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Current Insight into Anti-HPV Immune Responses and Lessons for Prophylactic and Therapeutic Vaccines

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1. Introduction

Human Papillomavirus (HPV) are epitheliotropic for stratified malpighian epithelia such as those of the cervix, vulva or anal canal. Mucosal papillomaviruses are responsible for several clinical lesions and can be classified as non oncogenic and oncogenic viruses. The first ones are responsible for benign lesions such as external genital warts (EGW) or condyloma due to HPV 6 and 11. The second ones include oncogenic viruses such as HPV 16 and 18, the most common ones, and HPV 45, 31, 33, 52 etc which are involved in invasive cancers preceded by cervical, vulvar, penile or anal intraepithelial neoplasia. Oncogenic HPV are detectable in 99.7% of cervical cancers (Bosch et al, 1995). Cellular immunity plays a key role in controlling and killing infected or transformed keratinocytes. Nevertheless, around 10% of women having infected cervical mucosa are not able to control oncogenic HPV and develop cervical intraepithelial neoplasia (CIN). High grade CIN (CIN3) require surgical treatment before their progression to invasive cancers in 30% of cases (McCredie et al, 2008; Ostor et al, 1993). A great priority is then to develop a preventive vaccine to protect against HPV infection. In women with CIN, therapeutic vaccine could be used to eliminate previously infected or transformed keratinocytes and avoid surgical treatment.

2. Virology

Following a breach in the malpighian pluristratified epithelium, HPVs infect basal stem cells of keratinocytes. The virus initially remains in episomal form with synthesis of E2 protein. This protein is a major regulator of viral vegetative cycle and is required for transcriptional regulation as well as viral DNA replication together with the E1 helicase (Desaintes et al, 1996). In contrast, E2 is generally undetectable in cancers due to a preferential integration of the viral genome in the cell genome and disruption of the E2 open reading frame (Berumen et al, 1994; Collins et al, 2009). Therefore E2 is a marker of viral infection and is specific for the early stages of the viral gene expression in infected cells. This was formally demonstrated in a recent work that showed a strong staining of the E2 protein in the intermediate differentiated layers of HPV16-infected tissues and low grade CIN (Xue et al,

2010). The high expression of HPV16 E2 in low grade lesions therefore represents a marker for HPV infection even before any clinical manifestation.

After integration of the genome of oncogenic HPVs into the host genome, viral oncogenic E6 and E7 proteins are synthesized in large quantities in the inner third of the epithelium. During maturation of keratinocytes from the basal layer to the epithelial surface, viral capsid proteins L1 and L2 are synthesized and expressed at the surface of mature keratinocytes in order to form a new viral particle which is able to infect adjacent healthy epithelium and to contaminate sexual partners.

3. Epidemiology of oncogenic HPV and related diseases

HPV infections occur preferentially in young women under 25 years of age (Boulanger et al, 2004). Several stages of lesions can be observed following oncogenic HPV infection. The first stage is a simple infection of keratinocytes that become koilocytes and develop into condyloma. The following stages are related to the transformation of infected keratinocytes into malignant cells. The depth at which malignant cells are found defines the disease stage: low (as CIN1) or high grade squamous intraepithelial lesions as CIN2/3. The latter is diagnosed on the basis of Pap smears, followed by colposcopy and biopsies and can evolve towards invasive cancer. HPV16 is found in more than 50% of cervical cancer cases and HPV18 in 17%. The incidence of cervical cancer remains very high with 500 000 new cases per year in the world, essentially in developing countries where the level of screening by Pap smear is very low. It annually leads to 230 000 deaths.

The premalignant lesions of HPV-related grade 3 usual vulvar intraepithelial neoplasia (usual VIN or VIN3) involve the mucosal and/or cutaneous epithelium of the vulva, perineal and perianal region. Usual VIN occurs in adult women and commonly resembles persistent anogenital warts that are often multifocal pigmented papular lesions disseminated on the vulva and/or the perianal skin. Usual VIN is characterized by the presence of poorly differentiated or undifferentiated basal cells and highly atypical squamous epithelial cells (McCluggage et al, 2009). The oncogenic HPV most frequently found in usual VIN is HPV16 that plays a direct role in up to 91% of the cases (Srodon et al, 2006).

The overall incidence rates of anal cancer has recently increased, particularly among men who have sex with men (MSM) and HIV-infected patients (Piketty et al, 2008). Combination antiretroviral therapy does not prevent nor revert anal cancer in the latter patients (Piketty et al, 2010). Despite several HPV coinfections in particular in HIV-infected patients, HPV16 is the most common one in anal cancer (Abramowitz et al, 2011).

4. Humoral immune response after HPV infection

Serum antibodies against HPV are directed against viral capsid antigens and in particular against L1 protein. Their synthesis is late (6 to 12 months after infection) and antibody concentration remains limited because of the absence of HPV viremia (Carter et al, 2000). However, these antibodies persist in many women for at least 10 years (af Geijerstam et al, 1998). Only 70% of women having persistent HPV16 DNA in the genital

mucosa have detectable antibodies (Ho et al, 2004; Kirnbauer et al, 1994). These antibodies do not play any neutralizing role against HPV after virus entry in basal stem cells of keratinocytes (de Gruijl et al, 1999) because L1 protein is not expressed at the surface of these cells.

5. Antibodies detected after HPV infection do not protect against a new infection

Antibodies synthesized after HPV infection do not protect against a new infection with the same HPV genotype, as observed in a cohort study of women with and without such antibodies (Viscidi et al, 2004, 2005). There was no difference over time between the two groups with respect to HPV16 DNA detection in the genital mucosa. This is not surprising since the level of the anti-HPV antibodies found in mucosal secretions is lower than in the serum where the level of antibodies is already very low (Lowe et al, 1997; Nardelli-Haeffliger et al, 2003). Local secretory IgA could not stop the spread of HPV infection (Bard et al, 2004).

6. Cellular immune response after HPV infection

Cellular immune responses play a critical role in HPV infections by controlling or eliminating the virus. The incidence of HPV-induced diseases is increased in T-cell immunodeficient individuals, such as HIV-infected (Sun et al, 1997), transplanted patients (Arends et al, 1997), patients treated by immunosuppressive drugs (Ulrich et al, 2008) or in primary immunodeficiencies (Lawrence et al, 2005). In patients with high-grade CIN 2/3 or invasive cervical carcinoma, blood cytotoxic T lymphocytes (CTL) directed against HPV-16 E6 or E7 proteins are barely detectable (Nakagawa et al, 1997, 2000). Proliferative responses of CD4-lymphocytes against these two proteins seem to correlate with the infection stage. Indeed, high frequency specific interleukin-2 (IL2)-producing CD4 lymphocytes have been observed in asymptomatic HPV-16-infected women (de Jong et al, 2002) whereas they decrease during disease progression toward high-grade CIN or invasive cancer (Tsukui et al, 1996).

In a woman who completely cleared usual vulvar intraepithelial neoplasia (VIN) lesions eight months after disease onset (Figure 1), an immunohistochemical study showed a marked dermal infiltrate containing a majority of CD4⁺ T lymphocytes and an epidermal infiltrate made up of both CD4⁺ and CD8⁺ T cells (Figure 2) (Bourgault Villada et al, 2004). Before clinical regression, high frequency anti-E6 and anti-E7 effector blood T-cells by *ex vivo* IFN γ ELISPOT assay was evidenced (Figure 3). This appears to be the first evidence of an association between spontaneous regression of usual VIN lesions and HPV-specific T cell responses detectable in the blood. Hence, an increase of HPV-specific effector T lymphocyte responses by vaccine-based therapeutic strategies might be useful to clear the lesions in usual VIN disease.

On the contrary, in chronic nonregressive CIN3, lymphocyte infiltrates in the epidermis mainly contain CD8⁺ lymphocytes and no CD4⁺ cells. It is likely that CD8⁺ lymphocytes play a major role in the defense against HPV infections by killing infected keratinocytes. However, CD4⁺ lymphocytes that synthesize IFN γ and IL2 are required for an optimal induction of high affinity tumor-specific memory CD8⁺ effector T-cells.



Fig. 1. Clinical lesions of multifocal pigmented usual VIN before spontaneous clinical regression

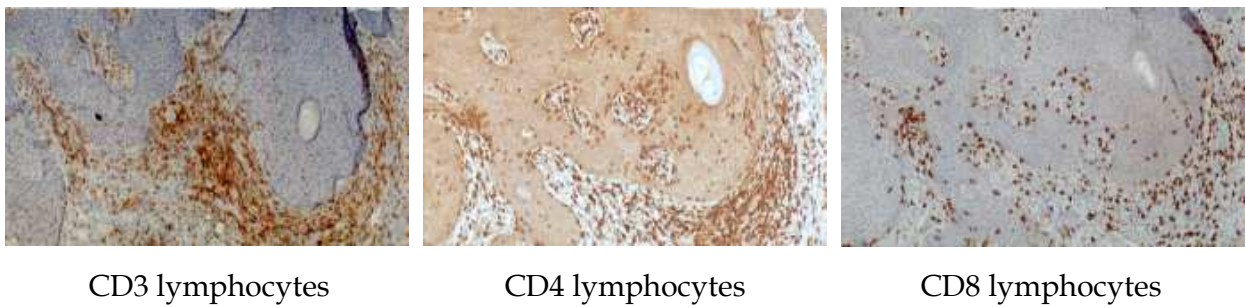


Fig. 2. Immunohistochemical study of the vulvar biopsy just before spontaneous regression

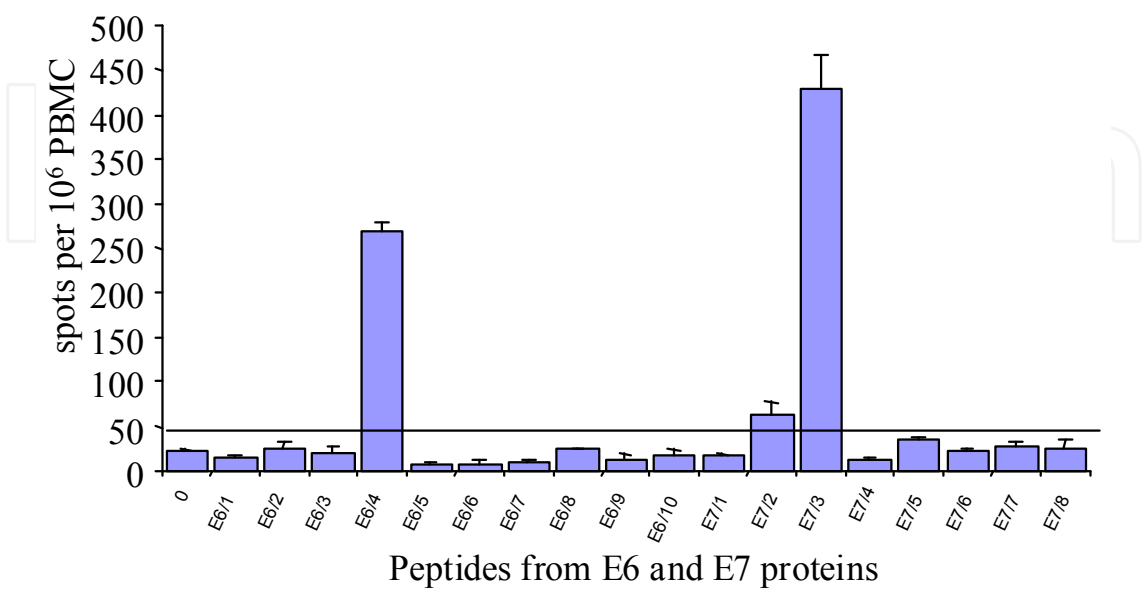


Fig. 3. IFN γ ELISPOT assay performed just before clinical regression

7. Presentation of HPV antigens to T and B lymphocytes after HPV infection

During infection, viral particles enter through epithelium up to basal stem cells of keratinocytes and sometimes can penetrate into the chorion. In the epithelium, they can be captured by Langerhans cells and they are quickly internalized (Bousarghin et al, 2005; Fausch et al, 2005; Malejczyk et al, 1997) and degraded into short and large peptides (Combadière et al, 2008; Herbst et al, 1996; Offringa et al, 2003; Yan et al, 2004). The classical view of the role of Langerhans cells is one of antigen uptake in the epidermis, and migration through the dermal lymphatics to the lymphoid organs, where they present antigen to lymphocytes that then home back to the tissue to carry out their effector function. Matthews et al (2003) have previously reported that Langerhans cells number is significantly reduced in HPV16 lesions without Langerhans cells depletion in the surrounding uninfected tissue. During HPV infection, the migration of Langerhans cells towards mucosal follicle is followed by presentation of short and large viral peptides by HLA class I and HLA class II molecules to CD4+ and CD8+ T lymphocytes, respectively. After stimulation, CD4+ and CD8+ T lymphocytes can circulate and migrate within the HPV-infected epithelium by using their surface molecules such as cutaneous lymphocyte antigen (Grover et al, 2006). The presentation of whole viral particles to B lymphocytes requires that HPV binds to dermal dendritic cells that are able to carry the whole virus to follicular dendritic cells present in mucosal follicle (Palucka et al, 2010).

8. Does a T-cell marker of viral control exist?

We recently tested by proliferative assays, intracellular cytokines synthesis and IFN γ ELISPOT the cellular immune responses against the HPV16 E2 protein that is early synthesized after HPV infection when the virus is episomal in eight women presenting with HPV16-related usual VIN and their healthy male partners (Jacobelli et al, 2011, unpublished data). We showed that anti-E2 polyfunctional CD4 T-cell responses (proliferative responses and synthesis of IFN γ and/or IL2) appear when the clinical lesions heal or when the HPV infection remains silent. Blood proliferative T-cell responses against HPV16 E2 peptides have been also observed in 50% of healthy women, who presumably previously cleared HPV16 infection (de Jong et al, 2004) and in 9 out of 22 regressive CIN3 cases (Dillon et al, 2007). In another studies, the lack of anti-E2 proliferative responses was reported in 16 of 18 patients (89%) affected with usual VIN lesions (Davidson et al, 2003) and in 7 of 8 and 9 of 12 women affected with CIN3 (Dillon et al, 2007; de Jong et al, 2004). These observations reinforce the strong role of T-cells in the control of HPV replication.

9. Why the male partners of women having CIN3 or usual VIN do not have any lesion?

Men are vectors of oncogenic HPV infection (Buckley et al, 1981). However, while HPV infection was found in 71 to 90% of the partners of HPV-infected women (Hippelainen et al, 1994; Nicolau et al, 2005), only 52% harbored the same HPV subtypes (Reiter et al, 2010). Moreover, penile intra-epithelial neoplasia is rare and detected in less than 2% of the men in contact with oncogenic HPV (Giraldo et al, 2008). We thus analyzed HPV infection and anti-HPV16 E2 blood T-cell responses in asymptomatic male partners chronically exposed to HPV16 during sexual intercourses with their wives affected with usual VIN (Jacobelli et al,

2011, , unpublished data). We had hypothesized that male partners exposed to replicative HPV16 could develop immunologic responses against the early E2 viral protein and thus clear infection. We indeed observed HPV16-E2-specific proliferative responses and intracellular synthesis of single IFN γ , dual IFN γ /IL2 and single IL2. These T-cell responses indicate a striking link between the absence of HPV-related lesions and the presence of spontaneous anti-E2 specific polyfunctional T-cell response in male partners. It is tempting to speculate that E2-specific responses prevent HPV16-related lesions. Since E2 protein is not encapsidated in the viral particle, the strong E2-specific T cells responses measured in partners of women with usual VIN demonstrates that the virus effectively replicates in males. Our results suggest that male are an important reservoir of genital HPVs and provide a strong argument in favor of prophylactic HPV vaccination of young men with Virus Like Particles to decrease HPV16 infection in men, and thus fight against the spread of mucosal HPV diseases in the population.

10. Balance between cellular immunity and infected / tumoral cells: Mechanisms of tumor escape

The impairment of HPV-16-specific CD4 lymphocytes and CTL responses can occur many years after infection / transformation of keratinocytes. It could be related to the tumor or to T-cell responses. The tumor cells can down-regulate their MHC class I molecules, synthesize TGF β or decrease the number of viral peptides on their surface. Mechanisms of T-cell tolerance to HPV includes presence of regulatory T-cells (Treg) at proximity of the tumor cells (van der Burg et al, 2007) and sometimes in the blood (Molling et al, 2007; Visser et al, 2007), engagement of PD1 or CTLA4 in the immune synapse and inhibition of CD3 zeta expression on infiltrating tumor T-cells (Patel et al, 2009; Zehbe et al, 2006).

11. Prophylactic vaccines can prevent infection

The vaccination against HPV can have two different purposes. The first one is a preventive strategy aiming at blocking primary infection by preventing the entry of the virus into their target cells i.e. the basal stem cells of keratinocytes. In this aim, a vaccination able to induce transudated serum antibodies at the epithelial surface is a good strategy and is obtained by using viral L1 particles as vaccine. Large quantities of L1 are produced in vitro by splicing the L1 gene into plasmids (for expression in yeast) or recombinant baculoviruses (for expression in insect cells). The Virus Like Particle is formed by self aggregation of 72 L1 capsomers into a sphere. This spherical structure is similar to the viral capsid, but it is empty without DNA or RNA and non infectious. After systemic immunization, it is able to induce the synthesis of neutralizing antibodies that can recognize the conformational structure of the real viral capsid. Inversely, after HPV infection, antibodies are ineffective and only therapeutic vaccines can be considered to induce T lymphocytes able to kill HPV infected keratinocytes. These therapeutic vaccines must target oncogenic E6 and E7 viral proteins early expressed in the basal epithelial cells.

Merck has developed Gardasil®, a vaccine directed against four HPV types. It contains Virus Like Particles (L1) from HPV-6, 11, 16, and 18, with aluminum hydroxyphosphate as an adjuvant. Cervarix® is a bivalent vaccine developed by GSK. It targets HPV-16/18 VLP (L1) and contains a novel adjuvant named ASO4 co-formulated with aluminum. ASO4 contains phospholipids from *Salmonella minnesota* membrane and binds to TLR4 at the

surface of dendritic cells. Activated dendritic cells synthesize type I IFN, IL6, IL12, TNF α that allows recruitment and stimulation of Th follicular cells that increase antibodies production by B lymphocytes. Both vaccines must be administrated by intramuscular route. Three injections should be performed, at M0, M2 and M6 for Gardasil® and M0, M1 and M6 for Cervarix®. Gardasil® and Cervarix® have been commercialized worldwide and each country should define their own recommendations.

Phase I trials of Gardasil® were performed with only HPV16 L1 VLP in 300 16 to 23 years old women, who had less than 5 sexual partners and had never been exposed to HPV16 (Harro et al, 2001). The immunogenicity of the vaccine was excellent with the induction of very high levels of blood anti-L1 antibodies, 50 to 100 times those observed after natural HPV infection. The tolerance of the vaccine was good with only a slight pain, swelling and erythema at the injection sites. Phase II trials included about 2 000 subjects, they showed preliminary proof of efficacy using also HPV16 VLP versus a placebo (Koutsky et al, 2002). Subsequent phase III trial were carried out on more than 25 000 subjects, using the quadrivalent vaccine containing VLP from HPV 6, 11, 16 and 18. 98 to 100% protection was obtained against HPV -6, 11, 16, and 18 related diseases such as CIN2/3, vulvar and vaginal condyloma, usual VIN and VaIN3 (Garland et al, 2007; Joura et al, 2007; Munoz et al, 2010). Protection against persistent infection (for 6 months) by HPV16 or 18 was obtained in 99% of cases. Gardasil® obtained an FDA approval for the vaccination of girls and women aged 9 to 26 years to prevent cervical cancer, precancerous genital lesions and genital warts. Recently, 90% protection was obtained against extragenital warts in males (Giuliano et al, 2011). Efficacy of Gardasil® obtained against HPV31-induced CIN2/3 is around 55%. Finally, 43% protection was obtained against CIN2/3 induced by 14 oncogenic and non oncogenic genotypes of HPV (HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) 3.7 years after vaccination.

As for Cervarix®, phases I and II (Harper et al, 2004, 2006) studies showed 98 to 100% protection against CIN 2/3 similar to that of Gardasil®. The phase III trial involved more than 20 000 subjects (Paavonen et al, 2007) and was approved by the FDA and European Medical Agency. 70% protection was obtained against CIN2/3 related to 14 oncogenic HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) 3 years following vaccination (Paavonen et al, 2009). The efficacy of Cervarix® against HPV31-induced CIN2/3 is around 92%. Protection against persistent infection (for 6 months) by HPV33 and 45 was obtained in 76 and 77% of cases respectively.

Nevertheless, many questions remain unanswered. How long will the protection last ? Does thresholds of antibodies exist to allow the protection ? What will be the impact of the vaccination on the ecology of other HPVs, HPV screening by Pap smears and on adolescent sexual behavior and their use of condoms for HIV protection ? Will parents, preteens, physicians and the public at large, accept vaccination of young girls ? Will it be interesting to vaccinate young men to decrease HPV16 infection in men, viral transmission from men to women and more importantly spread of mucosal HPV diseases in the population ? Are MSM the future candidates for vaccination against anal cancer ?

12. Protection by the quadrivalent prophylactic vaccine against cutaneous external genital warts

A paradox exists between the protection by prophylactic vaccines against cutaneous EGW (related to HPV6 and 11 replicative viruses) and the absence of detectable antibodies on the

keratinized skin surface. Following a breach in the epithelium, HPVs bind via L1 first to the basement membrane and then to the cellular receptor on the basal stem keratinocytes (Kines et al, 2009). Anti-L1 antibodies induced by prophylactic vaccines could block both of these interactions (Day et al, 2007). Indeed, this process of virus entry is slow, between 12 to 14 hours (Sapp et al, 2009) and, since the breach is accompanied by a serum exudate, exposure to serum antibodies is rapid. Another explanation for protection could be a stronger stimulation of anti-HPV CD4⁺ and CD8⁺ T-cells after infection. Indeed, in the presence of memory anti-L1 CD4⁺ T-cell, the CD8⁺ cytotoxic T-lymphocytes could be more strongly stimulated, with multiple specificities and higher affinity (Sauzet et al, 1995). The killing of infected keratinocytes could be then more effective.

13. Prevention of infection by other oncogenic HPVs by divalent prophylactic vaccine

Recently, it has been demonstrated that Cervarix® is able to prevent CIN2/3 induced by HPV 16 and 18 and also by HPV 31, 33 and 45 (<http://www.ema.europa.eu/>, cervarix®, summary of product characteristics). An efficacy of 70% and 43% was obtained against CIN2/3 related to 14 oncogenic HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) with Cervarix® and 12 oncogenic HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) with Gardasil®, respectively (<http://www.ema.europa.eu/>, gardasil®, summary of product characteristics). A high level of anti-HPV antibodies usually correlates with a broad recognition of B-cell epitopes. Such epitopes can be shared by several other closely related HPVs. In Cervarix®, the use of ASO4 adjuvant allows to obtain high anti-HPV16 and 18 antibodies levels, that are able to prevent infection by numerous other oncogenic HPVs (McKeage et al, 2011).

14. Alternative to L1 VLP vaccines

A particularly attractive strategy is to vaccinate with a linear N-terminal highly conserved sequence of the viral capsid L2 protein. In a properly immunogenic context, neutralizing antibodies to this B cell epitope-containing region are elicited and they have broadly neutralizing capacities against a wide range of HPVs (Conway et al, 2011). Immunization with adjuvanted chimeric HPV16L1-HPV16L2 VLP have also induced neutralization or cross-neutralization of HPV16, -18, -31, -45, -52, and -58; HPV6 and -11; and HPV5 (Schellenbacher et al, 2009). These new types of vaccine are very promising.

15. How to cure infection / tumorigenesis? Therapeutic vaccines

Preventive vaccines do not address the current need for better treatment for women previously infected by HPV 16 or 18. Other types of vaccines must be used to increase or induce new specific anti-HPV cellular immunity (CD4⁺ and CD8⁺ T lymphocytes) in order to kill transformed epithelial cells. Several approaches can be used in this aim. To stimulate cytotoxic or antiviral CD8⁺ T lymphocytes, the vaccines must target the cytoplasm of dendritic cells. The degradation of vaccine antigens by proteasomes results in short peptides that can bind to HLA class I molecules and migrate at the surface of dendritic cells. To stimulate CD4⁺ T lymphocytes, endocytosis of vaccinal antigens is essential, followed by degradation of antigens by lysosome/endosome in large peptides that associate with HLA

class II molecules before migrating at the surface of dendritic cells. All these therapeutic vaccines must target E6 and E7 viral proteins and contain recombinant viruses (vaccinia viruses for example), DNA or peptides.

Peptidic or lipopeptidic vaccines were tested within phases I-II for treatment of women having CIN3 or metastatic cervical cancer. The chosen peptides were E7 11-20 and E7 86-93, two peptides able to bind HLA-A2 molecules in association with a CD4+ epitope (PADRE) able to bind numerous HLA-DR molecules (Ressing et al, 2000; Steller et al, 1998; van Driel et al, 1999). In women with invasive cervical cancer, 25 to 30% of cellular immune responses were observed without any clinical improvement. Another trial in 18 women with CIN3 has shown a clinical improvement in 50% of them (Muderspach et al, 2000). Recently, an open clinical trial was performed by the Melief's group (Kenter et al, 2009) in twenty women presenting with usual VIN using 13 large peptides spanning the whole E6 and E7 proteins. Forty five percent of complete (9/20 women) and 25 % (5/20) of partial remission were observed 12 months after immunization. These important results would be even more interesting if the investigators had included a placebo group (Bourgault Villada, 2010a). A new trial with a placebo group is currently under way.

Vaccinia virus was used in a recombinant vaccine containing E6 and E7 genes from HPV16 and HPV18 (TA-HPV) to vaccinate usual VIN patients. A clinical complete or partial response was observed in 8/18 treated women (Davidson et al, 2003). More recently, vaccination against usual VIN was also performed with another recombinant vaccinia virus, TA-L2E6E7 from HPV16 (Daayana et al, 2010). Two months before vaccination, 19 women were treated by topical imiquimod and then vaccinated by intramuscular route with 3 doses of recombinant vaccinia virus. Imiquimod is an immunomodulator that increases the synthesis of type I IFN by dendritic cells after its fixation to the TLR7 in human dendritic cells. Complete remission was obtained in 58% of vaccinated women.

Vaccination against CIN 2/3 was also performed by Transgene using a recombinant vaccinia virus, MVA, associated with the genes coding for E6 and E7 proteins and IL2 cytokine in 18 women. The disappearance of the lesions was observed by colposcopy 6 months later in 10 patients, without any CIN at biopsy in 9 cases. The important conclusion of this trial was that the vaccine was clinically effective, thus avoiding conization for 50% of the women with HPV16-related CIN2/3. A phase II trial including a placebo group is presently undertaken.

A phase II clinical trial has also been performed to evaluate the potential use of the MVA-E2 in treating CIN 2/3 (Garcia-Hernandez et al, 2006). Thirty-four women received the therapeutic vaccine injected directly into the cervix once every week over a 6-week period. Nineteen patients (59%) showed no lesion nine weeks later and histological analysis showed total elimination of high-grade lesions in 20 patients. All patients developed Ab against the MVA-E2 vaccine and showed a specific cytotoxic response against papilloma-transformed cells.

DNA containing E6 and E7 genes from HPV 16 and 18 (ZYC101a) was administered on 86 women having CIN2/3 (Garcia et al, 2004). Conization was performed 6 months later. Resolution of CIN was observed in 73% of the younger (less than 25 years old) women with a significant difference compared to a control group. This therapeutic vaccine is also very

promising as CIN 2/3 treatment. New phase II trial is currently under way, testing DNA from E6 and E7 genes versus placebo.

All these results are very important and encouraging for the development of therapeutic vaccines for HPV induced cancers. Nevertheless the proof of efficacy in CIN 2/3 should be carefully demonstrated because therapeutic vaccine should be more efficient than surgery. It is important to note that these therapeutic vaccines should avoid relapse of HPV infection after treatment by increasing HPV-specific cellular immunity.

Other vaccines were tested to fight high grade anal intraepithelial neoplasia (AIN3) in HIV+ patients. HSPE7 including *Mycobacterium bovis* BCG heat-shock protein 65 (Hsp65) and HPV16 E7 protein was tested. Clinical complete and partial responses were observed in 5 vaccinated patients out of 15 (33% of efficacy) (Palefski et al, 2006). A better vaccine with adjuvant is presently developed.

The safety and immunogenicity of the human papillomavirus type 16 (HPV16) or HPV18 (HPV16/18) E7 protein-pulsed mature dendritic cell vaccination (phase I) were evaluated as adjuvant therapy for 10 patients with stage IB cervical cancer treated by radical hysterectomy (Santin et al, 2008). All patients developed CD4+ T-cell and Ab responses to DC vaccination and 8 of them E7-specific CD8+ T-cells. DC vaccination was well tolerated and no significant toxicity was recorded. New trials (phase II) in cervical cancer patients harboring a limited tumor burden or who are at significant risk of tumor recurrence are warranted to show an efficacy of this immunotherapy.

Condyloma have been also treated by immunotherapy. Two trials were performed using the VLP of HPV6 (Zhang et al, 2000) or the fusion protein L2E7 from HPV6 (Lacey et al, 1999; Thompson et al, 1999; Vandepapeliere et al, 2005) with a clearance of condyloma obtained in 75 and 20% of cases, respectively. In the absence of control group in these trials, it is too early to make conclusions about the efficacy of these vaccines. In a phase I/II trial, thirty males presenting with intraurethral flat condyloma were treated with either a recombinant vaccinia viral vaccine MVA-E2 (expressing the E2 gene of bovine papillomavirus) (Albarran et al, 2007). 28/30 patients treated with MVAE2 vaccine were free of clinical or histological lesion or HPV at 4 weeks.

16. How to determine the epitopic regions for a therapeutic vaccine?

In a study including 16 women presenting with usual VIN, we have determined the strongly immunogenic regions from HPV16 E6 and E7 proteins for CD4+ and/or CD8+ T lymphocytes (Bourgault Villada et al, 2010b). Among 18 large peptides of the proteins E6 and E7, two were recognized in proliferative assays as immunodominant by T cells from 10 out of 16 women (62%) at the entry in the study, namely E6/2 (aa 14-34) and E6/4 (aa 45-68) peptides. Four other peptides, E6/7 (aa 91-110), E7/2 (aa 7-27), E7/3 (aa 21-40) and E7/7 (aa 65-87) were recognized by only 12% of the women in proliferative or IFN γ ELISPOT tests. The regions of E6 and E7 proteins implicated in T cell recognition during HPV infection were not yet well defined because of the usually low frequency of anti-HPV blood T cell responses and of the difficulties of their study.

In protein E6, some peptides included in, including or overlapping our peptides E6/2 (aa 14-34) and E6/4 (aa 45-68) have already been described as preferentially recognized by

CD4⁺ T cells. Among them, peptide E6 42-57 that is restricted by HLA-DR7 has already been identified (Strang et al, 1990). Regions E6 1-31, 22-51 and 24-45 can be also immunogenic for CD4⁺ T cells as shown in CIN or sexually active healthy women (Kadish et al, 1997). The region E6 42-71, which includes peptide E6/4 (aa 45-68), has also been described as a target of proliferative responses in CIN patients (Kadish et al, 1997). Another E6 111-158 region was previously described as inducing proliferative responses in infected asymptomatic subjects or in patients with CIN3 (Kadish et al, 1997; Strang et al, 1990) as well as E6 127-141 peptide in healthy young women (Gallagher et al, 2007). Similarly, peptides E7 43-77, E7 50-62 and E7 58-68 which are restricted by DR3, DR15 and DR17, respectively, were defined as epitopic peptides for CD4⁺ T cells (Strang et al, 1990; van der Burg et al, 2001; Wang et al, 2009). E7 region 51-98, including our E7/7 (aa 65-87) peptide, is also very immunogenic for proliferating T lymphocytes (de Gruijl et al, 1998; Luxton et al, 1996; Nakagawa et al, 1996).

The characterization of E6 and E7 HPV-16 epitopes and the HLA restriction of their recognition by CD8⁺ T lymphocytes are more precise: E6 29-38, E7 11-20, E7 82-90 and E7 86-93 epitopes are presented by HLA-A2 (Evans et al, 2001; Rensing et al, 1995, 1996), E6 80-88 and E7 44-52 by HLA-B18 (Bourgault Villada et al, 2000) and E6 49-57 by HLA-A24 (Morishima et al, 2007). In women who cleared HPV 16 infection, cytotoxic T lymphocytes (CTL) responses are directed against epitopes preferentially located in the N-terminal half of the E6 protein (region 16-40) (Nakagawa et al, 2005). In this fragment, the dominant epitope E6 29-37 is restricted by HLA-B48, E6 31-38 by HLA-B4002 and the subdominant epitope E6 52-61 by HLA-B35 (Nakagawa et al, 2007). The same group had also shown that the peptide E6 33-42 is recognized by CD8⁺ T lymphocytes in association with HLA-A68, peptide E6 52-61 in association with HLA-B57 and -B35, peptide E6 75-83 in association with HLA-B62, peptide E7 7-15 in association with HLA-B48 and peptide E7 79-87 in association with HLA-B60 (Nakagawa et al, 2004, 2007; Wang et al, 2008). In addition, E7 7-15 is also able to bind HLA-A2 and -B8 to be recognized by CTL (Oerke et al, 2005; Rensing et al, 1995). From the latter results, two hot spots of CD8⁺ T-cell epitopes in protein E6 may be located in the regions E6 29-38 and 52-61 and another one in protein E7 (E7 7-15) (Nakagawa et al, 2007). Nevertheless, a poor immunogenicity of E7 protein was observed in many studies during both HPV 16 infection and after peptidic vaccination using long peptides spanning both E6 and E7 (Kenter et al, 2008; Welters et al, 2008) such as those used in our study.

The epitopes E6/2 (aa 14-34) and E6/4 (aa 45-68) hence could be strongly recognized by CD4⁺ and / or CD8⁺ T lymphocytes and could be particularly relevant in the design of a peptide vaccination. We may hypothesize that the T cell responses that we observed were able to contain the tumor cells into the epithelium. Therefore, E6/2 (aa 14-34) and E6/4 (aa 45-68) peptides could play a major role in the protection against invasive cancer by stimulating T lymphocytes.

17. Conclusion

HPV infections are very frequent. Eighty percent of women more than 25 years old have been infected. Two third of them have been infected by a oncogenic HPV and 10% of them will develop an intraepithelial neoplasia, mainly CIN. Preventive vaccines are very effective

means of avoiding CIN and cervical cancer with an efficacy of 70% against CIN 2/3 related to 14 oncogenic HPVs. Some questions persist about this preventive vaccine: How long will the protection last ? Boosts will be necessary ? Young boys should be also vaccinated ? What is the best age to perform the vaccine with the highest immunogenicity ?

Women previously infected by HPV 16 or 18 and presenting with intraepithelial neoplasia are not good candidate for prophylactic vaccines. Therapeutic vaccines should be good alternatives to surgery for CIN2/3, VIN3 and AIN3 and they are being continuously improved.

18. References

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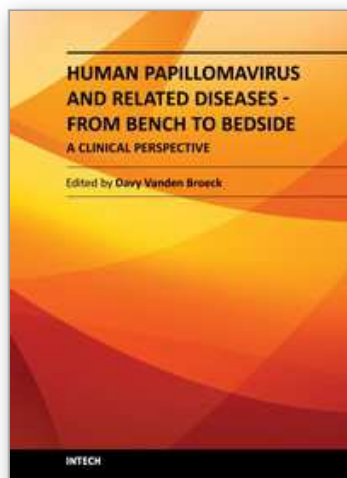
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Zhang, L. F., Zhou, J., Chen, S., Cai, L. L., Bao, Q. Y., Zheng, F. Y., Lu, J. Q., Padmanabha, J., Hengst, K., Malcolm, K., & Frazer, I. H. (2000). HPV6b virus like particles are potent immunogens without adjuvant in man. *Vaccine* 18:1051-8.

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Human Papillomavirus and Related Diseases - From Bench to Bedside - A Clinical Perspective

Edited by Dr. Davy Vanden Broeck

ISBN 978-953-307-860-1

Hard cover, 348 pages

Publisher InTech

Published online 20, January, 2012

Published in print edition January, 2012

Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Isabelle Bourgault-Villada and Simon Jacobelli (2012). Current Insight into Anti-HPV Immune Responses and Lessons for Prophylactic and Therapeutic Vaccines, Human Papillomavirus and Related Diseases - From Bench to Bedside - A Clinical Perspective, Dr. Davy Vanden Broeck (Ed.), ISBN: 978-953-307-860-1, InTech, Available from: <http://www.intechopen.com/books/human-papillomavirus-and-related-diseases-from-bench-to-bedside-a-clinical-perspective/current-insight-into-anti-hpv-immune-responses-and-lessons-for-prophylactic-and-therapeutic-vaccines>

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