



Published in final edited form as:

Antivir Ther. 2012 ; 17(3): . doi:10.3851/IMP1941.

New Approaches to Prophylactic Human Papillomavirus Vaccines for Cervical Cancer Prevention

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Abstract

The currently licensed human papillomavirus (HPV) vaccines are safe and highly effective at preventing HPV infection for a select number of papillomavirus types, thus decreasing the incidence of precursors to cervical cancer. It is expected that vaccination will also ultimately reduce the incidence of this cancer. However, the licensed HPV vaccines are type-restricted and expensive, and also require refrigeration, multiple doses, and intramuscular injection. Second generation vaccines are currently being developed to address these shortcomings. New expression systems, viral and bacterial vectors for HPV L1 capsid protein delivery, and use of the HPV L2 capsid protein will hopefully aid in decreasing cost and increasing ease of use and breadth of protection. These second generation vaccines also could allow affordable immunization of women in developing countries, where the incidence of cervical cancer is high.

Introduction

Infections with high-risk human papillomaviruses (HPV) are associated with the development of cervical cancer. In the world population, 530,000 women develop cervical cancer and 275,000 women die from this disease each year [1]. In developed countries, screening in the form of Pap smears and protection through vaccination is readily available and attainable. However, in developing countries, screening and vaccination efforts are less feasible due to high cost and difficulties in implementation. Generally, the incidence of various types of cancer in women is similar between more developed and less developed countries. Cervical cancer defies this trend. Cervical cancer is the second leading cause of cancer death in women worldwide after breast cancer, and the incidence of cervical cancer is four-fold greater in less developed countries than in more developed countries mainly as a result of a lack in screening for cervical dysplasia [2].

HPV is a non-enveloped double-stranded DNA virus that infects squamous epithelial cells. More than 100 types of HPV have been identified, and 15 of these are considered to be high-risk types, i.e., oncogenic genital types [3]. HPV types 16 and 18 are associated with 70% of all cervical cancers. Infection with high-risk types, such as HPV16 and 18, are present in cases of cervical cancer, high grade cervical dysplasia, other genital and anal cancers, and oropharyngeal tumors. Low-risk types, including types 6 and 11, are associated with genital warts, low grade cervical dysplasia, and recurrent respiratory papillomatosis.

Disclosure statement

RLG has intellectual property related to L1 capsomeres managed by the University of Colorado. LG is a consultant to Sanofi Pasteur MSD and GlaxoSmithKline and receives royalty payments from the sales of Gardasil™ and Cervarix™.

Prevention is the key to decreasing new cases of cervical cancer. Prevention can be achieved via vaccination (primary) or screening (secondary), and treatment of precursor lesions. In 2006 and 2007, two prophylactic HPV vaccines were first approved by the responsible agencies in different countries. In the United States, they are now recommended by the Center for Disease Control for girls at age 11 or 12 and are available for females from ages 9 to 26. In addition, catch-up immunizations are recommended for females from ages 13 to 26. One of these vaccines, which also protects against genital warts, is available for males from ages 9 to 26. To date, either one or both of these vaccines have been licensed in more than 100 countries throughout the world.

The two currently available HPV vaccines, Cervarix™ and Gardasil™, are composed of recombinant HPV L1 capsid proteins expressed in insect or yeast cells, respectively, that are assembled into virus-like particles (VLPs). HPV VLPs resemble HPV virions, but are non-infectious because they lack the viral genome. The use of VLPs as vaccines is becoming prevalent. A VLP-based vaccine against Hepatitis B virus has been approved by the FDA, and both Norwalk and Influenza virus VLP vaccines are currently in clinical trials (reviewed in [4]).

Cervarix™ (GlaxoSmithKline) protects against HPV16 and 18, the high-risk types associated with 70% of cervical cancer, while Gardasil™ (Merck) protects against HPV16 and 18 as well as HPV6 and 11, the types responsible for 90% of genital warts. They are administered through intramuscular injection in three doses at 0, 1 or 2, and 6 month intervals. The cost for the vaccination series is about US\$ 360 (not including additional visit costs), an amount that exceeds the annual income of many citizens in developing countries. Clinical trials have been performed to determine the efficacy of Cervarix™ and Gardasil™ relative to HPV infection and disease endpoints. The vaccines are safe, highly immunogenic, and effective in preventing HPV infection and high grade cervical intraepithelial lesions. In addition, follow-up studies showed that these vaccines are protective for at least 5 years after administration [5, 6]; the efficacy and high immunogenicity of Cervarix™, in particular, has been recently demonstrated to persist for at least 7.3 years [7].

The current VLP vaccines induce immune responses to L1, but their effects are not therapeutic [8]. L1 is not detectably expressed in the basal epithelium, the location where the HPV infection is maintained. However, the HPV protein products E6 and E7 are expressed at this site, and the therapeutic vaccines under study usually target these HPV oncogenes.

Immunogenicity testing of HPV vaccines involves the measurement of antibodies in the sera of vaccinated individuals. Generally, three types of assays are used: the enzyme-linked immunosorbent assay (ELISA), the competitive Luminex immunoassay (CLIA), and the *in vitro* neutralization assay (reviewed in [9, 10]). In the ELISA, HPV antibodies in the serum bind to a HPV antigen on an ELISA plate, and this interaction is quantified. In the CLIA, VLPs are fixed to Luminex microspheres, serum is added, and the ability of the serum to prevent binding of a specific HPV monoclonal antibody is determined. In the *in vitro* neutralization assay, serum and pseudovirions containing a marker gene whose product can be easily quantified are added to cells. If the serum contains neutralizing antibodies, the cells will not be infected and the marker gene will not be expressed. While these assays are useful to evaluate immunogenicity of the HPV vaccines and to perform bridging studies to avoid large-scale clinical trials, they do not yet provide an immune correlate of protection. Development of a standard assay is still required for accurate vaccine comparisons.

HPV cervicovaginal challenge models have been recently developed that may lead to an immune correlate of protection [11, 12]. These animal models for cervicovaginal challenge may allow for the direct comparison of HPV vaccines. In this system, HPV pseudoviruses

that encapsidate a reporter plasmid are used to infect vaccinated mice. The cervix and vagina of mice that are protected from HPV infection will not express the reporter. Passive transfer of sera from vaccinated humans to these mice and subsequent pseudovirus challenge could provide a possible method for comparison of sera from clinical trials.

The limitations for the licensed vaccines include cost and requirements for cold chain, intramuscular injection, and multiple doses. Although there is evidence that the protection against infection is not restricted to the HPV types in the vaccine formulations, but also partially includes genetically related HPV types (e.g., HPV31 and 45) [13], these vaccines will not prevent all cases of cervical cancer. Thus, screening is still necessary to monitor abnormalities due to the other high-risk HPV types. As a consequence, many second generation prophylactic vaccines are being developed to resolve one or more of the aforementioned shortcomings. These second generation vaccines include new VLP-based vaccines, recombinant fusion proteins, recombinant viral and bacterial vectors for the delivery of L1, L2 capsid protein-based vaccines, and DNA vaccines.

L1 capsomere vaccines

The HPV capsid is comprised of two structural proteins: the major capsid protein L1 and the minor capsid protein L2. The L1 protein oligomerizes into pentamers, termed capsomeres. Seventy-two of these capsomeres constitute the icosahedral HPV capsid. The capsid also consists of 12 to 72 copies of the L2 protein [14], which contacts the pentameric L1 structure [15] and, possibly, the viral DNA.

Capsomeres may provide an alternative to the current VLP-based vaccines. L1 capsomeres can be produced when recombinant L1 is expressed in bacteria, in contrast to VLPs, which form in eukaryotic expression systems. Capsomeres are more stable and likely easier to produce, and thus potentially less expensive.

Protection from HPV is mediated through neutralizing antibodies directed against conformational epitopes. Neutralizing antibodies were shown to be markers of protection through an experiment in which passive transfer of animals with L1 VLP-specific serum provided protection against challenge [16]. Neutralizing conformational epitopes were demonstrated to be preserved on the surface of a capsomere [17] and, thus, should elicit an immune response. In fact, vaccination with L1 capsomeres results in high titers of neutralizing antibodies against L1. HPV11 [18] and 33 [19] L1 capsomeres induce neutralizing antibodies in rabbits and mice, respectively. In addition, dogs immunized with canine oral papillomavirus (GST-COPV) capsomeres are protected from challenge with COPV infection [20]. Ohlschlager *et al.* [21] vaccinated mice either subcutaneously or intranasally with HPV16 L1 capsomeres. Vaccination elicited L1-specific antibody production as well as cytotoxic T cell responses and regression of L1-expressing tumors. In more recent studies [22, 23], HPV16 L1 capsomeres were shown to induce similar titers of neutralizing antibodies as VLPs in mice and non-human primates when the appropriate adjuvants were added.

Research to express and purify L1 capsomeres from *Escherichia coli* is ongoing [24, 25]. Expression and purification schemes focus on truncated versions of the L1 protein in which pentamer-pentamer contacts are prevented, the inclusion of purification tags, the elimination of the chaperone protein GroEL, and the formulation of a product that is suitable for human use. It is expected that an optimized protocol will lower production costs, while maintaining vaccine efficacy.

Alternative L1 VLP vaccine expression systems

Other expression systems are also being optimized for L1 VLP production. Transgenic plants provide the ability to produce L1 VLPs on an agricultural scale [26–29]. Early research has shown that HPV11 L1 expressed in potatoes assembles into VLPs [30]. Ingestion of transgenic potatoes elicited an anti-L1 VLP immune response in mice although the practicality of this approach for human vaccination is questionable. More recent studies have focused on the expression of HPV16 L1 in transgenic tobacco plants. The yield of soluble protein recombinantly expressed in plants is a limiting factor for this expression system. Therefore, targeting signals for various cellular compartments were added to the L1 protein sequence [31, 32]. L1 targeted to tobacco chloroplasts showed an increase in solubility and was able to successfully assemble into VLPs. Injection of crude tobacco plant extracts into mice provided high titers of neutralizing antibodies against HPV16 L1 [32].

Baculovirus-infected insect cells are the expression platform for the manufacture of Cervarix™. A new inducible expression system using *Drosophila* Schneider cells has been developed for the expression and secretion of HPV16 L1 VLPs [33]. In this approach, the secretion step allows for a more straightforward purification protocol, thus decreasing expenses for scaled-up purification.

Another option is the use of a multimeric expression cassette within the recombinant baculovirus that significantly increases the yield of HPV VLPs [34]. Other systems for L1 VLP expression include lactic acid bacteria [35–37], various yeasts [38–41], and *Trichoplusia ni* larvae [42].

Recombinant viral and bacterial vectors for L1 delivery

Recombinant vectors can be used for the delivery of foreign proteins for immunization. Some second generation prophylactic HPV vaccines use other viruses or bacteria as vehicles for L1 delivery.

The Gluek group and collaborators have employed measles virus in this approach [43]. They created a recombinant measles virus that expresses HPV16 L1 from an open reading frame (ORF) inserted into its genome. Expression of L1 was stable and did not hinder viral replication. Mice inoculated with the recombinant measles virus produced high antibody titers against L1. Because one vaccination with the standard measles virus vaccine protects patients against measles for life, this recombinant measles virus system may improve the duration of HPV protection above that of the current vaccines. Measles virus was suggested to be a better vehicle than other RNA virus vectors because of its ability to incorporate large sequences into its capsid and to maintain small inserts that may be lost in other viral vectors due to recombination. In addition, this recombinant vector vaccine would lower the production cost over the currently available HPV vaccines and utilizes a measles virus strain that is already in use and is safe and efficacious. A recombinant measles vaccine for the delivery of L1 could be used to immunize against both measles and HPV, and a measles virus vector cocktail that incorporates ORFs for other foreign proteins could be included in routine vaccination series in the future. It is important to note that the use of measles virus as a recombinant vector for the delivery of L1 may be problematic in immunocompromised patients, as the measles vaccine is comprised of live attenuated virus.

A recombinant live bacterial vector system utilizes attenuated *Salmonella*. Nardelli-Haeffliger *et al.* have shown that live attenuated *Salmonella* promotes mucosal and systemic immune responses and have used this system to express foreign antigens. Strains of the serovar Typhimurium transformed with a codon-optimized HPV16 L1 plasmid were orally administered to mice. This recombinant vector vaccine induced high titers of HPV16 L1

neutralizing antibodies after only a single oral dose [44]. In a subsequent study, mice were intravaginally immunized with *Salmonella* expressing HPV16 L1 [45]. *Salmonella* infection itself promoted transient inflammatory responses in the genital mucosa, and its expression of L1 induced humoral responses against HPV16 that were similar to those induced from oral administration. This intravaginally administered vector vaccine also partially protected the mice against challenge with implanted HPV16 L1-expressing tumor cells. A *Salmonella*-based HPV vaccine suitable for human use has also been prepared [46], and nasal vaccination of mice with this L1-expressing *Salmonella* strain promoted the production of conformation-dependent neutralizing epitopes. High HPV16 neutralizing titers were observed in both serum and genital secretions. In addition to its possible use as a HPV vaccine, this L1 vector can also be employed as a typhoid vaccine, as the efficacy of protection against *Salmonella* infection was maintained even with the expression of L1.

Additional recombinant viral vectors for the delivery of L1 include poliovirus, vesicular stomatitis virus, and adeno-associated viruses. In early work, the potential of picornaviruses, specifically poliovirus, as viral vectors for L1 delivery was investigated [47]. Although recombinant poliovirus containing the complete ORF of HPV16 L1 produced VLPs, only a modest immune response was obtained after infection of susceptible transgenic mice with this recombinant viral vector. In a study of an attenuated vesicular stomatitis virus vector, the L1 sequence of cottontail rabbit PV (CRPV) was inserted into various sites of the viral genome to control the level of L1 protein expression [48]. When inserted into an upstream site in the genome versus a downstream site, L1 was more highly expressed and was able to induce high levels of anti-L1 antibodies in vaccinated rabbits. This viral vector also completely protected the vaccinated rabbits against CRPV challenge. Injection of mice with a recombinant adeno-associated virus encoding HPV16 L1 induced a strong neutralizing antibody response [49]. In fact, when co-injected with adenovirus encoding granulocyte macrophage-colony stimulating factor, this viral vector was suggested to perform as well as the VLP vaccine, which requires three injections. Subsequently, another adeno-associated virus vector for L1 delivery was employed in a single intranasal vaccination of mice [50]. This potential vaccine yielded a prolonged systemic and mucosal immune response without adjuvant. More recently, this class of intranasal viral vector was modified to include E7 in addition to L1 to produce a promising prophylactic and therapeutic HPV vaccine [51].

Other recombinant bacterial vectors include Bacille Calmette-Guerin (BCG), live attenuated *Shigella*, and *Listeria monocytogenes*. Bacille Calmette-Guerin, the vaccine for tuberculosis, is a live attenuated bovine bacillus that is inexpensive, safe, effective, and a strong adjuvant. Recombinant BCG has been used to express HPV6b L1 with modest results [52] and CRPV L1 with more promising results [53]. Rabbits immunized with recombinant BCG expressing CRPV L1 were protected against CRPV challenge up to five weeks in a dose-dependent manner. Live attenuated *Shigella*, a species of bacteria that causes dysentery, has also been employed to deliver HPV16 L1 [54] and HPV58 L1 [55]. Immunization of guinea pigs with these bacterial vectors resulted in the production of conformation-dependent, neutralizing antibodies to L1. The intracellular pathogen *Listeria monocytogenes* secretes listeriolysin O (LLO) and other proteins, which are targeted by the cellular immune system. By creating recombinant *L. monocytogenes* in which secretable fragments of HPV16 L1 were fused to LLO and orally administering this bacterium to mice, systemic and mucosal immune responses and protection against a HPV challenge model were obtained [56].

Multivalent L1 vaccines

Results from the clinical trials demonstrate that prevention against infection and the development of precursor lesions is higher than expected from the prevalence of HPV16 and 18 in the study population [13, 57]. This effect is most likely based upon cross-

neutralization of non-vaccine types [58, 59]. It is unclear, however, whether this additional immunity is as durable as that against HPV16 and 18.

Therefore, in order to increase the breadth of protection, it appears necessary to develop multivalent vaccines that protect against more types than Cervarix™ and Gardasil™. To achieve 92% protection from cervical cancer on a global scale, L1 capsomeres or VLPs from HPV types 31, 33, 45, 52, and 58 must be included in the vaccines in addition to HPV16 and 18 [60]. Formulation with L1 from additional high-risk types could increase protection to almost 100%, thus reducing the expense of screening for cervical abnormalities. The formulation of multivalent vaccines is currently underway, including a nonavalent L1 VLP vaccine, which is in clinical trials [61].

Even with a nonavalent vaccine, total protection against HPV will never be achieved. Cervical cancer rates should decrease, but will take 20 to 30 years to do so. In the interim, cervical cancer screening will still be necessary although it will not be required as often. In countries where screening is not possible, immunization is high priority. As immunization continues, more studies of vaccinated populations will be required to test the effectiveness of these vaccines. The endpoint of most current studies is infection or the development of precursor lesions, not the development of cervical cancer. In the long term, protection should be evaluated in terms of cervical cancer development.

Regions differ in their prevalence of HPV types determined by HPV testing of cervical specimens from women in various countries. After HPV16 and 18, the next three most frequent types are, in decreasing order, HPV31, 33, and 45 in the United States, HPV45, 33, and 35 in India, and HPV51, 45, and 31 in Argentina [62]. This variation may motivate the development of region-specific multivalent vaccines based on the prevalence and distribution of HPV types in specific populations. It should be noted that there is overlap in the most prevalent types across different regions. A universal multivalent vaccine that protects against all of these prevalent types is another option. Ultimately, cost will drive the production of region-specific vaccines *versus* a universal multivalent vaccine, i.e., if it is less expensive to produce multiple three- or four-type vaccines, region-specific vaccines will be beneficial, while if one vaccine that includes eight or nine types is more cost effective, a universal multivalent vaccine that can be distributed worldwide might be preferable.

An increase in the components of a multivalent vaccine likely comes with an increase in formulation and clinical trial complexity, which increases production cost and, ultimately, cost for the health care system. To lower the cost for multivalent vaccines, production should ideally occur in the regions in which they will be dispensed. Another potential negative for multivalent L1-based vaccines is that the neutralization activity for one type may outcompete that of other types although this has not yet been shown to be this case.

L2 vaccines

One possible solution to the limitations of a VLP-based multivalent HPV vaccine utilizes the HPV minor capsid protein L2. HPV L1-based vaccines provide predominantly type-specific protection, but can also provide limited cross-protection against infection with related types. For example, the sera from women vaccinated with Gardasil™ or Cervarix™ were evaluated for their ability to cross-neutralize pseudovirions of HPV types that are closely related to the types in the vaccine formulations [58, 59]. Gardasil™ and Cervarix™ were found to confer cross-protection for HPV45, and Cervarix™ can further cross-protect against HPV31. HPV45 and 31 are closely related to HPV16 and 18, respectively, with regard to their L1 amino acid sequences.

However, L2 has been shown to be widely cross-protective in animal models due to its highly conserved N-terminus, which contains neutralizing epitopes directed against L2. The amino acid sequence of the N-terminal region of many L2 types is presumably conserved due to the importance of this region to steps in viral entry. This region has been shown to interact with a currently unidentified receptor on the cell surface that is critical for infection, and the presence of an anti-L2 antibody can prevent this interaction [63]. Recently, Kondo *et al.* [64] immunized rabbits with short synthetic peptides corresponding to segments the surface region of HPV16 L2 between amino acids 14 to 144. The sera from these animals were able to neutralize HPV16 pseudovirions and cross-neutralize HPV18, 31, and 58 pseudovirions. Immunization of animals with L2 can induce the production of cross-neutralizing antibodies that protect against papillomavirus infection. The protection gained by vaccination with L2 is diverse, also protecting against papillomavirus types from different species. For example, HPV16 L2 vaccination protects rabbits challenged with CRPV [65].

L2 appears to be less immunogenic than L1 VLPs, and the immunogenicity acquired from vaccination with L2 may diminish over time. Immunization with the L2 protein does not produce neutralizing antibody titers to the scale of those from immunization with L1 VLPs although the *in vitro* pseudovirus neutralization assay may not be appropriate to judge comparative responses. Rabbits and cows vaccinated with N-terminal CRPV or BPV L2 peptides, respectively, developed neutralization titers ranging only from 1:5 to 1:100, but were protected against PV infection [66–69]. This discrepancy may reflect differences between the mechanism of neutralization *in vivo* and that measured by the routinely-used pseudovirion-based neutralization assay [11].

When L2 is in the context of a L1 VLP, neutralizing antibodies are not induced at detectable levels. A reason for this phenomenon has been delineated by Day *et al.* [70]. L2 is not exposed on the surface of the HPV capsid until the native virion binds to a host cell for infection. The L1 pentamers undergo a conformational change that allows for the display and cleavage of L2. Thus, the relevant L2 epitopes may not be displayed for antibody induction in the environment of a VLP until this step. An additional possibility is that the L1 epitopes may dominate over those of L2. However, even though vaccination elicits only low levels of neutralizing antibodies, immunization with L2 is sufficient for protection against PV challenge [11, 66, 71, 72].

L2 DNA vaccines

The potential of a DNA vaccine with the L2 gene for HPV prevention has been investigated. HPV DNA vaccines are likely safe, stable, easily administered, and inexpensive, although they may lack potency relative to viral vaccines. Hitzerth and colleagues have described preliminary work in this area [73]. The HPV16 L2 gene was cloned into pTH mammalian expression vector, which has been used in trials for a HIV DNA vaccine. Intramuscular injection of mice with L2 DNA gave only low antibody titers, and the antibodies were found to be non-neutralizing. When challenged with L2-expressing tumor cells, the tumor volume was reduced to 50% of that in the control animals, providing only some protection against HPV-related tumor growth.

Another HPV DNA vaccine gave more promising results [74]. This DNA vaccine encodes calreticulin, to enhance T-cell and humoral immune responses, and the HPV16 proteins E6, E7, and L2. Immunization of mice with this DNA vaccine was suggested to be prophylactic, as it elicited L2-specific antibodies as shown by ELISA and neutralizing antibodies as shown by neutralization assays using HPV16 pseudovirions. Moreover, this vaccine was therapeutic to E6/E7 expressing tumor cells.

Recombinant L2 fusion proteins

Recombinant L2 fusion proteins have been created in which L2 peptide epitopes are added to unrelated proteins to increase L2 immunogenicity. In one approach, the N-terminal 120 residues of HPV16 L2 or six peptides within this region were used as either one or multiple epitope copies that were added to the active site of bacterial thioredoxin, which is an exposed loop on the protein surface [75]. Thioredoxin, which stimulates murine T-cell proliferation, with the L2 epitopes in its exposed active site elicited strong immunogenicity. The immune response to the fusion proteins with multiple L2 epitope copies was found to surpass that of the fusion proteins with the corresponding mono-peptides. Thioredoxin with L2 residues 20–38 showed the strongest response of all tested peptides. This fusion protein induced strong neutralizing antibodies to HPV16 as well as cross-neutralizing antibodies against HPV18, 45, and 58. This thioredoxin-L2 fusion protein is a candidate for a low cost, cross-protective HPV vaccine and could also be used as a DNA vaccine or within a bacterial vector, such as *Salmonella*.

Residues 17–36 of HPV16 L2 have also been chemically coupled to a T helper epitope and a Toll-like receptor ligand to form a potential lipopeptide vaccine [72]. When injected or intranasally administered to mice, this lipopeptide neutralized pseudovirions from HPV16 as well as HPV5, 18, and 45 and BPV1. Mice challenged with pseudovirions from HPV16 and 45 were protected, suggesting the ability to use this synthetic lipopeptide as another future HPV vaccine.

In addition to fusing L2 to unrelated proteins or peptides, L2 epitopes from multiple HPV types have been fused to each other in concatenated multi-type L2 fusion peptides. Three concatenated multi-type fusion peptides have been created with the N-terminal epitopes for three, five, and 22 different types, respectively [71]. In a later study, the five-type fusion peptide, which contains amino acids 11–88 of L2 from HPV1, 5, 6, 16, and 18, was expressed in *E. coli*, purified, adjuvanted, and finally mixed with L1 capsomeres [76]. Sera of mice vaccinated with L1 capsomeres and the L2 multimer demonstrated excellent cross-neutralization activity for HPV35, 45, and 58. Hence, using a combination of L1 capsomeres with L2 fusion proteins, multi-type protection could be achieved along with decreased production costs.

L2 display in VLPs

Another method for increasing L2 immunogenicity involves displaying the antigen in a dense context, such as on the surface of a virus or VLP. Protective epitopes from HPV16 L2 have been displayed on bovine PV 1 VLPs [77]. Various L2 N-terminal fragments were inserted into the DE surface loop of BPV L1. The VLP with an L2 peptide containing residues 17–36 performed the best, as immunization of rabbits and mice with this VLP elicited neutralization against both high- and low-risk PV types, including 5, 6, 11, 16, 18, 31, 45, 52, and 58.

In another approach, HPV58 L1/L2 pseudovirions that encode HPV31 L2 were created in an attempt to simultaneously induce high titers of neutralizing antibodies to L1, which have been demonstrated for L1 VLP vaccines, and increase L2 immunogenicity by expressing L2 at higher levels than those achieved in a VLP context [78]. Mice immunized with the L1/L2 pseudovirions produced low titers of cross-neutralizing antibodies, while those immunized with pseudovirions encoding the L2 protein produced significantly higher titers, demonstrating the promise of this system as a new HPV vaccine strategy.

VLPs of other viruses have also been employed to display L2. For example, L2 has been inserted into the surface loop of the RNA bacteriophage PP7 coat protein [79].

Intramuscular injection of mice with these VLPs generated anti-HPV16 L2 serum antibodies and protected the mice against genital infection with HPV16 and 45 pseudoviruses.

HPV vaccine administration

Alternate routes of HPV vaccine delivery, including nasal, vaginal, oral, and lower airway administration, have been investigated to eliminate the current need for multiple intramuscular injections. These new delivery methods can induce a mucosal immune response in the genital tract, i.e., locally secreted, specific, neutralizing IgA antibodies, as well as a systemic response. The mucosal response is important to prevent HPV infection because this infection occurs in the genital mucosal membranes of the cervix and vagina. Intramuscular injection of the HPV VLPs promotes a systemic response and the production of HPV-specific neutralizing IgG antibodies, but not a mucosal response. Thus, investigation of alternative delivery methods for the HPV vaccine is advantageous for increased protection and could also facilitate vaccine implementation, especially in developing countries.

Johansson *et al.* used a model antigen to examine the effects of nasal and vaginal vaccination in women [80]. Nasal administration induced high antibody titers in the vagina, while vaginal vaccination at specific times during the menstrual cycle induced high antibody titers in the cervix. Therefore, a combination of both types of vaccination strategies could be used to prevent both the initial infection and the spread of virus.

Other groups have examined different vaccine delivery routes for HPV L1 VLPs, L1 capsomeres, and L2 peptides. For example, aerosol administration of HPV16 VLPs into the lower airway, i.e., the bronchi, of human volunteers by nebulization produced a mucosal response in their genital secretions and serum antibody titers similar to those from intramuscular administration, and this route was more efficient than nasal administration [81]. A follow up study demonstrated that the immunogenicity from this type of HPV vaccine delivery could be enhanced with the addition of mucosal adjuvants [82]. Lower airway delivery could also be optimized by altering nebulized particle size and possible formulation of HPV antigens into a powder form.

Balmelli *et al.* compared the immune responses to HPV16 VLPs administered parenterally, orally, or intranasally by measuring levels of IgG and IgA in serum, saliva, and genital secretions [83]. Systemic immunization alone produced IgG in the serum and modest levels of IgG in the genital tract, but no IgA. Oral immunization was inefficient. However, three nasal doses of HPV16 VLPs in anesthetized mice promoted high, long lasting, HPV16-specific IgG and IgA antibodies in saliva and genital secretions.

Alternative vaccination routes for L1 capsomeres have also been described, including intranasal vaccination with adeno-associated virus expressing HPV16 L1 and that with vesicular stomatitis virus expressing CRPV L1. One intranasal dose of the adeno-associated virus vector in mice produced high titers of L1-specific antibodies in serum and mucosal antibodies in vaginal washes [50]. Intranasal delivery of the vesicular stomatitis virus vector completely protected rabbits against challenge with CRPV after vaccination and boost [84].

Kawana and colleagues nasally immunized mice with a peptide containing amino acids 108–120 of the minor capsid protein L2 from HPV16 [85]. This vaccination elicited IgG and IgA antibodies against HPV6, 16, and 18 L1/L2 capsids in sera and vaginal secretions as shown by ELISA, and these antibodies neutralized HPV16 pseudovirions and HPV11 authentic virions.

Conclusions

The development of new prophylactic HPV vaccines is rapidly progressing. In the future, more affordable, cross-protective, efficacious HPV vaccines will be available worldwide. These vaccines may take advantage of innovative expression systems, recombinant bacterial or viral vectors for HPV protein delivery, and/or combinations of HPV L1 capsomeres or VLPs and L2 capsid proteins or peptides. They may be delivered in fewer doses and without needles, through inhalation, or oral or intravaginal administration. Hopefully, they will be affordable for developing countries and will reduce the costs for expensive Pap screening programs and the burden of precursor lesions in developed countries. Ultimately, these second generation HPV vaccines could decrease the incidence of cervical cancer by preventing infection of multiple high-risk HPV types.

Acknowledgments

RLG and EDF are supported by grants from the National Cancer Institute (P50CA098252, RC2CA148499).

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