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Immunology

Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas

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ABSTRACT **Infection of mucosal epithelium by papillomaviruses is responsible for the induction of genital and oral warts and plays a critical role in the development of human cervical and oropharyngeal cancer. We have employed a canine model to develop a systemic vaccine that completely protects against experimentally induced oral mucosal papillomas. The major capsid protein, L1, of canine oral papillomavirus (COPV) was expressed in Sf9 insect cells in native conformation. L1 protein, which self-assembled into virus-like particles, was purified on CsCl gradients and injected intradermally into the foot pad of beagles. Vaccinated animals developed circulating antibodies against COPV and became completely resistant to experimental challenge with COPV. Successful immunization was strictly dependent upon native L1 protein conformation and L1 type. Partial protection was achieved with as little as 0.125 ng of L1 protein, and adjuvants appeared useful for prolonging the host immune response. Serum immunoglobulins passively transferred from COPV L1-immunized beagles to naive beagles conferred protection from experimental infection with COPV. Our results indicate the feasibility of developing a human vaccine to prevent mucosal papillomas, which can progress to malignancy.**

Papillomaviruses are small (55 nm), nonenveloped DNA viruses that induce epidermal and mucosal papillomas in humans and animals (1, 2). Canine, bovine, rabbit, and some human papillomas can progress to the malignant state (3–7). In humans, the development of cervical carcinoma is closely associated with genital mucosal infection by a small subset of human papillomaviruses (HPVs), including HPV-16 and HPV-18 (7, 8). In regions where mass cancer screening is inadequate (e.g., Southeast Asia and South America), cervical cancer represents the leading cause of death by cancer in women. Although the United States has fewer annual deaths (4000–6000) from cervical cancer, the medical health care costs are enormous for screening and treating early HPV-related lesions. The development of an effective prophylatic HPV vaccine could potentially reduce the occurrence of genital warts, cervical dysplasia, and neoplasia by an estimated 90%.

Currently there are no vaccines to prevent disease caused by HPVs. These viruses possess certain properties which make vaccine development difficult. First, HPVs are highly species specific, making it impossible to use animals for the direct evaluation of a vaccines' efficacy. Second, there is no reliable *in vivo* or *in vitro* source of intact papillomaviruses. Mucosal lesions caused by HPV-16 and HPV-18 yield small quantities of infectious virus, and papillomaviruses cannot be propagated efficiently *in vitro*.

Infection of animals with species-specific papillomaviruses offers the best opportunity for evaluating vaccines. We recently described the use of canine oral papillomavirus (COPV) infection of beagles as a model for the mucosotropic HPVs (9). Like the HPV types associated with anogenital cancer, COPV infects and induces lesions at a mucosal site. Although bovine papillomavirus type 4 (BPV-4) also infects and induces papillomas in mucosal epithelium (esophagus) (10), it is difficult to monitor the development and progression of these internal tumors. Cottontail rabbit papillomavirus (CRPV) and BPV-1 induce tumors which are easily monitored; however, they selectively induce cutaneous rather than mucosal lesions (1).

We have shown in beagles that formalin-inactivated wart extracts completely prevent both experimental and natural infections of oral mucosa by COPV (9). However, the active component in these vaccine preparations has not been identified. Papillomavirus virions are composed of two proteins, L1 and L2. L1 protein constitutes 90–95% of the virion protein content and L2 protein represents the remaining 5–10%. Papillomavirus L1 protein is capable of assembling independently into virus-like particles (VLPs) when expressed in eukaryotic cells infected with baculovirus (11–13) or vaccinia virus (14, 15) vectors. Coexpression of L1 and L2 resulted in VLPs containing both structural proteins (13, 15).

The intent of the current study was to determine whether the COPV L1 protein, independent of L2, was sufficient for successful vaccination and whether conformational properties of the L1 protein were important for immunization. The results of these studies provide a strong rationale for the development of similar VLP-based vaccines to prevent HPV-induced oral and genital warts and cervical cancer.

MATERIALS AND METHODS

Cell Culture and Virus Stocks. *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and recombinant viruses were propagated as described (16).

Construction of Transfer Vector Containing the COPV L1 Gene and Selection of Recombinant Baculovirus bacCOPV L1R. The COPV L1 open reading frame was amplified from a plasmid containing the entire COPV genome (17) by PCR using the primers 5′-CGCGCCATGGCGGTTTGGCTTCC-3′ (sense) and 5′-GACATGAAGCTTGTGCGGTGCCGG-TCA-3′ (antisense). The resulting DNA fragment was digested with *Nco* I and *Hin*dIII and cloned into the multiple cloning site in pBlueBacIII (Invitrogen), downstream of the polyhedrin promoter. Recombinant plasmid and *Bsu*36I-digested AcMNPV DNA (BacPAK6; Clontech) were cotransfected into Sf9 insect cells with Lipofectin (GIBCO/BRL). Recombinant baculovirus bacCOPV L1R was selected and plaque purified as described (16).

Preparation of VLPs and Analysis by Transmission Electron Microscopy. Sf9 cells (10^6 per ml) in a Cellagen stirred-

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Abbreviations: BPV, bovine papillomavirus; COPV, canine oral papillomavirus; CRPV, cottontail rabbit papillomavirus; HPV, human papillomavirus; VLP, virus-like particle.

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tank bioreactor (New Brunswick Scientific) were infected with recombinant baculovirus at a multiplicity of infection of 0.03. Cells were harvested when the viability of the culture dropped to ≈70%. Cells were resuspended in lysis buffer (10 mM Tris·HCl, pH 8.0/10 mM NaCl/1.5 mM $MgCl_2$) containing protease inhibitors (leupeptin, 5 μg/ml; aprotinin, 1 μg/ml; pepstatin, 1 μg/ml; and phenylmethanesulfonyl fluoride, 1 mM) and were lysed by mechanical disruption. Nuclei were pelleted by centrifugation at 3600 × *g* for 30 min at 4°C, suspended in phosphate-buffered saline (PBS: KCl, 0.2 mg/ml; KH_2PO_4, 0.2 mg/ml; NaCl, 80 mg/ml; $Na_2HPO_4·7H_2O$, 2.16 mg/ml) containing 1 mM $MgCl_2$ and protease inhibitors, and lysed in a microfluidizer (Microfluidics). Alternatively, nuclei were lysed by suspension in RIPA buffer (19). After nuclear lysis, Benzonase (Merck) was added and the lysate was incubated on ice for 2 hr. The nuclear lysate was then layered over 40% (wt/vol) sucrose in PBS containing 1 mM $MgCl_2$ and protease inhibitors and was centrifuged at 110,000 × *g* for 2.5 hr at 4°C. Pelleted material was resuspended in CsCl/PBS (1.33 g/cm^3) and centrifuged to equilibrium at 250,000 × *g* for 22 hr at 4°C. Material banding with a density of 1.29 g/cm^3 was pooled, diluted with CsCl/PBS, and rebanded as above. Bands with the desired density were pooled and dialyzed extensively against PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. VLPs were adsorbed to carbon-coated grids, stained with 2% phosphotungstic acid at pH 6.8, and examined under an electron microscopy (Jeol 1005).

Calculation of L1 Concentration and Purity. L1 concentration was determined by quantitative immunoblot assay (9). The total protein content of the VLP preparations was measured with the bicinchoninic acid reagent (Pierce). Purity was calculated as mg of L1 per mg of total protein.

Immunization of Dogs with COPV L1 VLPs and Challenged with COPV. Intradermal injection into the accessory carpal foot pad of 6- to 8-week-old beagle dogs was carried out as described (9). For COPV challenge, the dorsal buccal mucosa and maxillary mucosa of dogs were abraded with a wire brush. Wart homogenate was then applied to the excoriated mucosa with a cotton swab.

Passive Transfer of Serum Proteins. Pooled dog sera was fractionated by the addition of ammonium sulfate to 50% saturation. Precipitated protein was resuspended in PBS, dialyzed, and infused intravenously (200 mg of protein per kg of dog body weight) into naive dogs. Approximately 24 hr after infusion, dogs were challenged with COPV as described above.

ELISA. Authentic COPV particles were purified from warts (20). Virions were diluted in PBS and distributed into 96-well plates (10 ng of L1 per well). After incubation at 37°C for 1 hr, the wells were blocked with 5% nonfat milk protein in PBS. Dog serum samples were diluted with 1% nonfat milk protein in PBS prior to addition in the virion-coated wells. Horseradish peroxidase-labeled goat anti-dog IgG (Kirkegaard & Perry Laboratories) was used a secondary antibody. The averages of optical density measurements of duplicate wells were calculated as the final optical density values. Endpoint titers were calculated as the serum dilution giving an optical density 2-fold higher than that obtained with preimmune serum.

RESULTS

Expression and Isolation of L1 Protein. A recombinant baculovirus, bacCOPV L1R, was engineered to synthesize COPV L1. The 57-kDa COPV L1 protein was detected in lysates of bacCOPV L1R-infected Sf9 cells by Western blot analysis with an L1-specific mAb, AU1 (Fig. 1*B*, lane 2). AU1 antibody recognizes a linear epitope present in many L1 proteins, including that of COPV (21). COPV L1 protein was not found in lysates of uninfected cells (Fig. 1*B*, lane 1) or Sf9 cells infected with nonrecombinant baculovirus (data not shown).

Most of the L1 protein in bacCOPV L1R-infected Sf9 cells was found to be associated with the nucleus (data not shown). Extracts were prepared from bacCOPV L1R-infected Sf9 nuclei by either mechanical disruption or detergent extraction. These extracts were applied to CsCl gradients and centrifuged to equilibrium. Gradient fractions possessing a density of 1.29 g/cm^3, which is the density of authentic "empty" papillomavirions (22), contained the 57-kDa L1 protein (Fig. 1*A*, lanes 3 and 4). The presence of L1 was confirmed by Western blot analysis with monoclonal antibody AU-1 (Fig. 1*B*, lanes 3 and 4). When examined by electron microscopy (Fig. 1*C*), these fractions were found to contain VLPs that resembled authentic papillomavirus particles, with an average diameter of 55 nm.

The purity of the COPV L1 protein prepared from nuclei by detergent extraction was ≈20%, which represented a 10-fold increase over the purity of L1 obtained by mechanical disruption. However, recovery of the more highly purified COPV L1 VLPs was less than 1/10th that of the crude L1 VLPs prepared from equivalent numbers of infected nuclei.

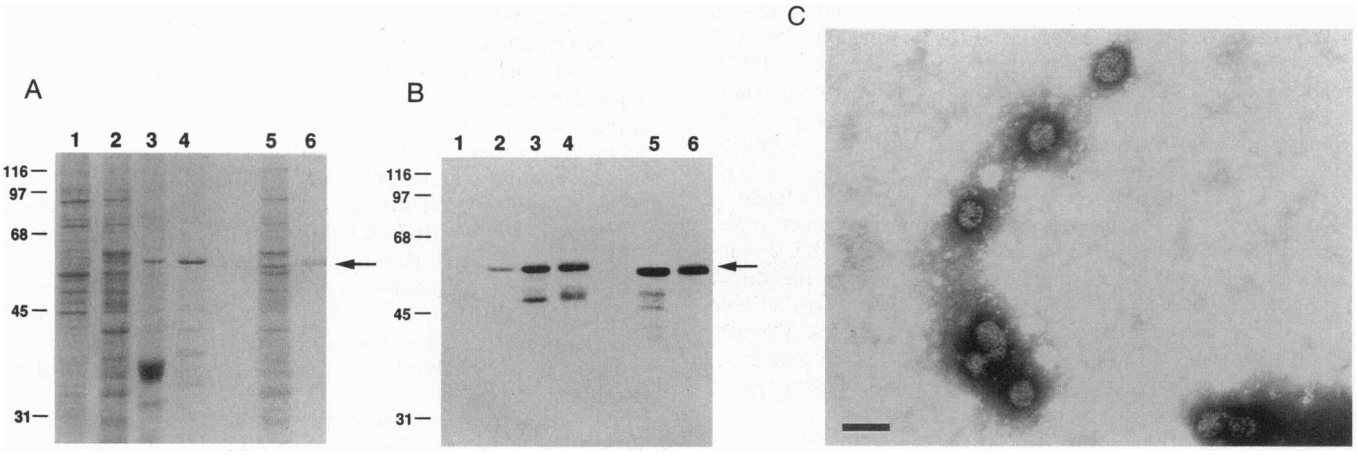

FIG. 1. Purification and visualization of L1 VLPs. (*A*) Coomassie blue-stained SDS/10% polyacrylamide gel of mock-infected Sf9 cell lysate (lane 1), bacCOPV L1R-infected Sf9 cell lysate (lane 2), COPV L1 VLPs prepared by mechanical disruption of bacCOPV L1R-infected Sf9 nuclei (lane 3), COPV L1 VLPs prepared by detergent extraction of bac COPV L1R-infected Sf9 nuclei (lane 4), bacHPV-11 L1-infected Sf9 cell lysate (lane 5), and HPV-11 L1 VLPs prepared by detergent extraction of bacHPV-11 L1-infected Sf9 nuclei (lane 6). Position and size (in kilodaltons) of protein standards are shown at left. (*B*) Immunoblot probed with mAb AU-1. Lane contents identical to those in *A*. (*C*) Transmission electron micrograph of COPV L1 VLPs prepared by mechanical disruption of bacCOPV L1R-infected Sf9 nuclei. (Bar = 100 nm.)

For use as a control in subsequent immunogenicity studies, HPV-11 L1 VLPs were also isolated. Sf9 cells were infected with a recombinant baculovirus encoding the HPV-11 L1 protein (23). HPV-11 L1 was purified from these cells by detergent extraction and CsCl density gradient centrifugation, as described above, and was estimated to be 60–80% pure (Fig. 1*A*, lane 6).

COPV L1 Protects Beagles from Oral Papillomas. Several vaccine studies were conducted in beagles to evaluate the protective efficacy of recombinant COPV L1 protein. A representative study is shown in Table 1. All immunogens were formulated without adjuvant and were injected intradermally into beagle footpads. Beagles were vaccinated at 8 weeks of age and received a booster injection 2 weeks later with an identical preparation of immunogen. Two weeks after boosting, the dogs were challenged with infectious COPV bilaterally on oral mucosa. Dogs were evaluated weekly for 13 weeks after challenge for the appearance of oral papillomas. As a positive control for protection from COPV infection, dogs in group 1 received the formalin-fixed wart extract vaccine (9). As anticipated, none of the group 1 animals developed papillomas. In contrast, all dogs that had been injected with PBS (group 2) developed papillomas ≈5 weeks postchallenge. The tumors in these animals persisted for ≈3 weeks and then regressed spontaneously.

Dogs immunized with either crude COPV L1 VLPs (group 4) or an enriched COPV L1 preparation (group 5) were completely protected from papilloma formation. To demonstrate that protection did not result from nonspecific immune stimulation, a group of beagles (group 3) was injected with a nuclear extract prepared from Sf9 cells infected with a recombinant baculovirus that encoded a human parvovirus antigen. Six of seven animals in this group developed COPV disease that followed the same course as in the PBS group (group 2). Immune protection was dependent, therefore, on the presence of COPV L1.

To determine whether or not L1 conformation was essential for inducing protective immunity, a group of dogs (group 6) was vaccinated with enriched COPV L1 VLPs that had been denatured by boiling in 1% SDS. In contrast to dogs in group 5, which had received identical amounts of native COPV L1 protein, none of the group 6 animals was resistant to viral challenge. We also determined whether unrelated, HPV-11 L1 VLPs could induce protection against COPV challenge. Dogs in group 7, which were vaccinated with HPV-11 L1 protein, were all susceptible to COPV infection, verifying previous data that protection against papillomaviruses is type specific (24, 25). Thus, only recombinant vaccine preparations that contained conformationally correct COPV L1 protein conferred protection from COPV infection.

L1-Vaccinated Beagles Develop COPV-Specific Antibodies. The dogs in the study described above were bled every 2 weeks for the duration of the experiment. Aliquots of serum from dogs in each group were pooled, diluted 1:100, and evaluated for the presence of COPV-specific IgG by ELISA. Serum immunoglobulins specific for intact COPV were detected in dogs immunized with the wart extract vaccine, the crude COPV L1 VLP preparation, and the enriched COPV L1 VLPs (Fig. 2*A*). The anti-COPV response in these dogs increased following primary immunization and peaked approximately at the time of virus challenge. In contrast, dogs immunized with either PBS, Sf9 nuclear extract, HPV-11 L1 protein, or SDS-denatured COPV L1 protein showed no increase in COPV antibody titers at the time of viral challenge (Fig. 2*B*). However, an increase in the COPV-specific immunoglobulins was detected in these dogs at the time of wart regression.

Examination of the COPV-specific antibody responses of individual dogs revealed very little animal-to-animal variation within each group (data not shown). The single exception was a dog immunized with baculovirus and Sf9 nuclear protein that failed to develop warts (Table 1, group 3). Examination of serum samples from this animal revealed an anti-COPV antibody response at the time of challenge (week 4) that was ≈2-fold above background. Furthermore, unlike the other dogs in this group, this animal demonstrated no increase in COPV-specific immunoglobulin in serum samples taken between weeks 10 and 14 of this study.

Serum pools from the dogs immunized with SDS-denatured COPV L1 (group 6) were tested in an ELISA for reactivity with denatured COPV L1 protein. At the time of challenge with COPV, these dogs were found to possess antibodies that recognized denatured COPV L1 (data not shown). Likewise, serum pools from animals immunized with HPV-11 L1 LVPs (group 7) were tested for antibody reactivity against HPV-11 L1. Prior to the time of challenge with COPV, these animals were found to possess anti-HPV-11 L1 antibodies (data not shown). Thus, both the SDS-denatured COPV L1 and the intact HPV-11 L1 VLPs, though not protective against COPV, were immunogenic.

Despite the dramatic protection from COPV disease provided by both the wart extract vaccine and the recombinant, conformationally correct COPV L1 protein, the antibody responses against intact COPV in these immunized dogs were relatively weak. Endpoint titers at the peak of the response were calculated to be <1:1000. In addition, by the end of the experiment, the anti-COPV response in dogs immunized with either the wart extract vaccine or the COPV L1 VLPs had diminished to near background levels (Fig. 2*A*). A repeat vaccine study was carried out to investigate whether formulation of COPV L1 VLPs with adjuvant could increase either the duration or the magnitude of the anti-COPV antibody responses. Groups of animals received two doses of the crude COPV L1 VLPs (20 μg of L1 per dose) either in PBS, or adsorbed to alum, or formulated with QS21 adjuvant (Cambridge Biotech). As controls, two additional groups of dogs received either the wart extract vaccine or PBS. Two weeks after the secondary immunization, all dogs were challenged with COPV. As anticipated, dogs injected with PBS developed oral warts whereas dogs that had received either the wart extract vaccine or the COPV L1 VLPs were protected from

Table 1. COPV vaccine study

Group	Immunogen	L1, μg per dose	Total protein, μg per dose	No. of dogs with warts
1	Wart extract vaccine	0.01–0.1	1	0/7
2	PBS	—	—	7/7
3	Baculovirus/Sf9 nuclear protein	—	860	6/7
4	Crude COPV L1 VLPs	20	860	0/7
5	Enriched COPV L1 VLPs	20	120	0/7
6	SDS-denatured COPV L1 VLPs	20	120	7/7
7	HPV-11 L1 VLPs	20	30	7/7

Dogs (seven per group) were immunized by intradermal injection at week 0 and week 2. Immunogens were administered without adjuvant. All dogs were challenged with infectious COPV at week 4. Animals were followed for wart development for 13 weeks after challenge.

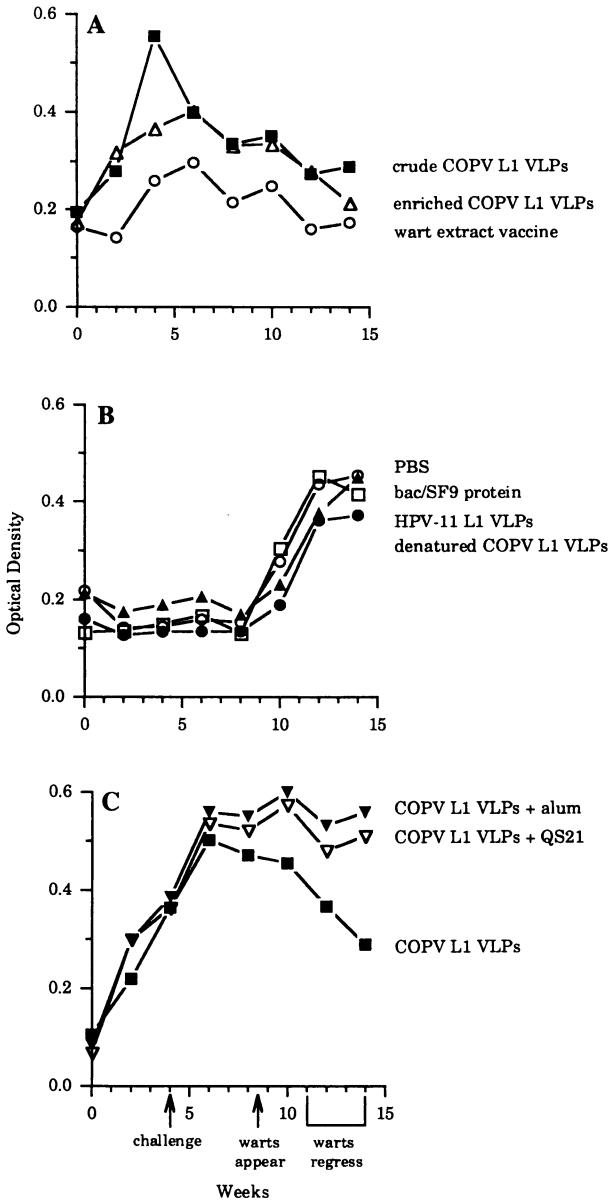

FIG. 2. COPV-specific antibody responses in immunized dogs. Serum samples from each group of animals in the vaccine studies were pooled and assayed for COPV-specific immunoglobulins as described in *Materials and Methods*. (*A*) Serum from dogs protected from COPV disease by immunization with the formalin-fixed wart extract vaccine (○), crude COPV L1 VLPs (■), and enriched COPV L1 VLPs (△). (*B*) Serum from dogs that developed oral warts after challenge with COPV. Animals were immunized with PBS (○); baculovirus and Sf9 nuclear protein (▲); HPV-11 L1 VLPs (□); and denatured COPV L1 VLPs (●). (*C*) Serum taken from dogs in a repeat vaccine study that were protected from COPV disease by immunization with crude COPV L1 VLPs in PBS (■); COPV L1 VLPs adsorbed to alum (▼); and COPV L1 VLPs formulated with adjuvant QS21 (▽).

COPV disease. Protected animals all exhibited COPV-specific serum immunoglobulins at the time of viral challenge (Fig. 2*C*). The antibody titers in dogs injected with L1 preparations without adjuvant began to decease after reaching a peak response just after viral challenge. In contrast, dogs receiving L1 protein with either QS21 or alum adjuvants maintained peak anti-COPV responses for the duration of the study. As in the previous experiment, however, the anti-COPV response in all dogs was relatively weak. Endpoint titers did not exceed 1:2000 even in serum samples taken from dogs immunized with COPV L1 formulated with alum or QS21.

Protection from COPV Can Be Conferred by Passive Transfer of Serum Immunoglobulins. Although successful vaccination of beagles with COPV L1 protein correlated with the appearance of antibodies to intact COPV in the serum of animals, it was uncertain whether humoral immune responses alone could confer protection from COPV. To directly assess the mechanism of protection, a passive-transfer experiment was conducted. Blood was obtained from dogs immunized with the irrelevant baculovirus and Sf9 nuclear protein and from those which had received intact COPV L1 VLPs (Table 1, groups 3 and 4, respectively), one week following secondary immunization and prior to challenge with COPV. Sera from the dogs in each of the two groups were pooled, and immunoglobulin fractions were prepared by ammonium sulfate precipitation. These serum proteins were infused intravenously into two groups of naive dogs (four dogs per group). Approximately 24 hr after infusion, the dogs were challenged with COPV. Animals were monitored for development of papillomas for 4 months following challenge. All the dogs that received serum immunoglobulins from animals immunized with the irrelevant Sf9 and baculovirus protein developed oral warts, whereas those infused with immunoglobulins from the COPV L1 VLP-immunized dogs remained free of oral papillomas.

COPV L1 Protein Is Efficacious at Low Dosages. In order to investigate the minimum dose of COPV L1 VLPs required to elicit a protective immune response, a vaccine study was conducted in which groups of dogs were immunized with concentrations of COPV L1 ranging from 20 μg to 0.125 ng per dose (Table 2). Immunogens were administered without adjuvant. As in previous experiments, each group of dogs received a primary and a secondary immunization and was challenged with infectious virus 2 weeks later. Complete protection from COPV disease was observed with vaccine preparations containing 20 μg, 1 μg, or 50 ng of COPV L1 protein per dose. Partial protection was observed even at the two lowest doses of COPV L1 VLPs tested (2.5 ng and 0.125 ng of COPV L1, respectively). Of the five animals immunized with 0.125 ng of COPV L1, two animals were wart-free throughout the course of the study (10 weeks postchallenge) and two other animals exhibited a 2-week delay in papilloma development compared with the PBS control group.

DISCUSSION

The present study demonstrates the efficacy of systemically administered COPV L1 for preventing COPV-induced oral mucosal papillomas. Immunization of dogs with COPV L1 VLPs, in the absence or presence of adjuvant, was shown to completely protect animals from experimental challenge with COPV. Further, the recombinant VLPs were found to be efficacious even at extremely low doses. These data strongly

Table 2. Protection achieved with various dosages of COPV L1 VLPs

Group	L1, μg per dose	No. of dogs with warts
1	20	0
2	1	0
3	0.05	0
4	0.0025	2
5	0.000125	3
6	—	5

Dogs (five per group) were immunized by intradermal injection with crude COPV L1 VLPs at the indicated dosages. Group 6 was injected with PBS. All dogs received primary and booster immunizations at weeks 0 and 2, respectively, and were challenged with infectious COPV at week 4. Animals were followed for wart development for 10 weeks after challenge.

support the feasibility of a similar L1-based vaccine for the prevention of papillomas and the malignant sequelae induced by the mucosotropic HPVs.

Our results indicate that an efficacious L1-based papillomavirus vaccine need not include the minor papillomavirus capsid component, L2 protein. Several previous studies have shown that VLPs composed of papillomavirus L1 protein alone are antigenically similar to authentic papillomavirus particles. Immunization of rabbits with HPV-11 L1 VLPs induced antibodies capable of neutralizing HPV-11 in a xenograft assay (24, 25). Similarly, VLPs composed of BPV-1 L1 protein induced the synthesis of BPV-1-neutralizing antibodies (11). VLPs composed of both the BPV-1 L1 and L2 proteins did not induce higher levels of BPV-neutralizing antibodies than did particles containing BPV L1 alone (26). Recently, VLPs containing either CRPV L1 protein alone or CRPV L1 and L2 were shown to protect rabbits from experimental infection with CRPV (27). However, somewhat higher levels of protection were obtained with VLPs composed of both the major and minor CRPV capsid antigens. Whether or not there is any advantage for a papillomavirus vaccine to contain both L1 and L2 is not known for the COPV model.

COPV-specific antibodies were detected in the serum of dogs vaccinated with COPV L1 protein prior to viral challenge; however, the titer of these antibodies, as measured by ELISA, was relatively low. Formulation of L1 with adjuvants had little effect on the peak anti-COPV antibody titers but did appear to prolong significantly the humoral response. ELISA titers reported in this study may underestimate neutralization titers. Such a discrepancy has been observed in the BPV model system, where serum antibodies from steers with regressing BPV-1-induced papillomas exhibited low anti-BPV-1 ELISA titers but possessed a high level of virus-neutralizing activity (28).

The importance of anti-COPV antibodies for the prevention of COPV-induced papillomas was demonstrated in a passive-transfer experiment. Whereas this passive-transfer result does not preclude a role for cell-mediated immune responses in papilloma regression, it clearly indicates that a systemic humoral immune response is sufficient for preventing papilloma formation.

The COPV/beagle model has demonstrated the utility of an L1-based vaccine for preventing infection by mucosotropic papillomaviruses. This model system can be used to investigate the duration of the protective immune response, protection from natural infection, and the optimization of vaccination protocols. Applications of these approaches should facilitate the development of an effective systemic vaccine against mucosotropic HPVs.

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