Concatenated Multitype L2 Fusion Proteins as Candidate Prophylactic Pan-Human Papillomavirus Vaccines

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Background Vaccination with minor capsid protein L2 induces antibodies that cross-neutralize diverse papillomavirus types. However, neutralizing antibody titers against the papillomavirus type from which the L2 vaccine was derived are generally higher than the titers against heterologous types, which could limit effectiveness against heterologous types. We hypothesized that vaccination with concatenated multitype L2 fusion proteins derived from known cross-protective epitopes of several divergent human papillomavirus (HPV) types might enhance immunity across clinically relevant HPV genotypes.

Methods Antibody responses of mice ($n=120$) and rabbits ($n=23$) to vaccination with HPV-16 amino-terminal L2 polypeptides or multitype L2 fusion proteins, namely, 11-200 × 3 (HPV types 6, 16, 18), 11-88 × 5 (HPV types 1, 5, 6, 16, 18), or 17-36 × 22 (five cutaneous, two mucosal low-risk, and 15 oncogenic types), that were formulated alone or in GPI-0100, alum, or 1018 ISS adjuvants were compared with vaccination with L1 virus-like particles (VLPs), including Gardasil, a licensed quadrivalent HPV L1 vaccine, and a negative control. Mice were challenged with HPV-16 pseudovirions 4 months after vaccination. Statistical tests were two-sided.

Results The HPV-16 L2 polypeptides generated robust HPV-16–neutralizing antibody responses, albeit lower than those to HPV-16 L1 VLPs, and lower responses against other HPVs. In contrast, the antisera to the multitype L2 fusion proteins 11-200 × 3 and 11-88 × 5 induced high neutralizing antibody titers against all heterologous HPVs tested. 11-200 × 3 formulated in GPI-0100 adjuvant or alum with 1018 ISS protected mice against HPV-16 challenge (reduction in HPV-16 infection vs phosphate-buffered saline control, $P < .001$) 4 months after vaccination as well as HPV-16 L1 VLPs, but 11-200 × 3 alone or formulated with either alum or 1018 ISS was less effective (reduction in HPV-16 infection, $P < .001$).

Conclusion Concatenated multitype L2 proteins in adjuvant have potential as pan-oncogenic HPV vaccines.

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The discovery that persistent infection with oncogenic human papillomavirus (HPV) types, of which 15 have been identified (1), is a necessary cause of cervical cancer has driven the development of prophylactic vaccines that are based on the capsid proteins L1 and L2 (2). Vaccination with L1 virus-like particles (VLPs) (3–5) elicits high, but type-restricted, titers of neutralizing antibodies, which appear to be the main mediators of protection (3,6–9). VLP vaccines confer a high degree of protection against infection and neoplastic disease caused by the papillomavirus types used to derive the vaccine (10–12). Current formulations of the two licensed L1 VLP vaccines (Gardasil, Merck & Co., Inc., and Cervarix, GlaxoSmithKline) contain two oncogenic HPV genotypes, HPV-16 and HPV-18, which together account for about 70% of cervical cancers (11,13). Gardasil also contains L1 VLP types that are derived from HPV-6 and HPV-11 and prevents benign genital warts caused by these viruses.

If protection induced by L1 VLP vaccines is predominantly HPV type specific, it would be necessary to incorporate VLPs from nine oncogenic HPV types to prevent greater than 90% of cervical cancers (14). Although L1 VLP vaccination may induce partial cross-protection against very closely related HPV types (12,15), which is likely mediated by relatively low levels of cross-type neutralizing antibodies (8,16), comprehensive vaccination against all oncogenic HPV types is challenging because of the cost and complexity of developing highly multivalent L1 VLP vaccines (17). The possibility of a single protein, inexpensive, pan-oncogenic HPV vaccine is an attractive alternative to highly multivalent and thus costly L1 VLP vaccines.

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Preclinical studies have shown that immunization of cows (18–20) or rabbits (21–24) with L2 polypeptides protects against experimental challenge by the homologous animal papillomavirus at mucosal sites in the bovine papillomavirus (BPV) type 4/cattle model and at cutaneous sites in the cottontail rabbit papillomavirus (CRPV)/rabbit model.

In addition to papillomavirus type–specific protection, vaccination with amino-terminal L2 polypeptides has also induced a remarkable degree of protection against animal challenge with heterologous papillomavirus types (24,25). Notably, vaccination with HPV-16 L2 11-200 protects against cutaneous and mucosal challenge with CRPV and the rabbit oral papillomavirus, respectively, both of which are evolutionarily divergent from HPV-16 (24). In addition, vaccination with HPV-16 11-200 or BPV-1 1-88 L2 peptides generated sera with cross-neutralizing antibodies against diverse HPV types (26). Protection induced by homologous and heterologous L2 polypeptides appears to be mediated by neutralizing antibodies because the induction of neutralizing antibodies against CRPV L2 was associated with protection against challenge with infectious CRPV virions in individual rabbits (24). Cell-mediated immunity does not appear to play a role because inoculation with CRPV DNA induced similar papilloma burdens in naive rabbits and animals that had been completely protected from challenge by CRPV virions by vaccination with L2.

These observations suggest that an L2-based vaccine might have potential as a pan-HPV vaccine, with activity against nongenital papillomaviruses in addition to those that cause cervical and other mucosal cancers and those that cause genital warts. However, a disadvantage of monovalent L2 immunogens is that the neutralizing titers and protection induced by them are greater for the homologous-type virus than for a heterologous-type papillomavirus, that is, although L2 induces antibodies that cross-neutralize diverse papillomavirus types, they neutralize the homologous type more effectively (26,27). The lower immune response to heterologous HPVs could severely limit the practical utility of an L2-based vaccine because its breadth and duration of protection are likely to depend on the ability to induce robust neutralizing activity against heterologous HPV types. To address this issue and provide broader immunity, we have here examined concatenated multitype L2 fusion proteins for their ability to induce cross-neutralizing antibodies against several clinically relevant HPV types.

Cross-linking of B-cell receptors by arrays of epitopes on VLPs (28,29) or polymers (30,31) potentiates B-cell activation. We hypothesized that B-cell receptors recognizing concatenated neutralization epitopes of L2 of multiple different HPV genotypes would be preferentially activated as compared with L2 type–specific B-cell receptors, and thus, B cells presenting the former would be more readily activated and bias the global repertoire of neutralizing antibody response to cross-reactive epitopes. We have tested this hypothesis by examining whether vaccination with concatenated multitype L2 fusion proteins enhances the breadth and titer of cross-neutralizing antibodies as compared with a monotypic L2 immunogen.

Methods

Antigen Preparation

HPV-16 L2 polypeptide expression constructs encompassing residues 1–88, 13–88, 1–107, 13–107, 11–200, 13–200, and 89–200 were generated by polymerase chain reaction as described previously (26). The multitype L2 constructs were codon optimized for *Escherichia coli* expression by lowest free-energy calculation and synthesized by Blue Heron Biotechnology, Inc. (Bothell, WA), with 5′ *Bam*HI and 3′ *Xho*I sites to facilitate cloning. The L2 genes were subcloned into the pET28a vector (Novagen, San Diego, CA) and the resulting hexahistidine (6His)-tagged recombinant polypeptides expressed in *E coli* BL21 (Rosetta cells; Novagen) (26). The recombinant L2 polypeptides were affinity purified by binding to a nickel–nitrilotriacetic acid column (Qiagen, Valencia, CA) in 8 M urea (using the QiaExpressionist standard purification protocol for denaturing conditions) and then dialyzed in cassettes (Pierce, Rockland, NJ) against phosphate-buffered saline (PBS, 137 mM NaCl, 12 mM phosphate, 2.7 mM KCl). Purity was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and protein concentration determined by bicinchoninic acid test (Pierce) using a bovine serum albumin (BSA) standard. HPV-16 and HPV-45 L1 VLPs were prepared in 293TT cells (32) as for pseudovirions but without L2.

Enzyme-Linked Immunosorbent Assays of L2 or HPV-16 Pseudovirion–Specific Serum Antibody Titers

Pseudovirions were prepared as previously described (32). Immobilon plates (Nunc, Rochester, NY) were coated overnight at 4°C with 100 ng/well of 6His–HPV-16 L2 prepared in *E coli* or HPV-16 L1/L2 pseudovirions produced in 293TT cells and diluted in PBS. Wells were then blocked with 1% BSA–PBS for 1 hour at room temperature and incubated with twofold dilutions

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CONTEXT AND CAVEATS

Prior knowledge

Current human papillomavirus (HPV) vaccines are based on capsid L1 proteins and appear to confer only HPV type–specific immunity. Although vaccination with minor capsid protein L2 induces antibodies that neutralize many types of papillomaviruses, the response to the specific virus type is usually higher than it is to other types.

Study design

Mice were vaccinated with HPV-16 L2 polypeptides, multitype L2 fusion proteins in different adjuvants, Gardasil, HPV-16 L1 virus-like particles (VLPs), or a negative control, followed by challenge with HPV-16 pseudovirions 4 months later.

Contributions

Vaccination with the multitype L2 fusion proteins induced antibody responses to all HPV types tested and protected mice against HPV-16 challenge as well as HPV-16 L1 VLPs.

Implications

Multitype L2 proteins have potential as pan-oncogenic HPV vaccines.

Limitations

To be effective in humans, the vaccine will need to protect against infection for several years; only short times were tested in this study.

From the Editors

of rabbit or mouse antisera for 1 hour at room temperature. Following a wash step with PBS/0.01% (vol/vol) Tween-20, peroxidase-labeled goat anti-rabbit IgG (KPL, Inc., Gaithersburg, MD) diluted 1:5000 in 1% BSA/PBS was added for 1 hour. The plates were then washed again and developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid solution (Roche Applied Science, Indianapolis, IN) for 10 minutes (33). The absorbance was measured at 405 nm (A_{405}) in a Benchmark Plus ELISA plate reader (Bio-Rad, Hercules, CA). Single assays were performed in parallel using preimmune sera as a negative control and mouse monoclonal antibody RG-1 or rabbit antiserum to the coating antigen as positive controls.

Antibody Neutralization Assays

The papillomavirus pseudovirion in vitro neutralization assays were performed as described earlier (32), and the secreted alkaline phosphatase activity in the cell-free supernatant was determined using *p*-nitrophenyl phosphate (Sigma Aldrich, St Louis, MO) dissolved in diethanolamine, with absorbance measured at 405 nm. Constructs and detailed protocols for the preparation of the pseudovirions can be found at http://home.ccr.cancer.gov/lco/. Titers were defined as the reciprocal of the highest dilution that caused a greater than 50% reduction in A_{405}, as described previously (32), and a titer less than 50 was considered not detected. Single assays were performed in parallel using preimmune sera as a negative control and mouse monoclonal antibody RG-1 or rabbit antiserum to L1 VLP as positive controls.

Vaccine Studies

Studies in animals were performed in accordance with institutional policies and with the approval of the Johns Hopkins Animal Care and Use Committee or the Animal Ethics committee of Shantha Biotechnics, Hyderabad, India, using standard protocols. Balb/c mice (n = 120, from NCI, Frederick, MD) were vaccinated in groups of five mice three times at 2-week intervals by subcutaneous injection with 10 µg of HPV-16 or HPV-45 L1 VLP, or the adjuvants alum (1.3 mg), or 1018 ISS alone (10 µg/mouse), or 25 µg of recombinant L2-based antigens including 11-200 × 1, 11-200 × 3, 1-88 × 1, 11-88 × 5, 17-36 × 22, or HPV-16 L2 17-36 peptide prepared by chemical synthesis (Sigma Aldrich, St Louis, MO) in the formulations indicated: PBS alone, or alum alone (1.3 mg), or 1018 ISS alone (10 µg/mouse), or 25 µg 11-200 × 3 alone, or formulated with alum (1.3 mg), or with 1018 ISS (10 µg/mouse), or with GPI-0100 (at either 50 or 200 µg/mouse), or with GPI-0100 (50 µg/mouse) + Tween-40 (1 mg/mouse), or with alum and 1018 ISS (10 µg/mouse). Blood samples were obtained from the tail vein 2 weeks after the final immunization. Rabbits (n = 15) were vaccinated on days 1, 28, 42, 60, and 76 with 300 µg of HPV-16 L2 polypeptide or polymeric L2 constructs in complete Freund's adjuvant (CFA) with the initial dose and incomplete Freund's adjuvant (IFA) thereafter per standard protocols. Rabbits (n = 8) were vaccinated with 12 or 30 µg of Gardasil on days 1, 21, 35, and 56, and blood samples were collected 1 week after the final vaccination. Blood was allowed to congeal overnight, and the serum was separated by centrifugation and stored at −20°C until use.

Cutaneous HPV-16 Challenge

At 4 months after vaccination, the above mice were challenged with HPV-16 pseudovirions as described previously (25,34). Briefly, all mice were anesthetized, and a patch of skin on their ventral torso was shaved with an electric razor while taking care not to traumatize the epithelium, before challenge by application of approximately 3×10^9 HPV-16 pseudovirion particles (100 ng protein) that encapsidated pYLUC, a plasmid carrying a luciferase gene that would be expressed upon pseudoinfection (http://home.ccr.cancer.gov/lco/) in 10 µL 0.6% carboxymethylcellulose (Sigma Aldrich) to the patch of shaved skin on each mouse [all parameters were defined previously (25,34)]. Three days later, all mice were again anesthetized by isoflurane inhalation (~1%), injected intraperitoneally with luciferin (100 µL at 7 mg/mL), and their image was acquired for 10 minutes [timing previously optimized (25,34)] with an IVIS 200 bioluminescence imaging system (Xenogen, Cranbury, NJ) to visualize the expression of luciferase by measuring light emission. Equal areas encompassing the site of virus inoculation were analyzed using Living Image 2.20 software (Xenogen). Bioluminescence levels above that of mice vaccinated with HPV L1 VLPs before challenge was determined.

Statistical Analysis

Comparison between groups for titers and levels of infection in the mouse model was made by multiway analysis of variance with Bonferroni adjustment (Statview 5.0; SAS Institute, Inc., Cary, NC). All statistical tests were two-sided, and *P* values less than .05 were considered statistically significant.

Results

We first sought to determine whether we could identify an optimal monovalent L2 immunogen. The L2 vaccines comprising residues 1–88 and 11–200 used in earlier studies to demonstrate induction of cross-neutralizing antibodies and protection against heterologous challenge had been selected based on the presence of convenient restriction sites in L2 DNA, rather than immunogenicity considerations (35,36). Although vaccination with either peptide was protective, there was some suggestion that the cross-neutralization and cross-protection might be more effective with the 11–200 peptide than with the 1–88 peptide (24). Consistent with this possibility, vaccination with L2 peptides from 94–112 and 107–122 had been found to be protective against homologous challenge (23). Therefore, to assess the potential benefit to immunogenicity of including these regions within an L2 vaccine, we generated amino-terminal L2 polypeptides that terminated at residues 88, 107, and 200 (Table 1). We also tested the influence on immunogenicity of starting the L2 polypeptide at various amino-terminal amino acid residues. During infection, L2 must be cleaved by furin to remove residues 1–13 (37), and this cleavage renders a conserved neutralization epitope between residues 17 and 36 (16) more accessible to monoclonal antibody RG-1 (38). For these vaccine studies, we therefore generated amino-terminal L2 polypeptides initiating at residues 1 and 11, as benchmark sites known from the earlier studies to be immunogenic; residue 13, as an immunogen whose amino terminus mimicked what would be present after furin cleavage; and residue 89, to assess the role of amino acids downstream from residues 1–88 (Table 1).

Table 1. Antibody responses of rabbits vaccinated with monomeric HPV-16 or multimeric HPV L2 polypeptides of different sizes*

Antigen	Rabbit	HPV-16 L2 ELISA	HPV-16 ELISA	HPV-16 IVN	HPV-18 IVN	HPV-31 IVN	HPV-45 IVN	HPV-58 IVN	HPV-6 IVN	HPV-5 IVN
HPV-16 L2 polypeptides										
1–88	a	409 600	204 800	409 600	200	3200	3200	12 800	1600	800
13–88†	a	51 200	6400	3200	None	None	None	200	—	200
1–107	a	204 800	102 400	409 600	6400	12 800	6400	102 400	400	25 600
	b	409 600	102 400	102 400	800	3200	400	6400	—	12 800
13–107	a	409 600	102 400	204 800	1600	3200	200	6400	—	6400
11–200	a	102 400	102 400	409 600	200	400	400	800	800	800
13–200	a	819 200	102 400	102 400	800	1600	3200	6400	—	12 800
89–200†	a	None	None	None	None	None	None	None	None	None
	b	204 800	12 800	3200	None	None	None	None	—	None
Multimeric L2 constructs										
17–36 x 22	a	409 600	102 400	204 800	12 800	800	12 800	25 600	800	3200
	b	409 600	102 400	12 800	6400	None	3200	3200	—	400
11–88 x 5	a	819 200	819 200	819 200	204 800	51 200	102 400	409 600	102 400	>102 400
	b	1638 400	819 200	819 200	102 400	102 400	102 400	409 600	—	>102 400
11–200 x 3	a	409 600	102 400	204 800	25 600	1600	12 800	25 600	6400	1600
	b	409 600	102 400	51 200	6400	1600	3200	12 800	—	800

* Individual rabbits were vaccinated five times with 300 µg of the HPV-16 L2 polypeptides or polymeric L2 constructs using Freund's adjuvant. Hyperimmune sera were collected at 1 week after the final vaccination and tested for L2-specific antibody by ELISA with microtiter plates coated with full-length HPV-16 L2 (HPV-16 L2 ELISA) or HPV-16 L1/L2 pseudovirions (HPV-16 ELISA). The sera were also tested for IVN titers for the HPV pseudovirion types indicated. Neutralization titers were not detected in the preimmune sera. HPV = human papillomavirus; None = less than 50% neutralization at the lowest dilution tested of 1:50; — = not tested; ELISA = enzyme-linked immunosorbent assay; IVN = in vitro neutralization.

† Protein exhibited substantial degradation in *Escherichia coli*.

Responses in Rabbits Vaccinated With Monomeric and Multimeric L2 Polypeptides

To map immunogenicity for homologous and cross-neutralization epitopes, seven HPV-16 L2 polypeptides (Table 1) were expressed in *E coli* with 6-His tags and affinity purified for vaccination studies. Although all the polypeptides were readily purified, two, HPV-16 L2 13–88 and 89–200, were unstable during manufacturing. Rabbits were vaccinated five times with 300 µg of each polypeptide, in CFA for the priming dose and in IFA for the booster vaccinations. The success of each vaccination was first verified by testing the hyperimmune sera in an enzyme-linked immunosorbent assay (ELISA) to detect full-length HPV-16 L2 and an ELISA to detect HPV-16 L1/L2 pseudovirions. High titers of serum antibodies were raised to each HPV-16 L2 polypeptide, although the titers against HPV-16 pseudovirions were lower for the antisera raised with the two unstable antigens, L2 13–88 and 89–200 (Table 1). HPV-16–neutralizing titers and HPV-6, HPV-18, HPV-31, HPV-45, and HPV-58 cross-neutralizing titers were then determined for each rabbit antiserum induced by the L2 polypeptides (Table 1). Consistent with earlier studies (24), the HPV-16 L2 1–88 and 11–200 peptides induced robust titers of HPV-16–neutralizing antibodies. Similarly, robust HPV-16–neutralizing antibody titers were observed for the antisera to HPV-16 L2 1–107, 13–107, and 13–200. Vaccination with HPV-16 L2 13–88 and 89–200 proteins that were unstable produced considerably weaker neutralizing responses.

The L2-specific antisera induced by the various HPV-16 L2 peptides neutralized not only HPV-16 but also the diverse range of heterologous papillomavirus types, including the tested oncogenic types, HPV-18, HPV-31, HPV-45, and HPV-58 (Table 1). However, neutralizing antibody titers against HPV-16 were higher than against other types, although there was no clear relationship between titers and evolutionary distance of the heterologous types from HPV-16 (HPV-31 and HPV-58 are more closely related to HPV-16 than are HPV-18 and HPV-45, which are closely related to each other).

Because none of the alternative HPV-16 L2 peptides substantially increased neutralizing titers to heterologous viruses, we examined concatenated fusion proteins, consisting of several homologous L2 peptides derived from different clinically relevant HPV genotypes. Based on the above results and previous studies, L2 polypeptides corresponding to HPV-16 L2 17–36, 11–88, and 11–200 were chosen for fusion constructs. We tested three multitype constructs: one with three copies of amino acids 11–200 (termed 11-200 × 3 and derived from the L2 proteins of HPV-6, HPV-16, and HPV-18), one with five copies of 11–88 (termed 11-88 × 5 and derived from L2 of HPV-1, HPV-5, HPV-6, HPV-16, and HPV-18), and one with 22 copies of 17–36 (termed 17-36 × 22; see Table 2 for the various HPV types used in this construct), with each L2 peptide being derived from clinically relevant and diverse HPV genotypes (Table 2) (39). The proteins were expressed in *E coli*, affinity purified under denaturing conditions, and used to vaccinate rabbits as described for the HPV-16 L2 polypeptides. In general, vaccination of each rabbit with the multitype L2 fusion proteins (11-200 × 3, 11-88 × 5, and 17-36 × 22) in Freund's adjuvant induced robust neutralization titers against the various HPVs in a given immunogen (Table 1), although the 17-36 × 22, which included a peptide from each HPV type tested for neutralization, had relatively low titers against HPV-6 and HPV-31. Notably, vaccination with 11-200 × 3

Table 2. A summary of the multitype HPV L2 constructs*

L2 residues × no. of HPV types	Molecular weight, kDa	Types of HPV (in order from amino to carboxyl terminus)
1–88 × 1	16	16
11–200 × 1	26	16
11–200 × 3	63	6, 16, 18
11–88 × 5	43	16, 18, 1, 5, 6
17–36 × 22	49	1, 2, 63, 5, 8 (cutaneous) 6, 11 (mucosal low risk) 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 (mucosal high risk)

* HPV = human papillomavirus.

induced strong cross-neutralizing titers against HPV-45, HPV-31, and HPV-58, although their peptides were not present in the immunogen (Table 1). The immunogenicity of 11-88 × 5 was particularly impressive because it induced remarkably high titers of neutralizing antibodies to all the tested HPV types, including the three (HPV-31, HPV-45, and HPV-58) that were not represented in this fusion protein (Table 1).

Responses in Rabbits Vaccinated With Gardasil

Vaccination with L1 VLPs can induce antibodies that cross-neutralize very closely related papillomavirus types, for example, HPV-18 and HPV-45 (16) (22,38). Therefore, we sought to compare the levels of cross-neutralizing antibodies generated by vaccination with Gardasil (which targets HPV-6, HPV-11, HPV-16, and HPV-18, and is formulated in aluminium salt), using two different concentrations, vs multitype L2 proteins formulated in CFA/IFA (Table 3). As expected, vaccination with Gardasil produced high titers of neutralizing antibodies to the oncogenic HPV types included in the vaccine, HPV-16 and HPV-18. Even higher HPV-16 and HPV-18 titers were generated with the L2 fusion protein, but it should be noted that the rabbits received a higher dose of antigen for L2 than Gardasil, and the CFA/IFA adjuvant used for the L2 immunogen is more potent than aluminium salt. The most noteworthy differences between the rabbits vaccinated with Gardasil and those vaccinated with the L2 fusion protein were seen in the neutralizing titers against the heterologous HPV types. The sera from those receiving Gardasil consistently contained low, but detectable, levels of neutralizing activity against HPV-45 and occasionally against HPV-31 but had no detectable activity against HPV-58. However, the titers against the heterologous HPV types were 2–3 orders of magnitude lower than those against HPV-16 or HPV-18. By contrast, the sera from rabbits that were vaccinated with the L2 fusion protein had titers against the heterologous HPVs that were less than 1 order of magnitude lower than against the homologous HPVs.

Responses of Mice Vaccinated With Monomeric and Multimeric L2 Polypeptides

We also evaluated the immune response induced by the L2 immunogens in mice, in part, to determine their neutralizing titers in a second animal species and in part as a prelude to vaccine protection studies because mice can be challenged with HPV pseudovirions and infection quantified by delivery of a reporter such as luciferase (34,40). Mice were vaccinated three times at 2-week intervals with monovalent HPV-16 L2 polypeptides containing residues 17–36, 1–88, or 11–200, or one of the three concatenated multitype L2 fusion proteins, 11-200 × 3, 11-88 × 5, or 17-36 × 22, using the saponin-based GPI-0100 adjuvant (41). Blood was collected 2 weeks later, and the in vitro neutralization titers were determined for HPV-16, HPV-18, HPV-45, HPV-58 (four common oncogenic HPV types), and HPV-6 (the most common type found in benign genital warts). As had been observed in the rabbits, vaccination of mice with HPV-16 L2 1–88 or HPV-16 L2 11–200 in GPI-0100 induced robust titers of neutralizing antibodies against the homologous virus type, HPV-16, although the titers were approximately 2 orders of magnitude lower than in the rabbits vaccinated using Freund's adjuvant (Figure 1). However, vaccination with a synthetic peptide comprising HPV-16 L2 residues 17–36 (which had not been tested in the rabbits) did not induce detectable neutralizing antibodies, or L2-specific antibodies (not shown), probably because it lacks a T-helper epitope for this mouse strain (25). The positive control, vaccination of mice with HPV-16 L1 VLPs in the absence of adjuvant, induced even higher titers of HPV-16–neutralizing antibodies than the HPV-16 L2 constructs ($P < .001$). In contrast, vaccination with HPV-45 L1 VLPs failed to induce HPV-16–neutralizing antibodies, consistent with the type-restricted response to L1 VLP vaccines (Figure 1). Vaccination with L2 11-88 × 5, 1-88 × 1, 11-200 × 3, and 11-200 × 1 constructs generated statistically similar HPV-16–neutralizing

Table 3. Antibody responses of rabbits vaccinated with Gardasil*

Antigen, μg	Rabbit	HPV-16 IVN	HPV-18 IVN	HPV-31 IVN	HPV-45 IVN	HPV-58 IVN
Gardasil, 30	a	51 200	51 200	50	100	None
	b	25 600	51 200	None	100	None
	c	25 600	25 600	None	800	None
	d	51 200	102 400	50	800	None
Gardasil, 12	a	12 800	25 600	None	None	None
	b	51 200	25 600	200	1600	None
	c	51 200	25 600	None	800	None
	d	102 400	51 200	50	400	None

* Rabbits were vaccinated four times with either 30 or 12 μg of Gardasil. Hyperimmune sera were collected at 1 week after the final immunization and tested for IVN titers for the HPV pseudovirion types indicated in comparison with a control serum of a rabbit that was vaccinated five times with 300 μg of the polymeric L2 constructs 11-88 × 5 using Freund's adjuvant, and shown in Table 1, rabbit a. Neutralization titers were not detected in the preimmune sera. None = less than 50% neutralization at the lowest dilution tested of 1:50; HPV = human papillomavirus; IVN = in vitro neutralization.

antibody titers in this small study. The 17-36 × 22 construct was less effective than 11-88 × 5 (*P* < .001; Figure 1), possibly as a result of weak CD4 T-cell help (25). The cross-neutralizing antibody responses observed in mice vaccinated with monotype HPV-16 L2 polypeptides in GPI-0100 were less robust than those generated in rabbits receiving the same vaccines in CFA/IFA. However, vaccination with 11-200 × 3 or 11-88 × 5 was very effective in inducing cross-neutralizing antibodies against HPV-45 and HPV-58, even though the fusion proteins do not contain sequences from either of these HPV types, in addition to inducing neutralizing antibodies against HPV-6 and HPV-18, which are represented in the fusion proteins (Figure 1).

Responses of Mice Vaccinated With L2 Multimeric Polypeptides in Adjuvant

Several adjuvants that are potentially more effective than, or can be complementary to, alum have shown promise in clinical vaccine trials, for example, the immunostimulatory sequence (ISS) 1018, an oligonucleotide that activates toll-like receptor 9 (42,43), and the saponin-based adjuvant GPI-0100 (44,45). To address whether a particular adjuvant was more effective at inducing HPV-neutralizing antibodies when formulated with a multitype L2 vaccine, we compared immune responses to 25 µg 11-200 × 3 formulated in a variety of adjuvants, and combinations thereof. However, when sera were obtained from mice 2 weeks after their third immunization and in vitro neutralization of HPV-16, HPV-18, HPV-45, and HPV-58 (Figure 2) were measured, the titers were remarkably similar across each adjuvant group, and none was notably superior to alum-adjuvanted 11-200 × 3 at this time point.

Adjuvants can increase peak titers and the longevity of antibody responses. To assess the possibility that differences between adjuvants might become clearer as humoral responses wane, the mice were challenged with HPV-16 pseudovirions at 4 months after vaccination. Cutaneous infection was detected as a bioluminescent signal 3 days after the administration of HPV-16 pseudovirions carrying a luciferase reporter, immediately after injection of the challenged mice with the reporter substrate, luciferin. HPV-16 infection was statistically significantly reduced after vaccination with 11-200 × 3 alone compared with after PBS control vaccination (*P* = .004; Figure 3); reduction with 11-200 × 3 in any of the adjuvants tested compared with PBS control vaccination was even greater (*P* < .001; Figure 3). In particular, the formulation of 11-200 × 3 with alum + 1018 ISS was more effective than 11-200 × 3 alone (*P* < .001) or with alum adjuvant (*P* = .001). GPI-0100 formulations tested with 11-200 × 3 were more effective than 11-200 × 3 alone (*P* < .001) or 11-200 × 3 in combination with alum (*P* < .001). We recently found that vaccination with GPI-0100 adjuvant alone had no statistically significant effect on infectivity after HPV-16 challenge (46). No statistically significant difference in protection was observed when using only alum or only 1018 ISS with 11-200 × 3 as compared with the protein alone.

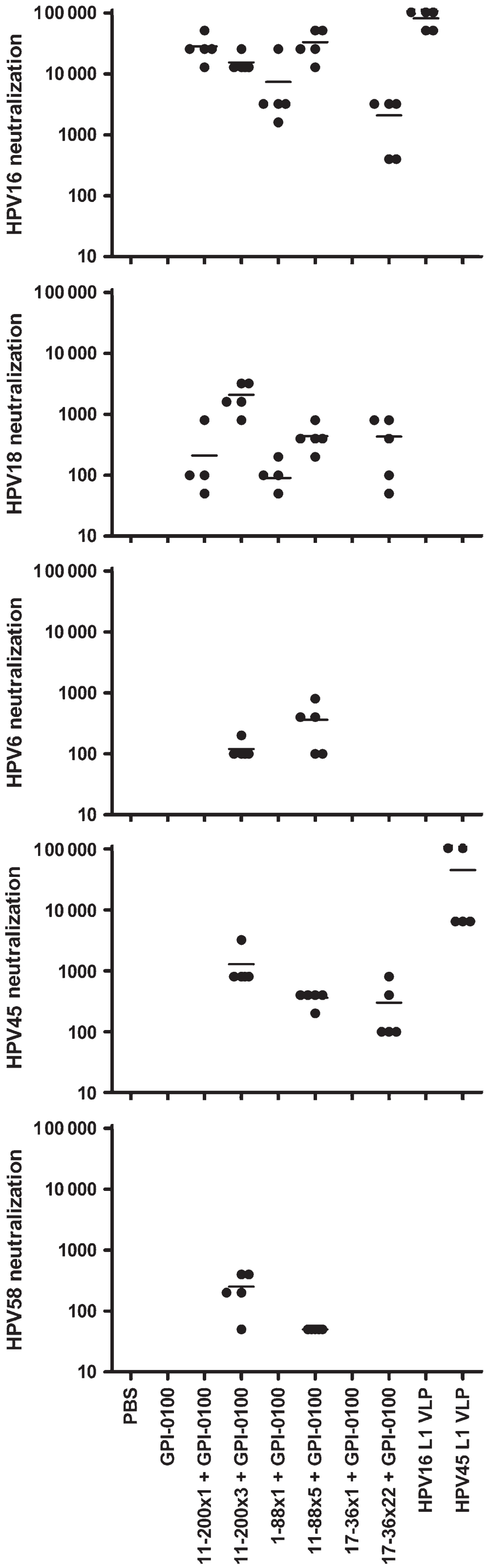

Figure 1. A comparison of neutralizing antibody responses of mice vaccinated with multitype or monomeric L2 vaccines or with L1 VLP. BALB/c mice were vaccinated subcutaneously on days 0, 15, and 30 with PBS or with 25 µg of different L2 monomeric and multitype constructs in GPI-0100 (50 µg) adjuvant or either HPV-16 L1 VLP or HPV-45 L1 VLP without an adjuvant (five mice per group). In vitro neutralization assays were performed using HPV pseudovirus for the genotypes indicated on twofold dilutions of the antisera collected from the mice 2 weeks after the final vaccination. Endpoint titers achieving 50% neutralization are plotted and the means shown as **horizontal lines**. HPV = human papillomavirus; PBS = phosphate-buffered saline; VLP = virus-like particle.

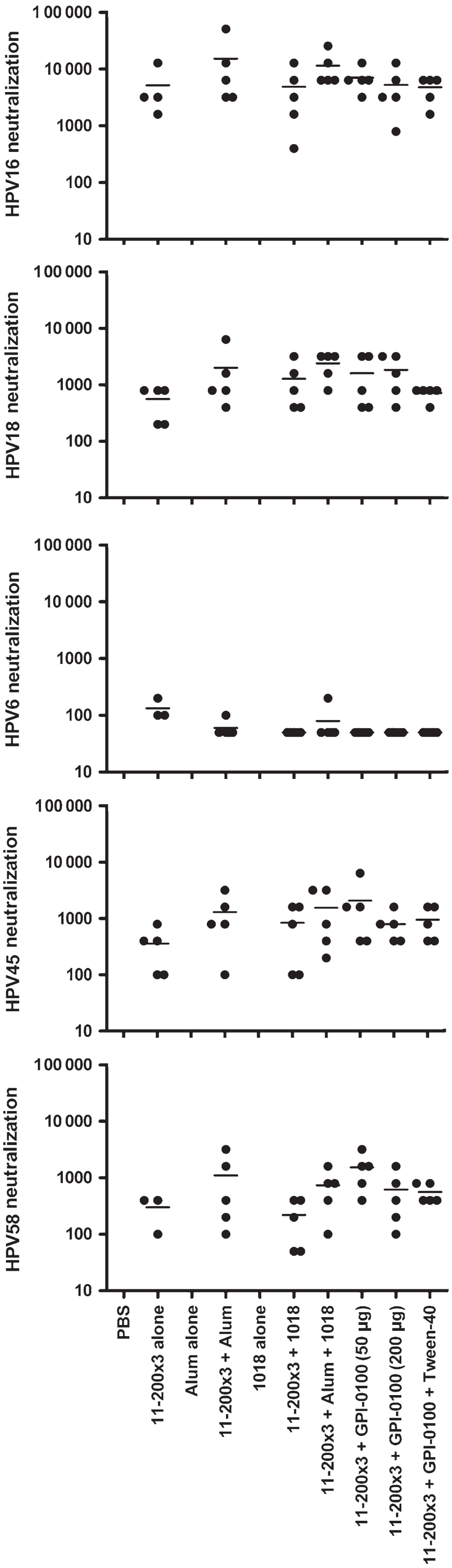

Figure 2. Neutralizing antibody responses of mice vaccinated with multitype L2 protein alone or in various adjuvant combinations. BALB/c mice (five mice per group) were vaccinated on days 0, 15, and 30 subcutaneously with alum alone (1.3 mg), or 1018 ISS alone (10 µg/mouse), or PBS, or 25 µg 11-200 × 3 alone, or formulated with alum (1.3 mg), or with 1018 ISS (10 µg/mouse), or with GPI-0100 (at either 50 or 200 µg/mouse), or with GPI-0100 (50 µg/mouse) + Tween-40 (1 mg/mouse), or with alum and 1018 ISS (10 µg/mouse). In vitro neutralization titers were performed with two dilutions of the antisera of mice collected 2 weeks after final immunization using HPV pseudovirus for the genotypes indicated. Endpoint titers for 50% neutralization are plotted and the means shown as **horizontal lines**. HPV = human papillomavirus; PBS = phosphate-buffered saline.

Vaccination with HPV-16 L1 VLP alone, but not with HPV-45 L1 VLP, also gave a similar level of protection against HPV-16 challenge as vaccination with 11-200 × 3 with alum + 1018 ISS or with GPI-0100 (*P* < .001; not shown). Therefore, we sought to compare the in vitro neutralizing antibody titers induced by vaccination of mice with Gardasil with those induced by vaccination of mice with either 11-200 × 3 or 11-8 × 5 in the adjuvant GPI-0100. The titers generated against HPV-16 and HPV-18, for which L1 VLPs are included in Gardasil, were substantially higher in the sera of mice that were vaccinated with Gardasil than in the sera of mice that were vaccinated with either multitype L2 construct. However, no HPV-45– or HPV-58–neutralizing antibodies were detected in the sera of mice vaccinated with Gardasil, although some neutralizing activity against HPV-45 had been seen in the rabbits immunized with Gardasil (Figure 4). In contrast, neutralizing antibody titers against HPV-45 and HPV-58 were readily detected in the sera of mice vaccinated with either 11-200 × 3 or 11-88 × 5, although neither construct contains L2 sequences derived from these two HPV types.

Discussion

We found that vaccination with the multimeric fusion proteins comprising the amino terminus of L2 of several HPV types induced robust neutralizing antibody titers, and when used with potent adjuvants, it also provided immunity from viral challenge even 4 months after immunization. Immunization with these multitype L2 fusion proteins also induced robust titers against all clinically relevant HPV types tested, as compared with the weaker cross-neutralizing responses to L2 of a single HPV type or the type-restricted responses to L1 VLP.

Several previous studies (18,19,24) showed that the amino terminus of L2 is protective in multiple animal models. Vaccination with L2 residues 94–122 provides relatively type-specific protection in rabbits (23,47), and studies in cattle (48) suggest the value of L2 101–120, 131–151, and 151–170 for protection against BPV-4. Other studies (48–51) support residues 108–120 as a cross-neutralization epitope. We recently defined L2 residues 17–36 as an important protective epitope (25,34), and others have defined neutralization epitopes between residues 36–49 (52) and 69–81 (48). Here, little difference was noted in the HPV-16 and heterologous type–neutralizing antibody titers produced by vaccination with HPV-16 L2 13–200 and 1–88. These findings are consistent with earlier reports that the first 88 residues of L2 contain important neutralization epitopes (26) and suggest that the amino-terminal 13 residues are dispensable for a robust neutralizing antibody response, probably because they would be eliminated by furin cleavage during infection (37,38). However, removal of this peptide did not increase the ability of amino-terminal L2 peptides to induce neutralizing antibodies.

Taking advantage of the presence of L2 epitopes that can induce broadly cross-reactive neutralizing antibodies could confer protection against a wide spectrum of HPV types. In support of this possibility, immunization with heterologous L2 polypeptides protects rabbits against CRPV, a model that was used in L1 VLP vaccine development (24,25). The production in bacteria of a single polypeptide, as with an L2 fusion protein, should be substantially less expensive to manufacture than a polyvalent L1 VLP vaccine. If an L2 vaccine were proven effective in people, its simpler manufacturing process could make the local production of such a vaccine highly feasible, which might achieve the goal of producing it at sustainable prices in emerging countries and lead to its widespread implementation in the developing world.

L1 VLPs induce high titer and long-lasting protective antibody responses even without adjuvant (53). A potentially serious limitation of the L2 approach has been that the level of cross-neutralizing antibodies against heterologous HPV types has been lower than that against the homologous virus, which has been associated with heterologous L2 vaccination inducing less effective protection than homologous L2 vaccination (24). To produce a practical vaccine, it is necessary for protection to last at least several years, and the less robust activity against heterologous HPV types raises doubts about whether sufficient protection of long duration would be induced by a monotype L2 immunogen and suggests the need for a potent adjuvant (54). Here, we examined multiple adjuvants (alum, GPI-0100, and 1018 ISS) that have been used in substantial numbers of patients and act via diverse mechanisms (44,55,56). GPI-0100 and 1018 ISS did not confer an obvious improvement in the peak humoral response to 11-200 × 3 as compared with alum, but there were statistically significant differences in protection from HPV-16 pseudovirus challenge at 4 months after immunization. Alum + 1018 ISS or GPI-0100 was more effective when used with 11-200 × 3 than alum alone or 1018 ISS alone and achieved long-term protection in this model on par with that of L1 VLP. McGarvie et al. (57) found that cattle were strongly protected from BPV-4 challenge 1 year after vaccination with 1 mg BPV-4 L2 in IFA twice, suggesting the potential for long-term immunity with other adjuvants.

The ability of an L2 fusion protein to increase the immune response to HPV types not present in the immunogen, although not as robust as its reactivity against those HPV types whose L2 peptides are represented in the fusion protein, strongly suggests that multitype fusion proteins may have the capacity to protect against a broad range of HPV types and that such protection could be of long duration. This more potent and broadly effective immune response may reflect enhanced cross-linking and activation of those B cells that recognize L2-specific neutralizing epitopes common to multiple HPV types in the fusion constructs and a resulting bias toward production of cross-neutralizing antibodies (30,31).

We made direct comparisons in two animal models between Gardasil, an L1 VLP vaccine that has been licensed in the United States, and the L2 fusion proteins. Although the responses were generally comparable, L2-specific cross-neutralization was weaker in mice than in rabbits. These analyses verified first that the L2 vaccines can induce strong neutralizing activity and protection against homologous HPV types, although the L2 immunogens are somewhat less potent immunologically than L1 VLPs against HPV types represented in the respective vaccine. However, the striking difference between L1 VLPs and the L2 fusion proteins was the limited cross-neutralizing activity induced by the VLP vaccine, in comparison with the robust activity displayed by the L2-based immunogens. Vaccination with Gardasil protects patients against HPV-6, HPV-11, HPV-16, and HPV-18, and efforts to assess cross-protection against types not used to make this vaccine are ongoing (58). Although there have been no clinical studies of an L2 vaccine in a prophylactic setting to date, vaccination of patients with HPV-16 L2 can trigger cross-neutralizing antibody responses even without an adjuvant (54).

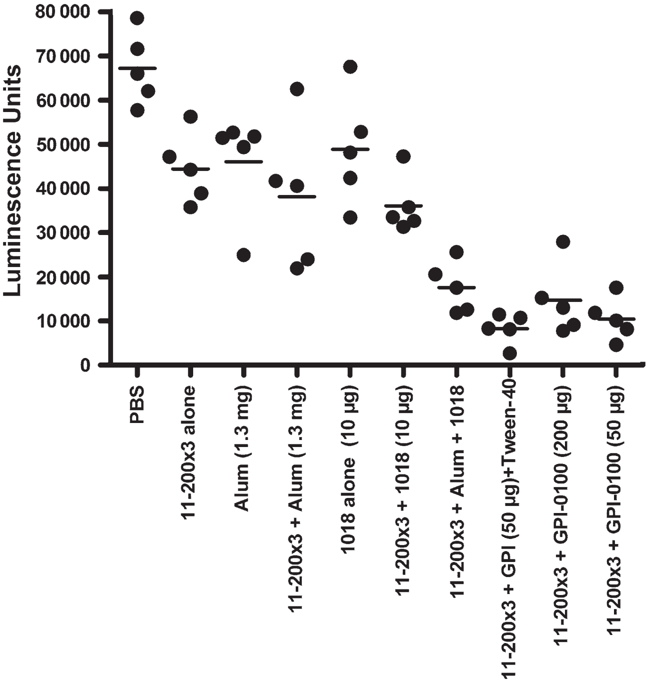

Figure 3. In vivo HPV-16 pseudovirus challenge of mice 4 months after vaccination with L2 11-200 × 3 in different adjuvant combinations. Mice (five per group, the same mice as in Figure 2) were vaccinated three times at 2-week intervals with PBS, 10 µg of L1 VLPs, or 25 µg of L2 11-200 × 3 in different adjuvants or adjuvant alone. Individual groups are as listed below from left to right: PBS alone, 11-200 × 3 alone, alum alone (1.3 mg), 11-200 × 3 + alum (1.3 mg), 1018 ISS alone (10 µg/mouse), 11-200 × 3+1018 ISS (10 µg/mouse), 11-200 × 3 + alum + 1018 (10 µg/mouse), 11-200 × 3 + GPI-0100 (50 µg/mouse), 11-200 × 3 + GPI-0100 (200 µg/mouse), 11-200 × 3 + GPI-0100 (50 µg/mouse) + Tween-40 (1 mg/mouse). Approximately 4 months after the vaccination, a patch on the belly of each anesthetized BALB/c mouse was shaved with an electric razor without traumatizing the epithelium. Mice were then challenged with 3 × 10^9 HPV-16 pseudovirions (100 ng) in 10 µL of 0.6% carboxymethylcellulose carrying a luciferase reporter construct. Three days later, the mice were anesthetized and injected with luciferin. Images were acquired for 10 minutes with a Xenogen IVIS 200 bioluminescence imaging system. Equal-sized areas encompassing the site of inoculation were analyzed using Living Image 2.20 software, and the luminescence units were plotted using levels in mice vaccinated with HPV-16 L1 VLP before challenge as baseline. Individual data points and means (**horizontal lines**) are shown. HPV = human papillomavirus; ISS = immunostimulatory sequence; PBS = phosphate-buffered saline; VLP = virus-like particle.

11-200 × 3 is composed of L2 peptides from HPVs that cause genital infection (HPV-6, HPV-16, and HPV-18), and 11-88 × 5 contains peptides from these three HPV types plus those from HPV-1, which is from a phylogenetic group that causes nongenital warts, and HPV-5, from another phylogenetic group that also

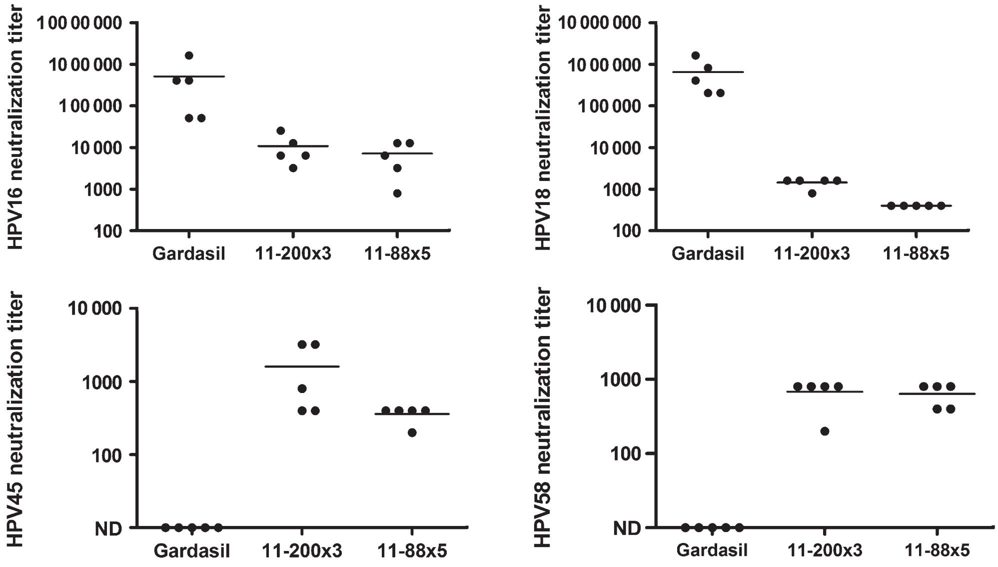

Figure 4. Neutralizing antibody responses of mice vaccinated with multitype L2 protein or Gardasil. Mice were vaccinated three times on days 0, 15, and 30 with Gardasil at one-fifth of a human dose or with 25 µg of L2 11-200 × 3 or 11-88 × 5 in GPI-0100 (50 µg) adjuvant. In vitro neutralization assays were performed with a twofold dilution series of the antisera of mice collected 2 weeks after final vaccination using pseudovirus of the HPV genotypes indicated. Endpoint titers for 50% neutralization are plotted and means shown as **horizontal lines**. HPV = human papillomavirus.

infects nongenital cutaneous sites. Infection by viruses related to HPV-5 may be asymptomatic, induce benign lesions, or be present in a subset of cutaneous squamous cell cancers. A potential advantage of 11-88 × 5 is that it might confer more potent protection against HPVs that infect nongenital cutaneous sites than 11-200 × 3, although both might be expected to confer a similar degree of protection against cutaneous and mucosal genital HPV infection. In mice, 11-88 × 5 and 11-200 × 3 showed similar potency against the HPVs that cause genital infection, but in rabbits, 11-88 × 5 induced stronger immunity to them than 11-200 × 3. In contrast to genital HPV infection, nongenital HPV infection is not usually sexually transmitted and frequently occurs in children. If future clinical testing of an immunogen similar to 11-88 × 5 were to show protection against infection at nongenital cutaneous sites in addition to protection against genital infection, it could provide a medical rationale for giving an L2-based vaccine in a time frame similar to that of childhood vaccines. Administration of an HPV vaccine to young children could render moot the theoretical issue that HPV vaccination of adolescents might influence their sexual behavior.

Oncogenic HPV infection causes approximately 5% of all cancer deaths globally. Its impact is greatest for women who are currently not reached by effective cervical cancer screening programs because approximately 80% of cervical cancers occur in low-resource settings in the developing world, and this malignancy accounts for the vast majority of cancer-related deaths attributable to HPV infection (59,60). If widely implemented, the current L1 VLP HPV vaccines have the potential to produce a dramatic drop in cervical cancer rates. However, these vaccines are currently too expensive for population-wide implementation in those settings that might benefit most from such a vaccine. Furthermore, because nearly a third of cervical cancer is caused by oncogenic HPV types not included in current HPV vaccine formulations, the type-restricted protection induced by the L1 VLP vaccines means that cervical cancer screening must remain in place in the industrialized world. In these settings, therefore, the cost of HPV vaccination must be borne in addition to that of screening, although cost savings might be achieved by altering the interval and/or modality by which vaccinated women are screened. Thus, it would be highly desirable to develop a more affordable vaccine for the developing world as well as to broaden protection against oncogenic HPV infections to a degree that would permit a major reduction in screening in the industrialized world. Although increasing the valency of the current L1 VLP vaccines represents a logical approach to overcome the latter problem (51), it seems likely that such a second-generation vaccine would, for many years, continue to be too expensive for widespread use in low-resource settings.

The weaker immune responses to multitype L2 vaccines as compared with L1 VLP raise concerns about the longevity of the response and the potential need to use adjuvant stronger than alum to achieve and maintain for the long-term comparable levels of immunity. Correlates of protection have not yet been defined for immunity in patients who have received HPV vaccines, although the presence of neutralizing antibody is likely important. Clinical studies are warranted to assess the safety and immunogenicity of multitype L2 vaccines in alum and other adjuvant formulations.

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