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Human papillomavirus-associated cervical cancer: Prophylactic and therapeutic vaccines

Review Article

Marcio O. Lasaro and Hildegund C.J. Ertl*

The Wistar Institute, 3601 Spruce St, Philadelphia, PA 19104

***Correspondence**: Hildegund C.J. Ertl, The Wistar Institute, 3601 Spruce St, Philadelphia, PA 19104; Tel: 215 898 3863; e-mail: ertl@wistar.upenn.edu

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Abbreviations: Adeno-associated virus, (AAV); bacilli Calmette-Guerin, (BCG); base pairs, (bp); cottontail rabbit papilloma virus, (CRPV); E6-associated protein, (E6-AP); epidermal growth factor receptor, (EGFR); histone deacetylases, (HDAC); human papillomavirus, (HPV); inverted terminal repeats, (ITRs); listerolysin O, (LLO); of lysosome-associated membrane protein, (LAMP); open reading frames, (ORF); Real-time reverse transcription polymerase chain reactions, (RT-PCR); signal sequence of LAMP, (Sig-LAMP); untranslated regions, (UTRs); virus like particles, (VLPs)

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Summary

Cervical cancer remains a leading cause of cancer-related mortality in reproductive age women. Although effective screening programs have decreased the incidence of cervical cancer in developed countries, they are too expensive for comprehensive use in developing countries. Since 60-80% of cervical cancers are associated with human papillomavirus (HPV)-16 and HPV-18 infections, antigens encoded by these viruses are obvious targets for prophylactic and therapeutic vaccines. Prophylactic vaccines aim to reduce the incidence of cervical cancer by preventing virus infection through induction of virus neutralizing antibodies against capsid proteins. Therapeutic vaccines are designed to induce cellular immune responses able to clear persistent infections with HPV or to eradicate cells transformed by HPV's oncoproteins. A number of candidate prophylactic and therapeutic HPV vaccines have been developed and tested in animal models and are now approaching clinical trials.

I. Introduction

Cervical cancer is the 2nd most common cause of cancer death in women worldwide claiming approximately 300.000 - 400.000 lives each year (Pisani et al, 1993). In the US ~ 4.800 women die each year from cervical cancer. Worldwide, cervical cancer affects ~ 1% of all women and is the most common cause of cancer death in women under the age of 50. Over 90% of cervical cancers are associated with human papillomavirus (HPV) infections. Prevalence of sexually transmitted infections with oncogenic types of HPV varies between 20-80% of sexually active adults depending on the study population. Most women infected with oncogenic types of HPV do not develop cancer but instead mount an immune response that resolves the infection. Women who fail to eliminate the infected cells for unknown reasons or due to underlying immunodeficiencies including infections with HIV-1 can develop progressive cancer. Although infections generally occur in young women (under 40 years of age) the mean age of diagnosis of cervical cancer is 54, suggesting a long latency between initial infection and development of malignant cell growth. Mortality due to cervical cancer decreased in the US by 70% between 1947 and 1984 due to aggressive implementation of Papanicolaou (Pap)-smear screening, which detects low or high-grade intraepithelial lesions. The sensitivity of Pap smear screening is limited leading to false negative results in up to 25% of the samples. Furthermore, screening programs are absent or minimal in less developed countries. Patients diagnosed early during stage I when the cancer is still confined to the cervix can generally be cured by simple hysterectomy (removal of the uterine corpus and cervix) with (stage IB) or without (stage IA) radiation therapy. More aggressive surgery including radical hysterectomy, which includes removal of the cervix, the uterus, the parametria, the uterosacral ligaments and a 2-3 cm cuff of the vagina, followed by radiation therapy cures (survival at 5 years) 80-90% of women with stage IIA cervical cancer. Women with stages IIB, III and IVA have in spite of aggressive therapy a more dismal prognosis with 5-year survival rates of 66, 40 and less than 20% respectively. Patients diagnosed with stage IVB (distant metastasis) cannot be cured. Approximately 30% of women with invasive cervical cancer either fail to enter

remission upon treatment or they relapse. Recurrent disease is treated with palliative chemotherapy, which generally fails to affect a cure. The viral etiology of cervical cancer invites for the development of preventative or therapeutic vaccines (**Table 1**).

II. The role of HPV in cervical cancer

Extensive epidemiologic data have firmly demonstrated the etiologic association of certain types of HPV with a variety of anogenital neoplasias, including condylomata (genital warts), cervical dysplasia, and cervical carcinoma. Over 100 types of HPVs have been classified on the basis of DNA sequence homology, correlating with biological behavior and tissue tropism (van Ranst et al, 1996). Mucosotropic HPVs are grouped into low- and high-risk categories on the basis of each genotype's association with benign or malignant disease. Low-risk HPV genotypes, such as HPV-6 and HPV-11, cause benign warts and low-grade pre-malignant lesions. Virtually 100% of cervical neoplasia is associated with HPV. Although a large number of high-risk HPV genotypes (including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) can be associated with cancer, HPV-16 and HPV-18 are the two most prevalent serotypes associated with cervical cancer; HPV-16 sequences are found in approximately 50-60% of cervical malignancies while HPV-18 sequences are present in 10-20% (Bosch et al, 1995; Walboomers et al, 1999). More recently, HPVs have also been linked to head and neck cancers (de Villiers et al, 1985).

III. Structure of HPV

HPVs are icosahedral non-enveloped viruses with double-stranded circular DNA of approximately 8,000 base pairs (bp); the genome has a non-coding region containing transcription regulatory sequences and the origin of replication, and eight open reading frames (ORF), encoding six early proteins (E1, E2, E4, E5, E6, and E7) and two late proteins (L1 and L2). The two late genes encode for the major (L1) and minor (L2) capsid proteins, whereas the early genes encode proteins with a variety of regulatory functions (zur Hausen, 1996). E1 and E2 are expressed first; they form a complex that recruits cellular transcription factors to the viral origin of replication to initiate DNA replication. E1 is a helicase that helps to unravel the viral double stranded DNA. E2 at low concentrations activates the early promoter of HPV while at high concentrations E2 has a negative effect by inhibiting binding of cellular transcription factors. E4 is expressed late before synthesis of L1 in differentiating layers of epithelium. One of its functions is to associate with the cellular cytokeratin causing disruption of its structure, which in turn may facilitate the release of virus particles (Doorbar et al, 1991).

E5 protein is expressed late during viral replication in differentiated cells. It increases phosphorylation of the epidermal growth factor receptor (EGFR) and reduces its degradation. The increased expression of EGFR initiates overexpression of a variety of proto-oncogenes and stimulates cell growth (Crusius et al, 1997). In addition, E5 inhibits the expression of the tumor suppressor gene p21 and impairs the control of cell cycle checkpoints (Tsao et al, 1996). E5 is highly expressed in all early stage cervical cancers. A significant percentage of cancer cells from women with late stage cervical cancer including metastatic disease continue to express E5 while in other patients E5 expression is extinguished after HPV genome integration. E5 thus apparently plays a role in the early stages of cell transformation (DiMaio and Mattoon, 2001) although it is dispensable for malignant transformation.

E6 protein forms a complex with E6-associated protein (E6-AP) and p53 protein leading to a rapid degradation of p53 through ubiquitin-directed proteolysis (Munger, 1995). E6 can also retain p53 in the cytoplasm, blocking its translocation to the nucleus thus inhibiting its function independent of degradation (Mantovani and Banks, 1999). E6 furthermore activates telomerase causing

Table 1

Approach	Antigen	Immune Response	Vaccine Carrier	Key References
Preventative Vaccines	Capsid antigens: L1, L2	Virus-neutralizing antibodies	VLPs	Rose et al, 1993; Koutsky et al, 2002
			DNA vaccines	Kowalczyk et al, 2001
			Adenovirus vector	Kowalczyk et al, 2001; Tobery et al, 2003
			Vaccinia virus vector	Zhou et al, 1992
			Salmonella Typhimurium	Nardelli-Haefliger et al, 1997
			Bacillus Calmette-Guerin	Jabbar et al, 2000
Therapeutic Vaccines	Oncoproteins: E7, E5, E2, E4	Cell-mediated immunity	Peptides	Tindle et al, 1995; Muderspach et al, 2000
			Adjuvanted Proteins	Hariharan et al, 1998; de Jong et al, 2002
			DNA vaccines	Chen et al, 2001; Liu et al, 2002; Kotecha et al, 2003
			Viral replicons	Daemen et al, 2003; Velders et al, 2001
			Poxvirus Vectors	Gao et al, 1994; Boursnell et al, 1996
			Adenovirus Vectors	He et al, 2000; Liu et al, 2000
			AAV Vectors	Liu et al, 2000
			Listeria monocytogenes	Gunn et al, 2001; Sewell et al, 2004
			Lactococcus lactis	Cortes-Perez et al; 2003
			Chimeric VLPs	Greenstone et al, 1998; Schafer et al, 1999
			Dendritic Cells	Okada et al, 1998; Tillman et al. 2000

an extension of the lifespan of infected cells (Klingelhutz et al, 1996).

E7 protein binds to the hypophosphorylated form of the retinoblastoma tumor suppressor protein (pRb), releasing the pRb-bound cellular transcription factor E2F. This results in the activation of E2F-dependent genes required for DNA synthesis. E7 interacts with others proteins that control cell growth, such as other retinoblastoma tumor suppressor family members (p107 and p130), histone deacetylases (HDAC), AP-1 transcription factors, cyclins, cyclin-dependent kinases (cdk) and cdk inhibitors (Brehm et al, 1999; Dyson, 1998; Funk and Kind, 1997; Munger et al, 1999).

The E6 and E7 oncoproteins of oncogenic types of HPV being causative for transformation of infected cells are natural targets for active immunotherapy of HPV-associated cervical cancer. Both proteins drive the malignant phenotype of cancer cells and are thus not eliminated during cancer progression. This is especially the case for E7. Some cervical cancers show mutation of p53; this may allow the cancer cells to progress in absence of E6.

Upon infection, the HPV genome persists mainly episomally. Integrated HPV DNA is found in the majority of advanced cervical lesions and invasive tumors. Integrated HPV-16 genome causes increased levels of expression of E6 and E7 transcripts, which may result from disruption of the E2 gene during integration (Frattini and Laimins, 1994) as E2 reduces transcription of E6 and E7 transcription by binding to palindromic sequences located close to regulatory elements in the untranscribed long control region (Desaintes et al, 1997).

L1, the major capsid protein of papillomaviruses assembles into capsomers, each of which contains 5 L1 molecules. In turn, 72 of L1 capsomers form the viral capsid (Baker et al, 1991; Galloway, 2003). The location of the minor coat protein L2 within the capsid remains undefined although it is thought that L2 is located at the vertices of the icosahedrons. Both L1 and L2 bind virus-neutralizing antibodies, which recognize conformation dependent epitopes on correctly folded proteins (Christensen and Kreider, 1991; Christensen et al, 1991; Hines et al, 1994).

IV. The lifecycle of oncogenic types of HPV

Upon sexual transmission, HPV enters the epithelium through microlesions and infects either basal or parabasal cells. Although the pathway of cell entry is thus far poorly understood, alpha integrins have been identified as candidate cellular receptors for HPV (Evander et al, 1997). The viral DNA replicates in cells of the basal layer to ~20-100 copies of genome, which are maintained at fairly stable numbers in undifferentiated cells. Production of mature infectious virus particles is restricted to differentiated suprabasal cells. Epithelium has a rapid turn over; cells migrate from the basal layers, differentiating as they progress to be at the end exfoliated from the surface and replaced by cells from below. Upon cell differentiation, one daughter cell remains part of the basal epithelium while the other daughter cell starts to differentiate. To optimize cellular conditions that allow HPV to progress through its replicative cycle, virally encoded early antigens induce proliferation of partially differentiated cells, which in turn causes cellular atypia followed by neoplasia.

V. Immune correlates of protection against HPV-associated cervical cancer

Serotype-specific HPV neutralizing antibodies directed against the two capsid proteins, most notably the more abundant L1 protein, prevent infections with HPV (Evans et al, 2001; Harro et al, 2001; Embers et al, 2002). Once an infection has occurred, humoral immune responses detectable in cervical cancer patients fail to affect viral clearance, nor do they inhibit development of malignancies. Consequently, women with humoral immunodeficiency do not have an increased susceptibility to cervical cancer while women with cell-mediated immunodeficiency, such as HIV patients, renal transplant patients or patients with genetic T cell deficiencies, have increased incident rates (Matas et al, 1975; Frisch et al, 2000; Lowy and Gillison, 2003; Moscicki et al, 2004). This together with extensive studies in animal models followed-up by clinical trials [reviewed in (Galloway, 2003)] implicates a crucial role for T cells in eliminating cells persistently infected with oncogenic types of HPVs.

VI. Preclinical models for testing of HPV vaccines

Development of vaccines to HPV has been hampered by the lack of suitable animal models. Cottontail rabbits, bovines and canines are naturally infected with papilloma viruses that cause malignant or benign tumors. Although these animal models have been used extensively they relay on surrogate viruses that although related to the oncogenic types of HPV each shown a unique biology. Testing for neutralizing antibodies to the capsid protein has been hindered by the lack of suitable cell lines that allow for propagation of oncogenic types of HPV. Surrogate neutralizing antibody assays have been established (Roden et al, 1996a; 1996b) and they have by now been validated by efficacy studies in pre-clinical and clinical trials (Galloway, 2003).

Initial pre-clinical testing of vaccines especially those that aim to induce protective T cell responses is generally conducted in inbred strain of mice which are genetically well defined and for which reagents for analyses of vaccine immunogenicity are in ample supply. Therapeutic vaccines to HPV have thus far been tested mainly in so-called transplantable tumor cell models. In these models, mouse fibroblasts (He et al, 2000) or epithelial cells (Ji et al, 1999) are transformed by transfection with vectors expressing E6 and E7 together with another oncoprotein such as v-Ha-ras. The transformed cells form tumors upon injection into syngeneic mice. Vaccine efficacy can be assessed by correlating the vaccine's immunogenicity such as its ability to induce $CD8^+$ or $CD4^+$ T cells to the HPV oncoproteins with its ability to protect vaccinated animals against formation of tumors or to cause clearance of already established tumors. The results vary depending on

the tumor cell line, on the number of tumor cells injected into mice, and on the route of application of the tumor cells. Overall, although immunogenicity studies conducted in experimental animals, such as testing for antibodies or frequencies of specific T cells, are likely to be relevant for the prospect of the vaccine's efficacy in a human population, protection achieved in transplantable tumor models most likely paints an unduly optimistic picture as such models fail to take into account that persistent viral infections, slowly developing into progressing tumors, have profound effects on the adaptive immune system.

To circumvent limitations of transplantable tumor models, transgenic mice that due to genetic engineering express oncoproteins, such as those derived from HPV-16, have been developed. Transgenic mice that express the entire HPV-16 genome or E6/E7 of HPV-16 under the control of permissive promoters develop a variety of different types of cancer, which apparently arise through multiple different pathways (Yang et al, 1995). Using tissue specific promoters allows for construction of mice prone to more predictable types of cancer. For example, mice transgenic for E6/E7 under the control of an alphaA-crystallin promoter, which targets expression to the lens, develop eye tumors as well as skin tumors, the latter due to ectopic expression of the oncoproteins (Hilditch-Maguire et al, 1999). Mice expressing E6/E7 under the control of the keratin 5 (K5) promoter developed lung carcinomas (Carraresi et al, 2001), while expression of E6/E7 under the beta-casein promoter causes mammary gland tumors (Hwang et al, 2000). Transgenic mice, in which the E6/E7 genes are controlled by the keratin 14 (K14) promoter restricting their expression to epithelial cells, develop skin cancer late in life (Sethi and Palefsky, 2004). Treatment of females of such transgenic strains of mice with sex hormones results in a high incidence of cervical cancers, which progress similar to those in women naturally infected with HPV-16 (Arbeit et al, 1996).

E6/E7 transgenic mice, especially those that develop cervical cancer upon hormone treatment are appealing for pre-clinical vaccine testing. They spontaneously develop cancer at a fairly predictable time after treatment with sex hormones. Mice can be screened histologically and therapeutic vaccination can then be administered at different stages of cancer progression. Unlike mice injected with transplantable tumors, E6/E7 transgenic mice are expected to be at least in part tolerant to the oncoproteins of HPV. Indeed it has been shown experimentally that E6/E7 transgenic mice can mount humoral but not T cell-mediated immunity to E7 (Borchers et al, 1999). Women, who develop cervical cancers due to persistent infections with HPV, are likely to become low or unresponsive to antigens of HPV that are expressed in infected cells due to induction of peripheral T cell tolerance. Only vaccines that can break this tolerance may succeed in clinical trials.

VII. HPV Vaccines

Most vaccines prevent infections through the induction of virus neutralizing antibodies. Such prophylactic vaccines can reduce the incidence of cancer as best exemplified by the vaccine to hepatitis B virus, which dramatically decreased the rate of hepatocellular carcinomas (Schafer and Sorrell, 1999). As cervical cancer is caused by viral infections, vaccine-induced neutralizing antibodies that block entry of HPV would be expected to reduce the incidence of cervical cancer.

Once an infection has occurred and caused cell transformation, a different type of vaccine is needed. The concept of active immunotherapy of cancer was first established in the 18th century without knowledge of the molecular events that contribute to cell transformation or the immune mechanisms that might halt tumor progression. In spite of the impressive advances in our understanding of molecular oncology and basic immunology, only limited progress has been achieved in generating efficacious vaccines for routine treatment of cancer patients. This has in part been related to the lack of well-defined tumor antigens suitable as targets for immunotherapy. Other obstacles such as immunosuppressive effects of established cancers remain to be overcome. Cancers associated with well-defined viral antigens required for the malignant phenotype such as E6 and E7 of HPV-16 or –18 furnish obvious targets for active immunotherapy. One would expect that the thus far frustrated field of therapeutic cancer vaccines would see its first success in cancers associated with persistent viral infections.

Advances in immunology have greatly facilitated the analysis of antigen-specific immune effector mechanisms (Altman et al, 1996; Crawford et al, 1998; Labalette-Houache et al, 1991; Taguchi et al, 1990) able to control tumor progression. Cytolytic T cells against a defined epitope can be quantified by intracellular cytokine staining of peptide-reactive $CD8^+$ T cells or by direct staining with MHC-peptide tetramers, thus allowing rapid and accurate assessment of vaccine-induced immune responses. Similar techniques are being developed for $CD4^+$ T cells against defined epitopes as well as for T cells against yet undefined antigens or mixtures of antigens. B and T cells can be quantified by ELISPOT assays. Real-time reverse transcription polymerase chain reactions (RT-PCR) or RNAse protection assays allow for detection of cytokine and chemokine transcripts. These quantitative methods should facilitate selection of vaccine candidates for further clinical development.

A. Prophylactic vaccines to HPV

Prophylactic vaccines to HPV have focused on the major capsid protein L1 of HPV given either alone or in combination with L2. L1 protein with or without L2 protein of HPV-16 self assembles into virus like particles (VLPs) (Hagensee et al, 1993; Kirnbauer et al, 1993) that are highly immunogenic (Kirnbauer et al, 1992; Harro et al, 2001). VLPs readily induce neutralizing antibodies to HPV, which are not induced by incorrectly folded L1 protein. VLPs purified from different expression systems have been used for immunization through injection or by the oral route. VLPs have been expressed in bacteria (Li et al, 1997), baculovirus transduced insect cells (Rose et al, 1993), yeast (Brown et al, 2001), as well as plant cells including potatoes and Tobacco leaves (Warzecha et al, 2003). Purified VLPs given without adjuvant, induced

neutralizing antibodies in mice and humans. Some investigators (Palker et al, 2001) included aluminum hydroxide as an adjuvant for intramuscular vaccination. For mucosal application, additives such as a thermosensitive mucoadhesive delivery system based on ploxamers and polyethylene oxide (Park et al, 2003), E. coli heat labile enterotoxin mutant R192G and CpG DNA were explored (Gerber et al, 2001). VLPs were shown to transduce dendritic cells, where they enter the MHC class I processing pathway (Lenz et al, 2001). This in turn triggers strong $CD8^+$ T cell responses. $CD8^+$ T cell responses may not be clinically useful, as L1 is expressed in fully differentiated epithelial cells that are destined to die. VLPs have been used as delivery systems for HPV oncoproteins; L1 fused to a small portion of E7 were shown to induce E7-specific $CD8^+$ T cell responses in experimental animals, which provided protection to tumor cell challenge (Greenstone et al, 1998; Schafer et al, 1999). Due to structural constrains, only small protein fragments can be fused to L1. Larger fragments would affect the structure of L1 and thus prevent formation of VLPs. It should be noted that small protein fragments may perform poorly in an outbred human population.

Genetic vaccines expressing L1 have been tested for the induction of neutralizing antibodies in pre-clinical animal models. DNA vaccines (Kowalczyk et al, 2001), bacterial expression systems such as recombinant *Salmonella* Typhimurium (Nardelli-Haefliger et al, 1997), recombinant Bacilli Calmette-Guerin (Jabbar et al, 2000) or viral vectors such as vaccinia (Zhou et al, 1992) or replication-defective adenoviral vectors (Kowalczyk et al, 2001; Tobery et al, 2003) carrying wild-type or codon-optimized forms of L1, given either alone or in prime boost regimens, induced HPV-16 antibodies with apparent neutralizing activity. Such constructs, which remain to be optimized and tested in more depth, may provide valid second generation preventative vaccines to oncogenic types of HPV.

Currently, purified VLPs are clearly leading the field. They were recently evaluated in a double-blinded, multicenter, randomized clinical trial to determine if they prevented HPV-16 infections in young women (Koutsky et al, 2002). A total of 2392 16-23 year old women were enrolled in the study. After vaccination with VLPs or placebo, biopsy tissue was evaluated for cervical intraepithelial neoplasia and analyzed for HPV-16 DNA by PCR. Three intramuscular administrations of 40_g of HPV-16 L1 VLP formulated with aluminum adjuvant reduced the incidence of both HPV-16 infection and HPV-16-related cervical intraepithelial neoplasia in tested women when assessed one year later. Although some questions such as vaccine stability and longevity of protection remain to be addressed, the results of this trial are very encouraging.

B. Therapeutic vaccines to HPV

Different types of vaccines have been tested for induction of cellular immune responses to HPV-16 transformed cells focusing mainly on immunization to E7 and to a lesser extent to E6, E5 and/or E2. These have included peptides, adjuvanted proteins, DNA vaccines and viral replicons, viral or bacterial recombinant vaccines and antigen-pulsed dendritic cells.

1. Peptide and protein vaccines

Peptides present a single epitope from a well-defined tumor antigen to the patients' immune system. Cell-mediated immune responses to individual epitopes are genetically restricted and only stimulate T cells in a subset of an out-bred population (Klein, 1987; Theze, 1984). This can be overcome by using mixtures of peptides. Peptides have a very short serum half-life and are thus commonly poorly immunogenic. This can in part be circumvented by their incorporation into microparticles (Ertl et al, 1996) or by the addition of terminal side chain modifications (Otvos et al, 1996), which inhibit degradation by peptidases.

Peptide vaccines carrying an immunodominant MHC class I epitope of E7 for mice of the $H\text{-}2^b$ haplotype were shown to induce a $CD8^+$ T cell response and protection against tumor cell challenge in the appropriate strains of mice (Feltkamp et al, 1993). Vaccination with longer peptides expressing the $CD8^+$ T cell E7 epitope caused regression of already established tumors (Zwaveling et al, 2002). A peptide vaccine expressing B, $CD4^+$ and $CD8^+$ T cell epitopes of E7 in tandem linked to an integral membrane protein of E. coli induced protective immunity in mice (Tindle et al, 1995). These studies led to Phase I clinical trials in HLA-$A0201^+$ cervical cancer patients with peptides expressing CD8 and CD4 epitopes of E7 of HPV-16 (Muderspach et al, 2000). Results were promising; the vaccine showed immunogenicity and induced complete or partial remission in some of the women. In another clinical trial, women with refractory cervical cancer were injected with a lipidated E7 peptide vaccine. Although some of the patients developed detectable $CD8^+$ T cell responses to E7, none of them showed a clinical response (Steller et al, 1998).

Protein vaccines to E7 given to mice in adjuvants, such as PROVAX (Hariharan et al, 1998), together with a cytokine such as interleukin (IL)-12 (Ahn et al, 2003) or as a fusion protein with the heat shock protein (hsp)-65 of *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) (Chu et al, 2000) induced protection against tumor growth if given before or after tumor cell challenge. Adjuvanted protein vaccines were tested in healthy volunteers; they were well tolerated and induced detectable B and T cell responses (de Jong et al, 2002).

2. DNA vaccines: bacterial expression vectors

DNA vaccines are bacterial expression vectors, which carry the gene of interest under the control of a suitable promoter such as the commonly used early promoter of cytomegalovirus. Plasmid vectors, when injected into animals, are taken up by cells close to the injection site including low numbers of antigen presenting cells. Uptake can be improved by using different formulations (Caputo et al, 2002) or by applying electric pulses (Otten et al, 2004). Alternatively, plasmid vectors coated to gold beads can be given by a so-called gene gun (Barry et al, 1995), which directly shoots the particles into cells of the skin including Langerhans' cells, which can

serve as antigen-presenting cells. Gene gun application is more efficient than application by syringe and allows for a reduction in the amount of DNA vaccine. Some reports suggest that gene gun immunization favors induction of T helper cells of the TH2 type while intramuscular immunization strongly supports TH1 mediated immunity (McCluskie et al, 1999) best suited to promote activation of $CD8^+$ T cell responses. Bacterial expression vectors provide their own adjuvant in form of unmethylated CpG sequences present in the bacterial backbone (Krieg et al, 1998). Such sequences, which are rare and generally methylated in mammalian genome, serve as pathogen-associated molecular patterns. They activate Toll-like receptor 9, which in turn induces an NF-κB response, leading to production of pro-inflammatory cytokines, and activation of immature dendritic cells (Akira and Hemmi, 2003). Due to the immunostimulatory effects of CpG sequences, DNA vaccines induce adaptive immune responses to the transgene product without requiring addition of an adjuvant. Nevertheless, addition of adjuvants can improve the efficacy of DNA vaccines (Ulmer et al, 1999). DNA vaccines induce a full spectrum of immune responses including $CD8^+$ T cells (Donnelly et al, 1995). They are easy to manufacture and clinical trials thus far indicate that they are well tolerated (MacGregor et al, 1998). DNA vaccines do not induce potent immune responses and their efficacy in clinical trials has been disappointing (MacGregor et al, 1998). Nevertheless, they may have one distinctive advantage as vaccine carriers for tumor-associated antigens. DNA vaccines, for reasons that are currently poorly understood, are apparently well suited to induce T cell-mediated immune responses to cryptic or subdominant epitopes as was initially shown with a DNA vaccine to hepatitis B virus surface antigen (Schirmbeck and Reimann, 2001). Tumor-associated antigens, including oncoproteins of HPV, are likely to induce tolerance of T cells directed to their dominant epitopes, during progression from pre-malignant lesions, to invasive cancer, due to constant inappropriate (that is non activating) antigen presentation. T cells to subdominant epitopes may escape tolerization and could hence be preferred targets for vaccine-induced activation.

DNA vaccines are exceedingly easy to manipulate and, consequently, a multitude of DNA vaccines expressing E7 or E6 have undergone pre-clinical testing. The results were mixed. In a rabbit model, a DNA vaccine encoding ubiquitin fused to E1, E2 and E7 prevented papilloma formation in rabbits subsequently challenged with cottontail rabbit papilloma virus (CRPV) (Leachman et al, 2002). In another study, DNA vaccines to E6 or E7 were administered to rabbits with established CRPV-induced papillomas (Han et al, 2000). The E6 vaccine had no effect while the E7 vaccine caused some delay in cancer development. The group of T.C. Wu tested a number of strategies to improve DNA vaccines to E7 in mice (Cheng et al, 2001; Hsu et al, 2001; Hung et al, 2003; Ji et al, 1999; Kim et al, 2004a; 2004b). Fusion of E7 to the sorting signal of lysosome-associated membrane protein (LAMP), Mycobacterium hsp-70, calreticulin, the translocation domain (dII) of *Pseudomonas aeruginosa*, or gamma tubulin, were all shown to increase $CD8^+$ T cell responses to E7 and protection against tumor cell challenge. Other investigators showed protection against tumor cell challenge with DNA vaccines linked to the VP22 of herpes simplex virus (Michel et al, 2002) or with DNA vaccines carrying a codon-optimized E7 gene (Liu et al, 2002). We tested DNA vaccines expressing E6 or E7 linked to various sequences that influence intracellular trafficking or stability of the proteins such as LAMP, an adenoviral leader sequence or ubiquitin (Wlazlo et al, 2004). Unlike other groups, we were unable to induce protective immunity with any of the E7 expressing DNA vaccines against a high dose tumor cell challenge but rather observed accelerated tumor growth. In our hands priming with the E7 vaccines also reduced the efficacy of a viral vector vaccine to E7 given as a boost. The DNA vaccines to E6 yielded more promising results and especially the vaccine that expressed E6 linked to a leader sequence induced partial protection to tumor challenge. Protection induced by the E6 DNA vaccine was most likely mediated by $CD4^+$ rather than $CD8^+$ T cells. Another group also reported that vaccination with a DNA vaccine expressing E7 accelerated progression of transplanted tumors (Kotecha et al, 2003). Variation in the efficacy of very similar vaccines ranging from induction of complete protection against challenge or even eradication of existing tumors to accelerated tumor progression most likely reflect differences in the challenge models.

A clinical trial with a DNA vaccine expressing multiple HLA-A2 restricted epitopes of E7 encapsidated into a biodegradable polymer microparticles was conducted in 12 patients with HPV-16 associated anal dysplasia; 10 subject showed an increase in their cell-mediated immune response to E7 and 3 subjects showed a partial histological response (Klencke et al, 2002). It is unclear if this response was mediated by an adaptive immune response to E7 or an innate immune response activated by the vaccine carrier.

3. DNA vaccines: viral replicons

Most viral replicons currently in pre-clinical testing are based on alpha viruses, which belong to the family of Togaviruses. The genus Alphavirideae contains 25 viruses, which are associated with a variety of diseases such as encephalitis (example: Venezuelan Encephalits virus [VEE]) or polyarthritis (examples: Sindbis and Semiliki Forest virus). The viral genome is transcribed and replicates in the cytoplasm. Togaviruses are small, enveloped viruses with a plus-strand RNA genome of ~ 12 kb that contains two ORFs flanked by 5' and 3' untranslated regions (UTRs). The 5' ORF encodes non-structural proteins needed for transcription and replication of the viral RNA and the 3' ORF encodes the capsid proteins. The 5' terminus as well as regions close to the 3' and 5' termini and in between the two ORFs have regulatory functions such as the subgenomic promoter located upstream of the 3'ORF. The genomic RNA serves as messenger RNA for translation of the non-structural proteins and as template for generation of the negative-stranded RNA and the so-called subgenomic RNA

composed of the 3' part of the viral genome including the subgenomic promoter, the 3' ORF and the 3' UTR.

Three different types of alphavirus-based vaccines have been developed [reviewed in (Schlesinger, 2001)]. One uses packaged replicon particles in which the 3' ORF that encodes the structural proteins is replaced with the gene of interest. Preparation of such vectors requires provision of the structural genes in trans in suitable packaging cell lines. These vectors have the advantage that they have a viral capsid that allows for efficient cell entry. Natural immunity to most alpha viruses is uncommon in the human population. This reduces the risk of interference of vector uptake due to pre-existing virus-neutralizing antibodies. A clear disadvantage of packaged replicon particles is that the structural proteins that coat the replicon's genome will induce an immune response including virus-neutralizing antibodies although they are not produced de novo by the transduced host cells. This in turn will limit the efficacy of repeated use of such constructs. Alternatively, alpha virus replicons can be used as naked RNA or DNA vaccines. Alpha virus-based RNA vaccines are self-replicating and self-limiting as they eventually induce apoptotic death of the transduced cells. They do not integrate and are thus safer than DNA-based replicon vaccines. In DNA-based replicon vectors part of the alpha virus' genome is incorporated into a bacterial plasmid vector. Transcription of the replicon RNA is driven by a RNA polymerase II-dependent promoter, which functions in the nucleus. The replicon RNA exists the nucleus and self-replicates and expresses of the gene of interest in the cytoplasm. Due to self-replication of the replicon RNA, alphavirus-based DNA vectors achieve in general higher expression than traditional DNA vaccines, which in turn translates into more potent transgene product-specific adaptive immune responses.

Both packaged and DNA-based alpha virus replicon vaccines have been used to elicit cell-mediated immune responses to HPV-16 oncoproteins. A Semiliki Forest virus-based packaged replicon vector expressing an E6/E7 fusion polypeptide induced transgene product-specific $CD8^+$ T cell responses and protection against tumor cell challenge (Daemen et al, 2002) and, upon further improvement of the vector, eradication of established tumors (Daemen et al, 2003). A VEE-based packaged replicon vaccine expressing E7 induced $CD8^+$ T cells responses, prevented tumor formation and caused partial regression of already established tumors (Velders et al, 2001). Similar results were obtained with a VEE replicon vaccine expressing E6 and E7 (Cassetti et al, 2004). A comparison study between a DNA vaccine, a naked RNA Sindbis replicon vaccine and a packaged Sindbis replicon vaccines all expressing E7 fused to VP22, a tegument protein of herpes simplex virus, showed that the packaged replicon vaccine outperformed the other two vaccines when tested in a therapeutic tumor model (Cheng et al, 2002). Vaccine efficacy was also achieved with a Semiliki Forest based DNA vaccine that in addition to E7 encoded BCL-xL to delay apoptosis of antigen-presenting cells (Kim et al, 2004).

More recently, flaviviruses, which are single stranded enveloped RNA viruses, have been vectored as self-replicating RNA vaccine vectors. One such vector derived from Kunjin virus, a flavivirus first isolated from mosquitoes in Australia that is closely related to Murray Valley Encephalitis virus, was tested in mice for induction of $CD8^+$ T cells. The vector expressed an epitope of E7 together with a number of other T cell epitopes (Herd et al, 2004). Comparing the efficacy of DNA vaccines, naked RNA replicon vectors and packaged Kunjin virus replicons, the packaged RNA replicon was found to induce superior T cell responses and protection to tumor cell challenge.

4. Virus vectors

Viral vectors similar to DNA vaccines induce a full spectrum of immune responses. Most viruses chosen for vaccine development have a broad tropism and hence achieve high levels of transgene product expression in large numbers of cells. Different types of viral vectors favor induction of different types of immune responses with for example poxvirus vectors promoting induction of potent $CD4^+$ T cell responses while replication-defective adenovirus vectors induce superior $CD8^+$ T cell responses (Fitzgerald et al, 2003). Virus vectors although in general by far more efficacious than DNA or peptide vaccines have two major disadvantages. Especially those that are replication competent such as poxvirus vectors based on vaccinia virus can cause serious reactions in immunocompromised hosts and potentially even in healthy adults as demonstrated by recent vaccination programs to protect military personnel against a bioterrorism attack with small poxvirus (Halsell et al, 2003). Even replication-defective vectors used at high doses, such as those based on E1-deleted adenovirus vectors, can cause complications due to induction of high and toxic levels of pro-inflammatory cytokines (Raper et al, 2003). A second disadvantage that is shared between virus vectors, packaged replicon vectors, and bacterial vectors discussed in the next paragraph is that pre-existing immunity, especially neutralizing antibodies to the vaccine carrier can severely dampen uptake of the vaccine vehicle (Fitzgerald et al, 2003), which is a pre-requisite for production of the vaccine antigen. Pre-existing immunity can be the consequence of a previous natural infection, such as with adenovirus of the human serotype 5, which infects nearly all humans during infancy, or it can be caused by vaccination, which is of concern for vectors based on vaccinia virus, used extensively in humans born before smallpox virus was eradicated. Induction of neutralizing antibodies to the antigens of a virus vector used for immunization to a heterologous protein also limits the repeated use of the same vector either for booster immunization against the same foreign protein or for immunization against an unrelated foreign protein. With advances in molecular microbiology, the repertoire of available virus vaccine vectors is expanding rapidly, offering solutions to the dilemma of pre-existing neutralizing antibodies to vaccine delivery vehicles.

4a. Poxvirus vectors

Poxviruses are large DNA viruses that cause a variety of diseases in different mammalian species.

Vectors based on vaccinia virus that originated from cowpox virus have been used widely in preclinical and clinical studies and a vaccinia virus recombinant expressing the glycoprotein of rabies virus was the first viral recombinant vector licensed for vaccination (Masson et al, 1999). The appeal of poxvirus vectors such as vaccinia virus, which can replicate in humans, modified vaccinia virus Ankara (Hodge et al, 2003), a highly attenuated variant of vaccinia virus, or avipox viruses (Marshall et al, 1999), which cannot replicate in mammals, is several fold. Poxviruses are highly immunogenic as was demonstrated with vaccinia virus during the successful smallpox virus eradication program. Poxviruses can be vectored readily through homologous recombination. The size of the poxvirus genome permits insertion of large foreign genes. Poxviruses replicate in the cytoplasm of infected cells and thus avoid problems associated with nuclear splicing of susceptible transcripts. Poxviruses cause lytic infections, they do not persist, and their genome does not integrate. Disadvantages of some types of poxviral vectors, e.g., those based on vaccinia virus, include their potential for serious complications. They also induce strong immune responses to their own antigens, which limits their usefulness for booster immunizations.

Vaccinia virus vectors expressing E6 of HPV-16 or E6 and E7 of HPV-16 and HPV-18 pioneered the development of therapeutic vaccines for cervical cancer by demonstrating induction of humoral and cell-mediated immune responses to E6 (Gao et al, 1994) and E7 (Boursnell et al, 1996) initially in mice. A phase I/II clinical trial with a vaccinia virus vector expressing E6 and E7 was conduced in 8 late stage cervical cancer patients, the vaccine induced a humoral response in 3/8 patients and a $CD8^+$ T cell response in 1/3 patients (Borysiewicz et al, 1996). The vaccine was then tested in 29 early stage cervical cancer patients (Kaufmann et al, 2002). Four out of 28 patients developed a $CD8^+$ T cell response after a single immunization. Patients could not be monitored for clinical responses as they underwent a radical hysterectomy within 2 weeks after the first vaccination.

In subsequent studies, vaccinia virus vectors were modified by fusing E7 to listerolysin O (LLO) of *Listeria monocytogenous* (Lamikanra et al, 2001) or to the signal sequence of LAMP (Lamikanra et al, 2001; Wu et al, 1995). Either vector was superior to vaccinia virus vectors expressing wild-type E7 and in a comparison study the LLO-E7 fusion protein-expressing vector outperformed the vaccine expressing E7 fused to the signal sequence of LAMP (Lamikanra et al, 2001).

4b. Adenovirus vectors

Adenoviruses are common in most species. They are medium-sized double-stranded DNA viruses with a genome of ~ 34-43 kb. Adenoviruses are species-specific and different serotypes have been isolated from a variety of mammalian species, such as humans, non-human primates, canines, ovines, porcines, avians and even frogs and snakes. Adenoviruses replicate in the nucleus of infected cells. In general, human adenoviruses cause mild upper respiratory or intestinal infections. They have a broad tropism and their small genome is amenable for easy manipulation. Adenoviruses can be vectored to express foreign genes within the E3 domain, which encodes several polypeptides that serve the virus to evade recognition by $CD8^+$ T cells. As E3 is nonessential for viral replication, such vectors are replication-competent in the appropriate species. Alternatively, foreign genes can be inserted into the E1 domain, which encodes polypeptides that are essential for viral replication. E1-deleted adenoviral vectors are thus replication-defective and can only be grown in packaging cell lines that provide E1 in trans. E1-deleted adenoviral vectors are highly immunogenic. They transduce dendritic cells and drive their maturation into competent antigen-presenting cells. They are non cytopathic and hence result in prolonged antigen presentation. On a practical note, they can be grown to high titers in appropriate cell lines, which is an important feature for eventual clinical development. Most studies conduced thus far have used vectors based on adenovirus of the human serotype 5 (AdHu5). AdHu5 is a common pathogen and ~ 45% of adult humans residing in the US carry virus-neutralizing antibodies to AdHu5 virus (Farina et al, 2001), which reduce the efficacy of such vaccines (Fitzgerald et al, 2003).

E1-deleted AdHu5 vectors expressing either E6 or E7 were tested for induction of transgene product-specific T cell-mediated immune responses in two different strains of inbred mice (He et al, 2000). The E7-expressing adenovirus vector-induced potent $CD8^+$ T cell responses in C57Bl/6 mice and completely protected these mice against subsequent challenge with syngeneic tumor cells expressing E7. The E6-expressing adenoviral vectors failed to induce T cells or protective immunity in C57Bl/6 mice but stimulated $CD4^+$ T cells in BALB/c mice (which in turn failed to respond to the E7 expressing vaccine). $CD4^+$ T cells to E6 provided partial protection to tumor cell challenge. These results stress the importance of genetic restriction of T cell responsiveness to the comparatively short oncoproteins of HPV and they show that both oncoprotein-specific $CD4^+$ and $CD8^+$ T cells can provide resistance to tumor development. Another group expressed E5 of HPV-16 by an adenoviral vector and reported induction of E5-specific $CD8^+$ T cells and protection against challenge with an E5-expressing tumor cell line (Liu et al, 2000).

4c. Adeno-associated virus (AAV) vectors

AAVs are dependo-viruses of the parvovirinae subfamily that in nature infect concomitantly with a helper virus such as adenovirus or herpes virus. Helper viruses are needed to allow for transcription of the AAV genome. So far, 6 distinct serotypes have been identified in humans, termed AAV1-6. Of those, AAV 2-based vectors have been used most commonly for gene replacement therapy (High, 2001) and as vaccine carriers (Manning et al, 1997). Recently a number of additional serotypes have been isolated from non-human primates (Gao et al, 2003). The AAV genome is composed of a 4.7 kb single stranded DNA flanked by inverted terminal repeats (ITRs). ITRs initiate DNA replication and are required for encapsidation of the genome. The genome contains two ORFs, one

encoding proteins needed for DNA replication and for integration, the other encoding the 3 capsid proteins, which are generated from a single ORF by alternative splicing and distinct translation initiation sites. In AAV vectors, the ORFs are removed and replaced with the gene of interest flanked by the ITRs. The resulting vector can be grown in cells that provide the proteins encoded by the AAV in trans and which furthermore provides helper virus functions commonly through the E1, E2, E4 and virus-associated RNA of adenovirus. AAV vectors can persist in vivo either episomally or upon integration into the host cell genome.

One study tested an AAV vector expressing the E7 epitope of HPV-16 fused to a heat shock protein. The vector induced an E7-specific $CD8^+$ T cell response and caused tumor regression in mice (Liu et al, 2000).

5. Bacterial vectors

A number of bacterial expression systems are being explored for vaccine delivery. Some of these systems use bacteria to deliver DNA vaccines [reviewed in (Dietrich et al, 1999)]. Alternatively, the genome of bacteria can be modified to produce the gene of interest.

Listeria monocytogenes is a Gram-positive facultatively intracellular bacterium that induces potent $CD8^+$ T cell responses (Gregory and Liu, 2000). *L. monocytogenes* vectors were constructed to either secrete E7 wild-type protein or E7 fused to LLO. Both vaccines induced $CD8^+$ T cell responses to E7 although the latter vaccine was markedly more effective in inducing tumor regression in mice (Gunn et al, 2001). Partial efficacy in causing regression of small, established tumors was also demonstrated with a *L. monocytogenes* vector expressing E7 fused to the Listeria protein ActA (Sewell et al, 2004).

Lactococcus lactis is a Gram-positive bacterium that does not cause disease in humans. Lactococcus vectors expressing E7 in a secreted form, a membrane anchored form, or a form that was retained intracellularly were compared for induction of cell-mediated immune responses to E7. The membrane anchored form of E7 induced the highest E7-specific cytokine responses (Cortes-Perez et al, 2003; Bermudez-Humaran et al, 2004).

6. Dendritic cell vaccines

Activation of naïve T cells requires expression of the antigen by dendritic cells [reviewed in (Mellman and Steinman, 2001)]. Dendritic cells derived from bone marrow precursors are present in tissues throughout an organism being especially numerous in the skin and at mucosal surfaces, the main entry sites for pathogens. Dendritic cells found in peripheral tissues are immature; they take up antigen but are not yet able to efficiently present this antigen to naïve T cells. Dendritic cells mature into professional antigen-presenting cells upon receiving a so-called danger signal. Once activated, dendritic cells start to express, upregulate or translocate molecules needed for efficient antigen presentation such as co-stimulatory molecules or MHC class II molecules. They cease to take up antigen and they change their chemokine receptor expression profile; this promotes their migration to lymphatic tissues, which provide a suitable environment for activation of adaptive immune responses.

Dendritic cell precursors can readily be harvested from bone marrow and be differentiated into immature dendritic cells by culture in appropriate growth factors such as granulocyte macrophage-colony stimulating factor and IL-4. Human dendritic cells can be isolated and expanded from peripheral blood. They can be pulsed in vitro with peptides, transduced with vectors or infected with viral recombinants and then tested in syngeneic hosts for induction of adaptive immune responses. Although antigen-presenting dendritic cells are exceedingly effective in inducing T cell-mediated immune responses, the obvious disadvantage of this approach is that for each and every patient a new vaccine based on her or his dendritic cells has to be constructed. This is only attainable in large medical centers in developed countries and thus out of reach for the vast majority of cancer patients.

Mouse dendritic cells pulsed with an immunodominant E7 peptide of HPV-16 induced in vivo an E7-specific $CD8^+$ T cell response and protection against challenge with an E7-expressing tumor cell line (Okada et al, 1998). Similar results were obtained with mouse dendritic cells pulse with E7 protein (Indrova et al, 2001), transduced with a DNA vaccine expressing E7 or infected with an adenoviral vector to E7 (Tillman et al, 2000).

7. Prime boost regimens

Prime boost regimens using different vaccine constructs sequentially has been explored for E7 vaccines in experimental animals and as expected resulted in E7-specific $CD8^+$ T cell frequencies well above those achieved with each individual vaccine applied once or repeatedly (Da Silva et al, 2003; Lin et al, 2003; Wlazlo et al, 2004).

VIII. Which therapeutic vaccine is best?

With so many vaccine modalities to choose from, the obvious question arises which of those that showed induction of cell-mediated immune responses and protection in animal challenge model is the most likely to succeed in the clinic.

The first question is which antigen is most suitable for incorporation into a cervical cancer vaccine. The oncoproteins of HPV are short proteins; E7 has ~ 100, E6 ~ 150 and E5 ~ 40-50 amino acids. It was already shown that E7 induces very potent $CD8^+$ T cell responses in some mouse strains but not in others (He et al, 2000). Use of a single HPV oncoprotein for a T cell-inducing vaccine in humans is expected to result in varied responses depending on the HLA haplotype of the patient. E7 is indispensable for the malignant phenotype of cervical cancer cells; E6 is present in most late stage cancer cells but is not essential for the malignant phenotype of cells with p53 loss-of-function mutations; E5 is expressed during early stages of cancer development but is commonly lacking in late stage disease. As a rule of thumb, vaccines that express multiple antigens induce broader immune responses, are more efficient to protect

against the outgrowth of escape mutants and are less affected by Ir-gene control; vaccine should thus express E6 and E7 potentially with E5, the latter especially if used in early stage cervical cancer. Several investigators explored oncoproteins of HPV linked to targeting sequences or immunomodulators. Whether or not such modifications will indeed increase vaccine efficacy in a clinical setting is likely to depend on the vaccine carrier: DNA vaccines or poxvirus vectors which are poor inducers of transgene product-specific $CD8^+$ T cell responses may benefit from such transgene modifications while their effect may be negligible in the context of more potent vaccine carriers such as E1-deleted adenoviral vectors. Codon-optimization of HPV genes dramatically increases protein expression levels (Liu et al, 2002) and is thus expected to augment the efficacy of most HPV vaccines independent of the carrier.

Choosing the best suitable vaccine carrier is more difficult. As detailed above an array of vaccine carriers expressing different oncoproteins of HPV has been tested in mice. The published reports are fairly consistent – the vaccines induce T cell responses, most commonly $CD8^+$ T cells and provide protection to tumor challenge given before or after vaccination. Only two studies deviated by reporting accelerated tumor progression upon vaccination of mice with DNA vaccines to E7. Protection experiments with transplantable tumor cell lines are habitually difficult to compare. The different studies used different tumor cell lines and even those that tested the same tumor cell line, such as TC-1 cells, an embryonal lung epithelial cell line that was transformed by transfection with vectors expressing E6, E7 and v-HA-ras (Ji et al, 1999), used different tumor cell doses and different routes of tumor cell challenge. An intravenous challenge with 10.000 tumor cells which form ~ 100 nodules in the lung can not be compared with a subcutaneous challenge with a million tumor cells that within 14 days form a 2-3 cm^3 tumor mass.

Comparing immune correlates of protection, i.e., activation of HPV-specific $CD8^+$ T cells may be more informative. Highly quantitative assays are available to assess T cell frequencies to a given epitope. Of those intracellular cytokines staining may be the most suitable; it has higher sensitivity than ELISPot assays, it only requires a 5 hr incubation period, which does not allow for proliferation of lymphocytes especially as a drug that inhibits secretion is added and it measures unlike MHC tetramer staining only biologically active T cells. Scanning the literature, many studies used ^{51}Cr-release assays to test for induction of $CD8^+$ T cells. These assays, which involve an in vitro expansion step, do not allow quantification of $CD8^+$ T cell frequencies. Other investigators used quantitative assays but first re-stimulated the T cells for several days in vitro. Some investigators used tetramer staining, which commonly overestimates frequencies of $CD8^+$ T effector/memory cells and only a few groups used intracellular cytokine staining. One group used intracellular cytokine staining but pre-incubated lymphocytes over night with the antigenic peptide, which may have allowed for some proliferation of antigen-reactive T cells (Kim et al, 2004a). The same group reports their results as numbers of antigen-reactive $CD8^+$ T cells (Kim et al, 2004a), while other groups show frequencies as a percentage of antigen-reactive $CD8^+$ T cells over all analyzed $CD8^+$ T cells (He et al, 2000; Lamikanra et al, 2001). One can attempt to compare such data (**Table 2**). Scanning the results obtained by different groups one detects a disquieting range of antigen-specific $CD8^+$ T cell frequencies induced in the same strain of inbred mice with very similar vectors such as vaccinia virus vectors expressing E7 or E7 as a fusion protein with the signal sequence of LAMP (Sig-LAMP).

It thus appears that it is currently impossible to objectively rate pre-clinical vaccine efficacy based on immunogenicity studies. Validated assays for pre-clinical vaccine testing are essential to focus clinical trials on the most promising vaccines. Now that antigen-specific T cells can be quantitated with high accuracy, standardized assays could and should be developed for pre-clinical testing of $CD8^+$ T cell inducing vaccines to HPV.

IX. Concluding remarks

Vaccines to oncogenic types of HPV are progressing towards the clinic. Till transmission of oncogenic HPVs has been controlled by preventative immunization, women with HPV-associated malignancies may benefit from therapeutic vaccines. Ideally, one day prophylactic vaccines could eradicate these viruses. This could only be achieved through worldwide preventative vaccination programs targeting teenagers before they become sexually active. Thus far preventative vaccines have focused on HPV-16 and –18, the two serotypes most commonly found in cervical malignancies. Nevertheless, other serotypes are also associated with cervical cancer and thus eventually need to be targeted by vaccines. Vaccination has focused

Table 1

Study	Vaccine type	Vaccine Antigen	Vaccine Doses	$CD8^+$ T Cell Frequencies (%)*
Chen et al, 2000	Vaccinia virus	E7-Sig-LAMP	1	0.07**
	Vaccinia virus	E7-Sig-LAMP	2	0.02**
Lamikanra et al, 2001	Vaccinia virus	E7	2	0.28
	Vaccinia virus	E7-Sig-LAMP	2	0.63
He et al, 2000	Vaccinia virus	E7	1	0.08

* Frequency of $CD8^+$ cells producing IFN-γ in response to the E7 peptide carrying the immunodominant epitope over all $CD8^+$ T cells in the sample. **Data were reported as number of IFN-γ producing $CD8^+$ T cells. Frequency was calculated estimating that 25% of the tested splenocytes reflected $CD8^+$ T cells.

on women, but, in order to eliminate the threat of HPV-associated genital cancers, men have to be included into preventative vaccination programs and it remains to be tested if vaccines that perform well in women have efficacy in men. Economical factors need to be considered as cervical cancer is by far more common in undeveloped countries than in the US, Canada or Europe. Current vaccine strategies under investigation do not yet address all of these issues.

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Group photo including Drs. Ertl (far left in the picture) and Lasaro (right, black hair, black T shirt)