Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms

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Abstract

Human papillomavirus virus-like particles (HPV VLP) can be generated by the synthesis and self-assembly in vitro of the major virus capsid protein L1. HPV L1 VLPs are morphologically and antigenically almost identical to native virions, and this technology has been exploited to produce HPV L1 VLP subunit vaccines. The vaccines elicit high titres of anti-L1 VLP antibodies that persist at levels 10 times that of natural infections for at least 48 months. At present the assumption is that the protection achieved by these vaccines against incident HPV infection and HPV-associated ano-genital pathology is mediated via serum neutralising Immunoglobulin G (IgG). However, since there have been very few vaccine failures thus far, immune correlates of protection have not been established. The available evidence is that the immunodominant neutralising antibodies generated by L1 VLPs are type-specific and are not cross-neutralising, although highly homologous HPV pairs share minor cross-neutralisation epitopes. Important issues remaining to be addressed include the duration of protection and genotype replacement.

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1. Rationale for HPV L1 vaccines

A successful immune response to genital HPV infections is characterised by strong, local cell-mediated immunity (CMI) that is associated with lesion regression and protection against a further infection with the same genotype of HPV. Humoral immunity (antibody) is generated in most, but not all, infected individuals [1] and is directed against conformational epitope(s) on the major coat or capsid protein L1 displayed on the outer surface of the intact virus particle. This antibody is held to be virus neutralising, as post-infection serum and monoclonal antibodies of the same specificity can each prevent infection by HPV in various in vitro and in vivo models. Serum-neutralising antibody levels in natural HPV infections, even at peak titres just after seroconversion, are low [2]. This low response probably reflects the exclusively intra-epithelial infectious cycle of the papillomaviruses and the consequent absence of a viraemia. Furthermore, L1 protein is expressed during a productive papillomavirus infection and virus particles are assembled in the cells of the surface layers of the epithelium, in the absence of cytolysis and inflammation, far from most antigen presenting cells (APCs) and patrolling macrophages. As a result, virus particles and their capsid proteins must be displayed in limited amounts in the draining lymph nodes and spleen, the classical sites for the initiation of antibody responses. Despite low antibody levels, at least in the animal models, seropositive individuals are protected, probably for life, against further viral challenge, thus suggesting that vaccines that generate neutralising antibodies to HPV capsid protein will be effective prophylactically. It is difficult to know the degree to which such protection is mediated against neutralising antibodies to capsid proteins or to CMI directed against structural or non-structural proteins. Patients with antibody immunodeficiency (e.g. common variable immunodeficiency) are no more susceptible to re-infection with cutaneous HPVs than healthy subjects, which suggests that while antibody may be sufficient for protection it is not necessary. However, early studies...
with the Shope cotton tail rabbit papillomavirus (CRPV) indicated that systemic immunisation with infectious extracts under conditions that did not induce visible lesions did induce neutralising antibody and could confer protection against CRPV challenge [3], thereby suggesting that vaccines that generate neutralising antibodies to HPV capsid protein might be effective prophylactically.

The immunodominant neutralising epitopes are located on the major capsid protein L1, which must be in the tertiary or native form assembled as a multimer for the generation of neutralising antibody. When expressed via recombinant yeast or viral vectors (Chapters 11 and 13), the L1 protein self-assembles into empty capsids or virus-like particles (VLPs) that are morphologically and antigenically almost identical to native virions (Fig. 1), and this technology has been exploited to produce HPV L1 VLP subunit vaccines.

2. Current HPV L1 VLP vaccines

Two HPV L1 VLP vaccines have been developed commercially and are, at present, in phase-III trials. Cervarix™ is a bivalent HPV-16/18 L1 VLP vaccine developed by GlaxoSmithKline (GlaxoSmithKline Biologicals, Rixensart, Belgium). In this preparation the L1 protein of each HPV type is expressed via a recombinant baculovirus vector; the VLPs of each HPV type are produced separately and then combined. The product consists of purified L1 VLPs of HPV types 16/18 at 20/20 μg per dose formulated on an ASO4 adjuvant comprising 500 μg of aluminium hydroxide and 50 μg of 3-deacylated monophosphoryl lipid A. The product is delivered by intra-muscular (i.m.) injection in a three-shot immunisation protocol at 0, 1 and 6 months as a 0.5-mL dose. Gardasil® is a quadrivalent HPV-16/18/6/11 L1 VLP vaccine developed by Merck and Co. Inc., West Point, Pennsylvania, USA. The L1 protein for each HPV VLP type is expressed via a recombinant Saccharomyces pombe vector and the product consists of purified L1 VLPs of HPV types 6/11/16/18 at 20/40/40/20 μg per dose formulated on a proprietary alum adjuvant. The product is delivered by i.m. injection as a 0.5-mL dose in a three-shot immunisation protocol at 0, 2 and 6 months. The critical issue of vaccine efficacy and how to ascertain this is discussed in Chapter 13 of this monograph.
3. Immune correlates of protection

Data from the published phase-II and phase-III trials of HPV L1 VLP vaccines demonstrate clearly that both the bivalent and quadrivalent vaccines protect the vaccinees from persistent cervical HPV-16/18 infection and cervical HPV-16/18-induced disease; the quadrivalent vaccine also protects women against HPV-6/11-induced mucosal and cutaneous genital disease (see Chapter 13). The mechanisms by which such protection is effected are not fully understood and at present there are no immune correlates that unequivocally denote protection since, to date, all VLP-immunised subjects have sero-converted and there have been very few vaccine failures to analyse. What is known, however, is that VLPs are highly immunogenic and in the studies reported to date VLP-immunised individuals have made anti-VLP antibody responses substantially greater (at least 1–3 logs) than that identified in natural infections. As discussed above, L1 antibody levels are low in natural infections and there is probably little or no viraemia and no evolutionary pressure on the virus to escape the antibody response. In contrast to natural infection, L1 VLP vaccines are delivered i.m., which allows them to gain access to the draining lymphatics and small vessels at the injection site, thereby effectively mimicking a viraemia. One can speculate that this explains, in part, the intensity of the antibody responses induced by these preparations.

3.1. Immunoassays

The measurement of specific serum immunoglobulin G (IgG) anti-L1 VLP antibodies by immunoassays in vaccinated and unvaccinated individuals is the main parameter used in the current vaccine trials to monitor vaccine-induced immune responses, therefore the technical details of the assays used to measure these are of interest. The methodologies used in the trials of the quadrivalent Gardasil® vaccine differ from those employed in the evaluation of the bivalent Cervarix™ vaccine and direct comparisons of antibody responses to the different vaccines are therefore not feasible.

For the quadrivalent vaccine serum antibodies to HPV-6, -11, -16 and -18 are measured using a competitive radioimmunoassay (cRIA) [4] or a competitive Luminex immunoassay (cLIA) [5]. In brief, in the cRIA, polystyrene beads are coated with a limiting amount of VLP antigen and incubated with the corresponding HPV type-specific, neutralising, mouse monoclonal antibody (M. Ab and the serum sample). The amount of M. Ab bound to the VLP antigen is measured after incubation with ¹²⁵I-labelled goat anti-IgG. In the cLIA, HPV L1 VLPs are conjugated to Luminex microspheres and incubated with phycoerythrin-labelled type-specific neutralising M. Ab and a serum sample. The amount of bound dye is quantitated with a Luminex Biopex instrument. These competitive assays, because they use a competitor M. Ab, measure only those serum antibodies that bind to, or compete with, the single neutralising epitope that binds the M. Ab on the specific VLPs and are not a measurement of total serum anti-L1 VLP IgG. A disadvantage of such assays is that, since affinities of binding and other parameters of antibody–antigen interaction will differ between the different monoclonal antibodies used, direct comparisons of antibody titres generated by the different VLPs (6, 11, 16, 18) in the vaccine cannot be made. The advantages of the competitive assays are that neutralising antibody is assayed specifically, the antibody response to each VLP is measured in the same test and, in general in such assays, backgrounds are low, sensitivity is enhanced and spurious cross-reactions are reduced.

For the bivalent HPV-16/18 vaccine, measurement of serum antibody to HPV-16 and -18 VLPs was performed using a conventional enzyme-linked immunoassay (ELISA) [6]. In this assay, microwell plates coated with purified recombinant HPV-16 or -18 VLPs are incubated with serial dilutions of sera, the bound antibody is reacted with horseradish peroxidase conjugated goat anti-human IgG and the colour intensity quantitated by optical density measurements. The advantages of such an assay are that total serum anti-VLP IgG is measured and direct comparisons can be made between responses to individual VLPs. A disadvantage is that the antibody response to individual VLPs is assayed in separate tests, the fraction of serum IgG attributable to neutralising antibody specifically cannot be quantitated, but the available data [7,8] suggest that neutralising and ELISA antibodies titres are usually highly correlated (see also Chapter 23).

4. Mechanisms of protection

4.1. Serum neutralising antibody

Currently, the best assumption is that the mechanism of protection elicited by VLPs is serum antibody mediated via the high titres of neutralising serum antibody induced by these vaccines [9,10]. In animal infections, seroconversion and the generation of anti-L1 neutralising IgG is associated with protection against challenge with infectious virus. However, the most unequivocal evidence for the notion that antibody is the protective mechanism comes from passive transfer experiments in rabbits [11] and dogs [12]. In rabbits, passive transfer of serum or purified IgG from hyperimmune animals immunised with CRPV L1/L2 VLPs completely protected the naïve recipients from challenge with 10¹⁰ infectious virus particles. Of the four animals challenged with 10¹¹ virus particles, three developed small papillomas that rapidly regressed. In a study by Ghim et al. [12], 12-week-old beagle dogs shown to be negative for maternal antibody to canine oral papillomavirus (COPV) were immunised with purified IgG or total hyperimmune IgG derived from hyperimmune serum from rabbits immunised with COPV L1 VLPs. The animals immunised with anti-COPV L1 IgG were completely protected from challenge with high levels of virus.
These studies demonstrate that serum IgG can confer protection, and the most likely explanation is that this is mediated via serum neutralising IgG. However, it should be noted that although there is no doubt that protection was via serum IgG in these experiments, the evidence that neutralising IgG mediates the effect was indirect in both. Nonetheless, the overall data consistently show that L1 VLPs induce high levels of serum neutralising IgG, and the results of VLP vaccination both in animal infections and clinical trials support the notion that it is this activity that is critical for protection. It seems unlikely that local mucosal IgA mediates significant protection in vaccinees since, although cervico-vaginal lavage fluid harvested from immunised primates neutralises HPV-11 particles [13], only 50% of women immunised with HPV-11 L1 VLPs were shown to develop local mucosal anti-HPV antibody [14]. IgG is the principal immunoglobulin in cervical secretions, and the assumption at the present, is that protection is mediated by serum IgG (predominantly neutralising IgG) that can transude across the cervical epithelium, particularly at the squamo-columnar junction, in high enough concentration to bind to virus particles and prevent infection. Systemic IgG levels are substantially higher than those in cervical secretions [15], and it is possible that potential sites of infection on cutaneous and mucosal epithelial surfaces may have access to systemic antibodies. This situation may arise because the establishment of infection requires that the virus come into direct contact with keratinocytes in the basal layer of the epithelium. Epithelial microtrauma, as can occur during intercourse, would increase the likelihood of such exposure, and in such a scenario the site of potential infection would have more direct access to systemic IgG. Consistent with this, the excellent efficacy of Gardasil® against external genital warts [16,17] that develop on a fully keratinised cutaneous epithelium, not bathed in mucosal secretions, would support the notion that: (1) microtrauma is important for the establishment of infection and (2) access to systemic IgG at such sites makes an important contribution to the protective activity of the vaccine.

5. **Virion structure**

Precise information about virus structure at the molecular level is central to any understanding of antibody-mediated immune protection. Papillomaviruses have an icosahedral structure and the outer shell, or capsid, contains 72 pentamers, or capsomers of L1, the major capsid protein, centred on the vertices of a $T = 7$ icosahedral lattice. The L2 protein is largely an internal protein and is present at about one-thirtieth of the abundance of L1. As discussed before, the L1 VLP is almost identical both morphologically (Fig. 1) and antigenically to the infectious virus particle [18]. Of its 72 pentamers, 60 interact with 6 neighbouring capsomers and 12 interact with 5 neighbours. The only HPV L1 crystal structure solved at the present is a $T = 1$ particle, a "small VLP" of 12 pentamers in which the pentamers are all in the pentavalent position and the capsomers interact via the C' tail [19].

A knowledge of the crystal structure of the protein is important for the prediction and identification of antigenic sites. The L1 proteins of the papillomaviruses are highly conserved and, indeed, the basis for classification of a new isolate as a new HPV type is that the L1 sequences differ by 10% or more compared to all known types. Chen et al. [19] aligned and compared the L1 amino acid sequences of 49 different HPV types, and it is evident from this analysis that the highly variable regions that classify these as different HPV types are interspersed among segments of conserved residues. When these sequences are displayed in three dimensions it is clear that the hypervariable regions lie on the outward or surface-exposed face of the capsomer in five loops, each 10–30 amino acids long, that protrude from a pillar comprised of the conserved regions more deeply embedded in the particle (Fig. 2). Both conformational and linear epitopes recognised

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**A MODEL OF THE L1 PENTAMER IN 3 DIMENSIONS**

![A three-dimensional appearance of the L1 pentamer](image)

**Fig. 2.** The three-dimensional appearance of the L1 pentamer [5]. (A) A side view of the pentamer. The red surface loops are the hypervariable regions poking out from the surface; these are obvious immune targets. (B) A view of the pentamer from the surface and slightly tilted; the red loops are protruding from the surface. (C) A view deep into the pentamer showing the blue core of conserved regions. The particle needs to be disrupted and degraded before these are seen by the immune system.
by neutralising monoclonal and polyclonal antisera have been mapped to these loops (see Ref. [20] and references therein). Furthermore, the loop domains can present non-L1 peptides and direct immune responses specifically to these sequences. These surface loops not only display potential virus-neutralising epitopes but the highly ordered, close-packed surface structure of the capsomer makes them highly immunogenic. All the evidence suggests that type specificity is determined by the loop epitopes. HPV-16 strains that have variant L1 sequences have been isolated, but evidence both from structural [19] and experimental studies [21] shows that neutralising antibodies directed against wild-type HPV-16 epitopes effectively neutralise the known HPV-16 L1 variants.

5.1. Type specificity

Serological studies with VLPs have shown that antibodies cross-reactive with multiple HPV VLP types recognise type-common epitopes and are, in general, linear and non-neutralising. The available evidence is that immunodominant neutralising antibodies generated by L1 VLPs are type-specific and are not cross-neutralising [22]. Highly homologous VLPs such as HPV-6/11 [23], HPV-31/33, HPV-18/45 [22] and HPV-16/31 [24] share one or more cross-neutralisation epitopes. However, these cross-neutralisation epitopes appear to be less immunogenic than the type-specific epitopes [24,25]. A linear, type-common neutralisation epitope has been described, but the levels of antibody generated against this determinant are considered to be too low to be effective in protection [25].

Since HPVs cannot be grown in adequate amounts in tissue culture, neutralisation assays have presented a challenge. Neutralisation assays were based initially on the nude/SCID mouse xenograft system in which tissue chips are infected in vitro with virus and then inserted under the renal capsule of a profoundly immunocompromised mouse [26]. This technology is technically demanding and expensive and the numbers of HPV types that have been grown successfully in this model are limited [27]. Most studies now rely on the generation of pseudovirions in vitro, an approach that can be a relatively high-throughput assay [28]. These pseudovirions are HPV L1/L2 VLPs that can package plasmid DNA (usually a reporter or marker gene) in both cellular and acellular systems. The pseudovirions are “infectious” and can transfer the marker plasmid (usually a reporter gene such as green fluorescent protein or luciferase) to cells, where it is expressed. Thus, measurement of reporter-gene expression quantitates neutralisation of the pseudovirion by the test antiserum [28]. As with authentic virus, pseudovirion infection depends upon both L1 and L2 and can be neutralised by L1 and L2 antibodies. The mechanism of L1-mediated antibody neutralisation may be heterogeneous, although there are some antibodies that, at high concentration, block cell attachment by steric interference with the virus receptor site or by binding across the groove between the pentamers, thus stabilising the particle and preventing virus entry [18].

5.2. Epitope mapping

Epitope mapping has been pursued in most studies using a domain-swapping approach, i.e. exchanging segments of L1 protein between different HPV types. This procedure generates hybrid VLPs or capsomers containing a putative epitope-expressing region of one HPV type on the L1 backbone of another HPV type, such as HPV-16 on HPV-11 [29] or HPV-16 on COPV [20]. These hybrid VLPs are then assayed in ELISA for reactivity with monoclonal antibodies known to recognise conformational and/or neutralising antibodies to specific HPV types. Using these approaches it has been shown that all conformation-dependent, type-specific M. Ab epitopes identified to date reside in one or more of the surface hypervariable loops (see Ref. [23] and references therein) or the C-terminus of L1. Fine mapping of the surface epitopes of HPV-6 L1 by mutating surface-exposed loops to corresponding sequences of HPV-11 L1 and using the resultant hybrid capsomers in ELISA with sera from HPV-6 infected-individuals [23] revealed that these individuals generate anti-L1 antibodies that target a complex set of epitopes that are both type-specific for HPV-6 and cross-reactive with HPV-11. There was no single immunodominant epitope recognised by all sera, and sera from different individuals targeted different loops of L1. Importantly, the patterns of epitope recognition did not change over time and remained stable even nine years after the first seropositive sample. This observation suggests, first, there could be cross-protection between HPV-6 and -11 and, second, that immune escape variants are unlikely to arise as a consequence of immune selection after vaccination.

The evidence is that HPV L1 VLPs generate serum-neutralising antibody responses that are, on the whole, type-specific and do not cross-neutralise. Further, the evidence from the experimental animal models is that these neutralising antibodies are crucial for protection against challenge with high levels of infectious virus of the same type. The antibody responses in humans to HPV VLP vaccination are not in conflict with these data. There is preliminary evidence [9] that HPV-16/18 vaccinees are partially protected against infection with HPV-31 and -45. Cross-reactive and cross-neutralising antibodies are generated after vaccination with the quadrivalent vaccine (N. Muñoz, personal communication, April 2006). The experimental evidence suggests that partial cross-protection between the closely related pairs 18 and 45, 6 and 11, and 16 and 31 is possible since each pair shares at least one cross-neutralisation epitope, albeit a minor one. Definitive evidence for or against cross-protection will need to come from trials with large statistical power since the prevalence of infection in the population with individual HPV types other than 16 is less than 1.0% (see Chapter 2).

6. Duration of protection

Duration of protection is a key issue that will influence vaccine implementation: how long will protection last and will
booster immunisation be necessary? There are both theoretical and evidence-based reasons for some optimism on this issue. The generation of memory B-cells and their response to antigen recall are crucial factors for the long-term efficacy of vaccine-induced humoral protection. B-cell memory and its generation are poorly understood. The rapid clonal expansion of B-cells in the lymph node follicle leads to the formation of the germinal centre where the key event of affinity maturation for the generation of high-affinity B-cell receptors (BCR), and thus high-affinity antibodies, occurs. This event is critical for vaccines, such as those under discussion, whose efficacy is dependent upon the generation of high-affinity neutralising serum IgG. At some point the maturing B-cells exit from the germinal centre and enter the long-term memory B-cell compartment. Upon antigen rechallenge, these memory cells rapidly expand and differentiate into plasma cells in a process controlled by memory Th2 cells and the cytokines they secrete. There is evidence that VLPs are ligands for toll-like receptor 4 (TLR4) and signal via the MyD88 pathway [30,31]. The generation of significant pools of memory T- and B-cells is exquisitely dependent upon the initial presentation of antigen by the dendritic cell to the naïve T lymphocyte and the downstream events that result. TLR activation and signalling are key events in this process and contribute both to the activation of dendritic cells for antigen presentation to naïve T cells and their differentiation down the Th2 path and the primary activation of B-cells and their differentiation into antibody-secreting plasma cells.

The available data from the HPV vaccine trials indicate that antibody levels fall from the peak levels after immunisation to a plateau level that is at least a log higher than those detected in natural infections and that persists for at least 48 months post-vaccination [9,10]. This is encouraging because it mirrors the situation in the animal models, where protection is long-lasting after VLP vaccination despite low levels of circulating antibody [32]. However, HPV infections may occur repeatedly over a number of years and the risk of acquiring new infections is closely linked to the sexual behaviour of the individual (see Chapter 6), which raises the possibility that vaccinated individuals may change their behaviour and increase the risk of acquisition of new infections. Relevant to this is the issue of whether exposure to virus post-vaccination will act as a natural booster. There is no unequivocal evidence for or against this from the trials, but there is some suggestion for natural boosting from sero-epidemiological studies. Capsid antibody levels in natural infections are generally stable over several years of follow-up [33] and 50% of women remain seropositive 10 years after the last detection of cervico-vaginal HPV-DNA (Carter and colleagues, personal communication, May 2005). The data from the vaccine trials cover a relatively short time-span (4.5 years) therefore there is no hard data that antibody persistence correlates with protection and, in reality, we do not know how long the protection induced by L1 VLPs will be.

7. Genotype replacement

A frequently asked question is whether currently less common HPV types will increase in incidence if infection with the common oncogenic types 16 and 18 is significantly reduced. In this context, it is important to remember that papillomaviruses are DNA viruses that are genetically very stable and have co-evolved with their specific vertebrate host over millennia. However, if the issue of genotype replacement is to be addressed, the key questions are whether:

- HPV infections are independent of each other;
- closely or distantly related HPV types compete for a specific cellular niche within the epithelium;
- persistence or clearance is dependent upon, or influenced by, co-infection with other HPV types.

Robust cell biological or epidemiological data to inform on these questions are not available. Several large, well-conducted and controlled studies indicate that the presence of pre-existing cervico-vaginal HPV infections increases the risk of acquisition of other HPV genotypes (see Chapters 5 and 6) both in women and men, which implies that niche competition is unlikely. On balance, the available evidence indicates that HPV infections are independent of each other and does not suggest that genotype replacement is probable. However, this issue can be addressed only in large, statistically powerful long-term studies, and post-vaccine surveillance will be critical in this regard.

8. Who and when to vaccinate

If effective prophylaxis is to be achieved with these vaccines, it is assumed that they will have to be delivered pre-exposure to the virus. Genital HPV infection is usually sexually transmitted, and immunisation to protect against the greatest number of infections and the resulting disease must therefore precede the sexual debut, which implies that the target population for vaccination will be pre-pubertal girls and young adolescents. There are sound immunological reasons to immunise before puberty since antibody responses induced by these (and other) vaccines are higher pre-puberty compared to post-puberty in both males and females [17]. Adolescent immunisation is not easy since, in general, adolescents are not very effectively targeted in vaccination programmes. In addition, there are major cultural and social differences between countries and communities that could significantly affect the acceptability of vaccination of female adolescents against genital HPV infection. In such societies, a childhood vaccine might be more acceptable but data on the safety, immunogenicity and duration of protection of the L1 VLP vaccines in young children will be required before this could be contemplated. There may be possible benefits of high levels of serum antibody in individuals already exposed to infection [17], and the effects of vaccinating women with
L1 VLP vaccines post exposure should be evaluated in long-term studies.

**Disclosed potential conflicts of interest**

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