Review

New approaches to prophylactic human papillomavirus vaccines for cervical cancer prevention

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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. The licensed HPV vaccines are, however, 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 could also 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. In developing countries, however, screening and vaccination efforts are less feasible because of the high cost and difficulties in implementation. Generally, the incidence of various types of cancer in women is similar in 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 fourfold 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, that is, oncogenic genital types [3]. HPV types 16 and 18 are associated with 70% of all cervical cancers. 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 tumours. Low-risk types, including types 6 and 11, are associated with genital warts, low-grade cervical dysplasia and recurrent respiratory papillomatosis.

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 HBV has been approved by the FDA, and both Norwalk (norovirus) 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, whereas 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 USD 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 end points. 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]. The L1 protein is not detectably expressed in the basal epithelium, the location where the HPV infection is maintained. The HPV protein products E6 and E7 are, however, 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: 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 an 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 is mixed with pseudovirions containing a marker gene and added to cells. If the serum contains neutralizing antibodies, the cells will not be infected and the marker gene will not be expressed. Although 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 might lead to an immune correlate of protection [11,12]. These animal models for cervicovaginal challenge might allow a 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 (for example, HPV31 and 45) [13], these vaccines will not prevent all cases of cervical cancer. Thus, screening is still necessary to monitor abnormalities resulting from the other high-risk HPV types. As a consequence, many second-generation prophylactic vaccines are being developed to resolve one or more of the previously mentioned 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 (Table 1).

L1 capsomere vaccines

The HPV capsid is composed of two structural proteins: the major capsid protein L1 and the minor capsid protein L2. The L1 protein oligomerizes into pentamers, termed capsomeres. In total, 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 might 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 probably easier to produce, and thus are 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 titres of neutralizing

Table 1. Strategies for second-generation prophylactic HPV vaccines

Strategy	Potential advantages	Potential disadvantages	Examples	References
L1 capsomere vaccines	Stability	Lower neutralizing antibody	HPV11, 16, 33 L1	[18,19,21–25]
	Bacterial production	titres than L1 VLPs	COPV L1	[20]
	Decrease in cost			
Alternative L1 VLP expression systems	Large-scale production in some systems	Decreased protein solubility in plants	Plants	[26–32]
			New insect cell systems	[33,34]
	Decrease in cost		Lactic acid bacteria	[35–37]
			Yeasts	[38–41]
			Insect larvae	[42]
Recombinant vectors for L1 delivery	Possible increase in duration of protection	Production cost	Measles	[43]
		Approval/acceptability	Poliovirus	[47]
	Use of vectors that are already in use, safe and effective		Vesicular stomatitis virus	[48]
			Adeno-associated viruses	[49–51]
	Combined immunization against vector and HPV		*Salmonella*	[44–46]
			Bacille Calmette-Guerin	[52,53]
	Possible decrease in dose number		*Shigella*	[54,55]
	Vector as adjuvant		*Listeria*	[56]
Multivalent L1 vaccines	Increase in breadth of protection	Increase in formulation and clinical trial complexity	Merck™ nonavalent V503	[61]
	Elimination of Pap screening			
	Region specificity	Production cost		
L2 vaccines	Cross-protection against multiple types	Less immunogenic than VLPs (although still protective)	L2 protein or N-terminal peptides	[64–69]
	Bacterial production			
Recombinant L2 fusion proteins	Possible increase in immunogenicity compared with L2 alone	Additional antigens might affect licensing	Thioredoxin +L2 peptides	[75]
			T-helper epitope +TLR ligand +L2 peptide	[72]
	Use as a DNA vaccine or vector vaccine		Concatenated L2 peptides	[71,76]
L2 display in VLPs	Increase in immunogenicity compared with L2 alone	Difficult to produce	Bovine PV1 L1 VLP +L2 peptide	[77]
		Additional antigens might affect licensing	HPV58 L1/L2 with encoded HPV31 L2	[78]
			RNA bacteriophage PP7+L2	[79]
HPV DNA vaccines	Stable	Decrease in immunogenicity	L2 DNA vaccine	[73]
	Easily administered		E6+E7+L2 DNA vaccine	[74]
	Inexpensive			
Alternative modes of vaccine delivery	Elimination of injection/needles	Decrease in immunogenicity	Nasal	[50,80–85]
	Induction of mucosal immune response	Decrease in duration of immune response	Vaginal	
			Oral	
	Facilitation of vaccine implementation		Lower airway	

COPV, canine oral papillomavirus; HPV, human papillomavirus; TLR, Toll-like receptor; VLP, virus-like particle.

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 (COPV) capsomeres fused to glutathione S-transferase are protected from challenge with COPV infection [20]. Ohlschläger *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 tumours. In more recent studies [22,23], HPV16 L1 capsomeres were shown to induce similar titres of neutralizing antibodies to those induced by 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. Targeting signals for various cellular compartments were therefore 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 titres 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 insect cells, specifically *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 [43] have used measles virus in this approach. 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 titres against L1. Because one vaccination with the standard measles virus vaccine protects patients against measles for life, this recombinant measles virus system might 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 could be lost in other viral vectors owing to recombination. In addition, the cost of production of this recombinant vector vaccine would be lower than that of the currently available HPV vaccines, and this recombinant system 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 could be problematic in immunocompromised patients because the measles vaccine is composed of live attenuated virus.

A recombinant live bacterial vector system utilizes attenuated *Salmonella*. Nardelli-Haefliger and coworkers 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 titres 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 by oral administration. This intravaginally administered vector vaccine also partially protected the mice against challenge with implanted HPV16 L1-expressing tumour 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 titres were observed in both serum and genital secretions. In addition to its possible use as an HPV vaccine, this L1 vector can also be employed as a typhoid vaccine because 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]. Indeed, 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*. BCG, 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 for up to 5 weeks in a dose-dependent manner. Live attenuated *Shigella*, a species of bacterium 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 that 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 probably based on 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.

To increase the breadth of protection, it therefore seems necessary to develop multivalent vaccines that protect against more types of HPV than do 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 end point 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 might motivate the development of region-specific multivalent vaccines on the basis of 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, that is, if it is less expensive to produce multiple three- or four-type vaccines, region-specific vaccines will be beneficial, whereas 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 probably comes with an increase in formulation and clinical trial complexity, which increases production cost and, ultimately, cost for the healthcare system. To lower the cost for multivalent vaccines, production should ideally occur in the regions in which they will be dispensed. Another potential

negative point for multivalent L1-based vaccines is that the neutralization activity for one type might out-compete that of other types, although this has not yet been shown to be the 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.

The L2 protein has, however, been shown to be widely cross-protective in animal models because of 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 because of the importance of this region to viral entry. This region has been shown to interact with a currently unidentified receptor on the cell surface that is crucial 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 of the surface region of HPV16 L2 between amino acids 14 and 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 seems to be less immunogenic than L1 VLPs, and the immunogenicity acquired from vaccination with L2 might diminish over time. Immunization with the L2 protein does not produce neutralizing antibody titres to the scale of those from immunization with L1 VLPs, although the *in vitro* pseudovirus neutralization assay might not be appropriate to judge comparative responses. Rabbits and cows vaccinated with N-terminal CRPV or bovine papillomavirus (BPV) L2 peptides, respectively, developed neutralization titres ranging only from 1:5 to 1:100, but were protected against

papillomavirus infection [66–69]. This discrepancy could 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 might not be displayed for antibody induction in the environment of a VLP until this step. An additional possibility is that the L1 epitopes might dominate over those of L2. However, even though vaccination elicits only low levels of neutralizing antibodies, immunization with L2 is sufficient for protection against papillomavirus 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 probably safe, stable, easily administered and inexpensive, although they could lack potency compared with viral vaccines. Hitzeroth and colleagues [73] have described preliminary work in this area. The HPV16 L2 gene was cloned into pTH mammalian expression vector, which has been used in trials for an HIV DNA vaccine. Intramuscular injection of mice with L2 DNA gave only low antibody titres, and the antibodies were found to be non-neutralizing. When challenged with L2-expressing tumour cells, the tumour volume was reduced to 50% of that in the control animals, providing only some protection against HPV-related tumour 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, because 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 tumour 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 monopeptides. 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 use of 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 3, 5 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 BPV1 L1 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, because immunization of rabbits and mice with this VLP elicited neutralization against both high- and low-risk papillomavirus 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 titres 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 titres of cross-neutralizing antibodies, whereas those immunized with pseudovirions encoding the L2 protein produced significantly higher titres, 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, that is, locally secreted, specific, neutralizing immunoglobulin A (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 immunoglobulin G (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*. [80] used a model antigen to examine the effects of nasal and vaginal vaccination in women. Nasal administration induced high antibody titres in the vagina, whereas vaginal vaccination at specific times during the menstrual cycle induced high antibody titres in the cervix. A combination of both types of vaccination strategies could therefore 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, that is, the bronchi, of human volunteers by nebulization produced a mucosal response in their genital secretions and serum antibody titres 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*. [83] 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. 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 anaesthetized 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 titres 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 [85] nasally immunized mice with a peptide containing amino acids 108–120 of the minor capsid protein L2 from HPV16. 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 could 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 might 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 programmes 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 by multiple high-risk HPV types.

References

1. WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre). Human papillomavirus and related cancers in the world. Summary Report 2010. (Updated 15 November 2010. Accessed 11 March 2011.) Available from http://apps.who.int/hpvcentre/statistics/dynamic/ico/country_pdf/XWX.pdf?CFID=4804166&CFTOKEN=32983136

2. Cohen J. Public health. High hopes and dilemmas for a cervical cancer vaccine. *Science* 2005; **308:**618–621.

3. Muñoz N, Bosch FX, Castellsague X, *et al*. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004; **111:**278–285.

4. Roldão A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM. Virus-like particles in vaccine development. *Expert Rev Vaccines* 2010; **9:**1149–1176.

5. Harper DM, Franco EL, Wheeler CM, *et al*. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 2006; **367:**1247–1255.

6. Villa LL, Costa RL, Petta CA, *et al*. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer* 2006; **95:**1459–1466.

7. De Carvalho N, Teixeira J, Roteli-Martins CM, *et al*. Sustained efficacy and immunogenicity of the HPV-16/18 AS04-adjuvanted vaccine up to 7.3 years in young adult women. *Vaccine* 2010; **28:**6247–6255.

8. Hildesheim A, Herrero R, Wacholder S, *et al*. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. *JAMA* 2007; **298:**743–753.

9. Schiller JT, Lowy DR. Immunogenicity testing in human papillomavirus virus-like-particle vaccine trials. *J Infect Dis* 2009; **200:**166–171.

10. Kwak K, Yemelyanova A, Roden RB. Prevention of cancer by prophylactic human papillomavirus vaccines. *Curr Opin Immunol* 2011; **23:**244–251.

11. Day PM, Kines RC, Thompson CD, *et al*. *In vivo* mechanisms of vaccine-induced protection against HPV infection. *Cell Host Microbe* 2010; **8:**260–270.

12. Roberts JN, Buck CB, Thompson CD, *et al*. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat Med* 2007; **13:**857–861.

13. Paavonen J, Naud P, Salmeron J, *et al*. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. *Lancet* 2009; **374:**301–314.

14. Buck CB, Cheng N, Thompson CD, *et al*. Arrangement of L2 within the papillomavirus capsid. *J Virol* 2008; **82**:5190–5197.

15. Finnen RL, Erickson KD, Chen XS, Garcea RL. Interactions between papillomavirus L1 and L2 capsid proteins. *J Virol* 2003; **77**:4818–4826.

16. Breitburd F, Kirnbauer R, Hubbert NL, *et al*. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* 1995; **69**:3959–3963.

17. Christensen ND, Kreider JW. Antibody-mediated neutralization *in vivo* of infectious papillomaviruses. *J Virol* 1990; **64**:3151–3156.

18. Rose RC, White WI, Li M, *et al*. Human papillomavirus type 11 recombinant L1 capsomeres induce virus-neutralizing antibodies. *J Virol* 1998; **72**:6151–6154.

19. Fligge C, Giroglou T, Streeck RE, Sapp M. Induction of type-specific neutralizing antibodies by capsomeres of human papillomavirus type 33. *Virology* 2001; **283**:353–357.

20. Yuan H, Estes PA, Chen Y, *et al*. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. *J Virol* 2001; **75**:7848–7853.

21. Ohlschläger P, Osen W, Dell K, *et al*. Human papillomavirus type 16 L1 capsomeres induce L1-specific cytotoxic T lymphocytes and tumor regression in C57BL/6 mice. *J Virol* 2003; **77**:4635–4645.

22. Thönes N, Muller M. Oral immunization with different assembly forms of the HPV 16 major capsid protein L1 induces neutralizing antibodies and cytotoxic T-lymphocytes. *Virology* 2007; **369**:375–388.

23. Stahl-Hennig C, Eisenblatter M, Jasny E, *et al*. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. *PLoS Pathog* 2009; **5**:e1000373.

24. Chen XS, Casini G, Harrison SC, Garcea RL. Papillomavirus capsid protein expression in *Escherichia coli*: purification and assembly of HPV11 and HPV16 L1. *J Mol Biol* 2001; **307**:173–182.

25. Schädlich L, Senger T, Kirschning CJ, Muller M, Gissmann L. Refining HPV 16 L1 purification from *E. coli*: reducing endotoxin contaminations and their impact on immunogenicity. *Vaccine* 2009; **27**:1511–1522.

26. Kohl TO, Hitzeroth II, Christensen ND, Rybicki EP. Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. *BMC Biotechnol* 2007; **7**:56.

27. Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. *Plant Biotechnol J* 2010; **8**:38–46.

28. Varsani A, Williamson AL, Stewart D, Rybicki EP. Transient expression of human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res* 2006; **120**:91–96.

29. Waheed MT, Thones N, Muller M, *et al*. Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines. *Transgenic Res* 2011; **20**:271–282.

30. Warzecha H, Mason HS, Lane C, *et al*. Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J Virol* 2003; **77**:8702–8711.

31. Lenzi P, Scotti N, Alagna F, *et al*. Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplastomic tobacco. *Transgenic Res* 2008; **17**:1091–1102.

32. Maclean J, Koekemoer M, Olivier AJ, *et al*. Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J Gen Virol* 2007; **88**:1460–1469.

33. Zheng J, Yang X, Sun Y, Lai B, Wang Y. Stable high-level expression of truncated human papillomavirus type 16 L1 protein in Drosophila Schneider-2 cells. *Acta Biochim Biophys Sin (Shanghai)* 2008; **40**:437–442.

34. Senger T, Schadlich L, Gissmann L, Muller M. Enhanced papillomavirus-like particle production in insect cells. *Virology* 2009; **388**:344–353.

35. Cortes-Perez NG, Kharrat P, Langella P, Bermudez-Humaran LG. Heterologous production of human papillomavirus type-16 L1 protein by a lactic acid bacterium. *BMC Res Notes* 2009; **2**:167.

36. Aires KA, Cianciarullo AM, Carneiro SM, *et al*. Production of human papillomavirus type 16 L1 virus-like particles by recombinant Lactobacillus casei cells. *Appl Environ Microbiol* 2006; **72**:745–752.

37. Cho HJ, Shin HJ, Han IK, *et al*. Induction of mucosal and systemic immune responses following oral immunization of mice with *Lactococcus lactis* expressing human papillomavirus type 16 L1. *Vaccine* 2007; **25**:8049–8057.

38. Bazan SB, de Alencar Muniz Chaves A, Aires KA, *et al*. Expression and characterization of HPV-16 L1 capsid protein in *Pichia pastoris*. *Arch Virol* 2009; **154**:1609–1617.

39. Park MA, Kim HJ. Optimum conditions for production and purification of human papillomavirus type 16 L1 protein from *Saccharomyces cerevisiae*. *Protein Expr Purif* 2008; **59**:175–181.

40. Kim SN, Jeong HS, Park SN, Kim HJ. Purification and immunogenicity study of human papillomavirus type 16 L1 protein in *Saccharomyces cerevisiae*. *J Virol Methods* 2007; **139**:24–30.

41. Sasagawa T, Tani M, Basha W, *et al*. A human papillomavirus type 16 vaccine by oral delivery of L1 protein. *Virus Res* 2005; **110**:81–90.

42. Millán AF, Gõmez-Sebastian S, Nuñez MC, Veramendi J, Escribano JM. Human papillomavirus-like particles vaccine efficiently produced in a non-fermentative system based on insect larva. *Protein Expr Purif* 2010; **74**:1–8.

43. Cantarella G, Liniger M, Zuniga A, *et al*. Recombinant measles virus-HPV vaccine candidates for prevention of cervical carcinoma. *Vaccine* 2009; **27**:3385–3390.

44. Baud D, Ponci F, Bobst M, De Grandi P, Nardelli-Haefliger D. Improved efficiency of a Salmonella-based vaccine against human papillomavirus type 16 virus-like particles achieved by using a codon-optimized version of L1. *J Virol* 2004; **78**:12901–12909.

45. Echchannaoui H, Bianchi M, Baud D, *et al*. Intravaginal immunization of mice with recombinant *Salmonella enterica* serovar Typhimurium expressing human papillomavirus type 16 antigens as a potential route of vaccination against cervical cancer. *Infect Immun* 2008; **76**:1940–1951.

46. Fraillery D, Baud D, Pang SY, *et al*. *Salmonella enterica* serovar Typhi Ty21a expressing human papillomavirus type 16 L1 as a potential live vaccine against cervical cancer and typhoid fever. *Clin Vaccine Immunol* 2007; **14**:1285–1295.

47. van Kuppeveld FJ, de Jong A, Dijkman HB, Andino R, Melchers WJ. Studies towards the potential of poliovirus as a vector for the expression of HPV 16 virus-like-particles. *FEMS Immunol Med Microbiol* 2002; **34**:201–208.

48. Roberts A, Reuter JD, Wilson JH, Baldwin S, Rose JK. Complete protection from papillomavirus challenge after a single vaccination with a vesicular stomatitis virus vector expressing high levels of L1 protein. *J Virol* 2004; **78**:3196–3199.

49. Liu DW, Chang JL, Tsao YP, *et al*. Co-vaccination with adeno-associated virus vectors encoding human papillomavirus 16 L1 proteins and adenovirus encoding murine GM-CSF can elicit strong and prolonged neutralizing antibody. *Int J Cancer* 2005; **113**:93–100.

50. Kuck D, Lau T, Leuchs B, *et al*. Intranasal vaccination with recombinant adeno-associated virus type 5 against human papillomavirus type 16 L1. *J Virol* 2006; **80**:2621–2630.

51. Nieto K, Kern A, Leuchs B, *et al*. Combined prophylactic and therapeutic intranasal vaccination against human papillomavirus type-16 using different adeno-associated virus serotype vectors. *Antivir Ther* 2009; **14**:1125–1137.

52. Jabbar IA, Fernando GJ, Saunders N, *et al*. Immune responses induced by BCG recombinant for human papillomavirus L1 and E7 proteins. *Vaccine* 2000; **18**:2444–2453.

53. Govan VA, Christensen ND, Berkower C, Jacobs WR, Jr., Williamson AL. Immunisation with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L1 gene provides protection from CRPV challenge. *Vaccine* 2006; **24**:2087–2093.

54. Yang XF, Qu XZ, Wang K, *et al*. Construction of prophylactic human papillomavirus type 16 L1 capsid protein vaccine delivered by live attenuated *Shigella flexneri* strain sh42. *Acta Biochim Biophys Sin (Shanghai)* 2005; **37**:743–750.

55. Li W, Liu H, Yang X, *et al*. Development of prophylactic recombinant HPV58-attenuated Shigella live vector vaccine and evaluation of its protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model. *Acta Biochim Biophys Sin (Shanghai)* 2009; **41**:137–145.

56. Mustafa W, Maciag PC, Pan ZK, *et al*. *Listeria monocytogenes* delivery of HPV-16 major capsid protein L1 induces systemic and mucosal cell-mediated CD4$^+$ and CD8$^+$ T-cell responses after oral immunization. *Viral Immunol* 2009; **22**:195–204.

57. Brown DR, Kjaer SK, Sigurdsson K, *et al*. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16-26 years. *J Infect Dis* 2009; **199**:926–935.

58. Smith JF, Brownlow M, Brown M, *et al*. Antibodies from women immunized with Gardasil cross-neutralize HPV 45 pseudovirions. *Hum Vaccin* 2007; **3**:109–115.

59. Kemp TJ, Hildesheim A, Safaeian M, *et al*. HPV16/18 L1 VLP vaccine induces cross-neutralizing antibodies that may mediate cross-protection. *Vaccine* 2011; **29**:2011–2014.

60. Bosch FX, Burchell AN, Schiffman M, *et al*. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine* 2008; **26 Suppl 10**:K1–K16.

61. Merck. Merck Pipeline. (Updated 16 February 2011. Accessed 13 April 2011.) Available from http://www.merck.com/research/pipeline/home.html

62. WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre). Human papillomavirus and related cancers, fact sheets 2010. (Updated 15 September 2010. Accessed 11 March 2011.) Available from http://apps.who.int/hpvcentre/statistics/dynamic/ico/SummaryReportsSelect.cfm

63. Gambhira R, Karanam B, Jagu S, *et al*. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. *J Virol* 2007; **81**:13927–13931.

64. Kondo K, Ishii Y, Ochi H, *et al*. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology* 2007; **358**:266–272.

65. Gambhira R, Jagu S, Karanam B, *et al*. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. *J Virol* 2007; **81**:11585–11592.

66. Campo MS, O'Neil BW, Grindlay GJ, *et al*. A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. *Virology* 1997; **234**:261–266.

67. Chandrachud LM, Grindlay GJ, McGarvie GM, *et al*. Vaccination of cattle with the N-terminus of L2 is necessary and sufficient for preventing infection by bovine papillomavirus-4. *Virology* 1995; **211**:204–208.

68. Embers ME, Budgeon LR, Pickel M, Christensen ND. Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. *J Virol* 2002; **76**:9798–9805.

69. Gaukroger JM, Chandrachud LM, O'Neil BW, *et al*. Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies. *J Gen Virol* 1996; **77**:1577–1583.

70. Day PM, Gambhira R, Roden RB, Lowy DR, Schiller JT. Mechanisms of human papillomavirus type 16 neutralization by L2 cross-neutralizing and L1 type-specific antibodies. *J Virol* 2008; **82**:4638–4646.

71. Jagu S, Karanam B, Gambhira R, *et al*. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. *J Natl Cancer Inst* 2009; **101**:782–792.

72. Alphs HH, Gambhira R, Karanam B, *et al*. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. *Proc Natl Acad Sci U S A* 2008; **105**:5850–5855.

73. Hitzeroth II, Passmore JA, Shephard E, *et al*. Immunogenicity of an HPV-16 L2 DNA vaccine. *Vaccine* 2009; **27**:6432–6434.

74. Kim D, Gambhira R, Karanam B, *et al*. Generation and characterization of a preventive and therapeutic HPV DNA vaccine. *Vaccine* 2008; **26**:351–360.

75. Rubio I, Bolchi A, Moretto N, *et al*. Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20–38) peptide displayed on bacterial thioredoxin. *Vaccine* 2009; **27**:1949–1956.

76. Jagu S, Kwak K, Garcea RL, Roden RB. Vaccination with multimeric L2 fusion protein and L1 VLP or capsomeres to broaden protection against HPV infection. *Vaccine* 2010; **28**:4478–4486.

77. Schellenbacher C, Roden R, Kirnbauer R. Chimeric L1-L2 virus-like particles as potential broad-spectrum human papillomavirus vaccines. *J Virol* 2009; **83**:10085–10095.

78. Combelas N, Saussereau E, Fleury MJ, *et al*. Papillomavirus pseudovirions packaged with the L2 gene induce cross-neutralizing antibodies. *J Transl Med* 2010; **8**:28.

79. do Carmo Caldeira J, Medford A, Kines RC, *et al*. Immunogenic display of diverse peptides, including a broadly cross-type neutralizing human papillomavirus L2 epitope, on virus-like particles of the RNA bacteriophage PP7. *Vaccine* 2010; **28**:4384–4393.

80. Johansson EL, Wassen L, Holmgren J, Jertborn M, Rudin A. Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans. *Infect Immun* 2001; **69**:7481–7486.

81. Nardelli-Haefliger D, Lurati F, Wirthner D, *et al*. Immune responses induced by lower airway mucosal immunisation with a human papillomavirus type 16 virus-like particle vaccine. *Vaccine* 2005; **23**:3634–3641.

82. Revaz V, Zurbriggen R, Moser C, *et al*. Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants. *Antiviral Res* 2007; **76**:75–85.

83. Balmelli C, Roden R, Potts A, *et al*. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *J Virol* 1998; **72**:8220–8229.

84. Reuter JD, Vivas-Gonzalez BE, Gomez D, *et al*. Intranasal vaccination with a recombinant vesicular stomatitis virus expressing cottontail rabbit papillomavirus L1 protein provides complete protection against papillomavirus-induced disease. *J Virol* 2002; **76**:8900–8909.

85. Kawana K, Kawana Y, Yoshikawa H, *et al*. Nasal immunization of mice with peptide having a cross-neutralization epitope on minor capsid protein L2 of human papillomavirus type 16 elicit systemic and mucosal antibodies. *Vaccine* 2001; **19**:1496–1502.