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Prevention of cancer by prophylactic human papillomavirus vaccines

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SUMMARY OF RECENT ADVANCES

Oncogenic human papillomaviruses (HPV) are exclusively mucosal pathogens that are non-cytopathic and the basal epithelial cells harboring and maintaining an infection do not produce either capsid antigen or virus. The efficacy of the licensed L1 virus-like particle (VLP) vaccines has encouraged development of several second generation vaccines aimed at expanding the coverage to all oncogenic HPV types and reducing barriers to global implementation. Currently there is no defined immune correlate of protection that can be used to determine if an individual patient is protected and for the evaluation of these second generation vaccines. Surprisingly, passive transfer of neutralizing serum antibody is protective in animal models. Recent studies suggest how neutralizing antibody mediates immunity against mucosal HPV and the possible impact of memory B cells.

INTRODUCTION

Like the hepatitis B vaccine, the development of the two licensed HPV vaccines is a medical triumph in both infectious disease and cancer prevention because the viruses targeted are each responsible for approximately 5% of all cancer cases worldwide. The two licensed HPV vaccines, Cervarix® (GSK) and Gardasil® (Merck), are composed of virus-like particles derived from the major capsid protein L1 of the most prevalent types in cancer, HPV16 and HPV18, and Gardasil also includes L1 VLP of the two benign types HPV6 and HPV11 that cause ~90% of genital warts. While L1 VLP are highly immunogenic alone, both vaccines utilize an adjuvant; Gardasil contains amorphous aluminum hydroxyphosphate sulphate (AAHS), whereas Cervarix utilizes aluminum hydroxide and a toll-like receptor (TLR)-4 agonist, 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL).

The primary etiologic role of persistent HPV infection in causing ~500,000 cervical cancer cases worldwide each year is firmly established, and thus with the advent of HPV vaccines and continued efforts in cytologic and viral DNA-based screening for precursor lesions

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(Figure 1) and ablative therapy of high grade squamous intraepithelial neoplasia (SIL), cervical cancer is a preventable disease [1,2]. Further, 40% of penile cancers, 40% of vaginal and vulval cancers, 90% of anal cancers, 3% of mouth cancers and 12% of oro-pharyngeal cancers are also triggered by HPV and potentially preventable through vaccination [3]. HPV is a small, non-enveloped DNA tumor virus and over 120 HPV genotypes have been described [2]. With increasingly sensitive new technologies investigators continue to find new types, but only approximately a dozen sexually-transmitted 'oncogenic' HPV types are responsible for more than 95% of cervical cancer cases [1].

IMMUNODOMINANT AND COFORMATIONALLY-DEPENDEDNT NEUTRALIZING EPITOPES OF L1 VLP

Since HPV utilizes the host DNA polymerase for replication of its genome at a low error rate, HPV sequences have remained very stable, although it is likely that the different HPV genotypes have emerged over eons based on distinct tissue tropism (e.g. cutaneous versus mucosal epithelia) and to escape immune responses [4]. Indeed, while the sequences of the internal regions of L1 are highly conserved, the surface loops are hypervariable between HPV types and correspond to type-restricted and immune-dominant neutralizing epitopes [5]. This suggests that the preferential accumulation of these changes in the hypervariable loops may reflect both selection to escape protective antibody responses and a lack of structural constraints in these loops. Thus L1 VLP-reactive neutralizing antibodies predominantly recognize only the type against which they were raised, and consequently most genotypes are different serotypes [6]. Another notable feature is that the neutralizing antibodies overwhelmingly recognize conformational/non-linear epitopes, and this may also contribute to the specificity of binding [7]. Thus the licensed vaccines target the two most common HPV types found in cervical cancer, HPV16 and HPV18 (and one also targets the two most prevalent types in benign genital warts, HPV6 and HPV11) and this produces robust protection against persistent infection and intraepithelial lesions caused by these HPV types, but variable efficacy of protection of potentially reduced duration for other related HPV types [8,9]. For example vaccination with HPV16 L1 VLPs can also provide strong protection against HPV31 [10,11]. This is presumably because the sequence of HPV31 L1 is evolutionarily most closely related to HPV16 [12]. However the cross-neutralizing antibody titer is orders of magnitude lower than to the HPV16-specific response (Kemp T *et al.*, Abstract BS2, 26th International Papillomavirus Conference, Montreal, Canada, July 2010). While this suggests that low titers of neutralizing antibody are sufficient for protection, it raises the question of the longevity of the cross-protective response. Similarly, HPV18 L1 VLPs can trigger an HPV45 cross-neutralizing and cross-protective response, but protection against other more divergent types is weak [10,11,13]. This contrasts the T cell responses to HPV L1 VLP which show some type-restriction but in general are very broad, likely reflecting recognition of epitopes within the conserved internal portions of the L1 capsid [14]. As a consequence of the type restricted nature of L1 VLP vaccines, there are ongoing clinical trials of a highly multivalent vaccine, comprising L1 VLPs of the two types found in ~90% of genital warts (HPV6 and HPV11) and the seven most prevalent HPV types detected in ~90% of cervical cancer cases (HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58) and efficacy data is expected in 2012.

While the licensed vaccines both utilize an adjuvant, L1 VLPs are remarkably immunogenic and produce robust neutralizing antibody responses even when patients are vaccinated without an adjuvant [15]. Indeed, a single dose of the licensed vaccine provides at least short term protection, although the standard regimen is three immunizations (Kreimer AR *et al.*, Abstract LBA, 26th International Papillomavirus Conference, Montreal, Canada, July 2010). It is likely that several features of L1 VLP contribute to this immunogenicity. Firstly, their

highly regular and close-packed display of neutralizing epitopes on the surface of the VLPs provides increased avidity for and cross-linking of reactive B cell receptors lowering the threshold for their activation compared to a monovalent antigen [16]. Secondly, L1 VLP can inherently activate both immature human myeloid and plasmacytoid dendritic cells (but not Langerhans cells) [17–19]. Thirdly, in contrast to typical protein antigens, L1 VLP are rapidly taken up by immune cells facilitating presentation of MHCI and II epitopes, and producing robust cellular immune responses to vaccination [20].

MECHANISM OF PROTECTION AND IMMUNE CORRELATES

An important unanswered question is how vaccination with L1 VLP mediates protection. This question has been addressed in animal models, notably cottontail rabbit papillomavirus (CRPV) challenge of rabbits and canine oral papillomavirus (COPV) challenge of dogs. Specifically, passive transfer of sera from L1 VLP immunized animals protected naïve animals from experimental viral challenge [21,22]. This implies that neutralizing antibodies are sufficient to mediate protection, but does not rule out a contribution of cell-mediated immunity in protection. However, parenteral vaccination with L1 VLP did not impact wart growth in animal models, suggesting minimal impact of L1-specific T cell responses on established lesions. It should also be noted that vaccination with L1 VLP induced type-specific neutralizing antibodies and protection in animals, and that vaccination with denatured L1 failed to induce significant titers of neutralizing antibodies and was not protective.

Clinical studies of potential immune correlates of protection have centered upon L1-specific serum antibody responses, as measured by Competitive Luminex-based Immuno-Assay (CLIA), L1 VLP ELISA, and in vitro neutralization studies [23]. Each of these assays indicates that almost all vaccinated patients generate a robust type-restricted serum antibody response to L1 VLP, corresponding with the high efficacy of type-restricted protection. The serum antibody titers wane to a plateau a few months after the final immunization and appear stable thereafter. In one study of the licensed quadrivalent HPV vaccine, HPV18 L1 VLP specific antibody titers determined by CLIA waned below the threshold of detection in 40% of patients by 48 months although the titers to the three other types in the vaccine remained detectable [24]. Despite the absence of CLIA-detectable HPV18 L1 VLP-specific antibodies, no new HPV18 infections were observed, suggesting that immunity remained [24]. This suggests that either immunity to HPV18 in these patients is cell-mediated, or reactivation of memory B cells or the titer of neutralizing antibody required for protection is very low and its measurement by CLIA lacks adequate sensitivity. Although L1 VLP vaccination induces robust T cell responses in patients, the absence of a profound therapeutic effect of the licensed vaccines upon established HPV infection suggest that it is unlikely that the protection against HPV18 in the absence of a CLIA-detectable antibody response is T cell mediated. Alternatively, it is possible that exposure to the viral inoculums triggers a reactivation of the B cell response and local production of neutralizing antibody in time to prevent an initial infection. However, several factors point to the limited sensitivity of the CLIA for detection of HPV18 L1-specific antibody as an explanation for this observation, and that low, but protective levels of neutralizing antibodies are maintained. Firstly, the threshold of this assay is set using antibody levels in natural infection, and it is likely that these titers are protective. Secondly, CLIA detects responses via competition of patient antibodies with a high avidity neutralizing monoclonal antibody to L1 VLP, and thus primarily detects high avidity responses to a single epitope. It is known that multiple neutralizing epitopes are displayed by L1 VLP, and thus these patients may have protective antibodies to other epitopes, and/or protective antibodies of low avidity that are poorly detected by CLIA.

While the L1 VLP ELISA assay is more sensitive than CLIA, it also detects non-neutralizing and presumably non-protective antibodies and may provide false positives. In vitro neutralization assays measure functional antibodies, but native HPV virions are hard to generate for all types and in sufficient quantities. Furthermore, the readout of infection is early spliced viral mRNA assayed by quantitative RT-PCR, and this is relatively cumbersome. A technology based on infection of 293TT cells with HPV pseudovirions that carry marker genes has greatly simplified the in vitro neutralization assay [25]. Nevertheless, recent passive transfer studies in mice suggest that the in vitro neutralization assay may lack the sensitivity to detect the minimal protective level of antibody, and that the passive transfer approach may provide a better approach, albeit low throughput [26]. This may reflect differing mechanisms of infection and thus neutralization in vivo within the genital mucosa [26] as compared to in vitro with 293TT target cells [27] (Figures 2 and 3 respectively). In vitro neutralization assays also depend upon extensive dilution to measure antibody levels, and it is possible that low avidity antibodies are protective and poorly detected by this in vitro assay [28]. Nevertheless, the range of HPV types cross-neutralized by the sera of L1 VLP-vaccinated patients appears to correspond to the breadth of protection.

That serum L1 VLP-specific antibody titer better correlates with protection than the robust L1-specific cell mediated response raises several questions. Firstly, why does the L1-specific cellular immune response not clear established HPV infections? This may be explained in part by the unique biology of HPV [29]. HPV does not produce viremia, but rather is confined to epithelial lesions above the basement membrane and systemic T cell responses may not reach these lesions or may be suppressed therein. Most importantly, while all HPV infected cells express early genes E6 and E7, the capsid proteins are only expressed in the upper differentiating and dying layers (Figure 1). Consequently, L1-specific cellular immune responses do not target the basal epithelial cells that harbor the infection, unless some type of bystander response can be triggered [29]. The second surprise is that serum neutralizing IgG titers are the relevant correlate for a purely mucosal infection and begs the question of how these antibodies reach the viral inoculum since systemic vaccination typical fails to induce a local IgA response (Figure 2). L1 VLP-specific IgG is detected in the vaginal fluid of vaccination patients and its level correlates with serum titer, although it varies with the menstrual cycle [30].

This indicates the occurrence of either active transport or passive transudation of the IgG into the vaginal fluid wherein it neutralizes the viral inoculum. However, it does not explain protection at cutaneous sites against HPV6 and HPV11 infections induced by vaccination. A second, not mutually exclusive, possibility is that the microtrauma that is associated with infection during intercourse and facilitating access for HPV to the basal epithelia may trigger a local exudation directly from plasma to the site of infection (Figure 2). It is unclear whether a minimal level of neutralizing antibody must be maintained sufficient to provide sterilizing immunity, or whether the viral inoculum can trigger a rapid anamnestic activation of memory B cells to produce neutralizing antibody locally [31]. The slow course of HPV infection and the ability to neutralize the virus many hours after binding to cell surfaces suggests that the latter is a possibility [32]. Indeed re-vaccination of individuals clearly triggers a robust anamnestic antibody response, but it is unclear if it happens within the window for neutralization [33].

CANDIDATES FOR SECOND GENERATION HPV VACCINES

The identification of a correlate of protection is important to identify whether an individual immunization is successful, to validate batches or sources of L1 VLP vaccine produced by different manufacturers and/or in different systems (e.g. bacteria or plants [34,35]) and also

in the development of second generation preventive HPV vaccines. One such potential second generation vaccine comprises L1 capsomers (the pentameric subunit of the VLP) that can be readily produced in bacteria [36]. The L1 capsomer-based vaccine offers potential advantages of reduced cost, stability at ambient temperature that could facilitate introduction into low resource settings where HPV vaccines are most needed. Although L1 capsomers induce lower neutralizing antibody titers than VLP, protection is robust even without adjuvant and the use of an adjuvant can potentially close this gap [36,37]. Another approach of HPV vaccination is the use of naked DNA vectors, which are simple to manufacture, heat stable and delivered by gene gun, tattoo or electroporation to express codon-modified L1 *in vivo* [38]. The use of needles for immunization also provides a barrier to widespread use, and there are efforts to use live recombinant vectors, such as an L1 recombinant version of the orally administered typhoid vaccine *S. typhi* [39]. The VLP vaccine could also be combined with other vaccines, either by mixing together, or by introducing the L1 gene, for example into measles vaccine, or the tuberculosis vaccine BCG etc, to deliver L1 [40,41].

In selecting a correlate of protection, it should be both simple to use, readily standardized into pre-defined international units and ideally not specific to L1. The latter point is relevant because of several efforts to second generation HPV vaccines based upon the minor capsid antigen L2 [42]. Vaccination with L2 protects animals from experimental viral challenge, but this immunity lacks the type restriction associated with L1 VLP vaccines [43,44]. The protection is mediated by broadly neutralizing antibodies that recognize conserved linear epitopes in the N-terminus of L2 [43,45]. Residues within the protective region of L2 play a critical role in viral infection and thus their sequence is conserved even in diverse HPV types [46,47] (Figure 3). The L2 cross-neutralizing epitope appears to be displayed on the capsid surface only during infection, and thus vaccination with virions or L1/L2 VLPs induces limited, if any, L2-specific antibody [27,44]. Unfortunately L2 does not form a particulate structure alone and therefore is significantly less immunogenic than L1 VLP, suggesting the need for an adjuvant for vaccination or for multimeric display of L2 in an immune-dominant epitope of a recombinant VLP [48,49].

CONCLUSIONS

The licensed HPV vaccines will dramatically reduce the incidence of cervical cancer in the years to come. However, the global impact upon HPV-associated cancer rates will depend greatly upon the extent of implementation of vaccination. Second generation HPV vaccines are being developed to overcome barriers to global implementation and expand the breath of protection. The development of robust immune correlate of protection with international units and a more detailed understanding of the immunologic mechanisms underlying the efficacy of the licensed HPV vaccines are important to further the development of such second generation HPV vaccines. It is also important to note that HPV vaccines targeting the viral capsid antigens alone do not appear to impact the course of preexisting infections, and that continued efforts to develop therapeutic HPV vaccines (or even combination preventive and therapeutic HPV vaccines) should remain a priority given the current high prevalence of HPV-associated disease.

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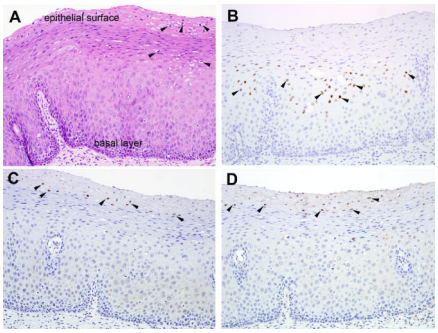

Figure 1. Localization of capsid protein expression and viral genomes in a productive HPV16 lesion (Low grade squamous intraepithelial lesion (LSIL/CIN1))

A. Hematoxylin and eosin-stained section of cervical squamous epithelium; Keratinocytes with markedly enlarged nuclei surrounded by cytoplasmic halo (arrowheads) in the upper epithelial layers characteristic of LSIL, **B**. In situ hybridization for HPV16 genome; discreet nuclear signals (arrowheads) predominantly in the middle zone of the epithelium where the proliferating cells are just beginning to differentiate, **C**. Immunohistochemistry of L1 using monoclonal antibody 1H8, **D**. Immunohistochemistry for L2 using monoclonal antibody RG-1. Positive immunolabeling in the occasional keratinocyte nuclei limited to the upper epithelial layers (arrowheads). The exquisite temporal/spacial regulation of viral genome copy number and late gene expression is apparent in this productive HPV16 lesion. In the basal epithelial cells the viral genome copy number is low (~100 episomes/nucleus), but as the cells divide and move up through the epithelium their differentiation triggers vegetative viral replication (producing a high copy number of ~10^4 episomes/cell), and in the uppermost regions of the lesion expression of the major L1 and minor L2 capsid proteins.

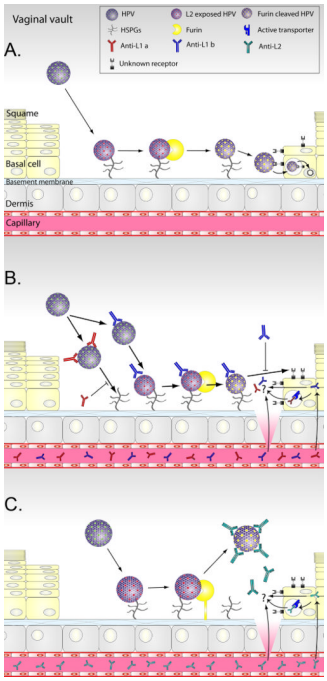

Figure 2. Models for the mechanisms of HPV infection in vivo and antibody-mediated protection

Day *et al.* have proposed models of infection and immunity based upon microscopic studies of HPV pseudovirions during vaginal challenge of naïve mice and those immunized with L1 or L2 [26]. **A**. Infection of vaginal epithelium. Microtrauma to the squamous epithelium of the vagina/and or cervix associated with intercourse provides HPV access to the basement membrane. HPV binds to the basement membrane via heparan sulphate glycosaminoglycans (HSPGs) and this triggers a conformational change in the capsid exposing L2 for N-terminal clipping by secreted furin. The furin-cleaved HPV binds to a viral receptor on the surface of basal epithelial cells during wound healing to initiate infection. **B**. L1 VLP-specific antibody-mediated protection against vaginal infection. In hosts vaccinated with L1 VLPs, neutralizing IgG passively transudates from the capillaries into the cervical/vaginal fluid and/or is actively exchanged. If the trauma is sufficient, direct exudation from plasma is possible at the site of wounding. High concentrations of L1 VLP-specific antibodies can prevent virions binding to the basement membrane. In the presence of lower antibody levels the binding of HPV to the basement membrane, and the cleavage of L2 by furin still occurs, but the virions are unable to transfer to the viral receptor on the basal epithelial cells and the virion-antibody complexes are released. **C**. L2-specific antibody-mediated protection against vaginal infection. In the presence of L2-specific antibody, the binding of HPV to the basement membrane, and the exposure of L2 occurs. However, the antibodies bind to epitopes in the N-terminus of L2 after its cleavage by furin and the virions are unable to transfer to the viral receptor on the basal epithelial cells, leading to release of the virion-antibody complexes.

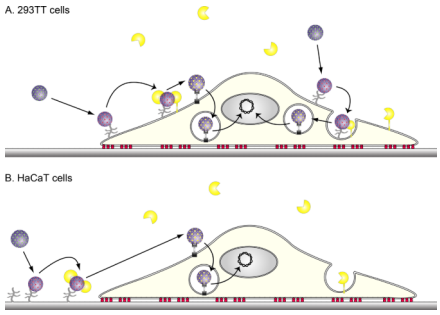

Figure 3. In vitro infection of 293TT and HaCaT cells by HPV

A. In vitro infection of 293TT cells. Early studies of in vitro infection by HPV utilized 293TT or other transformed cell lines as target cells. HPV binds directly to the 293TT cell surface via HSPGs which triggers a conformational change in the capsid such that the amino terminus minor capsid protein L2 becomes exposed. Exposure of L2 renders it accessible to cleavage by furin [50]. Furin-cleavage of L2 is essential to infection and is associated with escape of L2 and the viral genome from the endosome. γ secretase activity is also required for infection, but it is unclear what it cleaves [51]. The viral genome-L2 complex is too large to cross the nuclear envelope, but gains access to the nucleus as during mitosis while its membrane has dissolved [52]. **B**. In vitro infection of HaCaT cells or primary keratinocytes. In contrast to 293TT cells, HPV binds first to the laminin-5 associated extracellular matrix via HSPGs secreted upon the culture surface prior to infection of the immortalized keratinocyte line HaCaT or primary keratinocytes. Upon attachment to the extracellular matrix, the HPV virions undergo a conformational shift that exposes L2 for cleavage by secreted furin. Only then can the virions bind to a secondary receptor on the keratinocyte surface for subsequent uptake. Antibodies reacting with particular loops on the capsid surface (e.g. H16.V5 and H16.E70 monoclonal antibodies) can prevent virions binding to the extracellular matrix, but these antibody-bound virions can still bind the cell surface, although infection does not occur. In the presence of antibodies reacting with other surface L1 loops (e.g. H16.U4 monoclonal antibody), the binding of HPV to the basement membrane, and the cleavage of L2 by furin still occurs, but the virions are unable to transfer to the viral receptor on the basal epithelial cells [27]. Likewise, L2-specific antibodies (e.g. RG-1 monoclonal antibody) allow the binding of HPV to the basement membrane, and the exposure of L2. The antibody binds to the exposed L2 after its cleavage by furin and the virions are unable to transfer to the viral receptor on the basal epithelial cells [27].