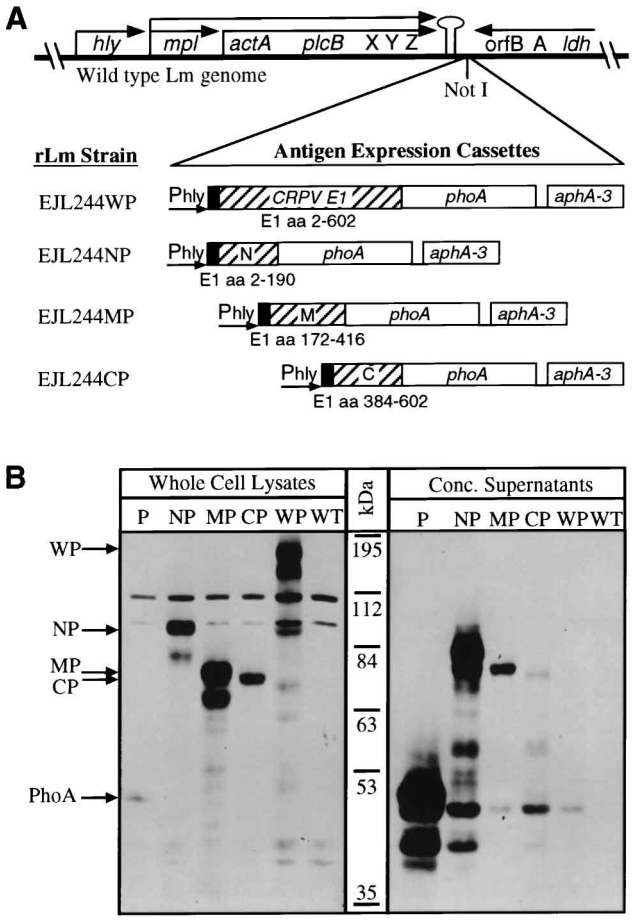

FIG. 1. Recombinant *L. monocytogenes* strains containing CRPV E1-PhoA antigen expression cassettes. (A) Antigen expression cassettes were introduced via homologous recombination into the wild-type *L. monocytogenes* 10403S genome (top) between two convergent transcription units and downstream from the transcriptional terminator following the Z open reading frame (⍑) (50). Insertion of foreign sequences at the indicated *Not*I site does not significantly affect in vitro or in vivo viability (51, 52). *hly* encodes the hemolysin (listeriolysin O) required for endosomal escape; *mpl* encodes a metalloprotease; *actA* encodes a surface protein (ActA) required for actin polymerization and cell-to-cell spread; *plcB* encodes a phospholipase; *X*, *Y*, *Z*, *orfB*, and *A* are uncharacterized open reading frames; and *ldh* encodes lactate dehydrogenase. Antigen expression and secretion are under the control of the *L. monocytogenes hly* promoter (Phly) and the translation initiation and signal sequence (black box). *aphA-3* confers kanamycin resistance. *phoA* designates an *E. coli* alkaline phosphatase gene lacking transcription initiation, translation initiation, and secretion signals. EJL244WP expresses the entire CRPV E1 protein translationally fused to PhoA. EJL244NP, -MP, and -CP express overlapping thirds of E1 fused to PhoA. aa, amino acids. (B) Anti-PhoA Western blot analysis of whole-cell lysates and concentrated supernatants from recombinant *L. monocytogenes* strains. Cell lysates and supernatants are designated as follows: P, EJL240P, a positive control strain which expresses PhoA; NP, EJL244NP; MP, EJL244MP; CP, EJL244CP; WP, EJL244WP; and WT, 10403S, the negative-control wild-type *L. monocytogenes* strain. All PhoA fusion proteins were of the predicted molecular mass, except for the NP fusion protein, which had an apparent size larger than the calculated molecular mass of 70.5 kDa. Arrows indicate the full-length fusion proteins. Degradation products are also detected in both whole-cell and supernatant fractions. The blot was scanned with a UMAX S-12 scanner, and the image was processed with Adobe Photoshop 4.0 for the Macintosh.

L. monocytogenes; however, PhoA itself was efficiently exported (Fig. 1B). We hypothesized that efficient antigen export from recombinant *L. monocytogenes* would significantly facilitate antigen processing and MHC class I presentation. To overcome the export incompetence of CRPV E1, we reasoned that portions of E1 may be more efficiently secreted than the entire protein. Accordingly, recombinant *L. monocytogenes* EJL244NP, EJL244MP, and EJL244CP were constructed. Each strain expresses an overlapping fragment of E1 to maintain potential T-cell epitopes (Fig. 1A). In contrast to full-length E1-PhoA, the partial E1-PhoA fusion proteins were secreted from recombinant *L. monocytogenes* (Fig. 1B). The predicted hydrophobicity of each E1 fragment inversely correlated with the secretion efficiency of the corresponding E1-PhoA fusion protein. Based on these results, we vaccinated rabbits with a cocktail, designated E1-rLm, which was equally composed of strains EJL244NP, EJL244MP, and EJL244CP.

E1-rLm immunization promotes papilloma regression. In two independent experiments, groups of six or seven rabbits were immunized with wild-type *L. monocytogenes* 10403S or with the E1-rLm cocktail and boosted twice with 10-fold-increasing doses at 2-week intervals. Throughout the vaccination schedule, all rabbits appeared normal and did not display any signs of distress. The ability of the rabbits to survive the final boost (10 50% lethal doses) indicates that protective anti-*L. monocytogenes* immunity was generated. Serum collected from rabbits after the final boosting immunization had high titers of anti-*L. monocytogenes* antibodies but did not contain detectable levels of E1-specific antibodies (data not shown). Three weeks after the final immunization, rabbits were challenged with CRPV at four scarified dorsal sites per animal. Papillomas developed on all rabbits following CRPV challenge (Fig. 2A and B); however, rabbits which responded to the E1-rLm vaccination had significantly fewer papillomas compared to control or E1-rLm-immunized nonresponder rabbits (one versus five per challenge site; $P < 0.001$). In contrast to control animals, which maintained papillomas throughout the course of the experiments, all papillomas were induced to completely regress from challenge sites on six of six and four of seven E1-rLm-vaccinated rabbits in the first and second experiments, respectively (Fig. 2A and B). E1-rLm responder animals remained papilloma free for at least 5 months. From the onset of regression, papillomas at each challenge site were simultaneously and rapidly eliminated from individual E1-rLm responder rabbits within 10 ± 2 days. The combined protective efficacy from both experiments was 77% (Fisher's exact *P* value, 0.0001; 95% confidence interval, 52 to 95%). Two animals did not significantly respond to the vaccination and failed to control or eliminate papillomas. Papilloma regression on a third animal progressed to near completion; however, at day 58 postchallenge, papilloma growth resumed at three of four regressed sites and continued for the duration of the experiment (Fig. 2B). These results demonstrate three effects of E1-rLm vaccination on CRPV-induced tumor formation in an outbred animal population: (i) complete regression, (ii) transient or partial regression, and (iii) no regression.

Papilloma growth at challenge sites. The rates of increase in total papilloma area per challenge site on vaccinated and control rabbits are shown in Fig. 3. During the first experiment (Fig. 3A), papillomas on control rabbits continuously increased in surface area, reaching a mean total papilloma area (MTPA) per site of 378 mm^2 at 93 days postchallenge. In contrast, the few papillomas which appeared on E1-rLm-immunized rabbits were significantly smaller, attaining a maximum MTPA per site of 15 mm^2 at 48 days postchallenge. Papilloma growth during the second experiment also demonstrated the dichotomy between protected and unprotected rabbits (Fig. 3B). Control and E1-rLm-immunized, nonresponder rabbits developed papillomas which grew at similar rates throughout the experiment, achieving MTPAs per site of 742 and 793 mm^2 at 88 days postchallenge, respectively. In contrast, E1-rLm responder rabbits were able to significantly control papilloma growth.

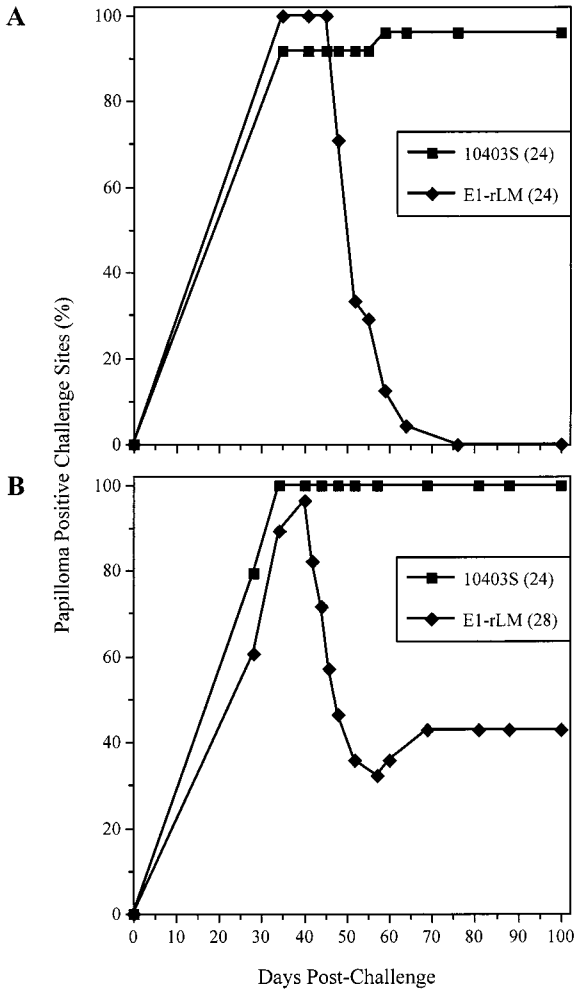

FIG. 2. Papilloma development and regression at CRPV challenge sites following E1-rLm immunization. Results from two independent experiments are shown. During the first (A) and second (B) experiments, groups of six or seven E1-rLm-immunized rabbits were challenged with CRPV at four dorsal sites per animal. Papilloma development or regression was monitored for each challenge site (total number indicated in parentheses).

Overall, in the majority of rabbits, E1-rLm vaccination stimulated quantitative control of papilloma formation and development and ultimately resulted in complete tumor regression.

Analysis of latent viral DNA in regressed CRPV challenge sites. The reappearance of papillomas at three completely regressed CRPV challenge sites on one of the E1-rLm responder rabbits suggested the potential for establishment and activation of latent infections. Therefore, regressed CRPV challenge sites were analyzed for the presence of CRPV DNA by PCR and Southern blot hybridization with an E7-specific probe (28). Skin biopsies were collected from 24 regressed CRPV challenge sites on six E1-rLm-immunized rabbits. Papilloma and normal skin biopsies were obtained from a 10403S-immunized control rabbit and served as CRPV-positive and -negative control biopsy samples, respectively. Total DNA was extracted from the tissue, and PCR was used to amplify a 344-bp fragment from the CRPV E7 gene and a 159-bp fragment from rabbit cytochrome P-450, which served as an internal control. PCR of all biopsy samples produced the internal control P-450 product, indicating that DNA was extracted and PCR could amplify a specific product (Table 1). The CRPV E7 PCR product was amplified from the CRPV-positive control but not from the negative controls or from 17 of 24 regression sites (71% CRPV negative) from E1-rLm-vaccinated rabbits. Additional biopsy samples from the same challenge sites were analyzed to reduce potential sampling errors and increase the area tested. These second samples confirmed the lack of detectable viral DNA at the CRPV E7-negative challenge sites. None of the challenge sites from the E1-rLm-immunized rabbit examined 36 weeks post-papilloma regression contained viral DNA, while animals examined at 19 weeks postregression each had at least one viral DNA-positive site (Table 1). Although only 10% of the total area of each challenge site was examined, two independent CRPV E7-negative biopsy samples from different locations in the same challenge site strongly suggest that papilloma regression was associated with the lack of latent infection from most CRPV challenge sites on E1-rLm-vaccinated regressor rabbits.

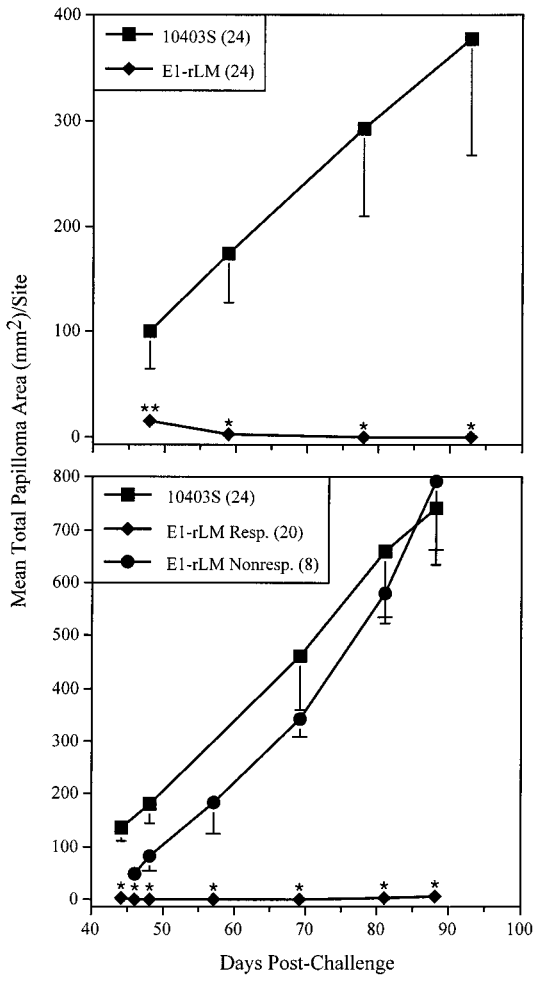

FIG. 3. Papilloma growth following CRPV challenge of E1-rLm vaccination. The total papilloma area per challenge site on rabbits from the first (A) and second (B) experiments was calculated from the smallest and largest diameters of each papilloma. The total numbers of challenge sites are indicated in parentheses. Data represent the means ± SEMs per challenge site. Papillomas on E1-rLm responder rabbits were significantly smaller than those on 10403S-immunized and E1-rLm nonresponder rabbits throughout both challenge experiments (**, $P < 0.042$; *, $P < 0.007$).

TABLE 1. PCR analysis of regressed CRPV challenge sites[a]

Rabbit no.	No. of wk postregression	No. of PCR-positive sites/ total no. of sites tested	
		P-450	CRPV E7[b]
38	19	4/4	1/4
41		4/4	3/4
42		4/4	1/4
44		4/4	1/4
53		4/4	1/4
28	36	4/4	0/4
Total		24/24	7/24

[a] Regression sites were determined to be CRPV DNA positive or negative following Southern blot hybridization as described in Materials and Methods.
[b] CRPV E7-negative challenge sites were confirmed by two independent CRPV E7-negative biopsy samples from different locations within the same site.

E1-rLm immunization protects against CRPV DNA-induced papillomas. Papilloma generation from CRPV DNA challenge is not affected by humoral immune responses, such as those which generate CRPV-specific neutralizing antibodies (15, 28). CRPV DNA-induced papilloma development, however, may be prevented by a CRPV antigen-specific cellular immune response. To further characterize the efficacy of E1-rLm vaccination and to investigate the immune mechanism of papilloma regression, rabbits in the second experiment were simultaneously challenged with recombinant CRPV DNA in addition to the viral challenge (Table 2). Papillomas developed on 10403S-immunized control rabbits at 18.5% of their 184 DNA challenge sites. Similarly, papillomas developed on E1-rLm-vaccinated nonresponder rabbits at 13.3% of their 60 DNA challenge sites. These papilloma development rates are relatively low compared with those for virus challenge but are characteristic of those obtained with this DNA delivery method (5, 28). In stark contrast to both control and nonresponder rabbits, papillomas did not develop on the five E1-rLm-immunized responder rabbits at any of their 163 DNA challenge sites ($P < 0.001$). Complete protection from recombinant CRPV DNA challenge suggests that a protective cell-mediated immune response was generated in the E1-rLm responder rabbits.

In vitro anti-E1 proliferative responses. To determine if an E1-specific cellular immune response existed, PBMC from 10403S and E1-rLm responder rabbits were isolated and analyzed in vitro for E1-specific proliferative responses. Previous analysis of cellular immune responses following CRPV infection demonstrated that PBMC from spontaneous regressor and nonregressor rabbits do not proliferate in response to CRPV E1 stimulation (46). PBMC were collected within 1 week of complete papilloma regression from five E1-rLm-vaccinated regressor rabbits and at the same time from two papilloma-positive 10403S-immunized control rabbits. PBMC were cultured over 6 days in medium only or medium containing ConA, TrpE-E1 fusion protein, or TrpE as a control, and proliferation was determined by [^{3}H]thymidine incorporation. PBMC from all rabbits responded on day 3 of culture to ConA stimulation (368.6 ± 39.3 kcpm) compared to that with medium only (0.8 ± 0.1 kcpm). Compared with TrpE stimulation, control PBMC did not significantly increase DNA replication in the presence of TrpE-E1 at any time during the experiment (Fig. 4). In contrast, PBMC from all five E1-rLm-vaccinated rabbits were able to specifically proliferate in response to TrpE-E1 during the assay, with mean kcpm of 18.2 ± 5.4 and 29.3 ± 8.6 on days 5 and 6, respectively. The TrpE-E1-positive proliferative response had SIs ranging from 2.2 to 4.9. PBMC from four of the five rabbits demonstrated E1-specific proliferation on day 5 of culture. In contrast to the lack of TrpE-E1-induced proliferation of PBMC from spontaneous regressor rabbits (46), the TrpE-E1-stimulated response of PBMC from E1-rLm regressor rabbits suggests that the vaccination generated an E1-specific cellular immune response.

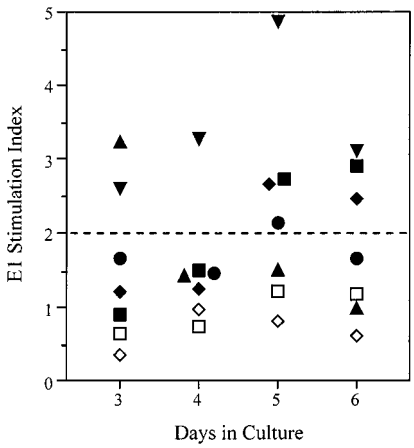

FIG. 4. In vitro anti-E1 proliferative response of PBMC from immunized rabbits. PBMC were collected within 1 week of papilloma regression from five E1-rLm-vaccinated rabbits (closed symbols) and at the same time from two papilloma-positive 10403S-immunized control rabbits (open symbols) and analyzed for in vitro anti-E1 proliferative responses over 3 to 6 days. Each symbol represents the data from an individual rabbit. SIs greater than 2 (dashed line) represent E1-specific proliferative responses (47).

TABLE 2. Papilloma development at CRPV DNA challenge sites

Group (no. of rabbits)	No. of papilloma-positive sites/ total no. of sites	% Papilloma-positive sites
10403S (6)	34/184	18.5
E1-rLm		
Nonresponder (2)	8/60	13.3
Responder (5)	0/163	0[a]

[a] Significantly different compared to 10403S or E1-rLm nonresponder rabbits ($P < 0.001$).

DISCUSSION

This study describes the first application of a recombinant *L. monocytogenes*-based live vaccine to eliminate tumors generated by oncogenic viral infection. We have demonstrated that prophylactic immunization of outbred rabbits with a cocktail of recombinant *L. monocytogenes* strains secreting the CRPV E1 protein stimulated an immune response which quantitatively affected CRPV-induced tumor development. Seventy-seven percent of E1-rLm-vaccinated rabbits decreased the number of epithelial tumors which developed, suppressed their growth, and eliminated all papillomas without having detectable E1-specific antibodies. Latent viral infection was undetectable at 71% of the regressed challenge sites 4.5 months postregression. Vaccinated rabbits which eliminated CRPV-induced papillomas were completely resistant to tumor formation generated by viral DNA challenge. PBMC from regressor rabbits mounted an in vitro anti-E1 proliferative response. Taken together, our results support the conclusion that rabbits vacci-

nated with E1-rLm generated a systemic, anti-E1 cell-mediated immune response which provided antitumor protection.

Previous studies have demonstrated that CRPV infection and subsequent tumor development can be affected by viral antigen-specific humoral and cellular immune responses, respectively (2, 15, 16, 28, 35, 48, 49). Several results indicate that recombinant *L. monocytogenes* vaccination stimulated anti-E1 CMI. E1-rLm immunization did not prevent the generation of papillomas by CRPV; however, the number of macroscopic tumors which developed at each scarification site was significantly reduced compared to those of control rabbits. In contrast to *L. monocytogenes* control rabbits and rabbits whose tumors spontaneously regress (46), PBMC isolated from E1-rLm-vaccinated animals following tumor regression specifically proliferated in response to E1 stimulation. In contrast to CRPV challenge, viral DNA did not generate any papillomas on E1-rLm responder rabbits. These results also suggest that the protective immune response stimulated by E1-rLm vaccination was directed against infected cells expressing viral genes, such as E1, and not directly against CRPV.

There is strong evidence that PV can establish latent or persistent infections in humans and other animals (3, 13, 32, 33, 41). Latent PV infection may be abrogated by interrupting the initial viral infection or by immune recognition of host cells containing viral DNA. Following PV infection, elimination of papillomas and latent infection will probably require cell-mediated immune responses directed against PV early gene products (49, 54). Rabbits which spontaneously regress or are induced to eliminate papillomas by TrpE-E1 immunization maintain viral DNA in the majority of papilloma regression sites (49). This indicates that establishment of a latent state is a common feature of CRPV infection following papilloma regression. In contrast, the anti-E1 CMI generated by E1-rLm vaccination was able to eliminate detectable latent CRPV DNA from the majority of challenge sites after papillomas had been eliminated. To our knowledge, this is the first study to demonstrate the ability of vaccination to prevent the establishment of a latent state following productive PV infection and suggests the potential for therapeutic treatment. Our preliminary results from therapeutic vaccination experiments indicate that established papillomas on *L. monocytogenes*-immune control rabbits can be substantially reduced following several high-dose E1-rLm immunizations. Therapeutic efficacy is likely to depend on multiple factors contributed by both the recombinant *L. monocytogenes*- and PV-host interactions. Our data indicate that targeting early proteins required for viral DNA replication and maintenance may be an appropriate vaccine strategy for generating immune responses against existing PV infection.

Several recombinant *L. monocytogenes* vaccines have been developed and tested in in vivo viral infection models. Recent reports have demonstrated the ability of recombinant *L. monocytogenes* to generate foreign-antigen-specific, MHC class I-restricted, $CD8^+$ cytotoxic T lymphocytes (19, 23, 44, 51). These memory cytotoxic T lymphocytes are capable of protecting mice against chronic or lethal viral challenge (19, 51). Recombinant *L. monocytogenes*-immunized mice have also been prophylactically and therapeutically protected against lethal challenge with transfected murine tumor cells which constitutively expressed the same viral antigen expressed by the recombinant *L. monocytogenes* vaccine (38, 39). In comparison to these systems based on inbred mice, CRPV infection of outbred rabbits presents an extremely stringent test of the protective efficacy of recombinant *L. monocytogenes* vaccination. CRPV infection of domestic rabbits is characterized by several important features: (i) CRPV infection is sequestered to the epithelium of the dermis, rather than being systemic or invasive; (ii) CRPV infection of the epithelium localizes with epithelial stem cells of hair follicles (45); (iii) expression of CRPV proteins is tightly controlled and linked to the terminal differentiation of infected epithelial cells; and (iv) CRPV infection can become latent and subject to reactivation (3). These characteristics provide possible explanations for the inability of most naive rabbits to generate protective immunity following CRPV infection. In spite of the obstacles imposed by the CRPV-host interaction, it appears that E1-specific effector cells, stimulated by E1-rLm vaccination, were surprisingly efficacious at controlling and eliminating CRPV infection. The observation that E1 expression is required for maintenance of viral DNA within infected cells likely contributed to the efficacy of our vaccine (7, 8, 54, 56).

Although the protective efficacy of E1-rLm vaccination is similar to that of TrpE-E1 immunization (48), the E1-rLm-generated immune response was more effective at preventing the establishment of latent CRPV infection following papilloma regression (49). Several features of the antigen delivery system may relate to its efficiency in stimulating cell-mediated antitumor immunity. Since *L. monocytogenes* can escape from endosomes and enter the cytoplasmic compartment of professional antigen-presenting cells, MHC class I and II antigen presentation can be accompanied by expression of the costimulatory molecules required to prime naive $CD8^+$ and $CD4^+$ T cells. *L. monocytogenes* induces interleukin 12 secretion by macrophages, leading to the development of Th1-type $CD4^+$ T cells, which promote cell-mediated immune responses (1, 9, 22, 55). Recombinant *L. monocytogenes* may therefore function simultaneously as an antigen delivery vehicle with access to the cytoplasmic compartment and as an adjuvant promoting appropriate T-cell help for the development of strong CMI.

The heterogeneity of MHC alleles within outbred NZW rabbits provides a possible explanation for the observed range of antitumor responses. The rabbits used in this study demonstrated three levels of immunity. E1-rLm stimulated protective immunity in the majority of animals. Two E1-rLm-vaccinated rabbits were nonresponsive. One rabbit, however, generated a transient, suboptimal response that did not completely eliminate CRPV-generated papillomas but was sufficient to prevent viral DNA-induced tumor formation. In contrast to experimental CRPV infection, viral DNA challenge is minimally traumatic, highly localized, and less efficient. The ability to eliminate localized cells transfected with viral DNA may be less challenging than protecting a large area (8 cm^2 per rabbit) of scarified skin. Considering the MHC heterogeneity of the rabbits, our use of a single CRPV antigen may have led to the variable antitumor protection. Future immunization studies using recombinant *L. monocytogenes* strains secreting multiple CRPV antigens will address the possibility of enhancing protective immunity, presumably by increasing the diversity of primed viral-antigen-specific T cells.

Our results and those of Pan et al. have established the efficacy of antitumor recombinant *L. monocytogenes* vaccines and provide the basis for further studies (38, 39). For prophylactic vaccination of healthy individuals or therapeutic treatment of patients to occur, the fundamental issue of safety must ultimately be addressed. Methods of attenuation which reduce virulence yet retain the highly immunogenic properties of wild-type *L. monocytogenes* have been developed (25). As such, the ability to escape from phagolysosomes into the cytoplasm is important for immunogenicity, while other aspects of the *L. monocytogenes* life cycle, such as intercellular spreading and host cell range, are targets for disruption. Perhaps the most exciting prospect for recombinant *L. monocytogenes* vaccina-

tion is its therapeutic potential against existing tumors and its potential for the prevention of carcinoma development and resolution of disease. The ability to successfully eliminate PV-generated tumors in their natural context suggests the possibility that recombinant *L. monocytogenes* vaccination may be able to affect other virus-associated cancers.

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