Immunization with Viruslike Particles Induces Long-Term Protection of Rabbits against Challenge with Cottontail Rabbit Papillomavirus

NEIL D. CHRISTENSEN,^{1,2*} CYNTHIA A. REED,¹ NANCY M. CLADEL,¹
RICAI HAN,¹ AND JOHN W. KREIDER^{1,2}

*Departments of Pathology¹ and Microbiology and Immunology,²
The Milton S. Hershey Medical Center,
Hershey, Pennsylvania 17033*

Received 5 September 1995/Accepted 29 October 1995

Rabbits were immunized with recombinant baculovirus-produced virus-like particles (VLPs) of cottontail rabbit papillomavirus (CRPV) to determine whether these antigens could induce long-term protection against experimental challenge with CRPV. Infectious CRPV and human papillomavirus type 11 L1 VLPs were used as positive and negative control immunogens, respectively. Three groups of immunized animals were challenged with 10-fold serial dilutions of infectious CRPV at 2 weeks, 6 months, and 12 months after immunizations. Antibody titers in serum reached 1:10,000 immediately after the final booster immunization and then decayed to 1:150 at 6 months and 1:100 at 12 months in unchallenged rabbits. Serum neutralization titers followed similar kinetics. Papillomas grew on control-immunized rabbits at sites challenged with 10^{-1} (100% of sites), 10^{-2} (96% of sites), 10^{-3} (63% of sites), and 10^{-4} (13% of sites) dilutions of virus. At 2 weeks after CRPV L1 VLP immunizations, the rabbits were completely protected against virus challenge. At both 6 and 12 months after CRPV L1 VLP immunizations, strong protection was also observed. In the last two groups, three of seven rabbits were completely protected and only 4 of 14 or 29% of sites challenged with 10^{-1} dilution of virus grew papillomas. Papillomas growing at these four sites were also reduced in size (3.5 ± 0.7 mm) at 50 days postchallenge compared with sites challenged with 10^{-1} dilution on control-immunized rabbits (13.2 ± 4.2 mm). The results demonstrate that strong and long-lasting protection against experimental challenge with papillomaviruses can be achieved with VLP immunogens.

Virus-like particles (VLPs) of papillomaviruses can be produced synthetically by over-expression of the major coat protein, L1, in various expression systems (9, 12, 14, 15, 21, 23, 25-27). The VLPs demonstrate surface topography indistinguishable from infectious viral particles (8) and retain surface conformational neutralizing epitopes as defined by neutralizing monoclonal antibodies (4, 20). Several groups have demonstrated that immunization with these VLPs can produce high titers of virus-neutralizing antibody (4, 14, 22), without addition of commercial adjuvant preparations (4), and can protect rabbits against challenge with cottontail rabbit papillomavirus (CRPV) (1, 10) and dogs against challenge with canine oral papillomavirus (7a). These vaccination studies examined the protective capacity of VLP immunogens by challenge soon after the final booster immunizations at a time when neutralizing antibody in serum is at a very high titer (4, 14). The potential for long-term protection by VLP immunogens, therefore, was not determined.

The purpose of the present study was to assess the long-term protective ability of CRPV L1 VLPs following immunization without commercial adjuvants. Separate cohorts of immunized rabbits were challenged at three different times after immunization, with a final challenge time point of 1 year postimmunization. Antibody titers in serum, virus neutralization titers in serum, and protection were measured, and the results demonstrate that the VLPs induced strong, long-lasting protection against experimental challenge with CRPV.

MATERIALS AND METHODS

Production of VLPs and immunizations. VLPs containing CRPV L1 (CRPV L1 VLPs) and VLPs containing human papillomavirus type 11 strain Hershey (HPV-11^{Hershey}) L1 (HPV-11 L1 VLPs) were prepared with recombinant baculoviruses as previously described (4, 14). Cultures of Sf9 insect cells were infected with recombinant baculoviruses at a multiplicity of infection of 10, and the cells were collected 72 h later, lysed by freeze-thaw, and disrupted with a Brinkman polytron for 60 s at the highest setting. Cell debris was removed by low-speed centrifugation, and the supernatant was mixed with CsCl and ultracentrifuged for 24 h at $246,000 \times g$. Visible bands containing VLPs were removed, tested by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies that detect only intact VLP, and stored frozen at -70°C . The content of the L1 protein in the VLP and virus preparations was estimated to be 20 to 30% by densitometric scanning of Coomassie blue-stained gels as previously described (4).

A total of 28 rabbits were divided into three groups, and individual rabbits in each group were given three injections, at monthly intervals, of infectious CRPV, CRPV L1 VLPs, and HPV-11 L1 VLPs antigens, respectively. Group 1 contained 4 rabbits that were given injections of infectious CRPV as a positive control antigen (24), group 2 contained 12 rabbits given injections of CRPV L1 VLPs, and group 3 contained 12 rabbits given injections of HPV-11 L1 VLPs as a negative control VLP antigen. Each rabbit received intramuscular injections (primary and booster immunizations) of 50 μg of protein diluted into 0.5 ml of phosphate-buffered saline (PBS; pH 7.0) without addition of commercial adjuvants. Rabbits receiving infectious CRPV as immunogen (50 μg per injection) were immunized through a small incision in the skin, and the area was flushed with 70% alcohol to prevent initiation of a papilloma at the injection site. The injection sites were examined throughout the experiment, and no papillomas were observed.

Collection of sera and ELISA. Sera were prepared from blood collected from the rabbits at 1- or 2-month intervals, as well as 1 week after each of the two booster immunizations. Only sera from unchallenged, uninfected rabbits were collected. Preimmunization bleeds were also taken. The sera were heat inactivated at 56°C for 30 min and stored at -70°C .

ELISAs with intact VLP and intact virus antigen were conducted as previously described (4). Serum samples were tested in duplicate for each dilution point, and the mean and standard error of optical density readings at 410 nm were plotted against the serum dilution.

* Corresponding author. Phone: (717) 531-6185. Fax: (717) 531-5634.

FIG. 1. Papilloma growth on the backs of New Zealand White rabbits following challenge with infectious CRPV. Three groups of rabbits were immunized (50 μ g of protein per rabbit) at monthly intervals with HPV-11 L1 VLP (A), CRPV L1 VLP (B), and CRPV (C). Two weeks after the last booster immunization, rabbits were challenged (two sites per dilution point) with 10-fold dilutions of infectious CRPV extract. Papilloma size was measured weekly beginning at day 14, and GMDs were calculated. Mean GMDs and SEM were plotted against time for sites challenged with 10^{-1} ($\circ$), 10^{-2} ($\square$), 10^{-3} ($\triangle$), and 10^{-4} ($\diamond$) dilutions of infectious CRPV.

Challenge of rabbits with infectious CRPV. The design of the challenge experiments was as follows. At 2 weeks after immunizations, 4 rabbits each (total of 12) from groups 1, 2, and 3 (immunized with infectious CRPV, CRPV L1 VLPs, and HPV-11 L1 VLPs, respectively) were challenged with infectious CRPV; at 6 months after immunizations, 4 rabbits each (total of 8) from groups 2 and 3 were challenged; and at 12 months after immunizations, the remaining 4 rabbits from each of groups 2 and 3 were challenged. Rabbits were challenged with 10-fold serial dilutions of infectious CRPV stock as previously described (5). The infectious titer of this stock was previously measured, and it was found that 50% of the challenged sites produced papillomas at a dilution of 10^{-3} ($ID_{50} = 10^{-3}$). Each rabbit was challenged at two sites (area, 2 by 2 cm) for each dilution of infectious virus stock from 10^{-1} to 10^{-4} . At weekly intervals starting on day 14, papilloma size was measured as length $\times$ width $\times$ height in millimeters and the geometric mean diameter (GMD) was calculated for each papilloma. Means and standard errors of the mean (SEMs) for the GMDs in each treatment group and viral dilution were plotted against time after challenge of virus.

Virus neutralization assay. Neutralization of CRPV was measured as previously described (5). Aliquots of infectious virus (10^{-2} final dilution of viral stock) were mixed with dilutions of rabbit sera and incubated at 37°C for 30 min. The virus-serum mixtures were placed onto scarified sites on the backs of New Zealand White rabbits, and papilloma growth was monitored weekly as described above. Papilloma size was plotted against serum dilution for each dilution point (three sites challenged per serum dilution), and neutralization titers were defined as the dilution of serum required to reduce papilloma size to 50% of the size at the untreated control sites (5).

Summary of immunization, challenge, and serum collection schedules. All rabbits in the study were immunized on days 0, 21, and 35. Three separate groups of rabbits were challenged on day 46 (2-week challenge group), day 213 (6-month challenge group), and day 403 (12-month challenge group). Serum bleeds were taken only from nonchallenged rabbits at day 0 (preimmunization bleed); days 14, 28, and 42 (1 to 2 weeks after each of the three immunizations); and days 74, 123, 182, 212, 276, 331, and 394 (representing sequential 1- or 2-monthly periods following the final booster immunization).

RESULTS

Challenge of immune rabbits with CRPV. Rabbits immunized with infectious CRPV, HPV-11 L1 VLPs, and CRPV L1 VLPs were separated into three groups and challenged with 10-fold dilutions of infectious CRPV as described in Materials and Methods. The challenge time points were 2 weeks, 6 months, and 12 months after the final booster immunizations. Papilloma growth was measured weekly beginning on day 21, and mean papilloma size (GMD) for each virus dilution (two sites per dilution per rabbit; four rabbits per group) was plotted

against time after virus challenge for each of the three groups (Fig. 1 to 3).

The results with rabbits challenged 2 weeks after the final booster immunization showed that rabbits immunized with either infectious CRPV (positive control antigen) or CRPV L1 VLPs were completely protected (Fig. 1). The HPV-11 L1-immunized control group (four rabbits) grew papillomas at sites challenged with 10^{-1} (8 of 8 sites; 100%), 10^{-2} (8 of 8 sites; 100%), 10^{-3} (5 of 8 sites; 63%) and 10^{-4} (0 of 8 sites; 0%) dilutions of virus, demonstrating an ID_{50} of 1.8×10^{-3} for this stock of infectious virus, which is in agreement with results of previous experiments in which nonimmune rabbits were used (data not shown).

Rabbits immunized with CRPV L1 VLPs and challenged with infectious CRPV at 6 months after the final booster immunization also demonstrated strong protection against virus (Fig. 2). Two of four rabbits were completely protected, and the remaining two rabbits each had one small papilloma at sites challenged with the highest dose (10^{-1}) of virus, which represents 3 log units of protection against this infectious virus preparation. Control immunized animals (four rabbits) had papillomas at frequencies similar to the control animals that were challenged 2 weeks after immunizations for virus dilutions of 10^{-1} (8 of 8 sites; 100%), 10^{-2} (8 of 8 sites; 100%), 10^{-3} (5 of 8 sites; 63%), 10^{-4} (1 of 8 sites; 13%).

Animals in the third group were challenged 12 months after the final booster immunization, and again there was strong protection against virus challenge for rabbits immunized with CRPV L1 VLPs (Fig. 3). One of three rabbits (one died prior to challenge) was completely protected, and the other two had one small papilloma each at one of the sites challenged with high-dose virus (10^{-1} dilution), again demonstrating almost 3 log units of protection against the challenge doses of CRPV. The four control immunized rabbits had papillomas at 10^{-1} (8 of 8 sites; 100%), 10^{-2} (7 of 8 sites; 88%), 10^{-3} (5 of 8 sites; 63%) and 10^{-4} (2 of 8 sites; 25%) dilutions of virus, in close agreement with results for the previous two control groups,

FIG. 2. Papilloma growth on the backs of New Zealand White rabbits 6 months after immunization with HPV-11 L1 VLP (A) and CRPV L1 VLP (B), as described in the legend to Fig. 1. Rabbits were challenged (two sites per dilution point) with serial 10-fold dilutions of infectious CRPV extract from the same virus stock used in the experiment in Fig. 1. Mean GMDs and SEM of papillomas were plotted against time for sites challenged with 10^{-1} ($\circ$), 10^{-2} ($\square$), 10^{-3} ($\triangle$), and 10^{-4} ($\diamond$) dilutions of infectious CRPV.

FIG. 3. Papilloma growth on the backs of New Zealand White rabbits 12 months after immunization with HPV-11 L1 VLP (A) and CRPV L1 VLP (B), as described in the legends to Fig. 1 and 2. Mean GMDs and SEM of papillomas were plotted against time for sites challenged with 10^{-1} ($\circ$), 10^{-2} ($\square$), 10^{-3} ($\triangle$), and 10^{-4} ($\diamond$) dilutions of infectious CRPV.

which were challenged 2 weeks and 6 months after immunizations.

Time course of antiviral antibody titers. Sera from immunized rabbits were collected at monthly intervals and after each of the three immunizations and were tested for antiviral antibody by ELISA as described in Materials and Methods. No sera were collected from rabbits after challenge with infectious CRPV. Antibody titers were calculated as the dilution of serum that gave 50% of the maximal optical density reading, and the results are plotted in Fig. 4. Antibody titers reached a maximum of 1:10,000 after the second and third immunizations with CRPV L1 VLPs and decayed to 1:100 12 months later (Fig. 4A). Similar antiviral titers were observed for rabbits immunized with HPV-11 L1 VLPs and tested by ELISA with HPV-11 L1 VLP antigen (Fig. 4B). Antibody titers for the rabbits immunized with infectious CRPV were slightly lower

than the titers obtained with VLP immunizations (mean titer after the first immunization, 185 ± 152 ; after the first booster immunization, $1,310 \pm 1,086$; and after the second booster immunization, 743 ± 489). These data are also summarized below (see Table 1).

Virus neutralization titers in serum. Selected sera were tested for CRPV neutralization as described in Materials and Methods. Sera from rabbits obtained immediately prior to virus challenge were chosen so that neutralization titers could be compared with the degree of protection. Serial dilutions of sera were mixed with aliquots of infectious virus (10^{-2} dilution of stock virus that was used for the challenges) and incubated at 37°C for 30 min. The virus-serum mixtures were then placed on scarified sites on the back of rabbits (each dilution point was tested in triplicate), and papilloma growth was measured weekly beginning on day 21. Papilloma size was plotted against serum dilution for one representative time point (day 42); the results are shown in Fig. 5. Neutralization titers were defined as the concentration of sera required to reduce papilloma size by 50% compared with the size at control sites that received no rabbit sera. Negative control sera included sera from rabbits immunized with HPV-11 L1 VLPs 1 week after the final booster and 12 months later (Fig. 5). The data demonstrated that high levels of virus-neutralizing antibodies (neutralization titer, 2,600 to 45,000) were present in sera from CRPV and CRPV L1 VLP immune animals 1 week after the final booster immunization but were reduced in sera from rabbits (neutralization titer, 92 to 1,200) taken 12 months after the last immunization (see Table 1).

Comparison between antibody and neutralization titers and degree of protection. Antibody titers to both CRPV and CRPV L1 VLP antigens in serum, as well as neutralization titers and degree of protection following virus challenge, were determined for selected rabbits; the results are summarized in Table 1. Antibody titers to both CRPV and CRPV L1 VLP were directly compared for each rabbit (Fig. 6), because these two immunogens are not antigenically identical. For example, immunization with VLPs may trigger antibodies to epitopes on VLPs that are not present on the surface of infectious CRPV, as well as to contaminating insect cell proteins. Additionally, immunization with CRPV may induce antibodies not detectable by CRPV L1 VLPs. When both antigen sources were

FIG. 4. Time course of serum antibody response to immunization with CRPV L1 VLP antigen (A) and HPV-11 VLP antigen (B) diluted into PBS and injected intramuscularly. Serum was taken from individual rabbits ($\circ$, $\square$, $\triangle$, ∇ , $\diamond$) at various time points after immunization but before virus challenge and tested in ELISA for reactivity to intact CRPV L1 VLP (A) and HPV-11 L1 VLP (B) antigen. Antibody titer was calculated as the concentration of serum required to give 50% of maximum optical density values. The mean rabbit titers and SEM ($\pm$) were plotted against time after primary immunization with antigen.

FIG. 5. Use of papilloma growth rates for assessing virus neutralizing antibody titers in serum. Dilutions of serum taken from rabbits 2 weeks after immunization with CRPV (○), 2 weeks after immunization with CRPV L1 VLP (□), and 6 (△) and 12 months (◇) after immunization with CRPV L1 VLP were mixed with CRPV and placed onto scarified sites (three per dilution point) on rabbits. Papilloma size was measured 42 days later and plotted against serum dilution. Negative control sera were taken from rabbits immunized with HPV-11 L1 VLP antigen 2 weeks (■) and 12 months (◆) after immunization. Neutralization titers were calculated as concentrations in serum required to reduce papilloma size to 50% of that at sites given untreated virus (●).

compared, however, titers were equivalent ($r = 0.866$; $P < 0.0001$ [Fig. 6]).

When neutralization titers in serum were compared with antibody titers, there was good correspondence for both CRPV ($r = 0.818$; $P = 0.0006$) and CRPV L1 VLP ($r = 0.898$; $P < 0.0001$) antigens (Fig. 7). However, when data from rabbits challenged 6 and 12 months after immunization only were analyzed ($n = 7$), the correspondence was not as strong ($r = 0.466$, $P = 0.146$ for CRPV antigen; and $r = 0.614$, $P = 0.07$ for CRPV L1 VLP antigen).

Finally, neutralizing and antibody titers in serum were com-

FIG. 6. Comparison of antibody titers in serum to intact CRPV and intact CRPV L1 VLP antigens for rabbits immunized with CRPV (△) and CRPV L1 VLP (○, □). Sera were taken from rabbits at 2 weeks (□, △) or 6 and 12 months (○) after immunization, and the results are plotted as the logarithm of antibody titer. The linear regression line of best fit is plotted with 95% confidence limits ($r = 0.866$; $P < 0.0001$).

pared with protection for animals challenged with CRPV for all challenge time points. All eight rabbits that were challenged immediately after immunizations were completely protected (Fig. 1). For these animals, neutralizing and antibody titers in serum were very high at the time of challenge, and the rabbits may have been resistant to even higher concentrations of virus. For animals challenged 6 and 12 months after immunizations, when neutralization and antibody titers were much lower, three of seven were completely protected (no papillomas)

TABLE 1. Summary of antibody and neutralization titers in serum and protection of rabbits immunized with viral antigens

Rabbit	Antigen	Time of serum collection ^a	No. of papillomas at dilution ^b :				Antibody titer ^c		Neutralization titer ^d
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	CRPV	CRPV L1 VLP	
C0793	CRPV	1 wk	0/2	0/2	0/2	0/2	215	270	2,600
C0794	CRPV	1 wk	0/2	0/2	0/2	0/2	6,100	400	ND ^e
C0807	CRPV	1 wk	0/2	0/2	0/2	0/2	4,700	1,000	12,000
C0808	CRPV	1 wk	0/2	0/2	0/2	0/2	3,100	1,300	ND
C0795	CRPV VLP	1 wk	0/2	0/2	0/2	0/2	2,600	900	45,000
C0796	CRPV VLP	1 wk	0/2	0/2	0/2	0/2	6,000	4,200	18,000
C0809	CRPV VLP	1 wk	0/2	0/2	0/2	0/2	3,900	4,400	ND
C0815	CRPV VLP	1 wk	0/2	0/2	0/2	0/2	4,400	3,700	39,000
C0801	CRPV VLP	6 mo	1/2	0/2	0/2	0/2	600	160	2,500
C0802	CRPV VLP	6 mo	0/2	0/2	0/2	0/2	290	80	115
C0810	CRPV VLP	6 mo	0/2	0/2	0/2	0/2	120	90	340
C0816	CRPV VLP	6 mo	1/2	0/2	0/2	0/2	600	190	280
C0797	CRPV VLP	12 mo	1/2	0/2	0/2	0/2	120	23	92
C0798	CRPV VLP	12 mo	1/2	0/2	0/2	0/2	800	145	190
C0818	CRPV VLP	12 mo	0/2	0/2	0/2	0/2	550	145	1,200
C0805	HPV-11 VLP	12 mo	2/2	2/2	1/2	0/2	ND	0	<10
C0806	HPV-11 VLP	12 mo	2/2	2/2	2/2	1/2	ND	0	<10
C0819	HPV-11 VLP	12 mo	2/2	2/2	2/2	1/2	ND	0	<10
C0820	HPV-11 VLP	12 mo	2/2	1/2	0/2	0/2	ND	0	ND

^a Time after final booster immunization.

^b Papillomas developing at sites challenged with 10-fold dilutions of CRPV (number of papillomas/number of sites challenged).

^c Dilution of serum calculated to give 50% of the maximum optical density reading to CRPV antigens only.

^d Dilution of serum calculated to give 50% reduction in papilloma size compared with sites established with untreated CRPV.

^e ND, not determined.

FIG. 7. Comparison of neutralizing and antibody titers in serum to CRPV L1 VLP antigen for rabbits immunized with CRPV (Δ) and CRPV L1 VLP ($\circ$, $\square$). Sera were taken from individual rabbits at 2 weeks (Δ , $\square$) or 6 and 12 months ($\circ$) after immunization, and the results are plotted with a calculated linear regression line of best fit ($r = 0.898$; $P < 0.0001$).

while the remaining four were also strongly but not completely protected, such that each rabbit had only one small papilloma developing at one of the two high-dose (10^{-1} dilution) virus challenge sites (Fig. 2 and 3). These seven animals therefore represent an immune status that is borderline protective for the highest dose of challenge virus. When neutralization and antibody titers were compared with the degree of protection for these animals (Fig. 8, where 0 papillomas represents complete protection and 1 papilloma represents partial protection), there was a poor correspondence ($r = 0.035$, $P = 0.47$; and $r = 0.323$, $P = 0.25$, respectively).

DISCUSSION

Papillomavirus VLPs have recently been produced in various expression systems and are now being tested as both antigen and immunogen in several animal papillomavirus and HPV models. One key feature of these VLPs is that they retain an important set of conformationally dependent neutralizing epitopes that cannot be duplicated easily by peptides and bacterially expressed viral coat proteins or protein fragments. Nonconformational neutralizing epitopes on papillomaviruses have been difficult to detect, and antigens containing such epitopes have induced only low titers of neutralizing antibody (6, 7, 16, 17, 19, 20).

In this study, we have examined the protective nature of CRPV L1 VLP antigens in New Zealand White domestic rabbits following both immediate (2 weeks) and delayed (6 and 12 months) challenge with infectious CRPV. Earlier studies had demonstrated that infectious CRPV and bovine papillomavirus antigen could induce strong protection (2, 3, 11, 18, 24), and, more recently, CRPV VLPs were also shown to be very protective (1, 10). These latter studies, however, examined protection soon after VLP immunization, at which time neutralizing antibodies in serum are at very high titers. Passive serum transfer experiments have demonstrated that neutralizing antibodies in serum can confer protection against CRPV challenge (1, 10, 13). It was of interest, therefore, to measure the degree of protection and levels of neutralizing antibody in serum at times long after immunization (6 and 12 months),

FIG. 8. Comparison of protection (number of developing papillomas) versus neutralizing titers (A) and antibody titers (B) in serum. Sera taken at 2 weeks after immunization with CRPV (Δ) or CRPV L1 VLP ($\square$) or at 6 and 12 months after immunization with CRPV L1 VLP ($\bullet$) were tested for virus neutralization and antiviral antibody titers in serum and plotted against the number of developing papillomas. Individual control immunized rabbits (12 rabbits) grew papillomas at 10^{-1} (24 of 24 sites), 10^{-2} (23 of 24 sites), 10^{-3} (15 of 24 sites), and 10^{-4} (3 of 24 sites) dilutions of virus, with a mean of 5.4 sites per rabbit and a range from 3 to 7 sites per rabbit.

when antibody levels in serum were greatly reduced. Animals in this latter category demonstrated strong but not complete protection (Fig. 2 and 3), such that three of seven were completely protected and four had only one small papilloma each. When neutralizing titers in serum were compared with numbers of papillomas (0 = protection; 1 = incomplete protection), there was a correlation (Fig. 8), although the sample size was small. The possible role for a recall response to the viral challenge (memory B cells and T_H cells) and the major histocompatibility complex haplotype of the outbred rabbits, however, should not be excluded as factors that may be additional to neutralizing antibodies in serum (1, 10, 13) in the resistance to delayed viral challenge.

In summary, CRPV L1 VLPs have been tested as immunogens for protection of rabbits against experimental challenge with CRPV. Complete protection was achieved when animals were challenged soon after immunization, which is in agreement with recently published reports (1, 10). In this study, we have added delayed challenge groups to test the long-term effectiveness of the VLP antigens. The results demonstrated that the VLP immunizations were strongly protective, with up to 3 log units of protection achieved at 12 months after immunization. Both CRPV-reactive and virus neutralizing antibodies were still detectable at 6 and 12 months after immunizations, although titers had decayed 10-fold. The mechanism for protection against delayed challenge most probably includes circulating neutralizing antibodies in serum (1, 13).

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant CA47622 from the National Institutes of Health and by the Jake Gittlen Memorial Golf Tournament.

We thank Linda Mylin for help in production of the figures.

REFERENCES

- Breitbart, F., R. Kirnbauer, N. L. Hubbert, B. Nonnenmacher, C. Trinh-Dinh-Desmarquet, G. Orth, J. T. Schiller, and D. R. Lowy. 1995. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J. Virol.* **69**:3959–3963.
- Campo, M. S. 1994. Vaccination against papillomavirus in cattle. *Curr. Top. Microbiol. Immunol.* **186**:255–266.
- Campo, M. S., G. J. Grindlay, B. W. O'Neil, L. M. Chandrachud, G. M. McGarvie, and W. F. H. Jarrett. 1993. Prophylactic and therapeutic vaccination against a mucosal papillomavirus. *J. Gen. Virol.* **74**:945–953.
- Christensen, N. D., R. Hopfl, S. L. DiAngelo, N. M. Cladel, S. D. Patrick, P. A. Welsh, L. R. Budgeon, C. A. Reed, and J. W. Kreider. 1994. Assembled baculovirus-expressed HPV-11 L1 virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titers of neutralizing antibodies. *J. Gen. Virol.* **75**:2271–2276.
- Christensen, N. D., and J. W. Kreider. 1990. Antibody-mediated neutralization in vivo of infectious papillomaviruses. *J. Virol.* **64**:3151–3156.
- Christensen, N. D., J. W. Kreider, N. C. Kan, and S. L. DiAngelo. 1991. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. *Virology* **181**:572–579.
- Ghim, S., N. D. Christensen, J. W. Kreider, and A. B. Jensen. 1991. Comparison of neutralization of BPV-1 infection of C127 cells and bovine fetal skin xenografts. *Int. J. Cancer* **49**:285–289.
- Ghim, S., A. B. Jensen, and R. Schlegel. Personal communication.
- Hagensee, M. E., N. H. Olson, T. S. Baker, and D. A. Galloway. 1994. Three-dimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. *J. Virol.* **68**:4503–4505.
- Hagensee, M. E., N. Yaegashi, and D. A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* **67**:315–322.
- Jansen, K. U., M. Rosolowsky, L. D. Schultz, H. Z. Markus, J. C. Cook, J. J. Donnelly, D. Martinez, R. W. Ellis, and A. R. Shaw. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. *Vaccine*, in press.
- Jarrett, W. F. H., B. W. O'Neil, J. M. Gaukroger, K. T. Smith, H. M. Laird, and M. S. Campo. 1990. Studies on vaccination against papillomaviruses: the immunity after infection and vaccination with bovine papillomaviruses of different types. *Vet. Rec.* **126**:473–475.
- Kelsall, S. R., and J. K. Kulski. 1995. Expression of the major capsid protein of human papillomavirus type 16 in *Escherichia coli*. *J. Virol. Methods* **53**:75–90.
- Kidd, J. G. 1941. Detection of "masked" virus (Shope papilloma virus) by means of immunization: results of immunization with mixtures containing virus and antibody. *J. Exp. Med.* **74**:321–344.
- Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* **89**:12180–12184.
- Le Cann, P., P. Coursaget, S. Iochmann, and A. Touze. 1994. Self-assembly of human papillomavirus type 16 capsids by expression of the L1 protein in insect cells. *FEMS Microbiol. Lett.* **117**:269–274.
- Lin, Y.-L., L. A. Borenstein, R. Ahmed, and F. O. Wettstein. 1993. Cottontail rabbit papillomavirus L1 protein-based vaccines: protection is achieved only with a full-length, nondenatured product. *J. Virol.* **67**:4154–4162.
- Lin, Y.-L., L. A. Borenstein, R. Selvakumar, R. Ahmed, and F. O. Wettstein. 1992. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. *Virology* **187**:612–619.
- Olson, C. 1963. Cutaneous papillomatosis in cattle and other animals. *Ann. N. Y. Acad. Sci.* **108**:1042–1056.
- Pilacinski, W. P., D. L. Glassman, R. A. Krzyzek, P. L. Sadowski, and A. K. Robbins. 1984. Cloning and expression in *Escherichia coli* of the bovine papillomavirus L1 and L2 open reading frames. *BioTechniques* **2**:356–360.
- Roden, R. B. S., E. M. Weissinger, D. W. Henderson, F. Booy, R. Kirnbauer, J. F. Mushinski, D. R. Lowy, and J. T. Schiller. 1994. Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *J. Virol.* **68**:7570–7574.
- Rose, R. C., W. Bonnez, R. C. Reichman, and R. L. Garcea. 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles. *J. Virol.* **67**:1936–1944.
- Rose, R. C., R. C. Reichman, and W. Bonnez. 1994. Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera. *J. Gen. Virol.* **75**:2075–2079.
- Sasagawa, T., P. Pushko, G. Steers, S. E. Gschmeissner, M. A. Hajibaghieri, J. Finch, L. Crawford, and M. Tommasino. 1995. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* **206**:116.
- Shope, R. E. 1937. Immunization of rabbits to infectious papillomatosis. *J. Exp. Med.* **65**:219–231.
- Volpers, C., P. Schirmacher, R. E. Streeck, and M. Sapp. 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. *Virology* **200**:504.
- Zhou, J., D. J. Stenzel, X.-Y. Sun, and I. H. Frazer. 1993. Synthesis and assembly of infectious bovine papillomavirus particles *in vitro*. *J. Gen. Virol.* **74**:763–768.
- Zhou, J., X. Y. Sun, D. J. Stenzel, and I. H. Frazer. 1991. Expression of vaccinia recombinant HPV-16, L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* **185**:251–257.