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Immunization with Viruslike Particles from Cottontail Rabbit Papillomavirus (CRPV) Can Protect against Experimental CRPV Infection

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We tested the ability of vaccination with virus-like particles (VLPs) to protect domestic rabbits against papillomas induced by the cottontail rabbit papillomavirus (CRPV). A recombinant baculovirus system that expressed only the L1 major papillomavirus structural protein or L1 plus the minor L2 protein was used in insect cells as the source of VLPs. Groups of 10 rabbits were immunized with native or denatured VLPs from CRPV or type 1 bovine papillomavirus by using Freund's adjuvant. Alum was used as the adjuvant for an additional group immunized with CRPV L1-L2 VLPs. Animals were challenged with 5×10^{10} and 2×10^{11} particles on opposing flanks. No protection was seen in rabbits immunized with native or denatured bovine papillomavirus L1-L2 or with denatured CRPV L1-L2. In these groups, the lower and higher challenge doses resulted in 27 of 30 animals with extensive papillomas, with each of the remaining animals having a smaller number of persistent papillomas. Progression to carcinoma developed in 20 rabbits. Animals inoculated with native CRPV VLPs composed of L1 alone or L1-L2 developed many fewer lesions; the lower and higher challenge doses resulted in 17 of 29 and 5 of 29 rabbits, respectively, with no lesions, and the remainder developed only one to eight papillomas, which all regressed except for those on 1 rabbit. None developed cancer within 1 year of infection. Rabbits vaccinated with native CRPV VLPs developed high-titer antibodies in an enzyme-linked immunosorbent assay based on native VLPs, and passive transfer of serum or immunoglobulin G from rabbits immunized with CRPV VLPs protected against CRPV challenge. We conclude that native VLPs can induce antibody-mediated, type-specific protection against experimental papillomavirus infection.

In addition to inducing benign epithelial hyperproliferation, infection by a subset of papillomaviruses (PV) can produce lesions that progress to malignancy. There is a particularly close association between infection with certain high-risk human papillomavirus (HPV) types and cervical cancer, which is the second most common cancer of women worldwide. Approximately 90% of cervical cancers contain HPV DNA, and a wealth of molecular biologic and epidemiologic data has provided strong evidence of an etiologic link between cervical HPV infection and the development of this malignancy (reviewed in references 28, 33, and 34).

The apparent importance of HPVs to cervical cancer, and to other tumors, has stimulated efforts to develop a vaccine to prevent infection by these viruses (reviewed in references 2, 6, 11, and 12). In contrast to those of most pathogenic human viruses, PV genomes contain viral oncogenes, which encode nonstructural proteins that stimulate abnormal cell growth (reviewed in references 7, 32, and 33). The presence of oncogenes in PVs represents a theoretical hazard that could restrict routine vaccination with an attenuated live PV or with an inactivated whole-virus preparation that contains viral DNA. This potential problem can be overcome by a subunit vaccine that does not contain the viral oncogenes.

Since PVs are species specific with respect to productive infection, and HPV infection of animals does not produce disease (reviewed in reference 25), preliminary testing of candidate vaccines must be carried out in animal PV models. Induction of warts in rabbits by the cottontail rabbit papillomavirus (CRPV) (5, 8, 9, 23, 29, 30) or in cows by several types of bovine papillomavirus (BPV) (14, 16, 26) represents a frequently used experimental PV system. Persistent papillomas induced by CRPV or BPV type 4 may progress to carcinomas (15, 21, 27).

PV contain two genes, L1 and L2, that encode the major and minor structural viral proteins, respectively, which bear both type-specific and group-specific epitopes (10, 24, 25). Infection with vaccinia virus vectors that express L1 or L2 can inhibit papilloma formation in rabbits (23), as can subunit vaccines composed of partially nondenatured L1 or L2 fusion proteins produced in bacteria despite low titers (<100) of neutralizing antibodies (5, 22, 23).

We have recently reported that a recombinant baculovirus vector system that expresses L1 alone or L1 plus L2 in insect cells leads to the self-assembly of PV capsids (18). BPV virus-like particles (VLPs), when inoculated into rabbits, induced high titers (>10,000) of neutralizing antibodies, as measured by an in vitro bioassay. The capacity to generate high-titer neutralizing sera depended upon conformational epitopes in the assembled particles, since denaturation of the capsids destroyed this ability.

To explore the possibility that VLPs might have potential as a subunit vaccine, we immunized rabbits with CRPV VLPs and challenged them with infectious CRPV. We also compared the

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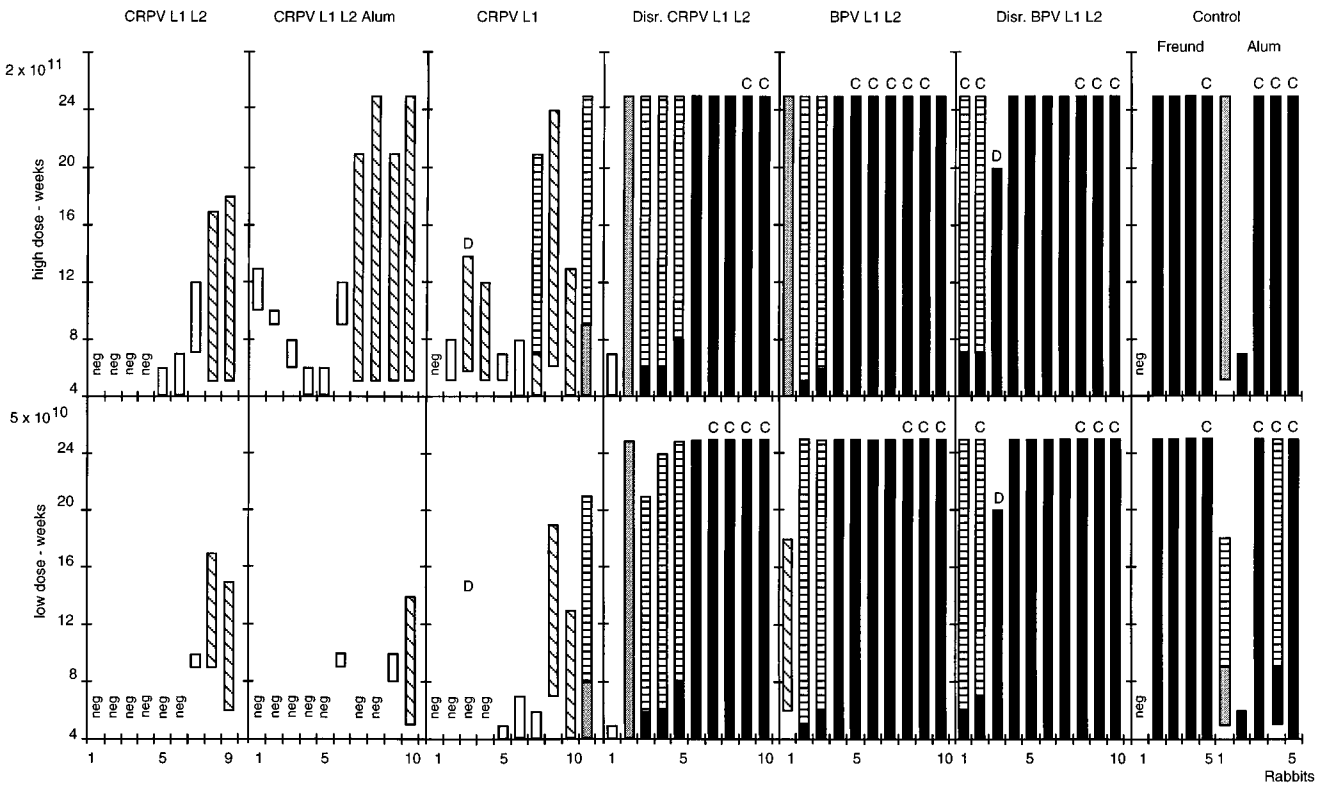

FIG. 1. Response to CRPV infection of rabbits immunized with native or denatured virus-like particles. Groups of rabbits were inoculated as indicated with native or sodium dodecyl sulfate-disrupted (Disr.) CRPV or BPV L1-L2 VLPs or with native CRPV L1 VLPs, together with Freund's adjuvant, or with alum (native CRPV L1-L2 and control). Rabbits were challenged with two doses, 5×10^{10} (lower panels) and 2×10^{11} (upper panels), of infectious CRPV particles applied to flank skin prepared by light scarification. Papilloma outgrowth in controls was usually observed 4 weeks after infection. The course of disease, which was monitored for 52 weeks after infection, is represented only for the first 24 weeks, since no further regression was observed at later times. Each bar represents one rabbit, and the length of a bar indicates disease duration in weeks. Response to infection was designated as follows: expected (■), which meant 10 to 100 papillomas for the low-dose flank and confluent for the high-dose flank, or stronger (confluent on both flanks); moderate (▨), fewer than 10 papillomas for the low dose and 20 to 30 for the high dose; mild (▧), 1 to 3 and 3 to 8 papillomas for the low and high doses, respectively; abortive (□), 1 to 3 and 1 to 12 minute, nongrowing papillomas; or negative (neg). Partial regression (▤) was observed in some rabbits. Malignant conversion (C) occurred in 20 rabbits, on one or both flanks, between 26 and 52 weeks after infection. Two rabbits died (D) with papillomas during the first 24 weeks, and one in the native CRPV L1-L2 group died prior to challenge. Nine rabbits with persistent papillomas died without cancer between 25 and 40 weeks after infection.

efficacy of intact versus denatured VLPs, examined the specificity of CRPV protection by challenging rabbits immunized with BPV VLPs, and tested the ability of serum to transfer protection from immune to nonimmune animals.

Immunization protocol and CRPV challenge. By infection of Sf9 insect cells with recombinant baculoviruses (31), we produced CRPV and BPV VLPs that were composed of L1 alone or L1 plus L2 (L1-L2). Generation of the CRPV and BPV baculoviruses and purification of the VLPs have been described previously (20). VLPs were denatured by boiling for 10 min in 3% sodium dodecyl sulfate with 5% 2-mercaptoethanol.

For immunization, 3- to 4-month-old New Zealand White rabbits were inoculated subcutaneously with 50 μg of intact or denatured VLPs in complete Freund's adjuvant or in alum (2% aluminum hydroxide; Serva). At 2 and 4 weeks after the first inoculation, they received booster injections with the same intact or denatured VLP preparation and adjuvant, except that incomplete Freund's adjuvant was used in the Freund's adjuvant groups. The alum groups received a third booster 2 weeks after the second booster.

Two weeks after the last booster, animals were challenged with a suspension of infectious CRPV virions prepared by successive low- and high-speed centrifugations of a 10% cottontail rabbit wart extract in phosphate-buffered saline–EDTA. Two doses of virus, 5×10^{10} particles (lower dose) and 2×10^{11} particles (higher dose), were applied to each animal on 20-cm^2 areas of shaved skin abraded with sandpaper while taking care to keep bleeding to a minimum (13). The development of papillomas was checked and photographed weekly for 24 weeks and monthly thereafter for a total observation period of 1 year. Serum was collected from the rabbits three times: before the first immunizing dose, 1 week after the last booster, and 2 weeks after CRPV challenge.

Each immunogen, with a given adjuvant, was administered to a group of 10 rabbits. Most groups were immunized with VLPs in Freund's adjuvant. These groups received native CRPV L1 or L1-L2 VLPs, native BPV L1-L2 VLPs, or denatured BPV L1-L2 or CRPV L1-L2, as well as adjuvant without viral protein (five rabbits). There were two alum groups: native CRPV L1-L2 and adjuvant without viral protein (five rabbits).

Lack of protection by native BPV L1-L2 or denatured CRPV L1-L2. The experiment (Fig. 1) was designed so that the lower challenge dose usually produced countable numbers of papillomas (10 to 100) and the higher dose produced confluent growth (>100 papillomas; Fig. 2). Five of the 10 control rabbits showed the expected number of papillomas, 3 gave confluent growth for both doses, 1 developed only a moderate infection (8 and 25 papillomas on the low- and high-dose sides, respectively), and 1 unexpectedly did not develop any papillomas despite mounting a significant serological response upon infec-

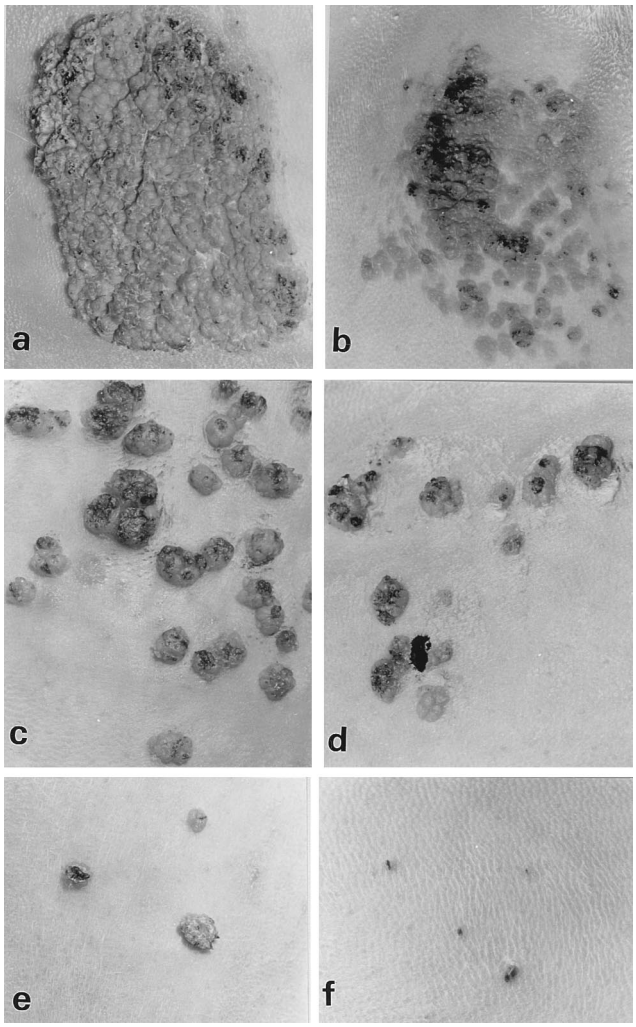

FIG. 2. Responses of rabbits to CRPV infection. Wart development after infection with CRPV at the higher dose (2×10^{11} particles; a, c, e, and f) and the lower dose (5×10^{10} particles; b and d) is shown. Lesions were classified as expected (a and b), moderate (c and d), mild (e), or abortive (f). Warts were photographed at 6 (a and f), 8 (b), 10 (c and d), and 11 (e) weeks after infection. Rabbits had been vaccinated with disrupted BPV L1-L2 (a to d), CRPV L1-L2 (e), and native CRPV L1 VLPs (f). Lesions are shown at their actual size.

tion (data not shown). These results illustrate the variation of the individual responses to infection.

Animals immunized with intact or disrupted BPV L1-L2 VLPs responded to the CRPV challenge as did control rabbits (Fig. 1). At least 9 of the 10 rabbits in each of these groups developed either the expected number of papillomas or an even greater number, i.e., confluent growth for the low-dose side. Eight of the 10 animals immunized with disrupted CRPV L1-L2 showed the expected or a stronger response to infection, 1 had a moderate response (8 and 30 papillomas for the low- and high-dose sides, respectively), and 1 had abortive disease (1 and 3 nongrowing papillomas lasting 1 and 3 weeks, respectively).

If the above four groups are considered together, only 2 of 40 rabbits had a complete regression and 9 of 40 had an incomplete regression of their lesions, i.e., clearing of about 90% of the warts, with the remaining warts persisting over the entire 1-year period of observation. Malignant tumors arose from the warts in 20 of 40 animals between 6 months and 1 year after infection (Fig. 1).

Native CRPV L1 and L1-L2 VLPs are protective. In contrast to the above results, a substantial rate of protection was observed for each group that received native CRPV VLPs, whether CRPV L1 in Freund's adjuvant or CRPV L1-L2 in Freund's adjuvant or alum (Fig. 1 and 2). Among the 29 animals in these three groups (there were only 9 animals in the CRPV L1-L2 group because 1 died shortly after being given the first immunizing dose), 28 had full protection, abortive disease, or mild disease and the remaining rabbit showed moderate disease (7 and 25 papillomas on the low- and high-dose sides, respectively; Fig. 2). Abortive disease was characterized by a small number (usually one to three) of minute, nongrowing, keratinized papillomas that appeared later than in control animals, with mean delays of 2.3 weeks for the low dose and 1.6 weeks for the high dose. Abortive papillomas regressed rapidly, with mean lesion durations of 1.7 and 2.7 weeks, respectively, on the low- and high-dose sides. Mild disease was characterized by the occurrence of a few actively growing papillomas, two to three for the low dose and three to eight for the high dose, appearing with mean delays of 2.2 and 1.0 weeks, respectively. Late papilloma regression was usually observed in these animals, with mean disease durations of 9.4 weeks for the low dose and 13.5 weeks for the high dose.

Combining the three groups, the low-dose side showed full protection (no papillomas) in 18 (62%) of 29 animals, abortive disease in 6 (21%), and mild disease in 5 (17%). On the high-dose side, we observed full protection in 5 (17%) of 29 animals, abortive infection in 12 (41%), and mild disease in 11 (37%); the best protection was achieved with CRPV L1-L2 in Freund's adjuvant. Thus, no animal had moderate or extensive disease, 55 (95%) of 58 sites of inoculation were free of warts after 24 weeks, and no animals developed cancer within 1 year after infection.

ELISA response of immunized rabbits. Sera from animals immunized with native and denatured L1-L2 VLPs were tested by a standard enzyme-linked immunosorbent assay (ELISA) that used native CRPV L1-L2 VLPs as the antigen and was carried out essentially as previously described for an HPV type 16 VLP ELISA (19). The cutoff point for a positive ELISA sample was set at an optical density of >1.0. Titers were defined as the reciprocal of the highest 10-fold serum dilution that gave a positive result.

Preimmune sera had a mean titer of less than 5, while the mean titer of animals inoculated with native CRPV L1 or L1-L2 VLPs in Freund's adjuvant was more than 10,000 1 week after the second booster. The mean titer was 5,000 when alum was used as the adjuvant. Animals that developed papillomas did not generally have lower titers in the native CRPV VLP ELISA than did those that did not develop papillomas. Control animals that received inoculations of adjuvant alone did not show a rise in titer until after CRPV challenge, which induced mean titers of 250 in the Freund's adjuvant-treated controls and 40 in the alum controls.

As with rabbits inoculated with native CRPV VLP, those inoculated with denatured CRPV L1-L2 demonstrated an antibody response in the native CRPV VLP ELISA. However, the mean titer for these animals was about 1 order of magnitude lower (1,000) than that for rabbits inoculated with native CRPV VLP.

When the sera were tested in an ELISA employing denatured CRPV L1-L2, the titers were much lower than those obtained with native VLPs. The mean titer of sera from animals that received denatured CRPV L1-L2 was 200, which was fivefold higher than that of those inoculated with native CRPV

L1-L2 VLPs (mean titer, 40). Taken together, the data indicate that native CRPV L1-L2 induced higher antibody titers in the native CRPV L1-L2 assay than did denatured CRPV L1-L2, and denatured CRPV L1-L2 induced higher titers than native CRPV L1-L2 in the denatured CRPV L1-L2 assay. As it is likely that the native CRPV ELISA antigen preparation also contains, to a lesser degree, the linear epitopes detected by the denatured CRPV ELISA, the former assay probably underestimates the differences in immunity against conformational epitopes induced by the native and denatured CRPV preparations.

None of the groups inoculated with native CRPV VLPs showed increased titers in an ELISA using native BPV L1-L2 VLPs (mean titer, less than 5). As previously described for neutralization by BPV L1 VLPs, animals inoculated with native BPV L1-L2 VLPs developed a mean titer of 6,300 in the native BPV VLP ELISA, while those that received denatured BPV L1-L2 had a mean titer of 13 in the same assay.

Protection from passive transfer of serum and immunoglobulin G (IgG). Passive transfer experiments were carried out to obtain some insight into the mechanisms underlying protection against CRPV. To determine if protection requires cellular or humoral immunity, serum or IgG from rabbits immunized with native CRPV L1-L2 VLPs was administered to nonimmune rabbits. The selected sera had an ELISA titer of at least 10^4. Control sera were pools of preimmune sera. IgG was purified from one-half of the volume of each serum pool by chromatography with a protein A-agarose column (Pharmacia).

Animals were inoculated intraperitoneally with 15 ml of serum or with the IgG purified from the same volume of serum. They were challenged 5 to 7 h later with two doses (10^{11} and 10^{10} particles) of infectious CRPV as described above. As expected, the three rabbits inoculated with either preimmune sera (one rabbit) or preimmune IgG (two rabbits) developed confluent papillomas for the higher dose and about 20 papillomas for the lower dose. Of the four animals inoculated with hyperimmune sera (two rabbits) or IgG from the hyperimmune sera (two rabbits), three were fully protected, while one of the IgG-treated animals developed three papillomas from the higher dose and none from the lower dose. Thus, passive transfer of serum and IgG was protective, which makes it likely that the protection observed in the vaccinated animals resulted from neutralizing antibodies.

Protection by native CRPV VLPs is type specific. The results indicate that immunization with preparations of CRPV VLPs can induce substantial protection against experimental challenge with infectious CRPV. These findings are consistent with early studies showing that intraperitoneal injection of infectious suspensions of CRPV virions actively immunized rabbits against papillomatosis (30). Effective vaccination required the use of native CRPV VLPs. Neither denatured CRPV nor native or denatured BPV VLPs conferred resistance to CRPV challenge, indicating that cross-reacting antibodies are not protective.

Despite the use of large CRPV challenge doses, protection against disease was achieved in almost all animals, although complete prevention of wart outgrowth was not observed in all animals. It is likely that challenging with a lower dose would have resulted in more complete protection. However, it should be stressed that disease, when it did occur in these animals, was abortive or mild, resulting in clearance of all warts within 24 weeks for the low dose, persistence of a few warts in only 3 of 29 rabbits for the high dose, and no cancers. These results were in contrast to the persistence of numerous warts in 36 of 40 control rabbits and the development of cancers in 20 of these animals within 1 year.

At the lower challenge dose, similar degrees of protection were induced by VLPs composed of L1 alone or L1-L2, and the degree of protection was independent of the adjuvant used. At the higher challenge dose, a greater degree of resistance was seen in animals immunized with L1-L2 VLPs in Freund's adjuvant than in those immunized with L1 VLPs in Freund's adjuvant or L1-L2 VLPs in alum (Fig. 1). Variations in the immunization and challenge protocol might improve the efficacy of VLPs as vaccine. A previous study with CRPV L1 and/or L2 proteins produced in bacteria or in vaccinia virus vectors reported protection against CRPV challenge (23). However, in another study involving fusion proteins from CRPV L1 and L2, protection was achieved only with L2 fusion protein and at the lower of the challenge doses tested (5).

Protection against CRPV infection correlated in general with high titers in the native CRPV VLP ELISA. Each native CRPV preparation, whether L1 or L1-L2, induced high titers. Denatured CRPV VLPs and native BPV VLPs induced lower levels. The correlation among the animals immunized with native CRPV VLPs may not, however, be absolute in that the rabbits in these groups that developed papillomas did not have lower ELISA titers than those that did not have lesions. In the ELISA that used denatured CRPV VLPs, animals inoculated with denatured CRPV VLPs developed higher antibody titers than those given native CRPV VLPs, which indicates that the levels achieved with this class of antibodies were not associated with resistance to CRPV infection. Taken together, the observations strongly suggest that protective immunity was conferred by type-specific, conformational epitopes and rule out the possibility of using cross-reactive antigens as universal vaccines. The apparent importance of conformational epitopes for protection against CRPV challenge has been suggested by earlier studies (22).

Protection is probably mediated by neutralizing antibodies. Our demonstration that passive transfer of serum from rabbits immunized with CRPV VLPs resulted in protection against CRPV challenge demonstrates directly that cell-mediated immunity is not essential for immunoprophylaxis under the experimental conditions employed. The obligatory role of nonlinear epitopes in the protection induced by VLPs is consistent with this conclusion, since cellular immune reactivity is usually directed against linear epitopes (1). The possibility has not been ruled out, however, that cell-mediated immunity might have contributed to the early regression observed in vaccinated animals that developed low numbers of papillomas. Indeed, early studies showed that neutralizing antibodies have no influence on the course of established papillomas (17).

The positive results obtained by passive transfer of IgG from hyperimmune rabbits make it likely that protection is conferred principally by neutralizing antibodies. The in vivo results obtained with CRPV VLPs are thus analogous to those obtained in experiments with BPV VLPs and neutralizing antibodies, in which inoculation with native BPV VLPs induced sera with high titers of antibodies that neutralized in vitro infection with BPV, while immunization with denatured BPV VLPs failed to induce these antibodies (18).

Implications for vaccination against HPV. It is difficult to know the degree to which the type specificity observed in our study can be extrapolated to the HPV situation for developing a vaccine against multiple HPV types, since several HPV types are more closely related to each other than are CRPV and BPV (3). The apparent importance of neutralizing antibodies for immunoprophylaxis suggests that analyses of cross-neutralization by different HPVs would provide highly relevant information. Such information will require infectious HPVs of various types and a reproducible infectivity assay for any HPV

type. The only HPV neutralization assay described thus far is an in vivo one for HPV type 11 (4). Until neutralization assays can be developed for other HPV types, VLP-based ELISAs for various HPV types might be used as surrogate assays, provided that they adequately distinguish between conformational and linear epitopes.

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