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DEVELOPMENT OF HPV VACCINES FOR HPV-ASSOCIATED HEAD AND NECK SQUAMOUS CELL CARCINOMA

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ABSTRACT: High-risk genotypes of the human papillomavirus (HPV), particularly HPV type 16, are found in a distinct subset of head and neck squamous cell carcinomas (HNSCC). Thus, these HPV-associated HNSCC may be prevented or treated by vaccines designed to induce appropriate HPV virus-specific immune responses. Infection by HPV may be prevented by neutralizing antibodies specific for the viral capsid proteins. In clinical trials, vaccines comprised of HPV virus-like particles (VLPs) have shown great promise as prophylactic HPV vaccines. However, given that capsid proteins are not expressed at detectable levels by infected basal keratinocytes, vaccines with therapeutic potential must target other non-structural viral antigens. Two HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are co-expressed in the majority of HPV-containing carcinomas. Therefore, therapeutic vaccines targeting these proteins may have potential to control HPV-associated malignancies. Various candidate therapeutic HPV vaccines are currently being tested whereby E6 and/or E7 is administered in live vectors, in peptides or protein, in nucleic acid form, as components of chimeric VLPs, or in cell-based vaccines. Encouraging results from experimental vaccination systems in animal models have led to several prophylactic and therapeutic vaccine clinical trials. Should they fulfill their promise, these vaccines may prevent HPV infection or control its potentially life-threatening consequences in humans.

Key words. HPV vaccines, head and neck squamous cell carcinoma, immunotherapy, tumor-specific antigens, cytotoxic T-lymphocyte, tumor immunology.

(1) Introduction

(1.1) The role of human papillomaviruses (HPVs) in human tumors

The human papillomaviruses (HPVs) represent a heterogeneous group of viruses that infect skin and mucosal epithelial tissues. Currently, over 100 genetically different HPV types have been isolated from humans. Humans are the only known reservoirs, and viruses are spread by direct human-to-human contact. "Low-risk" types (e.g., HPV 6 and 11) are associated with benign proliferative growths such as common, plantar, and genital warts. However, certain types are associated with lesions with potential to progress to carcinoma. For example, HPV types 5 and 8 commonly infect skin and, in the setting of lesions with potential to progress to carcinoma. However, given that capsid proteins are not expressed at detectable levels by infected basal keratinocytes, vaccines with therapeutic potential must target other non-structural viral antigens. Two HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are co-expressed in the majority of HPV-containing carcinomas. Therefore, therapeutic vaccines targeting these proteins may have potential to control HPV-associated malignancies. Various candidate therapeutic HPV vaccines are currently being tested whereby E6 and/or E7 is administered in live vectors, in peptides or protein, in nucleic acid form, as components of chimeric VLPs, or in cell-based vaccines. Encouraging results from experimental vaccination systems in animal models have led to several prophylactic and therapeutic vaccine clinical trials. Should they fulfill their promise, these vaccines may prevent HPV infection or control its potentially life-threatening consequences in humans.

(1.2) Human papillomavirus infection of the upper airway

The prevalence of asymptomatic HPV infection in the oral cavities of healthy adults and risk factors related to oral infection are topics of ongoing research. From the best available data, the
prevalence is estimated at 5 to 11% (Franceschi et al., 1996). HPV is associated with benign lesions of the upper airway, including focal epithelial hyperplasia, inverted papilloma, and juvenile- and adult-onset recurrent respiratory papillomatosis. The literature on respiratory papillomatosis provides the best available data on HPV transmission to the upper airway. This disease is characterized by multiple, recurrent, benign papillomatous growths initially arising most often from the vocal cords, with subsequent spread to other areas of the respiratory tract. The genome of HPV 6 or 11 has been detected in the majority of cases (Mounts et al., 1982). HPV infection of the larynx is thought to occur through two mechanisms: transmission during passage through an HPV-infected birth canal and through sexual contact. The observed bimodal age distribution of cases, with peaks at age < five and between 20 and 30 years of age, is thought to reflect these two different modes of transmission. Fifty percent of mothers of affected children report a history of genital HPV infection (Shah et al., 1998). In a recent retrospective cohort study based on data in the Danish National Registries, maternal genital warts during pregnancy conferred a greater than 200-fold risk of respiratory papilloma in the child (Silverberg et al., 2003). By contrast, disease among adults has been associated with sexual behaviors such as high numbers of lifetime sexual partners and a higher frequency of oral sex (Kashima et al., 1993).

(1.3) HUMAN PAPILLOMAVIRUS (HPV) AS AN ETIOLOGICAL FACTOR OF A DISTINCT SUBSET OF HEAD AND NECK CANCERS

Recently, high-risk HPV DNA has been found in a distinct subset of head and neck squamous cell carcinomas (HNSCC) (Gillison et al., 2000). HPV-associated head and neck squamous cell carcinoma (HPV-HNSCC) arose predominantly from the lingual and palatine tonsils in the oropharynx, had a distinct basoloid histopathology, occurred predominantly in the non-smoker and non-drinker, had wild-type p53, and had a favorable prognosis. High-risk HPV 16 was identified in the majority (90%) of the HPV-positive tumors (for review, see Gillison and Shah, 2001). The HPV viral oncoproteins, E6 and E7, are consistently expressed both in HPV-associated cancer cell lines (Nasseri et al., 1991) and in HPV-associated cancers (Schwarz et al., 1985). E6 and E7 expression has been demonstrated in HPV 16 DNA-positive HNSCC specimens by RNA in situ hybridization (Snijders et al., 1992; Wilczynski et al., 1998), Northern blot analysis (Snijders et al., 1992), and reverse-transcriptase/polymerase chain-reaction (RT-PCR) (van Houten et al., 2001). Furthermore, integration of HPV into the genome of HNSCC has been demonstrated by Southern blot (Gillison et al., 2000), two-dimensional gel electrophoresis (Snijders et al., 1992), sequencing (Steenbergen et al., 1995), and fluorescence in situ hybridization (Steenbergen et al., 1995). Recently, seropositivity to HPV 16 was demonstrated to confer a 14-fold increase in risk of oropharyngeal cancer, thus establishing that HPV exposure precedes cancer development (Mork et al., 2001). Analysis of these data provides compelling evidence that HPV 16 has a biological role in the development of a distinct molecular, clinical, and pathological disease entity within the head and neck, i.e., HPV-HNSCC (Gillison et al., 1999; Gillison and Shah, 2001).

It is estimated that 25% of HNSCC are HPV-associated, a prevalence estimate in agreement with the 11% to 25% prevalence reported in the literature (Brandwein et al., 1994; Snijders et al., 1996; Fouret et al., 1997; Paz et al., 1997; Pintos et al., 1999; Gillison et al., 2000). Therefore, an estimated 10,000 of the 40,000 cases of HNSCC that occur each year in the United States may be HPV-associated. In terms of morbidity, this is similar to the number of cases of invasive cervical cancer in the US each year. In developed Western countries, 50-70% of oropharyngeal cancers are estimated to be HPV-associated (Gillison et al., 1999; Gillison and Shah, 2001), with the majority arising from the lingual and palatine tonsils. Approximately 60% of tonsillar carcinomas are HPV-associated. According to Surveillance, Epidemiology & End Results (SEER) registry data, tonsillar squamous cell carcinoma accounts for 17% and 15% of all oral and oropharyngeal cancers among white men and women, respectively, and 22% and 23% among black men and women, respectively (Frisch et al., 2000b). Importantly, a significant increase in the incidence of tonsillar cancer occurred among men every year from 1973 through 1995 (Frisch et al., 2000b). This may possibly be explained by changes in sexual mores resulting in the increased transmission of HPV.

Analysis of preliminary data from a multinational case-control study of oral cavity and oropharyngeal cancers conducted by the International Agency for Research on Cancer (IARC) has confirmed that the majority of HPV-associated cancers are oropharyngeal squamous cell carcinomas and that HPV type 16 was present in the majority (95%) of the HPV-positive tumors. Given the high proportion of HPV-associated HNSCC that is attributed to HPV 16, these patients could obtain substantial benefit from the use of the same prophylactic and therapeutic vaccine strategies that have traditionally been developed to prevent and/or treat HPV-associated anogenital cancers (for review, see Ling et al., 2000). Given that > 90% of HPV-associated HNSCC are HPV-16-positive, as compared with cervical cancer patients, of whom 58% in North America are HPV-16-associated (Bosch et al., 1995), this population may be uniquely valuable for the study of these vaccines, the majority of which have been targeted specifically to HPV 16.

(1.4) IMPORTANCE OF HUMORAL IMMUNE RESPONSES IN PREVENTING HPV INFECTIONS

Animal studies suggest that virus-neutralizing antibodies can protect the host from infection. Recombinant HPV virus-like particles (VLPs) are generated by overexpression of L1, the major capsid protein of HPV, which displays neutralizing epitopes. Immunization of animals with VLP protects against experimental infection with the homologous animal papillomavirus (see Section 2.2). Indeed, passive transfer of sera from VLP-vaccinated mice to naïve mice is sufficient in generating protection, indicating that the protective effect is likely mediated by neutralizing antibodies. Experiments with VLPs constructed from the L1 genes of other viral types have demonstrated no protective immune effect, indicating the specifity and non-cross-reactivity of VLPs. Furthermore, denatured VLPs demonstrated no protective immune effect, indicating that protection required intact VLPs with conformational epitopes.

The other late gene is L2, which encodes the minor capsid protein of HPV. Although various studies have demonstrated that vaccination with L2 fusion proteins prevents experimental papillomavirus infections (Christensen et al., 1991; Lin et al., 1992), VLPs generate even higher titers of serum-neutralizing antibody. Thus, VLPs may be more efficacious than bacterially expressed L2 as prophylactic vaccines. However, vaccination
with L1/L2 VLP has been proven to be no more effective than that with L1 alone (Breitburd et al., 1995). Nevertheless, analysis of the experimental data in animals suggests that both VLP and L2 represent promising prophylactic vaccines (Lowy and Schiller, 1999; Schiller, 1999).

Unfortunately, immunization with capsid proteins fails to generate significant therapeutic effects for established or breakthrough HPV infections that have escaped antibody-mediated neutralization. This is likely because the capsid genes are expressed only upon terminal differentiation in the upper strata of the epidermis, but not in basal keratinocytes. Pre-existing HPV infection is highly prevalent and responsible for considerable morbidity and mortality. A different vaccination strategy is required to treat this infected population. Evidence suggests that cellular immunity, particularly antigen-specific T-cell-mediated immunity, is required for the treatment of established HPV infection. Therefore, vaccines that induce cell-mediated immunity specific for non-structural viral proteins are more likely to effect regression of established lesions or even malignant tumors.

**15 Importance of Cell-Mediated Immune Responses in Controlling Established HPV Infections and HPV-Associated Neoplasms**

Several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms (for review, see Wu, 1994). First, the prevalence of HPV-related diseases (infections and neoplasms) is increased in transplant recipients (Halpert et al., 1986) and human immunodeficiency virus (HIV)-infected patients (Schafer et al., 1991; Laga et al., 1992), both of which are known to have impaired cell-mediated immunity. The risk for in situ carcinomas in the genital tract increases with advancing immunosuppression in AIDS patients, indicating a gradual loss of control over HPV-infected keratinocytes (Frisch et al., 2000a). Second, animal studies have demonstrated that immunized animals are protected from papillomavirus infection and from the development of neoplasia. Immunization also facilitates the regression of existing lesions (Brandsma, 1994; Selvakumar et al., 1995). Third, infiltrating CD4+ (T helper cells) and CD8+ (cytotoxic T-cells) T-cells have been observed in spontaneously regressing warts (Tagami, 1983); and fourth, warts in patients who are on immunosuppressive therapy often disappear when treatment is discontinued (for review, see Benton et al., 1992). Therefore, HPV vaccines with potential for therapeutic efficacy should generate enhanced HPV-specific cell-mediated immune responses.

**16 HPV Vaccine Development Strategies**

Conceputally, there are three approaches to HPV vaccine development. The first approach seeks to block HPV-induced neoplasia by preventing the virus from establishing infection in the epithelium, mainly through the induction of neutralizing antibody via viral capsid protein. A prophylactic vaccine should stimulate the complete neutralization of free virus upon exposure before infection can occur. However, this may be of less benefit to those individuals who already have an established infection and dysplasia. The second approach of vaccine development addresses the needs of these patients by attempting to induce a cellular immune response (both CD4+ and CD8+) which may be able to both prevent and induce the regression of neoplastic lesions. The strategy used in this kind of therapeutic approach is known as antigen-specific immunotherapy, in which effector cells, particularly T-cells, are primed against HPV antigen epitopes known to be expressed by the neoplastic cells (tumor-specific antigens) such as E6 and E7. Finally, the third approach seeks to combine prophylaxis and therapy in one vaccine, in an attempt to provide total coverage for people who are newly exposed to high-risk virus and for people with current infections and dysplasia.

HPV vaccine development has been complicated by the lack of animal models for HPV infections and the difficulty in propagating HPVVs in cultures. As a result, the majority of prophylactic vaccine development was initially conducted with the use of cutaneous and mucosal animal papillomaviruses such as cottontail rabbit papillomavirus (CRPV), canine oral papillomavirus (COPV), and bovine papillomavirus (BPV). The discovery and development of virus-like particles (VLPs) have helped overcome some of these difficulties and resulted in a promising and exciting candidate for future prophylactic and therapeutic vaccines. (VLP vaccines are discussed at length in Section 2.2.) HPV vaccine development has also benefited from the development and use of tumorigenic mouse cell lines, such as C3 and TC-1, that express HPV 16 oncogenes E6 and E7. The C3 (Feltkamp et al., 1993) and TC-1 (Lin et al., 1996) cell lines are derived from mouse cells immortalized by HPV-16 E6 and E7 oncogenic proteins in vitro. Since these cell lines continuously express HPV-16 E6 and E7 oncogenic proteins and are capable of growing tumors in mice, they can be used in pre-clinical tumor models for the development of HPV therapeutic vaccines targeting E6 and/or E7 oncogenic proteins. The Table summarizes the various HPV vaccine strategies described in this review.

**Table: HPV Vaccine Strategies**

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*VLPs: virus-like particles.
(2.0) Prophylactic HPV Vaccines

(2.1) STRATEGIC AND IMMUNOLOGIC CONSIDERATIONS

The principle underlying a preventive or prophylactic vaccine is to prime the immune system so that it is able to induce enough neutralizing antibody production prior to, or upon exposure to, high-risk HPVs, to prevent infection from being established. This in turn would prevent the development of invasive cancer caused by the high-risk HPVs. Several requirements must be met for this goal to be fulfilled. First, the vaccine must prime an antibody response that is specific for neutralizing epitopes on HPV. Second, the vaccine must induce an immune response that is long-lasting, to maintain persistently high titers of neutralizing antibody. Third, the vaccine must be successful at thwarting infection of the mucosal epithelium, the natural target for HPV. In other words, induction of systemic IgG may not be sufficiently protective. The plasma cell precursors that migrate to and protect mucosal surfaces secrete mainly IgA and originate from mucosa-associated lymphoid tissue (MALT). Therefore, a vaccine should be able to induce local MALT to produce local antibody protection. Fourth, the vaccine must either be multivalent or possess cross-neutralizing properties to protect an individual from infection by all or most of the high-risk HPVs. Active investigation is ongoing to address these important issues.

(2.2) HPV L1 CAPSID PROTEIN-BASED VACCINES

The role of neutralizing antibodies in preventing infection is to bind certain regions tightly to the surface of the virus (neutralizing epitopes) to physically prevent the virus from docking with and attaching to a host cell. HPV capsid structural proteins are the logical target for such antibodies. The majority of prophylactic vaccine research has focused on using the papillomavirus major capsid protein L1 as the immunogen and target. Early attempts to use L1 protein subunits, denatured L1 protein, or L1 peptides were not successful and led to the observation that a protective antibody response to L1 requires intact conformational L1 epitopes (Liu et al., 1993). The landmark discovery that L1 capsid protein spontaneously assembles to form empty capsids, known as virus-like particles (VLPs) when expressed in mammalian (Zhou et al., 1991; Hagensee et al., 1993), insect (Kimbauer et al., 1993), yeast (Sasagawa et al., 1995), or bacterial cells (Nardelli-Haefliger et al., 1997), initiated a new line of prophylactic vaccine research. Parenteral injection of these VLPs elicits high titers of serum-neutralizing antibodies and protection from experimental challenge with infectious virus in several animal papillomavirus models (Breitburd et al., 1995; Jansen et al., 1995; Suzich et al., 1995; Christensen et al., 1996; Kimbauer et al., 1996). Protection from experimental infection by cottontail rabbit papillomavirus (CRPV) or canine oral papillomavirus (COPV) following passive transfer of IgG from immunized animals to naïve animals has been demonstrated in rabbits and dogs, respectively (Breitburd et al., 1995; Suzich et al., 1995).

Although VLP vaccination provides immunity from experimental inoculation, it is unclear whether this extends to protection against natural transmission of mucosal HPV. For complete prevention of sexual transmission of genital HPV infection, neutralizing antibodies must act at mucosal surfaces that are the natural site of infection. Antibodies not only pass from plasma into mucosal secretions but are also synthesized by local plasma cells. The plasma cell precursors that migrate to the genital or oral tract predominantly secrete IgA. Induction of these cells requires direct immunization of the mucosa-associated lymphoid tissue, and, in several experimental systems, nasal instillation was found to be the most effective route of immunization to generate specific antibodies in genital secretions in mice and in monkeys (Russell et al., 1996; Balmelli et al., 1998). In previous studies, systemic immunization of mice with purified HPV VLPs induced no detectable mucosal IgA antibodies and low titers of IgG (Hagensee et al., 1995; Balmelli et al., 1998). Further, low titers of VLP-specific IgG, and no IgA, were detected in cervico-vaginal lavage of parenterally immunized monkeys (Lowe et al., 1997). Although these experiments in monkeys showed that transudated IgG alone partially neutralized HPV 11 in vitro, the mucosal antibody response was short-lived (Lowe et al., 1997). Local, sustained production of secretory IgA (sIgA) and/or specific IgG is likely required for long-lasting sterilizing immunity.

So far, intranasal immunization with HPV 16 VLPs has been shown to induce significant and sustained titers of HPV-16-neutralizing antibodies in both serum and mucosal secretions of mice (Nardelli-Haefliger et al., 1997; Balmelli et al., 1998). Furthermore, vaginal immunization with HPV 6bL1 DNA has been shown to induce long-lasting IgA responses with neutralizing activity in vaginal secretions of vaccinated rabbits (Schreckenberger et al., 2000). Thus, the route of administration may be important for generating protective humoral immune responses.

The use of adjuvants has also proved to be important, since it was demonstrated that detoxified Escherichia coli heat-labile enterotoxin (LT R192G), when orally co-administered with HPV 16 L1 VLP, significantly enhanced a specific and long-lasting IgA mucosal immune response (Gerber et al., 2001). This finding supports the feasibility of oral immunization of humans against mucosal HPVs, which has several practical advantages over other routes of vaccine administration.

A Phase I/II randomized double-blind clinical trial to test the safety and immunogenicity of an HPV-16 L1 VLP vaccine in human adult male and female subjects was recently completed by The Johns Hopkins University Center for Immunization Research in Baltimore, MD (Harro et al., 2001). Overall, the authors concluded that the vaccine was very well-tolerated (Harro et al., 2001). All vaccinated subjects seroconverted to HPV-neutralizing IgG within one month of a second vaccination, with adjuvant significantly increasing antibody titer in a low vaccine dose group. However, the trial collaborators did not evaluate local mucosal immune response, and although most of the vaccine recipients were found to have become seropositive for HPV-16-specific serum IgA, they reported neither the IgA titer nor the presence of sIgA. The efficacy of this HPV-16 L1 VLP vaccine in preventing infection remains to be evaluated in the ongoing follow-up Phase III trial, currently being conducted in Costa Rica (John T. Schiller, personal communication). In a recently reported randomized, double-blind placebo controlled trial, three injections of an HPV-16 VLP vaccine successfully reduced the incidence of both HPV-16 infection and HPV-16-related cervical intra-epithelial neoplasia. Women administered the vaccine had an incidence of persistent HPV 16 cervical infection of 0 per 100 woman-years, as compared with 3.8 per 100 woman-years in the placebo group (Koutsy et al., 2002). This trial has demonstrated the tremen-
dous potential for VLP-based vaccines in the prevention of HPV infection.

Although most L1 capsid vaccination work has been done with VLPs, alternatives do exist. Pentameric GST-COPV L1 fusion protein expressed in E. coli was immunogenic in canines and induced a protective immune response against COPV challenge (Yuan et al., 2001). A pCMV-L1 plasmid which contains the complete COPV L1 open reading frame under control of a cytomegalovirus promoter was administered intra-epidermally by means of a particle-mediated DNA delivery (PMDD) system, and it induced a humoral antibody response protective against challenge with high doses of COPV (Stanley et al., 2001). Furthermore, vaginal immunization with DNA vaccine encoding the HPV 6bL1 gene has been shown to induce long-lasting IgA responses with neutralizing activity in vaginal secretions of vaccinated rabbits (Schreckenberger et al., 2000).

(2.3) HPV L2 CAPSID PROTEIN-BASED VACCINES

The L2 minor capsid protein has largely been ignored as a potential vaccine target. This is probably due to several early studies showing that the L2 protein was less immunogenic than L1, which contains most of the antigenic epitopes (Breitbart and Coursaget, 1999), and that when L1 and L2 co-self-assembled into L1/L2 VLPs, the resulting vaccine was no more immunogenic and protective than L1 VLPs alone (Kirnbauer et al., 1996). Although much of L2 is internal to the capsid and not required for binding of virions to cell surfaces, a small portion of L2 is exposed with epitopes accessible to neutralizing antibodies (Roden et al., 2000). It was discovered that monoclonal antibodies raised against L2 were able to neutralize pseudovirions of both HPV 6, a type commonly responsible for cutaneous warts, and HPV 16, suggesting that immunization with L2 protein may be cross-protective across HPV types (Kawana et al., 1999). Subsequent studies in sheep (Roden et al., 2000) and mice (Kawana et al., 2001) have confirmed the cross-neutralization capability of HPV L2 capsid protein. This is especially important, considering that L1 VLPs do not afford cross-protection between types.

(3.0) Therapeutic HPV Vaccines

(3.1) STRATEGIC AND IMMUNOLOGIC CONSIDERATIONS

There are several possible goals for a therapeutic vaccine: to clear existing HPV infection, to prevent the formation and progression of lesions, and to cause regression of or eliminate existing lesions of any grade, including invasive cancers. A therapeutic HPV vaccine must stimulate an immune response that mimics those that are capable of clearing virally infected and virally induced lesions. This approach involves stimulating a specific cell-mediated immune response using various HPV-associated antigens as targets.

Since E6 and E7 are consistently expressed in most HPV-associated cancers, they represent promising targets for the development of antigen-specific therapeutic vaccines. While most tumor-specific antigens are derived from normal or mutated endogenous proteins, E6 and E7 are completely foreign viral proteins, and therefore may harbor more antigenic peptides and epitopes than a mutant (i.e., p53) or re-activated embryonic protein (i.e., MAGE-1). Furthermore, since E6 and E7 are required for the induction and maintenance of malignant phenotypes of cancer cells (Crook et al., 1989), HPV-associated cancer cells most likely cannot evade an immune response through antigen loss. Finally, studies in animal models suggest that vaccination targeting early papillomavirus proteins (i.e., E7) can generate therapeutic as well as protective effects (Campo et al., 1993). Therefore, E6 and E7 proteins represent good targets for the development of antigen-specific immunotherapies or vaccines for the control of HPV-associated cancers. Various forms of vaccines—such as vector-based vaccines, tumor-based vaccines, DNA-based vaccines, and protein/peptide-based vaccines—have been described in experimental systems targeting HPV-16 E6 and/or E7 proteins (Meneguzzi et al., 1991; Chen et al., 1992; Feltkamp et al., 1993; Lin et al., 1996; Greenstone et al., 1998; Chu et al., 2000; Daemen et al., 2000; Liu et al., 2000; Gerard et al., 2001; Gunn et al., 2001; Smahel et al., 2001). Since HPV-16 E7 is more abundant, better conserved (Zehbe et al., 1998), and better characterized immunologically than E6, most of the studies focus on E7 for HPV therapeutic vaccine development.

(3.2) VIRAL VECTOR VACCINES

(3.2.1) Vaccinia virus vaccines

Vaccinia viruses (vV) are members of the poxvirus family. Vaccinia vaccines offer several appealing features, including high efficiency of infection and high levels of recombinant gene expression. Several studies have shown that E6- and/or E7-specific immunotherapy with vaccinia vectors generates strong CTL activity (Gao et al., 1994; Boursnell et al., 1996) and anti-tumor responses in pre-clinical studies (Meneguzzi et al., 1991; Lin et al., 1996; Ji et al., 1998; Chen et al., 2000b). Results of phase I/II clinical trials using recombinant vV encoding HPV-16 and -18 E6/E7 (also called TA-HPV) indicated that some patients with advanced cervical cancer, CIN3, or early invasive cervical cancer developed T-cell immune responses after vaccination (Borysiewicz et al., 1996; Adams et al., 2001). No significant complications or environmental spread of vV was noted in these trials.

vV has also been utilized to explore tumor vaccine strategies that use intracellular sorting signals. An increased understanding of intracellular pathways for antigen presentation has facilitated the design of novel strategies to enhance vaccine potency. For example, endosomal and lysosomal compartments are associated with MHC class II processing and presentation and are characterized by the presence of several compartment-specific membrane proteins, including the lysosomal-associated membrane protein (LAMP-1). A study by Wu et al. (1995) demonstrated that the linkage of the sorting signal of lysosome-associated protein (LAMP-1) to the HPV-16 E7 antigen (creating recombinant Sig/E7/LAMP-1 vV) targets E7 to endosomal and lysosomal compartments and enhances MHC class II presentation to CD4+ T-cells as compared with vV expressing wild-type E7. Furthermore, the Sig/E7/LAMP-1 vV vaccine cures established E7-expressing tumors in mice, while wild-type E7 vV shows no effect on established tumors (Lin et al., 1996). Another strategy for vaccinia vaccination against cervical cancer is the fusion of E7 to a non-hemolytic portion of listeriolysin O (LLO) (Lamikanra et al., 2001). Vaccination with LLO-E7 vaccinia induces a potent CD8+ T-cell-mediated immune response, causing regression of established HPV-16 immortalized tumors in mice. This effect may be mediated by...
rapid proteolysis in the cytosol or enhanced trafficking to the proteosomes (Lamikanra et al., 2001). These studies suggest that strategies that re-route antigen or modify antigen processing may be able to improve the in vivo therapeutic potency of recombinant vaccinia vaccines against cervical cancer.

### (3.2.2) Adenovirus and adeno-associated virus vaccines

Recombinant adenoviruses (AdV) are widely used vectors with a cloning capacity of approximately 8 kb, allowing for the insertion of a relatively large gene. They can be prepared easily in high titer. AdV can transduce a wide range of cell types with remarkable transduction efficiency without integrating into the host genome, eliminating the safety concern of insertional mutagenesis. Recombinant AdV vectors encoding tumor-specific antigen such as P815A (Warnier et al., 1996), β-gal (Chen et al., 1996), or gp100 (Zhai et al., 1996) can induce an antigen-specific CTL response and anti-tumor effect. One study compared modified adenovirus (with AdV) E1 oncogene deleted and vaccinia virus expressing HPV-16 E6 or E7 and found that these vaccines enhanced antigen-specific CD8+ and/or CD4+ T-cell immune responses and anti-tumor effects in a murine model (He et al., 2000). Another promising application of adenovirus is ex vivo preparation of dendritic cell-based vaccines. AdV vectors encoding E7 and targeted to CD40 by means of bispecific antibodies enhance E7 gene transfer to murine dendritic cells (DCs) (Tillman et al., 2000). Vaccination with AdV-modified E7-transduced DCs resulted in a CD8-dependent E7-specific therapeutic anti-tumor effect. The major concern for immunization is the production of anti-AdV antibodies by the host, which may inhibit repeat vaccination and thereby compromise the therapeutic effect.

Adeno-associated virus (AAV) is a parvovirus that is non-pathogenic in humans. Replication-defective forms of AAV are useful as vectors for delivering therapeutic genes to a wide range of cell types (Flotte and Carter, 1995). One study found that vaccination of mice with AAV encoding HPV-16 E7 fused to heat-shock protein 70 (HSP70) induced CD4- and CD8-dependent CTL activity and anti-tumor effects in vitro (Liu et al., 2000). HSPs are described in more detail in Section 3.3. These studies indicate that AdV and AAV vectors may be safe and effective for the development of therapeutic HPV vaccines.

### (3.2.3) Alphavirus vectors

Alphaviruses and their derivative vectors—such as Sindbis virus (Xiong et al., 1989; Hariharana et al., 1998b), Semliki Forest virus (Berglund et al., 1997; Ying et al., 1999), and Venezuelan equine encephalitis (VEE) virus (Pushko et al., 1997)—are attractive candidates for vaccine development (naked alphavirus RNA replicon vaccines are discussed in Section 3.6). The alphaviral vector is also called "replicon" because of the self-replicating nature of the alphavirus genome. The cytoplasmic replication of the RNA genome is mediated by four viral-encoded, non-structural proteins (these proteins together are called replicase). Replacement of genes encoding structure proteins with antigenic gene in the alphaviral vector can lead to a high level of antigen expression in infected cells. Alphavirus replicon-packaging cell lines can be used to produce replication-defective alphavirus replicon particles that are free of detectable replication-competent virus yet efficient at gene delivery (Polo et al., 1999). The availability of these packaging cell lines allows for large-scale vector production of infectious replication-defective virus that may be useful in vaccine applications. One study found that vaccination of mice with a replication-defective Venezuelan equine encephalitis (VEE) virus replicon particle vector containing HPV-16 E7 RNA enhanced E7-specific CD8+ T-cell immune responses and eliminated established tumors (Velders et al., 2001a). Another study found that replication-defective Sindbis virus replicon particles encoding an unique intercellular spreading protein, herpes simplex virus type-1 (HSV-1) VP22 (described in Section 3.5), linked to HPV-16 E7, generated improved E7-specific CD8+ T-cell immune responses, have a potent anti-tumor effect in vaccinated mice (Cheng et al., 2002), and were more potent than HSV-1 VP22-containing vaccinia and DNA vaccines. The use of replication-defective alphavirus replicon particles thus holds promise for efficient delivery of antigen genes or immunogen/antigen fusion genes to target cells with low toxicity.

### (3.3) Bacterial vector vaccines

#### (3.3.1) Listeria vaccines

Listeria monocytogenes has emerged as a promising bacterial vector for use as a recombinant vaccine for human cancers. L. monocytogenes is a Gram-positive intracellular bacterium that usually infects macrophages. When L. monocytogenes is phagocytosed by macrophages, it is taken up in a phagosome. However, unlike other intracellular bacteria, it escapes into the cytoplasm of the macrophage by secreting listeriolysin O, a factor that disrupts the phagosomal membrane. Because of its presence in the endosomes and in the cytoplasm, L. monocytogenes can deliver its antigens or carry foreign antigens into both the MHC-I and MHC-II pathways and induce strong cellular immune responses. A recent study found that vaccination with recombinant L. monocytogenes secreting HPV-16 E7 can lead to regression of pre-existing E7-expressing tumors using an E7-expressing murine tumor model, TC-1 (Gunn et al., 2001). Antigen-specific L. monocytogenes vaccines may also be administered orally in mice without losing efficacy (Pan et al., 1995).

#### (3.3.2) Other bacterial vaccines

Mammalian expression vectors containing genes of interest can be transformed into attenuated bacteria, such as mutant strains of Shigella, E. coli, or Salmonella, which can serve as bacterial carriers to deliver plasmid-encoding genes of interest to antigen-presenting cells (APCs). Among these mutant bacteria, Salmonella has already been used as a live vaccine in humans. The advantage of using Salmonella as a carrier is its natural route of infection, which allows for oral vaccination (Darji et al., 1997). After leaving the intestinal lumen, Salmonella migrates into the lymph nodes and the spleen, where it encounters macrophages and DCs. The attenuated Salmonella then releases multiple copies of antigen-coding plasmid inside phagocytes, which leads to expression of the antigen and elicits strong immune responses. Alternatively, genes of interest (i.e., antigen) can be cloned into a prokaryotic expression vector and transformed into Salmonella to induce expression of antigen in the bacteria. The bacteria can then be used as a carrier for the antigen in protein form as a vaccine. This approach has been used to deliver HPV-16 E7 (Krut et al., 1996) or E7 epitopes harbored in hepatitis B virus core antigen particles to generate E7-specific immune responses (Londono et al., 1996). Salmonella can also be engineered to express HPV-16 VLPs and used as a
therapeutic vaccine against the development of HPV-16-expressing tumors in mice (Revaz et al., 2001). Another bacterial vaccine vector is Bacille Calmette-Guerin (BCG, Mycobacterium bovis). BCG is safe, since it is used for widespread vaccination against tuberculosis, and it may also induce prolonged immune responses. One study found that BCG encoding L1 and E7 induced E7-specific antibody and immune responses (Jabbar et al., 2000). Targeting to the right type of immune cell, such as dendritic cells, as well as the ability to enhance antigen presentation and prolong expression of antigen are the reasons to consider the use of bacterial carrier systems to deliver HPV vaccines.

(3.4) Peptide/protein vaccines

(3.4.1) Peptide vaccines

The identification and characterization of CTL epitopes for HPV have promoted the development of peptide vaccines against cervical cancer. For example, several HPV-16 E7-specific CTL epitopes have been characterized for the HLA-A2.1 haplotype (Kast et al., 1993; Ressing et al., 1995). Immunization with a peptide derived from HPV 16 leads to protection of mice against a lethal dose of HPV-16 transformed tumor cells (Feltkamp et al., 1993). Peptide vaccines also have potential clinical applicability. At least three HLA-A2.1-restricted peptide CTL epitopes derived from HPV-16 E7, including aa 11-20 (YMLDLQPFETT), aa 82-90 (LMLGTLGIV), and aa 86-93 (TLGIVCIPI), are able to induce CTL responses in vivo in HLA-A2.1 transgenic mice and induce lysis of HPV-16 E7-containing HLA-A2.1-positive CaSki cells (Ressing et al., 1995). Peptides relevant to other HPV types (i.e., HPV 18) (Rudolf et al., 2001b) and other HLA backgrounds (i.e., HLA-B18) (Bourgault Villada et al., 2000) are also under investigation. In human studies, CTL responses were observed in some HPV-associated cancer patients after vaccination with lipitated peptides derived from HPV-16 E7 (Steller et al., 1998) or HPV-18 E6 (Yoon et al., 1998). In one phase 1/II study, no adverse side-effects of peptide-based HPV vaccine were observed in patients (van Driel et al., 1999). In another study, HPV-16- and HLA-A2.2-positive patients with high-grade cervical or vulvar intra-epithelial neoplasia were vaccinated with epitope aa 12-20 or aa 86-93; 10 of 16 patients exhibited measurable enhancement in cytokine release and cytokolyis mediated by CTLs derived from peripheral blood mononuclear cells (PBMCs), and some patients had partial clearance of virus and regression of lesions (Muderspach et al., 2000). Studies have also identified human MHC class I-restricted T-helper cell epitopes and T-helper immune responses to peptide vaccination (Ressing et al., 2000; van der Burg et al., 2001b).

The potency of HPV-16 E7 peptide-based vaccines can be further enhanced by the use of adjuvants such as immune stimulatory complexes (ISCOMs) (Fernando et al., 1995) and immunostimulatory carriers (ISCAR) (Tindle et al., 1995). Phase I clinical trials of HPV 16 peptide vaccines with adjuvant have been conducted with the use of various adjuvants, including incomplete Freund’s adjuvant (Muderspach et al., 2000) and Montanide ISA 51 adjuvant (van Driel et al., 1999). These vaccines and adjuvants produce noticeably positive immunologic and pathologic effects. Another adjuvant strategy involves the modification of CTL epitopes by lipid conjugation, tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P3CSS) to form an immunogenic lipopeptide vaccine (Deres et al., 1989; Schild et al., 1991). Two similar strategies have been adapted for the treatment of HPV-associated cancer, including an E7 (aa 86-93) lipopeptide vaccine (Steller et al., 1998) and two dipalmitoyl-lysine-glycine-glycine (P2-KGG) lipid-tailed E6 and E7 peptide-based vaccines (Sarkar et al., 1995), both of which generated enhanced CTL responses.

Heat-shock proteins are a family of chaperone proteins that facilitate delivery of non-covalently bound peptide to MHC class I molecules and induce peptide-specific CTL responses. Immunization with HSP-peptide complexes isolated from tumor or virus-infected cells (Srivastava and Udono, 1994) or peptides fused with HSPs (Srivastava et al., 1998) can induce potent anti-tumor or anti-viral immunity. HSPs thus serve as effective immunogens and have also been used in viral vaccines (see Section 3.2), protein vaccines (see Section 3.4.2), and DNA vaccines (see Section 3.5). Another strategy to enhance the potency of peptide vaccines is to modify anchor residues in CTL epitope-bearing peptides. Anchor-modified peptide epitopes can efficiently induce CTLs that are capable of recognizing wild-type epitope and can also protect against HPV-16 E7-expressing tumors (Vierboom et al., 1998). Modifications of epitopes may enhance the ability of peptide to be bound and transported by MHC class I and transporter associated with antigen (TAP).

(3.4.2) Protein vaccines

The convenience of utilizing peptide-based vaccines is limited by MHC restriction and the necessity to define specific CTL epitopes. Most CTL epitopes of HPV-16 E6 and/or E7 in patients with HLA other than HLA-A2.2 remain undefined, making it difficult to use peptide-based vaccines in such situations. In addition, the preparation of peptide-based vaccines for use on a large scale is inefficient and laborious. These limitations can potentially be overcome by using protein-based vaccines, which can present all possible epitopes of a protein to the immune system, thus bypassing the MHC restriction. In addition, protein vaccines offer certain safety advantages, since insertional gene activation and transformation, a potential concern with certain recombinant virus vaccines and DNA vaccines, are not issues.

Growing numbers of modified exogenous protein antigens, including HPV-16 E7, have been found to generate enhanced MHC-I restricted CTL responses. Association of E7 protein with adjuvants—such as PROVAX (Harirahan et al., 1998a), incomplete Freund’s adjuvant (De Brujin et al., 1998a), saponin QS21, and monophosphoryl lipid A (MPL) (Gerard et al., 2001)—is able to enhance E7-specific CTL activities. Studies have demonstrated that injection of heat-aggregated HPV-16 E7 antigen can prime CTLs (Schimpke et al., 1995), TA-GW fusion protein, which consists of HPV-6 L2 fused to E7 protein, has been tested for clinical treatment of genital warts (Lacey et al., 1999; Thompson et al., 1999), and TA-CIN fusion protein, which consists of HPV-16 L2/E6/E7, can induce E7-specific CD8+ T-cell immune responses and tumor protection (van der Burg et al., 2001a).

Various strategies have been developed to enhance the potency of protein-based strategies, some of which have yet to be tested in the HPV context. The fusion of antigen with heat-shock proteins (HSPs) is one such strategy for enhancing CTL priming. This strategy has been tested using Mycobacterium bovis bacille Calmette-Guerin (BCG) HSP65 fused to HPV-16
E7, which led to CD8-dependent, CD4-independent regression of HPV-16 E7-expressing tumors in mice (Chu et al., 2000). Studies have demonstrated that GM-CSF linked to an antigen can target the antigen to dendritic cells and other GM-CSF-responsive cells after the chimeric molecule binds to the GM-CSF receptor. Vaccination with GM-CSF chimeric molecules led to enhanced immune responses in the vaccinee (Tao and Levy, 1993; Chen et al., 1994). Immunostimulatory CpG oligodeoxynucleotides (ODNs) that contain unmethylated CpG motifs are also able to enhance the potency of protein vaccines by inducing macrophages to secrete IL-12 and shifting cytokine profiles to Th1-type immunity (Chu et al., 1997; Roman et al., 1997). CpG ODNs are a promising alternative to complete Freund's adjuvant because they lack significant toxicity (Weiner et al., 1997), making them an attractive option for enhancing HPV protein-based vaccines.

### 3.5 DNA Vaccines

Naked DNA vaccines are useful because of their purity, simplicity of preparation, and stability. DNA vaccines allow for sustained expression of antigen on MHC-peptide complexes compared with peptide or protein vaccines. Furthermore, the MHC restriction of peptide-based vaccines may be bypassed with approaches that directly transduce DNA coding for antigen to APCs so that synthesized peptides can be presented by the patient's own HLA molecules. Since DNA vaccines targeting different HPV types can be administered together, DNA vaccines may be effective for treating a variety of HPV-associated infections and tumors. These advantages have spurred interest in the development of DNA vaccines to treat cancers. DNA vaccines can be administered to the host by intramuscular injection, intradermal injection via hypodermic needle or gene gun (a ballistic device for delivering DNA-coated gold particles into the epidermis), intravenous injection, intranasal delivery, or biojector delivery (for review, see Donnelly et al., 1997; Robinson and Torres, 1997).

Studies have investigated the mechanisms involved with intramuscular injection or gene-gun delivery of DNA vaccines. Following intramuscular injection, myocytes can uptake DNA, allowing them to produce protein and transfer antigen to bone marrow-derived professional APCs (Carr et al., 1996). "Cross-priming", the processing of exogenous antigen transferred from another cell (i.e., secreted from DCs or in apoptotic bodies) via the MHC class I pathway (Huang et al., 1994; Albert et al., 1998), provides an explanation for the transfer of antigen from cells initially transfected by intramuscular immunization (i.e., myocytes) to professional APCs.

After gene-gun delivery, epidermal Langerhans cells uptake DNA and function as APCs. DCs in the skin carry antigen from the skin to the draining lymph nodes, where the antigen-loaded DCs activate naïve T-cells (Condon et al., 1996). Intradermal vaccination with DNA facilitates direct priming, whereby antigen expressed in DCs is directly processed within the cell and presented on MHC class I molecules to CD8+ T-cells (Forgador et al., 1998). The method of DNA inoculation (gene gun vs. intramuscular injection) and the form of the DNA-expressed antigen (cytoplasmic vs. secreted) can also influence the type of T-cell help (Th1 or Th2) (for review, see Robinson, 1997).

The delivery of DNA vaccines intradermally via gene gun allows for direct targeting of genes of interest into professional APCs in vivo. Gene-gun immunization has been used to test several intracellular targeting strategies that enhance MHC class I and/or class II presentation of antigen. For example, MHC class I presentation of HPV-16 E7 can be significantly enhanced by linkage with Mycobacterium tuberculosis heat-shock protein 70 (HSP70) (Chen et al., 2000a), calreticulin (Cheng et al., 2001a), or the translocation domain (domain II) of Pseudomonas aeruginosa exotoxin A [ETA(dll)] (Hung et al., 2001b) in the context of a DNA vaccine. The linkage of these molecules to E7 results in augmentation of the E7-specific CD8+ T-cell immune response in vaccinated mice. (HSPs are described in more detail in Section 3.4.) Furthermore, the use of DNA encoding a signal sequence linked to E7 and the sorting signal of the lysosome-associated membrane protein (LAMP-1) to create the Sig/E7/LAMP-1 chimera can enhance MHC class II antigen processing (Wu et al., 1995) (see Section 3.2). Expression of this DNA vaccine in vitro and in vivo targets E7 to endosomal and lysosomal compartments and enhances MHC class II presentation to CD4+ T-cells compared with DNA encoding wild-type E7 (Ji et al., 1999). While chimeric E7/HSP70, ETA(dll)/E7, or CRT/E7 DNA generates potent CD8+ T-cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T-cell responses. This realization raises the notion of co-administration of vaccines such as E7/HSP70 and Sig/E7/LAMP-1 in a synergistic fashion. Such an approach may directly enhance both MHC class I and class II presentation of E7 and lead to significantly enhanced E7-specific CD4+ and CD8+ T-cell responses and potent anti-tumor effects.

Although DNA vaccines that use intracellular targeting strategies can significantly enhance MHC class I and class II presentation of antigen in transfected DCs, they may generate only a limited number of antigen-expressing DCs, since naked DNA vaccines lack the intrinsic ability to amplify and spread in vivo. This significantly limits the potency of DNA vaccines. Therefore, a strategy that facilitates the spread of antigen to more DCs may significantly enhance the potency of naked DNA vaccines. The potency of DNA vaccines may be enhanced through the use of herpes simplex virus (HSV-1) VP22, an HSV-1 tegument protein capable of intercellular transport and useful in spreading protein to surrounding cells (Elliott and O'Hare, 1997). HSV-1 VP22 (HVP22) has been shown to be capable of enhancing intercellular spreading of the linked protein. Furthermore, mice vaccinated with HVP22/E7 DNA generate a significantly greater number of E7 specific CD8+ T-cell precursors (Hung et al., 2001a; Osen et al., 2001; Michel et al., 2002) and a stronger anti-tumor effect than wild-type E7 DNA (Hung et al., 2001a). The success of the chimeric HSV-1 VP22/E7 DNA vaccine warrants the consideration of other proteins with similar trafficking properties. Marek's disease virus VP22 (MVP22) shares about 17% amino acid identity with human herpesvirus VP22 and may be capable of intercellular transport after exogenous application (Dorange et al., 2000). MVP22/E7 DNA generates a significantly greater number of E7 specific CD8+ T-cell precursors and a stronger anti-tumor effect in vaccinated mice than does wild-type E7 DNA (Hung et al., 2002).

It is well-known that co-stimulators are required to generate a CTL response. Methods that use cytokines or co-stimulatory molecules may enhance the potency of DNA vaccines (Irvine et al., 1996; Caff et al., 1997; Tuting, 1999). Leachman et al. (2000) demonstrated that priming the E6 DNA vaccination
site with a GM-CSF-expressing vector greatly enhances the effects of cottontail rabbit papillomavirus (CRPV) E6 vaccination and increases tumor regression frequency and the probability of rabbits remaining disease-free after CRPV challenge. Tan et al. (1999) have shown that administration of IL-12 at the vaccination site of gene-gun-administered plasmid DNA encoding E7 increased vaccine-induced therapeutic efficacy. Thus, cytokines and co-stimulatory molecules may act as useful adjuvants for HPV DNA vaccines.

The immune response elicited by DNA vaccines may be augmented by the manipulation of pathways for intercellular protein degradation (Leachman et al., 2002). Ubiquitin, a small protein co-factor, targets conjugated protein for recognition and degradation within the proteasome. Velders et al. (2001b) have shown that a multi-epitope vaccine for HPV protected 100% of vaccinated mice against challenge with HPV 16 when ubiquitin and certain flanking sequences were included in the gene insert. Similarly, Liu et al. (2001a) observed enhancement of E7-specific CTL activity and protection against E7-expressing tumors in mice given a DNA vaccine with a ubiquitinated L1-E7 gene insert. Although ubiquitin may be a useful molecule for expediting protein degradation and antigen processing, it is not the only means of enhancing intercellular protein degradation. Shi et al. (1999) engineered mutations into two zinc-binding motifs of an HPV-16 E7 DNA vaccine to generate a rapidly degraded E7 protein. This mutated E7 protein elicited a significantly enhanced E7-specific CTL response and better protection compared with that elicited by a wild-type E7 DNA vaccine. These studies suggest that the enhancement of intercellular degradation of the antigen of interest may increase the immunogenicity of DNA vaccines.

Although the efficacy of DNA vaccination is important, safety is also a critical issue. DNA present in the vaccine may integrate into the host genome, potentially inactivating tumor-suppressor genes or activating oncogenes, thereby inducing malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation, and integration should not pose any real risk. A second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell-cycle regulation by binding to pRb, a tumor-suppressor protein in nuclei (Lukas et al., 1994). The presence of E7 in the nuclei may lead to accumulation of genetic aberrations and eventual malignant transformation of the host cells. For such problems to be avoided, strategies such as the endosomal/lysosomal-targeting Sig/E7/LAMP-1 DNA vaccine may be used to divert E7 away from the nucleus to regions such as the lysosomal and endosomal compartments, thus physically separating E7 from pRb. In addition, detailed mutational analysis of E7 has led to the identification of several mutations that abrogate the transformation activity of E7 (Edmonds and Vosden, 1989; Heck et al., 1992; Jewers et al., 1992; Phelps et al., 1992). One recent study demonstrated that a DNA vaccine encoding E7, with a mutation which inactivated the Rb-binding site, was able to enhance CTL activity and E7-specific anti-tumor effects compared with wild-type E7 (Shi et al., 1999). DNA vaccines using a “shuffled” E7 gene may also alleviate concerns of oncogenicity associated with E7 (Osen et al., 2001). Another strategy to avoid the problem of the oncogenicity of E7 is to use a DNA vaccine encoding a string of multiple epitopes surrounded by defined flanking sequences; this may be safe and promising for tumor protection and therapy, particularly if epitopes are targeted to the protein degradation pathway (Velders et al., 2001b). Ultimately, DNA vectors in human clinical trials may use a minimally mutated E7 gene or multi-epitope gene approach in which critical epitopes are preserved while potential oncogenic activity is eliminated.

### (3.6) RNA replicon vaccines

Naked RNA is another strategy for cancer vaccine development, although RNA is typically less stable than DNA and often has lower transfection efficiency. To improve the immunogenicity of RNA vaccines, one can use self-replicating RNA replicon vectors (also see Section 3.2.3). These non-infectious, self-replicating, and self-limiting RNAs can be launched in RNA or DNA form, followed by transcription into RNA replicons in transfected cells or in vivo (Berglund et al., 1998). Self-replication allows for expression of the antigen of interest at high levels for an extended period of time, thereby enhancing vaccine potency. Since RNA-launched or DNA-launched RNA replicons eventually cause lysis of transfected cells (Ying et al., 1999; Leitner et al., 2000), concerns about integration into the host genome associated with naked DNA vaccines are alleviated. This is particularly important for vaccine development targeting E6 and E7, since HPV-16 E6 and E7 are oncogenic proteins. The RNA replicon system has recently been applied to the development of HPV vaccines. Studies have demonstrated that the potency of HPV-16 E7-specific self-replicating RNA vaccines can be enhanced by application of the LAMP-1 targeting strategy (Cheng et al., 2001c), the Mycobacterium tuberculosis HSP70 strategy (Cheng et al., 2001b), or the HSV-1 VP22 strategy (Cheng et al., 2002) (see Section 3.5). Self-replicating and self-limiting RNA replicon vaccines may be administered as DNA (Berglund et al., 1998). DNA-based RNA replicons, also known as “suicidal” DNA, share the advantages of both RNA replicons and naked DNA vaccines without the disadvantages of either form of vaccine. Not only are they as stable and easily prepared as conventional naked DNA vaccines, but they may also be more potent than conventional naked DNA vaccines (Berglund et al., 1998). Since cells transfected with DNA-launched RNA replicons eventually undergo lysis (hence the term “suicidal”), there is little concern for the malignant transformation commonly associated with naked DNA vaccines. Hsu et al. (2001) recently used DNA-launched RNA replicons for the development of HPV vaccines and demonstrated significant E7-specific CTL activity and anti-tumor effects. Thus, RNA- and DNA-launched RNA replicon vaccines are promising therapeutic options for the treatment of HPV-associated cervical cancer.

### (3.7) Cell-based vaccines

Cell-based vaccines for cancer immunotherapy can be conceptually divided into two broad categories: dendritic cell-based vaccines and cytokine-transduced tumor cell-based vaccines.

#### (3.7.1) Dendritic cell-based vaccines

The generation of large numbers of DCs was previously hindered by a lack of information about DC maturation and the lineage-specific markers that define their cellular differentiation state. Recent advances have revealed the origin of DCs, their antigen uptake mechanisms, and the signals that stimulate their migration and maturation into immunostimulatory APCs (for review, see Cella et al., 1997; Hart, 1997). Several strategies for the generation of large numbers of active DCs ex
indicate that the potency of DC-based vaccines may depend on the specific route of administration.

(3.7.2) Tumor-cell-based vaccines

The use of tumor-cell-based vaccines may not be suitable for the treatment of early-stage, pre-cancerous HPV-associated lesions because of the risks and controversy associated with administering modified tumor cells to patients. Therefore, tumor-cell-based vaccination is likely reserved for patients with advanced HPV-associated cancer. Transduction of tumor cells with genes encoding co-stimulatory molecules or cytokines may enhance immunogenicity, leading to T-cell activation and anti-tumor effects after vaccination (for review, see Chen and Wu, 1998). Several HPV-related tumor-cell-based vaccines have been reported in pre-clinical model systems. For example, vaccines involving HPV-transformed tumor cells transduced with cytokine genes such as IL-12 (Hallez et al., 1999) and IL-2 (Bubenik et al., 1999) have been demonstrated to generate strong anti-tumor effects in mice. Recently, it has been shown that an E7-expressing GM-CSF gene-transduced allogeneic tumor-cell-based vaccine can generate E7-specific CTL activities and protective anti-tumor immunity in immunized mice (Chang et al., 2000). Analysis of these pre-clinical data indicates that tumor-cell-based vaccines may be useful for the control of minimal residual diseases in patients with advanced HPV-associated cervical cancers.

(4.0) Combined Prophylactic and Therapeutic Vaccines

(4.1) HPV chimeric VLPs

Chimeric HPV virus-like particle (VLP) vaccines represent innovative protein-based HPV vaccines. Immunization with HPV VLPs induces high-titer neutralizing antibodies in the serum and can protect animals from experimental papillomavirus infections. However, VLPs do not generate therapeutic effects for established or breakthrough HPV infections, which are prevalent in high-risk sexually active populations. Treatment of established infections requires the induction of T-cell-mediated immune responses. This can be achieved with the use of chimeric VLPs that carry E2 and/or E7 antigen (Greenstone et al., 1998; Peng et al., 1998; Jochmus et al., 1999; Schafer et al., 1999). E7 chimeric VLPs can also elicit high titers of neutralizing antibodies (Greenstone et al., 1998) and activate dendritic cells (Rudolf et al., 2001a). Currently, clinical-grade HPV-16 L1/L2-E2-E7 chimeric VLPs, which contain four HPV-encoded proteins (L1, L2, E2, and E7) as target antigens, are under preparation for a phase I clinical trial (John T. Schiller, personal communication).

(4.2) HPV pseudovirion vaccines

Naked DNA can be encapsulated in papillomavirus capsids by a variety of expression systems–including recombinant vaccinia viruses (Zhao et al., 1998), Semliki Forest virus (Rodent et al., 1996), and baculovirus (Touze and Coursaget, 1998)–to form non-replicative pseudovirions. More recently, infectious virus particles containing a mammalian-expressing DNA vector have been generated in Saccharomyces cerevisiae (Rossi et al., 2000). The target DNA plasmid can be packaged into HPV-16 VLPs expressed in yeast and transduced into different primary and established cells in culture and in vivo via receptor-mediated endocytosis. This method provides a quantitative system for
the assessment of HPV-16 VLP infection. Non-replicative papil-
lomavirus pseudovirions also allow for safe and improved
delivery of therapeutic DNA to target cells. Touze and
Coursaget (1998) have demonstrated higher frequency of gene
transfer with HPV pseudovirions than with DNA alone or with
liposome. One recent study found that vaccination with papil-
lomavirus-like particles containing mutated E7 DNA is able to
induce mucosal and systemic E7-specific CTL responses (Shi et
al., 1999). These studies demonstrate that it is possible to gen-
erate papillomavirus pseudovirions in an in vitro system, and
that such pseudovirions can deliver packaged DNA into differ-
cent cell lines and induce antigen-specific CTL responses. The
ability of pseudovirion to generate both neutralizing antibodies
and antigen-specific CTL responses makes it a potentially
desirable vaccine for combining the advantages of preventive
and therapeutic HPV vaccines.

(5.0) Potential Application of Vaccine Strategies
to the Head and Neck Cancer Population

The target population for administration of an effective vaccine
to prevent HPV infection is a matter of considerable ongoing
debate. Although women bear the greatest burden in terms of
morbidity and mortality from HPV infection, men make a con-
siderable contribution to the spread of the virus and are also at
risk for penile, anal, and tonsillar carcinomas. HPV infection is
the most common sexually transmitted disease and is most
often asymptomatic, and therefore all sexually active individu-
als should be considered at risk. Although tobacco use is an
independent risk factor for many HPV-associated cancers,
including HNSCC and cervical cancer, the relative risk associ-
ated with HPV infection after adjustment for tobacco use is
considerable. Therefore, vaccine administration should be
independent of other known co-factors. Given that 15% of all
incident malignancies worldwide have been attributed to HPV
infection, an effective vaccine would have a substantial impact
on worldwide morbidity and mortality. Ideally, the vaccine
would be capable of generating protective neutralizing anti-
bodies against all 17 of the high-risk HPV types. It is likely that
HPV vaccination will eventually be recommended to all indi-
viduals prior to onset of sexual activity. The incidence of all
HPV-associated malignancies, including HPV-associated
HNSCC, would then be expected to decline thereafter.

The target populations for therapeutic vaccines would
include individuals with established HPV infection and HPV-
associated malignancy. The natural history of oral HPV infec-
tion is unclear. Considerable research must be done to identify
the population at risk for oral HPV infection and methods for
screening (including how to optimize oral sample collection).
In the cervical cancer model, it is clear that individuals with a
persistent HPV-16 infection are at greatest risk for progression
to an in situ or invasive cervical cancer, and the same is likely
to be true for oral infection. However, until this information is
available, vaccine protocols for patients with oral HPV infec-
tion cannot be designed.

Individuals with HPV-associated HNSCC are an ideal
population for the study of therapeutic HPV-specific vaccines,
for several reasons. Most therapeutic vaccines have been
developed with HPV 16 as a model and are designed to gen-
erate an immune response to HPV-16 oncoproteins. The
overwhelming majority of HPV-associated HNSCC are HPV-16-
positive. Although phase I studies are designed to evaluate
toxicity, and phase II studies incorporate primarily immuno-
logical outcomes, these trials often include initial measure-
ments of possible vaccine efficacy, such as tumor infiltration of
HPV-16-specific T-cells. Oropharyngeal tumors or lymph node
metastases in the head and neck are far more amenable to
biopsy than pelvic masses and lymph nodes in cervical cancer
patients. There is evidence that patients with HPV-associated
HNSCC have improved survival over individuals with HPV-
negative HNSCC (Gillon et al., 2000; Mellin et al., 2000; Lindel
et al., 2001; Schwartz et al., 2001). The reason for this is as yet
unclear. It has been hypothesized that the difference in sur-
vival might be attributed to an immunological response to the
virus during therapy. There are in vitro data that radiation
therapy induces increased E6 and E7 transcription and MHC
Class II expression in the HPV-infected cell. Generation of a
cell-mediated immune response in these patients may there-
fore hold particular promise. Given that therapeutic vaccines
are designed to generate a systemic immune response, there
would be no particular advantage to local injection into the
tumor. Analysis of animal model data suggests that the therape-
utic vaccines hold greatest potential for low-volume dis-
ease. Therefore, the greatest potential utility for these vaccines
would be in the post-surgical/post-primary-radiation therapy
setting as adjuvant therapy to stimulate an immune response
capable of clearing microscopic residual disease.

(6.0) Conclusion

In the past decade, significant progress has been made in the
field of HPV vaccine development. The determination that
HPV is an etiological agent for a subset of head and neck squa-
mous cell carcinoma and their precursor lesions has paved the
way for the development of preventive and therapeutic HPV
vaccines that may lead to the control of HPV-associated mali-
gancies and their potentially lethal consequences. An under-
standing of the molecular progression of HPV-associated can-
cer has led to the realization that HPV E6 and E7 are important
targets for the development of HPV therapeutic vaccines for
the control of established HPV infections and HPV-associated
lesions. Several experimental HPV vaccine strategies—includ-
ing vector-based vaccines, peptide-based vaccines, protein-
based vaccines, nucleic-acid-based vaccines, chimeric VLP-
based vaccines, cell-based vaccines, pseudovirions, and RNA
replicons—have been shown to enhance HPV-specific immune
cell activity and anti-tumor responses in murine tumor sys-
tems. Several clinical trials, including trials with HPV-HNSCC
patients, are currently under way, based on encouraging pre-
clinical results from these preventive and therapeutic HPV vac-
cines. A side-by-side comparison of these vaccines will help to
identify the most potent preventive and therapeutic HPV vac-
cine with minimal negative side-effects. Clinical HPV vaccine
trials provide a unique opportunity to identify the characteris-
tics and mechanisms of immune response that best correlate
with clinical vaccine potency. Such immunological parameters
will help define protective immune mechanisms for controlling
HPV infections and HPV-related disease in the head and neck
and anogenital regions of the body. Rational development of
more effective vaccines for HPV infections would be greatly
facilitated by comprehensive information on these protective
immune mechanisms in humans. Therapeutic vaccines may be
a practical option for patients with HPV-associated cancers,
such as those of the head and neck, with attention being paid to
efficacy, safety, and cost. Combining immunotherapy with
traditional treatment such as chemotherapy and/or surgery
may be another option for improving the prognosis and quality of life of those with HPV-associated cancers. With continued endeavors in HPV vaccine development, we may soon be able to implement a variety of safe and effective preventive and therapeutic vaccine strategies for the control of HPV-associated cancers, including those of the head and neck.

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