MEDICAL INTELLIGENCE UNIT **14**

Robert W. Tindle

Vaccines for Human Papillomavirus Infection and Anogenital Disease

R.G. LANDES COMPANY

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COMPANY
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PREFACE

The anogenital epithelium of a majority of the sexually active population is likely to experience infection with a number of human papillomavirus types. In most, infection will be asymptomatic and resolve, with or without transient seroconversion. In some, infection will progress to low grade anogenital lesions associated with episomal HPV virions, which may or may not resolve spontaneously. In a very few individuals, higher grade dysplasia which may progress to squamous cell carcinoma, associated with integration of HPV DNA into the host genome occurs. There is little understanding of factors predisposing to disease onset and progression, though the infecting HPV genotype(s) ('high risk' versus 'low risk'), and immunosuppression are clearly two such factors. The limitations of drug and ablative therapies warrant an immunomodulatory approach to control of disease. HPV evades rather than subverts the immune system, which leaves HPV open to attack by an appropriately vaccine-primed immune response. The biology of HPV provides several distinct avenues of attack, namely prevention of infection, therapy for infection, and therapy for HPV-associated neoplasia, and each demands a different vaccine strategy. Which strategy will prevail at the population level is likely to depend on considerations which are fiscal and cultural as well as scientific.

HPV vaccinology is not easy; derivation of vaccines capable of preventing or curing infection by a number of immunologically distinct genotypes at a mucosal surface, or capable of killing HPV infected epithelially-derived tumor cells, in the absence of a) a convenient tissue culture system for propagating virus, b) an animal model for HPV infection, or c) an animal papillomavirus model which mimics HPV-associated neoplastic disease, has tested the ingenuity of papillomavirus vaccinologists to the full. This book recounts the outcome of their endeavors. The number of commercial companies involved in bringing to the market place vaccines to prevent infection, cure genital warts and dysplasia or cervical cancer, reflects the pecuniary prizes to be won.

Inevitably some colleagues may feel that their work has not been adequately cited or emphasized. However, in the many reviews referenced by the chapter authors, it is unlikely that any significant work has gone unacknowledged.

Robert W. Tindle
April 1999

Dedication

to the memory of Jian, and for Xiao-Yi and Andy

Chapter 1

Immunomodulation of HPV Infection and Disease: An Overview

Robert W. Tindle

About 50 of the approximately 100 (the number continues to grow) genotypes of human papillomaviruses (HPV) infect the mucous epithelium of the genital tract. HPV genotypes 6, 11, 43 and 44, in particular, predispose individuals to noninvasive flat or hyperkeratotic condylomata (genital warts) and low grade dysplasia. New reported cases of genital warts exceed one million per annum in the USA. Current drug therapies include local injection of interferon-α, topical podiphyllotoxin and imiquimod with or without cryotherapy, and laser ablation or surgical excision. The limitations of the current ablative and drug approaches (recurrence rates are typically 25-50%) in control of disease indicates that immunomodulatory approaches to prevent infection and disease progression are warranted. It is less than clear, however, how this might be approached, since a majority of the sexually active population is infected with anogenital-associated HPV, asymptomatically.[1] The quest for HPV vaccine(s) is also driven by the association of HPV infection with carcinoma of the uterine cervix.[2] HPV DNA (predominantly genotypes 16, 18, 45) is detected in more than 90% of all uterine cervical tumors.[3] Some 500,000 new cases of cervical carcinoma are reported annually worldwide. It is estimated that 0.1-1% of HPV infections would progress to neoplastic disease if left untreated. However, while recurrence of genital warts is a problem in the developed world, 80% of cervical cancer occurs in developing countries (where it is frequently the major cause of mortality in women) which lack full availability of Pap screening programs, surgery and radiotherapy. Typically, cervical cancer ranks fourth to eighth as the cause of death in countries where these services are available. It is clear that immunomodulatory approaches will only make inroads into the global burden of cervical cancer if immunotherapy is combined with immunoprophylaxis.

The observation of the rapid progression of HPV-associated premalignant cervical lesions and florid wart development in immunocompromized individuals (iatrogenically, genetically, and from other diseases)[4] clearly suggests an immunological component in the outcome of infection, and this is supported by successes with autologous wart vaccines.[5] There is little evidence to indicate that immunodeficiency predisposes to infection, (in contrast to disease). On the other hand, epidemiological evidence suggests that resistance to reinfection with genital genotypes can be acquired, and this correlates with the incidence of HPV-directed serum antibody.[6] Furthermore, evidence from animal papillomaviruses (PVs) shows protection against infection afforded by PV Immunization.

This book is concerned with immunomodulatory strategies for the control of HPV-associated anogenital disease. The present chapter provides an overview of these strategies and of current vaccine trials . The groundwork for the book is laid by a consideration of the

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immunobiology of HPV infection (chapter 2, Drs. Leggatt and Frazer). The immunology and molecular biology of HPV have been reviewed elsewhere.[7,8]

Prospects for Vaccines for HPV: Theoretical Considerations

Successful viral vaccines hitherto developed have a number of features in common (Table 1.1). While evidence is accumulating that vaccine-induced and naturally acquired antibody may hinder HPV infection, HPV shares none of the other features listed in Table 1.1, suggesting that the road to successful vaccine development for HPV may be arduous. The daunting scenario facing an aspiring HPV vaccinologist is weak immunogenicity during natural infection, a range of genetically distinct but morphologically similar virus types, each producing a similar disease, and a range of diseases which can be produced by a given virus type. Furthermore, infection is chronic, and disease onset insidious, and so the epidemiology of infection has been particularly difficult to define. Add to this strict species specificity (precluding animal experiments with HPV), the small numbers of virus particles available from lesions and the inability to grow the virus in vitro (thus precluding a source of live virus for experiment or attenuation), and the enormity of the task of creating a vaccine becomes apparent.

On the other hand, restricted cladistic evolution[10] and relative lack of intratypic variation suggest that HPV is evolutionarily stable and not prone to variation as a result of immunoselective pressures, and this augurs well for a vaccine. It is with this background that a number of pharmaceutical companies have set HPV vaccine(s) as a priority; genotypes commonly associated with genital warts and those associated with cervical cancer are both being targeted.

Three Stages for Immune Intervention

There are three stages in the biology of anogenital HPV infection at which vaccine mediated immune intervention may be attempted:

1. Prior to HPV infection of anogenital epithelium—a prophylactic vaccine designed to prevent HPV entry at the mucosal surface;
2. During viral replication in the basal cells of the epithelium—a therapeutic vaccine designed to eliminate infection;
3. After viral integration (of oncogenic HPVs), cell transformation and subepidermal invasion;
4. A therapeutic vaccine designed to control HPV-induced tumors.

The target viral antigens and the type of immune response one wishes to evoke will be clearly different in each of these three scenarios, and this will be reflected in the vaccine strategy employed.

Prophylactic Vaccination to Prevent Infection

Natural HPV infection at genitomucosal surfaces is poorly immunogenic,[11,12] presumably reflecting the lack of productive infection, lack of an inflammatory response and the limitations of keratinocytes as antigen-presenting cells. (In the absence of B7/CD28 second signaling,[13] keratinocytes transmit a tolerogenic rather than an immunogenic signal from internally processed foreign (e.g., viral) antigens. Specific immunological tolerance, or immunological 'ignorance', is a likely outcome of antigen presentation through keratinocytes.[14]) Nonetheless, antibody responses to natural infection do occur, suggesting that HPV antigens find their way to draining lymph nodes where, in the context of professional antigen-presenting cells (APCs), antibody inductive mechanisms are set in train by cross-presentation.[15]

Table 1.1 Characteristics of Viral Infections for which Successful Vaccines Have Been Developed (Adapted from ref. 9)

Obligatory viraemia
Circulating antibody protects against infection and disease
No latency
Self-limiting illness (though death may supervene)
Following elimination of virus, immune protection is complete
An animal model reproducing human disease

The rationale for a prophylactic vaccine is that it would circumvent the constraints on immunogenicity seen in natural infection and induce a protective antibody response. For epitheliotropic viruses with no systemic phase of infection (though there are isolated reports of HPV DNA in peripheral blood mononuclear cells[16]), the relevance of neutralizing antibodies in blood will be restricted to their availability to the genitomucosal surface. A vaccine to prevent HPV infection must seek to establish an immunological barrier at the portal of entry, i.e., anogenital epithelium. In women, this would be immunoglobulin at the cervicovaginal surface directed to conformational determinants on L1 and/or L2 viral capsid structural proteins.

Proponents of prophylactic vaccination are encouraged by recent epidemiological data indicating:

1. Seroconversion within approximately 6 months of first infection, with antibody persisting for about a year;[1] and
2. That older women with fulsome sexual histories are less susceptible to infection than younger women whose limited sexual experiences presumably have not provided a solid seroimmunity.

The advent of technology to genetically engineer large quantities of infectious, but DNA-free, HPV virus-like particles (VLPs) has been a major breakthrough in the HPV prophylactic vaccine approach, and has circumvented the inability to propagate HPV in vitro to create an attenuated/killed vaccine. VLP vaccination also overcomes the finding that isolated L1 and L2 proteins are generally suboptimal in inducing protective immune responses, presumably because a lack of tertiary structure precludes formation of neutralizing conformational epitopes. 'Empty' VLPs (i.e., composed only of capsid proteins), may serve as vehicles for the delivery of additionally incorporated immunomodulatory components, either DNA or protein. Thus, incorporation of appropriate immune response modifier proteins (cytokines), may selectively stimulate the desired 'arm' of the immune response (Th1 vs. Th2). Or, a combined prophylactic and therapeutic vaccine featuring tumor-associated viral proteins (e.g., HPV16 E7) encapsulated in VLPs may be envisaged.[17] Dr. Kast's group (personal communication) has shown that Immunization of mice with chimeric VLPs of the HPV16 L1 protein plus the entire HPV16 E7 protein fused to HPV L2 protein protected mice from challenge with the E7-expressing tumor cell line TC-1. A single injection of an HPV16 E7-containing VLP consisting of either HPV L1/L2, BPV L1/L2, or CRPV L1/L2 eliminated existing tumors. Enhanced E7-directed immunotherapy was obtained by injecting these three VLPs one week apart sequentially, thereby avoiding possible negative effects of species-specific L1/L2-directed neutralizing antibodies. Furthermore, the Kast group has shown that delivery of peptide in VLPs may abrogate the tolerizing effect seen following Immunization with some 'naked' peptides. The chimeric VLP principle may be extended to the incorporation of immunogenic moieties of other

sexually transmitted disease organisms, to create a multi-STD vaccine. Analysts predict that vaccination of adolescents against STDs is likely to be a major growth area in the vaccine market in the next 10 years (but warn that parents may be unwilling to allow their teenage children to be vaccinated, as this may be seen to be condoning sexual activity.)

The use of VLPs as prophylactic vaccines for HPV is discussed by Dr. Jansen in chapter 3. Perhaps the most encouraging finding from animal models of PV VLP vaccination is that there is clear evidence, from canine oral papillomavirus (COPV) and bovine papillomavirus (BPV), that parenteral Immunization will induce an antibody-mediated response which will protect against PV infection at a mucosal surface (discussed by Drs. Leggatt and Frazer, chapter 2). It had hitherto been thought that protection against HPV at the mucosal surface would be best achieved by Immunization which targeted that site and was mediated by secretory IgA. Since IgA-secreting precursor B cells have the capacity to home to any mucosal subepithelial sites,[18] antigenic experience at one mucosal site can be reflected in immune effector response at a distant surface,[19-21] and so oral rather than parenteral Immunization has been mooted to be preferable for response in the genital mucosa. For papillomavirus VLPs, the question becomes whether to supplement parenteral Immunization with oral Immunization. Direct vaginal Immunization is a possibility, though there is little firm data to support the efficacy of this route.[23]

DNA vaccination to control HPV infection at the genitomucosal surface should be effective,[24] as it is in protecting cottontailed rabbits against skin infection when immunized with cottontailed rabbit papillomavirus (CRPV) L1 capsid DNA. DNA vaccines produce good antibody and cell-mediated immune responses, are easily constructed, heat stable, contain no adventitious organisms, and are cheaper to produce than other new generation vaccines. The DNA vaccine approach is discussed by Drs. Leachman and Brandsma in chapter 8. If mucosal delivery proves necessary, the lower efficiency of transfection at the mucosal surface may be offset by conjugating the HPV plasmid DNA to cationic liposomes before oral[25] or aerosol[26] delivery. A DNA approach offers an easier route to the production of a multivalent vaccine by combining DNA-encoding viral capsid proteins of different HPV genotypes. This concept is of particular import, since it is apparent that HPV capsid-induced antibodies are generally genotype-specific,[27] which will confound attempts to produce a vaccine protective against multiple HPV genotypes with a single VLP. The two generic concerns relating to DNA vaccine technology, namely the danger of integration into the host genome, and the possibility of generation of anti-DNA antibodies, are likely to prove unfounded, but may be regulatory stumbling blocks.

Therapeutic Vaccination for HPV Infection

Complete sterile immunity is unlikely to be achieved by even the most efficacious neutralizing antibody response. Those HPV virions which escape are likely to be safe from antibody interference and able to embark on the replicative cycle once inside basal/suprabasal epithelial cells. To eliminate replicating virus, a vaccine should prime for cytotoxic T lymphocytes (CTLs) directed to CTL epitopes derived from intracellularly processed viral proteins presented in the context of any of the MHC class I alleles expressed by the host. Conceivably, a vaccine-induced CTL response to L1, L2 and/or E4 proteins expressed in distal differentiating layers of the epidermis may serve to obliterate these cells. This, however, would be of little benefit since infected proximal stem cells not expressing these antigens would provide a self-renewing reservoir of virus. To eliminate cycling stem cells, E1 and E2 are the putative target antigens, since these are the major HPV proteins expressed in the suprabasal self-renewing epithelial stem cell population.[29] Since these proteins are likely to be expressed below the threshold level of 100-500 viral epitopes presented to MHC class I per cell, strategies may have to be concomitantly invoked to upregulate their expression in

order to render the cell susceptible to specific CTL attack. E7 protein as a target antigen in undifferentiated keratinocytes is a possibility.

Dr. St. Clair Roberts and colleagues describe therapeutic vaccines for HPV infection and genital warts, particularly the TA-GW vaccine directed to HPV6 L2 and E7 proteins (chapter 4). Positive clinical findings encourage pursuit of TA-GW, particularly when incorporated into novel adjuvants to improve Th1-type immune responses in recipients.

A subtle option for immunological control of intracellularly replicating HPV would be vaccine-induced delayed-type hypersensitivity (DTH) effector T cells , with the capacity to eliminate epithelial cells bearing viral antigens and induced MHC class II molecules by cytokine-mediated mechanisms.

Therapeutic Vaccination to Control HPV-Associated Tumors

Persistence of E6 and E7 oncoproteins in the neoplastic cell is required to maintain its transformed state.[28] A corollary is that cervical cancer vaccinologists have two tumor-specific antigens (tsa) to which to direct HPV-specific CTL induction strategies. Tumor cells should be susceptible to vaccine-induced E7 and/or E6-directed CTL effector function, even though selective pressure in immunocompetent hosts has worked to minimize presentation of HPV16 E6 and E7 to the inductive arm of the immune response, for example, by the loss of expression of MHC class I alleles,[30] by point mutations in CTL epitopes,[31] or by downregulation of endogenous peptide processing machinery for MHC presentation.[32] Phase I/II trials of full length E6 and/or E7-based vaccines for cervical cancer are described by Dr. Man and colleagues (chapter 6), and Dr. Frazer and colleagues (chapter 7). Concerns over administration of an oncogene product (i.e., E6, E7) may be overcome by vaccination with CTL epitopes defined by algorithm prediction and/or elution from MHC class I molecules of oncogene-expressing cells. This approach is discussed by Dr. Ressing and colleagues in chapter 5. 'Polytope' vaccines consisting of multiple CTL epitopes strung together as a linear polypeptide[33] or as DNA,[34] and 'matched' to the HLA class 1 alleles expressed by the patient, can be envisaged. A 'generic' polytope vaccine encoding multiple CTL epitopes functional in the context of the more common HLA haplotypes would be generally applicable to the outbred human population. Concern over immunodominance issues (i.e., response to one epitope, or set of epitopes, precluding the development of response to others) is likely to prove unfounded, as it has been in DNA 'polytope' vaccines in an animal model.[34]

Three areas require attention if therapeutic vaccines for infection or for cervical neoplasia are to proceed to phase II/III trials. Firstly, adjuvants or delivery systems must be optimized to elicit Th1-type CTL rather than Th2-type responses, since it is unlikely that antibody will be effective in control of disease. An optimal therapeutic immune response would be directed to tsa, and would include CTL and DTH components. Secondly, measurement of CTL activity in humans is less than straightforward; it requires autologous (or at the very least, HLA restriction element-matched) target cells which have antigen (E6 or E7)-derived peptides that are noncovalently bound in cell surface class I molecules. Phytohemagglutinin(PHA)-derived PBMC blasts, or Epstein-Barr virus (EBV)-transformed lymphoblastoid lines have been traditionally used, but E6 and/or E7-expressing cells of epithelial origin would make more appropriate targets to mimic the in vivo situation. Staining of vaccine-induced effector cell populations with fluorescent MHC tetramers[35] containing cognate peptide 'in the groove', may remove the difficulties of having appropriate target cells, and enhance sensitivity of quantification of specific CTL effectors. This, and other surrogate assays of cell-mediated immunity must be viewed with caution, since they may be nonquantitative and/or irrelevant to clinical outcome. Thirdly, it is necessary to establish the duration of HPV-specific CTL memory.

While proof of concept in the form of clinical efficacy in phase III trials has yet to be confirmed for any therapeutic vaccine, nonetheless, a vaccine to induce an anti-tumor response to clear HPV-associated residual tumor following conventional ablative therapy at primary diagnosis should be an achievable goal. Immunomodulation as an effective primary therapy for untreated tumor is likely to prove too difficult.

Delivery Systems: How Best to Deliver the Vaccines

While it is clear that parenteral administration of VLPs elicits functional antibody responses (i.e., protection) at the mucosal surface, this may be augmented by concomitant oral administration. Were this to be so, microencapsulation technology should provide an optimal oral delivery vehicle for HPV VLPs. Polymer microcapsules protect antigen from gastric degradation, and are selectively absorbed by professional antigen-presenting M cells, rather than villus epithelial cells which generate a predominantly suppressor response.[22] Furthermore, polymer microcapsules are effective at remote site mucosal stimulation, being preferable to liposomes in this regard.[36]

Where the goal is to elicit CTL and DTH, a replacement for the traditional Alum-based adjuvants, (which predominantly elicit Th2-type antibody responses), is likely to emerge from the plethora of adjuvant candidates awaiting full characterization by the drug companies. Frontrunners include Smith-Kline Beecham's QS-21 (licensed from Cambridge Biotech). QS-21 appears to be synergistic with monophosphoryl lipid A (MPL) (also of Smith Kline Beecham), which is already in phase III trials in an HSV-2 vaccine. Iscom (immune stimulating complex) licensed by CSL and Chiron Biocine, and MF-29, also of Chiron Biocine, are further candidates. The novel adjuvant SBAS2 is being used with TA-GW (St. Clair Roberts and colleagues, chapter 4).

It is noteworthy, however, that VLPs elicit CTL in the absence of adjuvant, presumably gaining access to the alternative MHC class I antigen processing pathway.[37,38]

Several strategies lend themselves to circumventing the constraints on naturally acquired tumor specific immunity for the immunomodulatory control of cervical cancer. These include multiple subcutaneous infusions with tumor antigen (e.g., E7)-pulsed autologous dendritic cells,[39] administration of ex vivo-induced tumor-specific CTLs engineered to secrete immune-enhancing cytokine (e.g., IL-2[40]), rerouting the immunogen (e.g., E7 protein) to the appropriate compartments of the MHC class II pathway using a lysosome-associated membrane protein (LAMP) sorting signal, and vaccination with autologous tumor cells engineered ex vivo to secrete cytokines (particularly GM-CSF). These strategies and others are discussed by Drs. Stevenson and Wu in chapter 9.

The efficacy of live recombinant vectors to deliver HPV antigens is discussed by Dr. Paterson and colleagues in chapter 10. The decisive factor in vector efficacy is likely to be the capacity to deliver HPV proteins intracytosolically in professional APCs for entry into the TAP-MHC class I processing pathway and the B7.1/2 presentation pathway, and also to generate HPV-specific CTL memory. All live vectors run a certain risk of reversion to virulence. Vaccination strategies may need to be tailored to overcome vector-directed responses in vaccine recipients, which can diminish subsequent 'take' by the same vector if repeated dosing is necessary.

What the Animal Models Teach

HPV does not infect animals, and animal PVs produce dissimilar diseases. In particular, there is no animal equivalent of viral integration into host cell DNA, and oncogenesis at the anogenital surface following a long latency, as is seen in HPV. Nonetheless, three animal models have been particularly informative; the cogent findings are discussed by Drs. Leggatt and Frazer (chapter 2). In a nutshell, PV capsid protein vaccines given as VLPs or as DNA

protected their respective animal hosts against challenge with CRPV, BPV or COPV. As previously mentioned, the latter two are particularly relevant to the human situation since parenteral Immunization protected at a mucosal surface (oral mucosa and/or alimentary canal in cows,[41] and oral mucosa in dogs[42]). In the case of COPV, protection could be adoptively transferred with serum, indicating a role for antibodies in protection. In addition, VLP vaccination was associated with regression of existing lesions in CRPV[43] and BPV.[41] There are potential drawbacks to the use of VLPs in humans; they may not protect against infection by cell-associated virus, and difficulties in obtaining VLPs free from contaminating host cell DNA may become a regulatory issue.

Neutralization of infection with HPV virions of human foreskin keratinocytes transplanted under the renal capsules of athymic nude mice, by blocking with anti-virion antibody, has hitherto been the 'gold standard' model of serological immunoprotection.[44] The elicitation of cervicovaginal antibody in nonhuman primates by HPV VLP Immunization (described by Dr. Jansen in chapter 3), brings immunoprophylaxis for HPV infection a step nearer.

While of importance in establishing that the tenets of cell-mediated immunity and surveillance are applicable to tumors expressing HPV antigens, animal models of HPV-directed tumor immunity provide no information on immunogenic epitopes functional in the context of human MHC haplotypes and T-cell repertoires. An exception is studies in HLA transgenic mice, where the elegant work of Kast and Melief and colleagues (summarized in chapter 5) has demonstrated surrogate HLA-restricted responses.

An aim of animal tumor models for HPV is to describe the parameters of tsa-directed protection in epithelium. Insights will come from three in vivo models:

1. The use of transgenic mice expressing E6/E7 uniquely in epithelium in a non-inflammatory fashion. These mice have the potential to provide a model of progressive tumor development for vaccine efficacy studies more apt than that of direct challenge with a tumor inoculum. Recent work from our laboratory indicates that epithelially-expressed E7 transgene tolerizes the E7-directed CTL arm of the immune response.[45] Were this to occur in patients constitutively expressing E7 in their epithelially-derived cervical tumors, a challenge for E7-directed immunotherapy would be to overcome specific peripheral tolerance.
2. The grafting of keratinocyte cell lines expressing HPV16 E6 and/or E7 proteins on to syngeneic mice to form a differentiated epithelium overlying a granulation tissue bed, thus modeling the presentation of these viral antigens to the immune system in a way that is analogous to human infection.[46]
3. The adoptive transfer of immune effector cells of human origin into severe combined immunodeficiency (SCID) mice that have been grafted with HPV-associated human anogenital lesions, and that display a differentiated neo-epithelium exhibiting features of HPV infection.[47]

Strategies for Clinical Trials: Who to Vaccinate? What is the Read-Out for Vaccine Efficacy?

Since a majority of sexually active adults are positive (by PCR) for HPV DNA, but remain asymptomatic with or without serological evidence of infection, and since epidemiological data indicate cycles of infection and 'cure' with little understanding of the factors leading to progression to clinical disease in a minority of individuals, (although immunosuppression is clearly one such factor), the questions to be addressed are:

1. Who to immunize?
2. What will be the read-out for vaccine efficacy and when?
3. How long should vaccine-mediated immunity persist?

The design of trials is complicated by the natural history of HPV infection, the plethora of distinct but sometimes serologically crossreactive HPV genotypes producing overlapping diseases, and the long time delay between infection and disease onset. Evaluation of vaccines against low risk types (e.g., HPVs 6 and 11), which produce readily visible low risk genital warts should be easier and quicker that those against high risk oncogenic types which require colposcopical, cytological and histological examination of the uterine cervix. The later the stage of the disease chosen, the smaller the proportion of HPV-infected individuals who will meet the diagnostic criteria. Such considerations, plus the long period between prophylactic vaccination and endpoint (up to 25 years) in the case of vaccines for high-risk types, make it of questionable attractiveness to drug companies. Nonetheless, several are now proceeding with the development and production of VLPs at the standards required for human trials.

Clearly, there are a number of distinct goals which HPV vaccines might address.[49] These are:

1. Prevention of genital warts;
2. Immunotherapy for genital warts;
3. Prevention of diffuse symptomatic anogenital disease;
4. Prevention of cervical dysplasia/neoplasia;
5. Immunotherapy for cervical dysplasia/neoplasia.

A different trial strategy is required for each goal.

Prevention of Genital Warts

Warts appear within 6-12 weeks after exposure to (mainly) HPVs 6 or 11, and beyond 3 months, spontaneously regress at a rate of 30% per 3 month period. Infection usually coincides with the onset of sexual activity, and by age 20 about 30% of sexually active women test positive for HPV DNA, of which 10% will have, or have had, clinical genital warts, of which they may or may not be aware.

The natural history of the disease indicates a randomized placebo-controlled double-blind study on a cohort of young males and females, from which is excluded those with, or with a history of, genital warts, and those who are immunocompromised. College freshmen would ideally constitute such a cohort, since they are likely to be amenable to follow up over a number of years. A volunteer process may self-select for those likely to be sexually active, though those motivated to participate might also be motivated to practice safe sex, which may reduce the risk of warts. A 5% per annum incidence of visible warts , and an anticipated 50% dropout rate indicate a cohort of 2000 students.

Since it is unlikely that any vaccine produces full sterile immunity, HPV DNA status as an endpoint for the trial is too stringent a test. Rather, the disease endpoint should be visible warts, confirmed as HPV associated. Patients presenting with warts during the first 12 weeks should be analyzed separately. The no-disease endpoint will be freedom from genital warts. Analysis of efficacy will be substantially biased against the vaccine if dropouts are assumed to be prophylactic failures.

Immunotherapy for HPV6/11 Genital Warts

Destructive or antiproliferative therapies will speed spontaneous regression. Immunotherapy for HPV infection is predicated on the assumption that it can do significantly better than current treatment modalities. There is a choice whether immunotherapy is given as an adjunct after ablative therapy to prevent disease recurrence, or as a single primary therapy. The former would be most appropriate for first clinical trials, since the chance for successful outcome of the vaccine is improved, and the patients will be aware of receiving the best available therapy. A blinded, randomized, placebo-controlled trial is required, on patients

with visible genital warts, which have not been recently treated, and a sample wart should be biopsied on study entry for HPV-DNA analysis and histology. A cohort of ~350 would be desirable. ~50% of subjects will have recurrent disease by 3 months in the placebo arm, and a 50% improvement in the vaccination arm might be appropriate for study design. Outcome would be evaluated at 6 months.

A Prophylactic Vaccine for HPV-Related Cervical Dysplasia

The disease to be prevented must be one that in current opinion must be treated as part of a prevention program for cervical cancer, and this may vary between low grade intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) from country to country. CIN3 as a cancer precursor is HPV16-related. For HPV18-associated adenocarcinoma, there is no precursor lesion with a long natural history to follow. CIN1 is frequently not HPV16-related and may spontaneously regress, or regress and return. HPV16-positive CIN1 is not necessarily the result of HPV16 infection, since 30% of 20-25 year old women with histologically normal cervices carry HPV16 as a passenger in the cervical mucosa. CIN1 may represent two different processes:

1. A repair process following viral exposure in the cervix; and
2. Late in HPV16 infection, a dysplasia likely to progress.

Diagnosis of CIN is prone to biopsy sampling error, and histology has proved less than optimally reproducible for this lesion. All these complicating factors will reduce the power of a trial of a vaccine for HPV16/18 prophylaxis, if the endpoint is CIN1, and the expectation of large uncertainty in sample size calculations. The scenario argues for a good prospective study of the prevalence and natural history of CIN1. Without such a study, there is a risk that a significant real benefit from the vaccine for the prevention of HPV-induced CIN1 could be lost in the 'noise' generated by the aforementioned uncertainties.

Recruitment to the trial will be of women who have a normal cervix that tests negative for HPV DNA, and who are already sexually active , since cervical sampling at time of entry will be required. Follow up will involve multiple site cervical biopsy and DNA screening at intervals. Failure of prophylaxis may be defined as the development of HPV16 DNA^+ CIN1 after vaccination, which requires treatment before the end of the study because of progression to CIN3, or which is still present at the end of the study. Successful prophylaxis might be either no $HPV16^+$ CIN1, or $HPV16^+$ CIN1 which develops and regresses.

HPV Vaccines: Recent Developments

In addition to those reported in later chapters of this book, other HPV vaccines in trial, or about to be trialed, include: MedImmune's recombinant HPV11 L1 VLP vaccine (MEDI-501) in Alum, in phase I trials; Merck's multivalent HPV6, 11, 16, 18 recombinant VLP in Alum for which phase I trials are planned; Medimmune intends to trial HPV18 VLP (MEDI-504) and HPV16 VLP (MEDI-503) in collaboration with SB Biologicals; Medigene (GmbH) is developing a HPV16 L1-E7 fusion product; CSL's recombinant HPV16 E7 and E6/E7 protein in Iscom's adjuvant; Merck/Vical's plasmid DNA and Wyeth-Lederle/Apollon's plasmid DNA (GENEVAX), both at the preclinical stage; Smith Kline Beecham Biologics are reportedly developing a therapeutic vaccine based on HPV16 sequence; Transgene (SA) are targeting late-stage cervical cancer with a genetically modified vaccinia virus which expresses HPV16 E6, E7, L1 and L2. Trials from a number of laboratories are proceeding in South America, Africa, and Asia.

Cantab Pharmaceuticals has recently completed enrollment for a phase II trial of TA-HPV (vaccinia expressing E6 and E7 genes of HPV16 and HPV18) in addition to that reported by Dr. Man and colleagues (chapter 6). Two Immunizations with TA-HPV are being given to women with stage Ib or IIa cervical cancer at seven centers in the United Kingdom and

continental Europe (Hickling J, personal communication). In addition, Cantab are targeting CIN with their TA-CIN vaccine, a recombinant fusion protein comprising HPV16, L2, E7 and E6 formulated with a novel liposomal adjuvant.

Dr. Michael Steller and colleagues are conducting a trial using a lipidated version of the HPV16E7 HLA-A*0201-restricted epitope 86-93. Anecdotal outcomes in individual patients are encouraging (Steller M, personal communication). A second generation trial using both E7 lipopeptide and autologous dendritic cells pulsed with E7 epitope 86-93 is planned.

StressGen Biotechnologies Corporation is seeking approval for the use of an adjuvant-free recombinant heat-shock fusion protein encoding *Mycobacterium bovis* BCG and HPV16 E7 (Hsp65E7), for the treatment of cervical dysplasia and cancer. This is based on data showing that a single subcutaneous injection of Hsp65E7 in saline protects mice against tumor challenge , and when used as a therapy induces regression of preexisting subcutaneous tumor. Protection is accompanied by interferon γ and E7-specific CTL.[48]

Conclusions

Data from PV models using VLPs as immunogens validate a prophylactic approach for preneoplastic disease and for cervical cancer, with the caveat that HPV-neutralizing antibody in humans remains to be demonstrated. The design of clinical trials is complicated by who to vaccinate and what the read-out for vaccine efficacy will be. A therapeutic approach to existing HPV infection should target cell-mediated immunity to early PV proteins in infected epithelium. Self-renewing suprabasal stem cells must be targeted, as well as distal differentiating keratinocytes. The persistence of E6 and E7 oncoproteins as tumor-specific antigens allows a vaccine approach directed to these proteins. Engineering subunit vaccines to E6 and E7 T-epitopes restricted through common HLA haplotypes, and combining epitopes into 'polytopes,' may prove efficacious. Strategies to preferentially induce or switch to a Th1-type responses and to direct the responses to mucosal epithelium need to be invoked.

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CHAPTER 2

Immunobiology of HPV Infection

Graham R. Leggatt and Ian H. Frazer

This chapter reviews current concepts of the immunobiology of papillomavirus infection of epithelial cells. An understanding of the viral life cycle, models of infection and the host immune response is an essential first step in the development of therapeutic or prophylactic vaccines. The literature suggests that strategies to elicit neutralizing anti-capsid protein antibodies will be useful in prophylactic vaccines for HPV, while generation of CTL specific for E6/E7 may be more useful for therapeutic vaccines. Targeting of the immune system to the infected epithelium and provision of an immunostimulatory environment will also be important.

HPV Infection

Papillomaviruses form a large group of double stranded DNA viruses with over 80 different genetic groupings.[1] Common to all genetic groupings is a strict tropism for squamous epithelium of the skin or mucous membranes, where viral particles enter via minor trauma to the epithelial surface. Different subtypes of HPV will vary in the ability to infect particular epithelial sites such that HPV6, 11, 16 and 18 are most commonly associated with infection of the anogenital tract, while HPV1 and 2 are associated with common skin warts[2] and HPV5 and 8 with skin carcinomas in patients with EV.[3] Since most HPV infection is subclinical or produces spontaneously regressing warts, the focus has been on genotypes associated with clinical disease, particularly of the anogenital tract. HPV6 and 11 are primarily associated with genital warts and have been termed "low risk" genotypes because they are rarely associated with invasive cancer. In contrast, HPV16 and 18 are termed "high risk" genotypes because of their association with cervical cancer.[4,5] Cervical cancer is the second most common cancer in women and recent epidemiological studies suggest that HPV DNA can be detected in 93% of cervical tumors.[6] Among these tumors isolated from patients worldwide, 50% contain HPV16 and 14% contain HPV18, with greater than 20 other HPV genotypes isolated from the remaining DNA-positive tumors. Combinations of DNA/RNA testing and serology have determined that 75% of the US population in 1994 had been infected at some time with HPV.[4] Prior exposure was determined by the presence of anti-HPV antibody and so this figure could be an underestimate, as individuals with reduced antibody would be missed. Consequently, the high prevalence of HPV infection and its association with cervical cancer represents a worldwide problem which may be amenable to vaccine strategies.

To understand the immunological processes associated with HPV infection, the biological mechanism by which papillomavirus infects cells and the range of expressed proteins must first be reviewed. The 8 kb HPV genome encodes for six early proteins (E1, E2, E4, E5, E6 and E7) which function during the intracellular replication cycle and two late proteins (L1 and L2) which encode the capsid proteins of the mature virion.[7,8] All genotypes of

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HPV share the same genetic organization, and coding information is located on one of the two strands. The genome can be divided into three regions:

1. Control region;
2. Early region;
3. Late region;

depending on the function or time of expression during replication.[9]

The control region or long control region (LCR) contains regulatory sequence elements and DNA binding sites for the E1 and E2 viral proteins.[10] It is from here that transcription is initiated and controlled due to the interaction of viral and host transcription factors. The coordinate regulation of epithelial cell differentiation and viral replication, as well as the basis for the epithelial specificity of HPV, is likely to be influenced by events occurring within the control region. In this regard, an epithelial differentiation factor, Epoc-1/skn-1a, associated with HPV transcription has recently been identified.[11] Viral transcription rather than replication appears to dictate the epithelial specificity and linkage to cell cycle since various undifferentiated cells can support HPV replication provided the E1 and E2 proteins are expressed.[12]

The early region encodes for six different HPV proteins (E1, E2, E4, E5, E6, E7) each of which contributes to viral replication. E1 is a nuclear phosphoprotein, having ATPase and helicase activity,[13,14] which binds to the origin of replication in the LCR,[15] either as a monomer or trimer,[16] where it associates with DNA polymerase alpha to help initiate transcription.[17] E2 is also a nuclear phosphoprotein which regulates viral transcription/replication and associates with the E1 monomeric protein at the viral origin of replication.[15,18] E2 binding to the LCR also causes physical displacement of the host transcription factors (SP1 and TFIID) from the E6/E7 promoter, resulting in repression of transcription from that promoter but at the same time enhancing E1-dependent initiation of DNA replication.[19,20] Consequently, E2 acts as a negative regulator of the transforming factors E6 and E7, as recently observed in the E6/E7 transformed HeLa cell line where transfection of E2 led to apoptotic death.[21] E4 is a nonstructural protein which is often considered to be a late protein because it is expressed in differentiated epithelium.[22] The functions of the E4 protein are largely unknown, although this protein does associate with keratin intermediate filaments[23,24] as well as forming cytoplasmic and nuclear inclusions[22] which probably interfere with normal epithelial differentiation, allowing the virus to complete its life cycle or, alternatively, disrupt cell integrity leading to the release of mature HPV virions. The E5 gene encodes a small, hydrophobic, membrane-associated protein which can regulate cell signaling pathways,[25] interfere with cell-cell communication[26] and associate with surface growth factor receptors.[27] HPV E5 is a weak transforming protein which is not usually expressed in genital cancers. It is, however, the major transforming protein in bovine papillomavirus infection and may play a role in the early stages of transformation in the human.[9] The extent to which these four intracellular proteins are available to the immune system is not well documented. Presumably, through their endogenous expression, each is available to the class I pathway and therefore could be recognized by CTL, but the transient nature of their expression may limit their usefulness as immune targets.

The E6 protein, in conjunction with E7, is a major transforming protein in HPV infection. The ability to transform cells has been linked to its binding to the tumor suppressor protein p53, in conjunction with a third host protein, E6 associated protein or E6AP, and subsequent targeting of p53 to the ubiquitin-dependent degradation pathway.[28,29] p53 protein suppresses cell growth in response to DNA damage by contributing to the G1 arrest of the cell cycle, allowing DNA repair or cell death by apoptosis. Degradation of p53 results in genetic instability and accumulation of mutations which contribute to the transformation process. "Low risk" HPV types which do not transform epithelial cells have E6 proteins with lower

binding affinity for the p53 protein.[30] One report suggests that only "high risk" HPV E6 is capable of binding to a second site on the p53 protein which is involved in its degradation.[31] From an immunological standpoint, it will be interesting to determine whether p53 protein, through its degradation, is overexpressed in the class I pathway and therefore a potential target for low avidity CTL specific for self protein.

The E7 transforming protein also binds to an anti-oncogenic cell factor called retinoblastoma protein (pRb).[32,33] As for E6, the E7 proteins of low risk HPV types have a ten-fold lower binding efficiency to pRb than those of the high risk types, and this difference in affinity appears to be controlled by a single amino acid residue within E7.[34] Continued E6 and E7 expression is essential to the maintenance of the transformed state of the epithelial cell and consequently these proteins provide attractive targets for anti-tumor therapy.[35] Transfection of E6/E7 can sometimes lead to immortalization of the cell,[36,37] but in other cases it appears not to be sufficient.[38]

The late region contains the genes encoding the two capsid proteins L1 and L2. L1 is the major capsid protein and can self assemble into virus-like particles (VLPs) which resemble the mature virion,[39,40] while the L2 protein anchors the HPV genome to the viral capsid.[41] Both structural proteins, being surface exposed, are potential targets for antibody binding and consequently their role as diagnostic tools and targets for immune based therapy will be discussed.

Life Cycle

Viral particles enter the skin, infect keratinocytes via cell surface receptors, one of which has recently been identified as the α_6 integrin molecule,[42] and undergo limited replication in the suprabasal layers of the epithelium where the early proteins such as E1, E2, E4 and E5 each play a role in viral replication. E4 and E5 may play an early role in directing the viral particle from the cell surface to the nucleus, since E5 was shown to inhibit the acidification of lysosomes[43] while E4 was able to aggregate cytokeratins or form filament-like structures. Within benign HPV skin lesions, the HPV genome replicates autonomously from the host chromosome, while in malignant lesions integration into the host chromosome is observed.[44] Recent evidence suggests that the viral genome must remain extrachromosomal for expression of the late viral genes to occur.[45] In the host cell nucleus of a benign lesion, the E1 protein initiates viral DNA replication by binding the DNA polymerase **a** while E2 acts as a transacting transcriptional control. Low level E6 and E7 expression may assist viral replication in terminally differentiated keratinocytes where DNA replication is normally switched off. The viral life cycle is then tightly linked to the proliferation and differentiation of the keratinocyte as it moves distally toward the cell surface.[8] Production of the capsid proteins L1 and L2, and thus mature virions, is coordinated with the appearance of differentiated keratinocytes. Viral particles are shed from the surface without cell rupture, and with little evidence for viral shedding within the epithelium.

Occasionally, oncogenic HPV DNA will integrate into the host cell genome where the oncogenic proteins E6 and E7 will be aberrantly activated to transform the cell, due to disruption of the normal E2 transcriptional regulation of these genes. In accordance with the disruption of E2 function, breaks in the viral DNA, leading to integration into the host genome, are most commonly seen in the E1/E2 coding region of transformed cells.[46] Inactivation of the E2 gene then permits upregulated expression of the E6 and E7 transforming genes which, in conjunction with cofactors, leads to cell transformation. Viral integration sites on the host genome vary widely, suggesting that the site of integration is not a chief contributor to malignancy.[47] Integrated viral DNA may also produce a latent infection state with neither viral replication or transformation.

Although our knowledge of the life cycle of HPV has dramatically increased, several problems still remain:

1. The HPV life cycle is difficult to maintain in vitro;
2. No current rodent model exists for HPV infection.

Raft cultures of keratinocytes are beginning to provide conditions for the in vitro examination of the HPV life cycle, as are transfected keratinocytes. In vivo, the cottontail rabbit papillomavirus provided one of the earliest models for HPV infection, followed closely by the bovine PV model. Understandably, maintenance of the PV life cycle for experiments in these animals is expensive, and rodent models would be more convenient for future vaccine studies. Although reviewed in later chapters in more detail, a brief examination of each of these models provides further clues to the immunobiology of papillomavirus and suggests vaccine strategies.

Models of HPV Immunobiology

Cottontail Rabbit

The (Shope) cottontail rabbit papillomavirus (CRPV) was the first model for the study of viral carcinogenesis in mammals,[48] leading to important observations such as the role of E6 and E7 in oncogenesis.[49] CRPV results in skin warts which spontaneously regress or develop into carcinomas, either benign or invasive. Skin warts are commonly observed on cottontail rabbits trapped in the USA, and inoculation of wart extracts onto scarified skin results in transmission of the disease.[50,51]

During both natural and experimental infections, neutralizing antibodies targeted at conformational epitopes on the L1 and L2 proteins have been detected in serum.[52,53] Neutralizing antibody probably targets the initial infection phase, as antibody directed against L1 can protect against papilloma formation induced by virus but not by viral DNA.[52] In addition, rabbits are resistant to reinfection, but no major difference was seen in antibody responses to capsid proteins during regressing or persisting papillomas. Interestingly, anti-capsid antibody dramatically increases during progression to carcinoma, although the expression level of L1 and L2 is not increased, suggesting that immune recognition of these proteins is more efficient during carcinogenesis.[54] L1 protein in the form of virus-like particles provides type-specific protection against live viral challenge, and the protection can be transferred to a naive animal by serum IgG antibody.[55] Antibody responses to E1, E2, E6 and E7 have been detected in rabbits with papillomas and carcinomas, albeit infrequently.[56]

In vitro proliferation of lymphocytes to L1 was increased in frequency and strength in rabbits with carcinomas and was in agreement with antibody responses.[57] Proliferative responses to E2 were seen in about 45% of rabbits with regressing skin warts, while T cell responses against the other nonstructural proteins were infrequent.[58] Regressing warts were associated with infiltrating macrophages and T cells, expression of class II by the keratinocyte and production of TNF-α transcripts.[59-61] The role of cytotoxic T cells in CRPV infection is largely unknown, although one recent report suggests that $CD8^+$ T cell infiltrates are associated with active regression of rabbit papillomas.[62]

L1 and L2, whether expressed as bacterial fusion proteins, in vaccinia virus vectors, as virus-like particles, in baculovirus or yeast expression systems or as a polynucleotide vaccine (DNA Immunization), are protective against CRPV infection when given as a prophylactic vaccine.[52,55,63-65] VLP-based vaccines elicit long term protection.[66] In contrast, prophylactic vaccines based on nonstructural proteins (E1, E2, E6 and E7) have been ineffective in protecting rabbits against warts. One study, based on E1 and E2, did show accelerated regression of papillomas and increased numbers of regressor rabbits.[67] A $\gamma\delta$ T

cell line cytotoxic for Shope carcinoma cells has also been isolated from the peripheral blood of rabbits with CRPV papillomas, but the antigen specificity has not yet been determined.[68]

Taken together, data from this model suggest that structural protein may provide the best target for prophylactic vaccines.

The development and control of wart growth may be related to certain epitopes of the virus and their association with particular MHC molecules. Although the genetics of the MHC locus in rabbits is not as defined as for mice, one study has determined a linkage between particular MHC class II gene expression and papilloma development.[69] Further development of inbred rabbit strains and characterization of the rabbit MHC complex will be required to examine this issue in more detail.

Tissue Xenografts

The transplantation of human graft tissue into immunocompromized mice allows the growth of human tumors in an environment more closely resembling its natural surroundings. Most success with xenografting has come from transplant of human cervical epithelium or foreskin (infected with various HPV) under the renal capsule of severe combined immunodeficient (SCID) mice.

The xenograft model has been important in several ways:

1. Examination of viral replication in human tissue;[70-72]
2. Production of viral stocks of high titer;[73]
3. Detection of neutralizing antibody in serum;[74,75]
4. Determining neutralizing antibody epitopes using monoclonals;[76,77]
5. Testing of antiviral or antineoplastic drugs;[78]
6. Analysis of the interaction of HPV genotypes in a single tissue.[79]

The chief advantage of this model is the use of human tissues infected with relevant human papillomaviruses. To date, it has not been useful for vaccine studies because of the difficulties of reconstituting the human immune system in a mouse. However, even this limitation may soon be overcome, given the advances in SCID-hu mice development in relation to HIV infection.[80]

Bovine Papillomavirus

Papillomavirus infection of cattle has provided a further important model for examining the natural host/pathogen interaction. Six different types of bovine papillomavirus (BPV) infecting either dermal fibroblasts and keratinocytes (BPV1, 2, 5) or keratinocytes (BPV3, 6) exclusively or the mucosal epithelium of the upper alimentary canal (BPV4) have been well studied.[81] Progression to cancer is common after infection with BPV4, 2 and 1, and BPV6 infection of the udder can lead to problems in milk production.[82,83] There are many similarities with HPV, including genomic organization and function of the gene products, disease progression to carcinoma and the existence of multiple genotypes. A key difference is that BPV commonly causes fibropapillomas involving infection of fibroblasts, while HPV is strictly an epithelial infection.[81] Given that cattle are an outbred population and a natural host of PV, these animals can be used to test vaccines and study immunological correlates of protection.

BPV2 and 4 have been the target of many immunological studies, since they represent cutaneous and mucosal PV models. Several different vaccine preparations using whole virus or L1 and L2 proteins have been tested. Vaccination with whole virus leads to protection from challenge infection and to the presence of neutralizing antibodies.[84] Importantly, immunity is BPV type-specific, as has been seen for both HPV in humans and CRPV in rabbits.[85] Because of difficulties in growing virus, recombinant capsid proteins have also been utilized as vaccines. The L1 protein of BPV2 produced neutralizing antibody and

was protective in calves, while the L2 protein of BPV4 was also found to be protective.[86,87] Prophylactic Immunization of calves with VLPs containing either BPV4 L1 or L1 and L2 produced protective immunity against viral challenge.[88] Further studies on BPV4 L2 indicated that neutralizing epitopes were located in the N-terminal 200 amino acids and that this peptide was sufficient to provide protection.[89] Three dominant B cell epitopes have been mapped in the N-terminus and one of these is highly conserved among mucosal BPV.[90] Additional studies demonstrated that a combination of the three B epitopes, given as a mixture of peptides, gave better protection than any single epitope, although one peptide (aa 131-151) prevented disease progression to papilloma when given in isolation.[91] Interestingly, protection provided by anti-L2 antibodies may not prevent viral entry into the cell, but instead operate at a distal event in viral replication.[92] Consequently, protective, prophylactic immunity is conferred by either L1 or L2 alone in this model, though it remains to be clarified whether protection is mediated by the same or differing mechanisms.

The bovine PV model has also provided hope that therapeutic vaccination may be viable. Both BPV2 virus and the L2 protein were able to cause accelerated regression of established BPV2 warts associated with induction of cell mediated immunity.[81] In BPV4 infection, the major therapeutic antigen was E7, where both antibodies and E7-specific T cells could be detected.[93,94] There was some suggestion that BPV4 VLPs also affect the replication of papillomavirus.

Activated lymphocytes were found to accumulate in the dermis below regressing BPV4 papillomas, with the predominant phenotype being CD4 cells followed by γδ T cells and CD8 T cells.[95] Amongst the keratinocytes and basal layer, the γδ T cells and CD8 lymphocytes were found to predominate. This suggests that the latter cells are involved in the clearance of the BPV papilloma. BPV vaccines were effective in different breeds, suggesting that MHC restriction will not have an effect on multicomponent vaccines.

Canine Oral Papillomavirus (COPV)

Infection of beagle dogs with canine oral PV has provided a useful model for mucosal infection by HPV. Other animal infection models are not suited to the study of mucosal infection, since both CRPV and BPV1 and 2 produce cutaneous rather than mucosal lesions, while BPV4 produces mucosal lesions at an internal site which is more difficult to monitor than the mouth. Vaccine studies in the COPV system have indicated that formalin-inactivated, systemically administered COPV can protect beagles from a challenge infection.[96] Furthermore, COPV L1 protein alone was protective, and serum immunoglobulins from immunized animals were able to transfer this protection to naive dogs challenged with virus.[97] This data suggests that systemic administration of vaccine can give rise to local mucosal immunity, although it remains to be seen whether the intradermal injections used in these studies stimulate the mucosal immune system in addition to the systemic effect. This model does emphasize the importance of generating anti-capsid antibodies in prophylactic vaccines.

Epithelial Raft Cultures

The ability to manipulate and examine the events involved in HPV replication in a controlled environment in vitro is an attractive option. Since viral replication is tightly linked to keratinocyte differentiation, the differentiation of these epithelial cells must be replicated in vitro in order to study the viral life cycle. "Raft" cultures provide a variation on conventional keratinocyte cultures whereby the epithelial layer stratifies and differentiates.[98] Epithelial tissue from a variety of different anatomical sites will retain the general characteristics of in vivo epithelium in raft cultures.[98,99] HPV DNA sequences are introduced to the raft cultures via lipofection or retroviral vectors, and the function of many HPV control sequences can

be determined by mutational analysis.[100,101] The raft culture also permits an analysis of the molecular events involved in epithelial differentiation, as well as allowing antiviral compounds and gene therapies to be tested.[98,102] In the future, an analysis of the migration of lymphoid cells into a HPV-infected raft epithelium may shed light on adhesion molecules and chemoattractants required for lymphocytic infiltration into infected tissue.

Transgenic Mouse Models

Mice transgenic for the early region genes of HPV16/18 have been developed as a means to study the transformation process in epithelial cells or to provide constitutive HPV protein expression for the study of immune responses. Multistage progression to carcinoma has been consistently observed in the epithelium of mice transgenic for HPV proteins under the control of the K14 promoter, and these experiments have established an important role for E6/E7 in the cell transformation process.[103,104] Further studies will be required to understand the mechanisms underlying carcinogenesis in these models. Immunologically, E7 transgenic mice have reinforced the concept that E7 expression in the keratinocyte is largely ignored. One study, using the αA crystallin promoter to direct E6/E7 expression, showed that skin grafts from these animals were unable to be rejected from naive or E7-immunized, syngeneic, recipient mice.[105] In addition, the transgenic mice themselves appeared "ignorant" of the transgene early in life, since immune responses to E7 Immunization were no different from littermate controls, although with age, spontaneous immune responses to E7 did eventually appear.[106,107] Immunological "ignorance" of E7 expressed in keratinized epithelia (K14 promoter) was also seen in another transgenic line.[108a] In contrast, our laboratory has observed split tolerance in K14-E7 transgenic mice such that $CD4^+$ T cell and B cell responses to E7 remain intact while CTL activity is reduced or nonexistent.[108b] This suggests that tolerance in the CTL compartment exists via E7 recognition in the thymus or peripheral keratinocytes.[109] Although the combined data suggests discrepancies in the ability of the immune system to respond to E7 protein expressed in keratinocytes, the generation of E7 transgenic mice and natural infection with HPV will involve varied levels of protein expression. Consequently, a comparison of protein expression in these situations may suggest dosage thresholds for activation of different immune compartments. It may also be necessary to provide an inflammatory environment in order to achieve immune stimulation.

Immune Response to HPV

As a target for an effective immune response, the regular infection cycle allows few points for immune intervention, since productively infected epithelial cells are removed by normal desquamation of the skin cells without spread of the virus. In contrast, keratinocytes that are transformed by the HPV oncogenes present an invasive tumor target expressing well defined tumor antigens. In this regard, E6 and E7, which are essential to the transformed state of the cell, represent major target antigens for immune intervention.

Cross-priming of the immune system may explain the presence of cell-mediated and humoral immunity during natural infection, where the viral proteins are largely confined within nonprofessional APC (keratinocytes) and excluded from the draining lymph nodes.[110] In this scenario, HPV proteins or protein fragments "leaked" from the keratinocyte would be taken to the draining lymph by scavenging, professional APC (e.g., Langerhans cells) for priming of the naive immune response. In HPV infection some level of immune control is seen, since patients who become immunocompromized have warts or tumors that rapidly progress.[111] Conversely, regressing warts have infiltrates of lymphocytes and macrophages.[112] A role for the immune system in control of HPV is also suggested by the various HLA associations with disease.[113] Consequently, the relative roles of humoral and cellular

immunity to HPV clearance, as well as the innate immune system, must be considered in searching for vaccines. Vaccines have the potential to present any epitope in an immunogenic form to the immune system, thus circumventing any possible defects in antigen presentation for immune priming during the natural infection. More important is to identify relevant epitopes and events which modulate the activity of the effector response by reference to the viral life cycle and cellular transformation. It is possible that the natural immune response controls the outgrowth of occasionally transformed keratinocytes and that other immune factors which deviate or inactivate the immune response play a major part in the progression from viral infection to stably transformed cell.

Humoral Responses

As evidenced by the biology of HPV and its replication cycle, there is limited contact of viral capsid proteins with the immune system, since no viremic stage exists in the epidermal layer. Despite this, human patients with recurrent or persistent infection do produce antibody to the major capsid protein, suggesting that although sufficient antigen exists to stimulate antibody production there appears to be no protective effect.[114] This suggests that therapeutic vaccines based on anti-capsid antibodies may not be viable, but does not rule out the usefulness of prophylactic vaccines in which preformed antibody may prevent the initial infection. As previously described, animal models have suggested that pre-immunization can protect against subsequent challenge, with several studies showing that the L1 capsid protein is a strong immunogen.[115] Perhaps consistent with the limited exposure of the immune system to HPV is the very long period to seroconversion based on antibody to capsid proteins. In one study, the median time to seroconversion following HPV16 infection was 8.3 months, with most patients seroconverting.[116] If there is a high seroconversion rate following HPV infection, antibodies against capsid proteins may be a good diagnostic marker of past HPV infection, but the long time to seroconversion argues against use of antibody to screen for current infection.

Prevalence of anti-capsid antibody is variable among patients with invasive cervical cancer.[117,118] Given the loss of capsid expression occurring when HPV integrates into the host chromosome during transformation, the presence of antibodies directed against capsid proteins may reflect persistence of existing antibodies rather than an ongoing immune response.

During viral integration and the formation of malignancy, it is apparent that there is an upregulation of E6 and E7 protein expression allowing these molecules to become a target for antibody responses. Antibody against the E6 and E7 proteins has been associated with the progression to cervical cancer, with more seroconverters appearing towards the later stages of disease.[119] It is not clear whether the formation of antibodies to E6 and E7 is simply a clinical marker of disease progress. While it is difficult to imagine that antibody against two nonmembrane-associated, intracellular proteins could be directly protective given the cellular barrier, perhaps formation of antibody-antigen complexes and uptake by professional APC helps prime the cellular arm of the immune system.

Several studies have also indicated the presence of serum and tissue IgA antibodies to other early region genes, especially E2, in patients with various stages of cancer.[120]

Given that animal models suggest that antibody responses would be desirable in a prophylactic vaccine, it is important to determine the degree of crossreactivity between HPV genotypes or within the genotype (intratypic variation). Anti-capsid antibody responses, in general, appear to be type-specific, as evidenced by the fact that women are often infected with multiple HPV genotypes.[121] However, within a particular genotype, sequence variants induced largely crossreactive antibodies, as evidenced by the dominance of a particular HPV sequence during infection.[122] In one study, two diverse L1 capsid

proteins, representing intratypic variants and varying in seven amino acids, gave the same serological result when tested in immunoassay against 220 patient sera.[123] While type-specific antibodies may dominate, it has been possible to isolate monoclonal antibodies against capsid epitopes which are crossreactive between HPV6 and 11.[124] Consequently, incorporation of capsid antibody epitopes in vaccines will likely be type-specific and may require addition of several epitopes for cross-protection.

An important consideration for vaccine development targeted at antibody responses is the elicitation of neutralizing antibody and mucosal immune responses. Human neutralizing antibody has been difficult to assess, since few infection models exist in vitro or in vivo. Two promising approaches utilized a mouse xenograft system and pseudovirions, but attention in the future should be paid to improving methods for detecting neutralizing antibody.[125,126] Most work on HPV serology has focused on IgG responses, with very little attention on subclasses or mucosally important isotypes such as IgA. Some reports do indicate the presence of IgA antibodies specific for HPV antigens during the course of natural infection.[127] Vector systems which target the elicitation of neutralizing, mucosal antibody responses would be ideal for genital HPV infections. Encouraging results have recently been provided for HPV proteins expressed in *Salmonella* vectors.[128] Once it has been demonstrated that an antibody response can be generated, further studies on how to maintain desirable antibody responses will be required.

Cellular Responses

Nonlytic viral infections are usually targets of the cellular immune response. The immune system has nonspecific defense mechanisms which comprise innate immunity and highly evolved, antigen-specific components which form the adaptive immune response.

The innate immune response involving components such as acute phase proteins, complement, polymorphonuclear leukocytes and macrophages contributes to local inflammation and thereby recruits the adaptive immune response by the release of soluble attractants such as chemokines.[129,130] These proteins are activated by local cellular damage and the protein products of foreign pathogens. In the case of HPV, epithelial cells/keratinocytes, in the noninflamed state, are nonprofessional antigen-presenting cells[131] and so antigens must be delivered to the local lymph nodes by local dendritic cells (Langerhans cells) in order to initiate the immune response. Presumably, the Langerhans cells take up HPV antigens present in cell debris as actively secreted proteins and through the process of cross-priming.[110] Once the specific immune response is invoked, the innate system plays a role in maintaining and promoting the adaptive response at the local site, as well as removing debris from regressing disease.

Natural killer cells and macrophages are frequently observed in immune infiltrates during regression of HPV-associated warts.[132,133] Conversely, natural killer cell activity against infected keratinocytes was significantly impaired in patients with persistent anogenital lesions associated with HPV16 infection.[134] NK cells are activated for effector function by the absence of surface MHC class I molecules and therefore may play an important role in HPV-associated carcinomas where MHC expression is downregulated or lost completely.[135]

Cytokines produced by the keratinocytes, macrophages, NK cells and T cells will aid in promoting and maintaining the immune response, in addition to a direct effect on HPV replication and protein expression. Cytokines such as TNF-α are constitutively secreted by keratinocytes, while other factors such as IL-1, TGF-β and interferons have all been found to downregulate the expression of HPV in combination with their involvement in the recruitment and maintenance of the specific immune response.[136-139] The proinflammatory cytokine IL-6 was able to promote the growth of HPV-harboring carcinoma cell lines via an indirect pathway involving the epidermal growth factor receptor, while also stimulating the

NK-mediated lysis of HPV-associated neoplastic cells.[140,141] For therapeutic use, it will be important to dissociate immune recruitment from tumor promotion for each cytokine. Interferons (α, β and γ) are potent antiviral molecules and have been the basis of several clinical trials for HPV infection.[142] The success of IFN treatment has been difficult to ascertain, given the variety of delivery routes, doses and disease states in the trials. Placebo-controlled studies using intralesional interferon showed that approximately 60% of treated lesions remit compared with 20% in controls, while in other studies no difference was observed.[115] Similar studies with systemic interferon indicated that while remission may be accelerated, there was no real difference in overall cure rate.

The antigen-specific immune response is a very important arm of the immune response, since it contains receptors which enable specific recognition of a wide variety of pathogenic structures, allows maturation of the ongoing immune response and provides memory which enables a quicker response to the pathogen during a second exposure. Antigen-specific immune responses consist of both B cell and T cell responses. While B cell or antibody responses have been previously discussed, T cells can be divided into two compartments, based on the expression of either the CD4 or CD8 protein.

In general, CD4 T cells recognize foreign peptides bound to MHC class II molecules and constitute the helper T cell response, since they provide contact and soluble factors which promote the growth and development of B cells and other T cells. CD4 T cells appear to be a control point in the immune response, being required for most antibody responses and probably necessary in the development of the cytotoxic T cell response. CD4 T cells have been found in regressing lesions associated with HPV.[95,143] Helper T cells can be further divided into two subsets based on their secretion of "helper" factors or cytokines. Th1 cells produce large amounts of IFN-γ and IL-2 and are generally associated with an inflammatory response, while Th2 cells produce IL-4 and IL-10, which are associated with the development of antibody responses. Although not an absolute dichotomy, clearance of pathogens can often be associated with either a Th1 or Th2 response. One recent report examining immunoglobulin subclasses directed to E7 and associated with viral clearance suggested that a Th1 response may predominate during clearance of HPV.[144] Another study analyzing cytokine secretion from PBMC of patients with CIN revealed that a switch from Th1 to Th 2 was associated with more extensive HPV infection.[145] Subsequent studies will be needed to confirm these findings and determine the cytokine secretion patterns of isolated CD4 T cells. It is interesting that several studies have now showed that decreases in helper T cell responses to HPV proteins are associated with progression to cervical cancer.[146-148] The basis for this correlation has yet to be fully determined, but may reflect general immunosuppression or Th1 to Th2 shifts. CD4 T cell epitopes from the E6, E7, E2, E4 and L1 proteins have been mapped in both humans and mice.[149-154]

Cytotoxic T cells ($CD8^+$ T cells) are being increasingly recognized as a key factor in the control of intracellular viral infections. The class I antigen-presenting pathway specializes in sampling the internal environment of the cell, with subsequent display of peptide/MHC class I complexes at the cell surface and targeting by CTL. The majority of cell types, including keratinocytes, express class I constitutively, although it can be further upregulated by cytokines such as IFN-γ. CTL have been implicated in the regression of PV both in animal models and humans as demonstrated by immunohistochemistry.[62,112] Several studies in mice have demonstrated rejection of E6/E7-expressing tumor cell lines by CTL.[155,156] Human studies of CTL have been complicated by the technical inability to consistently grow CTL in vitro, although some studies have successfully isolated CTL from the peripheral blood or tumor site.[157,158] Human vaccine trials using E7-expressing vaccinia vectors have demonstrated that CTL can be induced in HPV patients.[159] In fact several vaccine studies are targeting the intracellular delivery of peptides and proteins from HPV as

a means to elicit CTL, and several CTL epitopes have now been mapped in both humans and mice.[160-162] Presumably, an effective disease vaccine will optimally stimulate helper T cells and CTL, but it remains to be seen whether this type of vaccine can completely clear HPV or whether latent virus will remain. We must also be aware that HPV has also developed mechanisms to escape the cellular immune system, including the downregulation or deletion of components of the class I pathway such as TAP and HLA.[135,163] Understanding the deficits in the class I presentation pathway in cervical tumors will be critical if immunotherapy based on CTL is to be effective. Alternatively, protective CTL will have to be generated at an early stage in tumor development before such escape mechanisms are employed.

Future Directions

Although much progress has been made in the understanding of the immunobiology of HPV infection and associated carcinomas, much is still to be learned. Lessons from the animal models suggest that prophylactic vaccines based on the capsid proteins L1 and L2 will be the most effective. It remains to be seen whether neutralizing antibody or cell-mediated immunity against the capsid proteins are involved in viral clearance. Presumably, this immunity operates at an early stage in infection either before the virus enters the cell or shortly after. For the anogenital HPV, it will clearly be important to elicit local mucosal immunity and this requires further examination. For established carcinomas, the preferred target for immunotherapy would be CTL directed against E6 or E7, since these proteins are absolutely required for continued transformation of the cell. Given that CTL can be important in tumor clearance, it will be critical to more thoroughly define the characteristics of "protective" CTL, including their specificity and avidity, expression of adhesion/skin homing molecules, optimal cytokine microenvironment and long term maintenance. The mechanism underlying downregulation or deletion of components of the class I presentation pathway requires further attention if CTL-based therapies are to be successful. Continued development of models for HPV infection, particularly in the well defined genetic background of inbred mice, will be necessary to test vaccine preparations and further dissect virus-host interactions. Papillomavirus also provides an opportunity to study the recruitment of antigen-specific lymphocytes to the skin, a field which is still poorly understood but clearly important, given that most pathogens enter via an epithelial site.

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CHAPTER 3

HPV Vaccines for Protection Against Infection

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The purpose of this chapter is to describe the rationale and scientific evidence for the development of prophylactic human papillomavirus (HPV) vaccines based on the use of recombinant HPV virus-like particles.

HPV-Associated Diseases

Human papillomaviruses (HPVs) infect cutaneous, genital and respiratory epithelia in a tissue-specific manner. Infection with HPVs is widespread throughout the world, and viral infection is closely associated with both benign and malignant lesions. There are now over 80 HPV types described; from a public health standpoint, however, only a small number of these different HPV types cause the majority of clinically important diseases. HPV16 and 18 are strongly associated with high-grade anogenital lesions and invasive cancers[1] and are found in ~70% of all cervical carcinomas[2] which, together with the less prevalent oncogenic types like HPV31, 33, 45, 52, and 58, contribute to an overall prevalence of HPV in cervical carcinoma of >90%.[2]

The incidence for cervical cancer in the US is ~14,000 cases/year, and ~5,000 women will die from the disease. In developing countries where access to routine cervical cytological screening is nonexistent or difficult, cervical cancer is the most common malignancy, with an estimate of ~500,000 new cases and ~200,000 associated deaths annually.[3] Two other HPV types, HPV6 and 11, while only rarely associated with malignancies and only rarely life-threatening with the exception of certain laryngeal papillomatosis cases which they cause, are responsible for >90% of condyloma acuminata, benign lesions of the respiratory and genital mucosa.[4] It is estimated that in the US alone one percent of all men and women between ages 15 to 49 present to physicians with condyloma acuminatum.[3] The overall percentage of HPV infection (either current or previously encountered) in a population could be as high as 75%.

While the number of HPV-related diseases appears to be increasing, there is, unfortunately, no effective treatment available. Treatment for most clinically apparent HPV-related lesions is limited to physical or chemical destruction of the visible lesions, which is uncomfortable and inefficient, with a high relapse rate. Therefore, a prophylactic vaccine which could prevent HPV infection in the first place, or at least reduce viral loads to levels where disease cannot manifest itself, would be highly desirable.

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HPV Proteins as Targets for Prophylactic Vaccination

HPV L Proteins

The L proteins, L1 and L2 , are the viral structural proteins and constitute the viral capsid. Sixty hexavalent and twelve pentavalent capsomeres composed of the L1 protein (5 L1/capsomere) form an icosahedral structure into which the L2 protein is also incorporated.[5] Vaccine development has been slowed by the inability to grow HPV in tissue culture or to infect species other than humans. Until recently, only HPV11 had been successfully propagated in the athymic mouse xenograft system developed by Kreider and colleagues.[6,7] With one isolate of HPV11, Kreider and colleagues were able to infect human foreskin tissue pieces in vitro, which they transplanted under the renal capsule of nude mice. After several weeks of growth, the tissue implants were harvested and new, infectious HPV11 virus stock was prepared to initiate another cycle of virus infection, replication and virus-related pathology. Even though this model is time consuming and labor intensive, it provided the first convincing clue that HPV11 infection is dependent on the viral capsid proteins and therefore makes the capsid proteins excellent targets for a vaccine approach geared to prevent infection.[8]

Expression of the L1 capsid protein alone or with the minor capsid protein L2 in recombinant systems has been demonstrated to result in the formation of virus-like particles (VLPs) which resemble native virions.[9-14] Immunization with these VLPs elicits virus-neutralizing antibodies which recognize conformational epitopes on the surface of native virions or VLPs.[13,15,16] While there is no doubt that the L1 capsid protein plays the predominant role in eliciting a virus-neutralizing antibody response, there is some evidence that the minor capsid protein L2 may also be partially involved in virus neutralization.[17,18] However, the ability of L2 to induce virus-neutralizing antibodies may be dependent on the virus type.[19] The neutralizing antibody responses elicited against the L1 VLPs has been shown to be predominantly type specific,[16,20] which still presents a challenge for any vaccine approach with the goal of covering the majority of clinically important HPV types.

Proof of the ability of VLPs to induce a neutralizing antibody response as well as to protect from viral challenge and disease has come from the study of three animal papillomaviruses: the cottontail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV) and canine oral papillomavirus (COPV). To date, it has been demonstrated in all three systems that systemic vaccination with recombinant VLPs consisting of L1 alone or L1 and L2 does prevent disease after experimental virus challenge.[21-24] Furthermore, we could show in the CRPV model that Immunization with CRPV VLPs was able to prevent infection with CRPV by in situ hybridization of thin sections obtained from biopsies of skin areas challenged with CRPV (Jansen K, unpublished observation).

While all the animal models have convincingly demonstrated that VLP Immunization protects from disease, none of the above described models is adequate to address the question of whether systemic Immunization is sufficient to elicit a protective antibody response in the genital mucosa. Such a response may be necessary to protect the cervical epithelium from infection with genital HPV types and subsequent disease. We have recently conducted a study where non-human primates were systemically vaccinated with highly purified HPV11 VLPs expressed in the yeast *Saccharomyces cerevisiae*. The study showed that high titer HPV11 VLP-specific serum antibodies were elicited not only in serum, but also in cervicovaginal secretions of these animals. Using the mouse xenograft system, we further demonstrated that high levels of neutralizing antibodies were present in the serum of immunized monkeys and significant neutralizing antibodies were also present in cervicovaginal secretions.[25] The antibody response persisted in serum and cervicovaginal secretions for more than six months after the booster Immunization. Since the antibody

response in cervicovaginal secretions followed closely the antibody response seen in serum and was predominantly of the IgG isotype (no VLP-specific IgA or sIgA was detectable), protection from HPV infection may be possible in the absence of mucosal immunity, defined by an antigen-specific sIgA response. Parenteral Immunization against genital HPVs could actually be an advantage, because it might overcome several potential obstacles associated with generating a local immune response in the genital tract. First, protein antigens are generally not very immunogenic when administered locally, necessitating frequent booster Immunizations which could potentially induce tolerance. In addition, local responses against protein antigens have been generally short lived and do not effectively induce local immune memory.[26]

HPV L-E Protein Chimeras

Despite the promising results obtained with animal models described above, it is not clear whether it may be possible to sustain a protective antibody response on a mucosal surface long enough to protect people for many years. Also, there is evidence that most vaccines currently used to protect against a plethora of diseases may actually not confer sterilizing immunity, i.e., completely prevent infection with the infectious agent. Until proof of the effectiveness of an HPV vaccine strategy based solely on L1 or L1 + L2-containing VLPs has been demonstrated in people, it seems prudent to also evaluate related back-up approaches. I will focus on subunit vaccine approaches based on VLPs, since different approaches are topics of other chapters in this book. Assuming that HPV can evade the neutralizing antibody responses and establish an infection, one would wish to also be able to induce immunity targeting the intracellular HPV. It seems clear that VLPs composed of the late proteins alone are not the likely candidates to induce a cellular immune response capable of eradicating HPV-infected cells, since these proteins are only expressed in terminally differentiated cells of productive lesions.[5] Also, late proteins are rarely detected in high grade cervical intraepithelial lesions. However, there is accumulating evidence that VLPs by themselves, without the addition of adjuvants, are capable of inducing CTL responses in mice,[27] suggesting that they could be used as a convenient vehicle to deliver other viral proteins expressed early in the life cycle of the virus. E proteins, on the other hand, are necessary to establish and to assure the maintenance of the viral infection, since they are expressed very early in the life cycle of HPV and are therefore good targets to induce cellular immunity. There has been evidence from animal models as well as tumor challenge models using tumor cells expressing E proteins that Immunization with E proteins E1, E2, E6 or E7 can induce cellular immunity which plays a significant role in early and faster regression of lesions and eradication of virally infected cells.[28-30] The feasibility of producing chimeras of VLPs with E proteins has been recently demonstrated.[31] Parts of the HPV16 E7 protein were fused to the C-terminal portion of the HPV16 L1 protein without abolishing its ability to form VLPs. The chimeric VLPs were able to induce a VLP-specific and neutralizing antibody response and were able to bind to and enter target cells in vitro. After uptake of the chimeric VLPs, chimeric proteins were detectable in the Golgi system, suggesting that they may be processed via the MHC class I pathway. Immunization with these chimeric VLPs was further able to induce strong HPV16 E7-specific CTL responses in immunized mice which protected the mice from challenge with tumor cells expressing the HPV16 E7 protein (Gissmann et al, personal communication). The HPV16 E7-specific CTL response was potent enough to prevent the outgrowth of E7-expressing tumors when the animals were immunized after tumor challenge. While the chimeric VLP approach seems promising not only to protect from viral infection but also to eradicate any breakthrough infection, the concept has to be proven in non-human primates and ultimately humans.

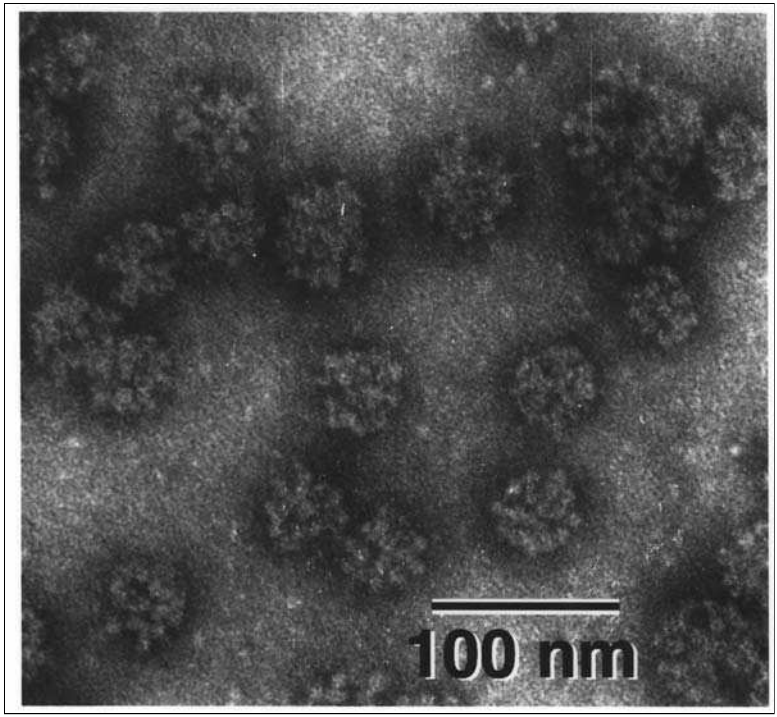

Fig. 3.1. Visualization of HPV16 L1 VLPs expressed in yeast. Purified HPV16 L1 VLPs expressed in yeast were placed on a 200-mesh carbon-coated copper grid. Two percent phosphotungstic acid (PTA), pH 7.0 was added to the grid for 20 sec. The grid was allowed to air dry prior to transmission EM examination. All microscopy was done using a JEOL 100CX transmission electron microscope (JEOL USA, Inc.) at an accelerating voltage of 100 kV. The micrographs generated have a final magnification of x100,000 (Bar = 100 nm).

VLP Expression Systems

The successful expression of HPV VLPs has been demonstrated in a variety of expression systems. VLPs have been expressed in mammalian and insect cells using viral expression vectors, yeast (*Saccharomyces* and *Schizosaccharomyces*) and bacteria like *E. coli* and attenuated *Salmonella typhimurium* .[9-14,32-34] Most of these expression systems have been very useful as research tools. However, *S. cerevisiae* offers many potential advantages for vaccine development. The efficient, high-level and stable expression of large quantities of a variety of proteins including VLPs in their native conformation (Fig. 3.1) has been well demonstrated.[21,34-36] Yeast has the apparatus for some posttranslational modifications similar to those of mammalian cells. The production of proteins in yeast is more cost effective than using mammalian and insect cell cultures and fermentations can be easily scaled up to thousands of liters. Furthermore, there are less regulatory and safety concerns associated with yeast, such as the presence of adventitious agents which can be found in cells of mammalian or other origin. Yeast-derived products for human use (e.g., hepatitis B vaccines) have gained regulatory approval and have excellent safety records in millions of people. Taken together, yeast offers the best chance to produce sufficient quantities of a safe prophylactic vaccine.

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CHAPTER 4

Vaccine for Genital Warts

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Genital warts are benign proliferative lesions caused by certain genotypes of human papillomavirus (HPV), principally HPV types 6 and 11.[1] They are one of the most common clinically recognized sexually transmitted diseases worldwide, occurring at incidence rates of 1.5-2.5% per year in men and women aged 20-24 years in populations in the developed world.[2,3] For example, in the USA between 500,000 and 1 million new cases of genital warts are reported annually.[4] Many treatments are described, but immediate treatment failure or recurrence of warts after initial clearance is seen with all treatment modalities.[5] Total prevalent cases of genital warts exceed incident cases by a factor of 1.8, illustrating the frequency of recurrence.[6] The economic burden of genital warts is consequently substantial, and estimated to exceed $3.8 billion in the U.S. for 1997.[4]

Biberstein first described the concept of immunotherapy as a treatment for genital warts in 1925.[7] This approach to treatment used excised genital wart material from the subjects themselves prepared in various manners as an 'autogenous' vaccine. Such treatment remained in use for many years without any rigorous evaluation. However, a double-blind controlled crossover study, published in 1982, showed no difference in outcome for such therapy versus placebo[8] and such therapy subsequently ceased. In regressing genital warts there is evidence that cell-mediated immunity plays a central role, characterized by an activated $CD4^+$ T cell and macrophage infiltration of the epidermis.[9]

A number of animal papillomavirus infection systems have been used for vaccine research, including the cottontail rabbit papillomavirus (CRPV) and the bovine papillomaviruses (BPV). Such vaccine research, again in former years, used relatively crude clinically-derived antigenic material. However, the development of recombinant proteins led to experiments using BPV2 and 4 which demonstrated successful prophylactic and therapeutic vaccination against these papillomavirus diseases.[10,11] In BPV4, Immunization with E7 was shown to mediate regression and L2 to provide protection; we therefore proposed to explore a similar immunotherapeutic strategy as a potential treatment for genital warts. Given that HPV6 and 11 show a high degree of sequence homology[12] and that the majority of genital warts are caused by HPV6[1] we have developed a therapeutic vaccine, designated 'TA-GW,' based on a fusion protein of HPV6 L2 and E7 proteins.

Development of TA-GW

Generation of L2E7 Gene Construct

HPV6 L2 and E7 gene sequences were amplified from viral DNA and subcloned into vectors for expression of the recombinant synthetic protein in *E. coli* .

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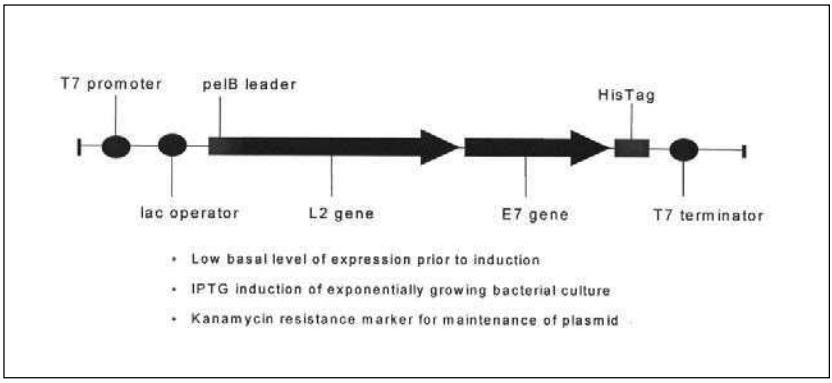

Fig. 4.1. Structure of the L2E7 bacterial expression cassette.

In vitro transcription and translation indicated that there were sequences present within the open reading frames which led to the production of truncated proteins in *E. coli* . Expression of full length protein was optimized and the final construct was produced as follows:

1. Production of truncated proteins was the result of premature termination of transcription at long stretches of thymidine residues in the HPV6 L2 gene sequence. Specific alterations of the T-rich sequences were made by site-directed mutagenesis. This procedure did not change the integrity of the protein sequence but did result in a direct improvement in the expression of full-length protein both in vitro and in vivo.
2. The modified sequences were subcloned into a plasmid vector based on bacterio phage T7 promoter for high level expression in *E. coli.* The final expression construct, pGW53, featured a pelB leader sequence upstream of the HPV6 L2E7 gene sequence (Fig. 4.1). A 'His.Tag' sequence was incorporated downstream and in frame with the 3' end of the HPV6 E7 gene sequence.

Fermentation

Bacteria (*E. coli* host strain HMS 174) were grown in 50 liters of 2YT growth medium supplemented with 25 μg/ml kanamycin in fermenters to a biomass of 0.3 g/l, at which point IPTG was added to a final concentration of 1.0 mM. The cultures were grown for a further 2 hours, harvested as 2 L batches and washed in buffer. The final yield of the fermentation was at least 20 mg L2E7/l.

Purification of Protein

Downstream process batches were derived from batch cell harvests. Inclusion bodies from several batches were concentrated by tangential cross-flow filtration in wash buffer and then in disruption buffer prior to the addition of solubilization buffer (50 mM Tris pH 8.0, 10 mM urea, 12.5 mM DTT).

Inclusion bodies were solubilized in a final solution containing 50 mM Tris pH8.0, 8 M urea, 10 mM DTT. The solubilized product was then purified by successive chromatography steps on a Poros HQ50 anion exchange column, a Poros HS50 cation exchange column and a Superdex 200 size exclusion column. The eluate from the Superdex 200 column was concentrated by chromatography on a Poros HQ50 anion exchange column and then passed

over a G25 gel filtration column to remove the urea. The bulk purified product (in 42.86 mM Tris pH 8.3, 0.2 mM DTT) was stored aseptically at –40°C prior to formulation with an adjuvant. L2E7 protein was found to be particulate in nature with two populations of particle, one of between 30 and 50 nm and the other of 100 nm particle size.

Formulation

Alhydrogel® was selected as the initial adjuvant for formulation with the L2E7 protein, as it is currently the only vaccine adjuvant licensed for human use. However, whilst recognizing that Aluminium based adjuvants are known to generate poor cell-mediated immune (CMI) responses, the inclusion of Alhydrogel® enabled us to establish baseline immune and clinical responses. More potent stimulators of T cell (Th1)-mediated immunity, adjuvants such as monophosphoryl lipid A (MPL), were subsequently evaluated.

Preclinical Characterization of TA-GW

The objectives of the preclinical characterization of TA-GW were:

1. To determine the variability of specific immune responses in different inbred strains of mice;
2. To establish the level of immune response generated using L2E7/Alhydrogel®;
3. To determine whether MPL enhances the immune response;
4. To determine reactogenicity of the formulations.

Immune Response in Different Mouse Strains

The antibody response to any antigen varies according to the MHC type of the individual vaccinated. A preliminary study was performed to determine the antibody response towards the fusion protein adjuvanted with Alhydrogel® in five different strains of mouse.

A prime and boost protocol was used and specific antibody was measured by ELISA using serum collected on day 21 or day 28. IgG and IgG1 titers generated by Immunization of B6/CBA F1 mice were significantly higher than those generated by Immunization of CBA/Ca, Balb/c and C3H/He mice. The IgG titer generated by Immunization of C57/BL6 mice was not significantly different from the B6/CBA F1 titer. B6/CBA F1 mice ($H\text{-}2^b$ and $H\text{-}2^k$) were used in all subsequent preclinical studies.

Immune Response to L2E7/Alhydrogel®

B6/CBA F1 mice were immunized with free L2E7, L2E7/Alhydrogel® or L2E7 in Freund's adjuvant (complete for the first Immunization and incomplete for the boost).

Immunization of mice as described showed that the use of Alhydrogel® increased the IgG1 titer by over 1 $\log_{10}$ compared with the titer when free L2E7 was used. Immunization of mice with L2E7 in Freund's adjuvant did not increase the IgG1 titer but did broaden the response to include antibodies of both IgG2a and IgG2b subclasses.

In vivo DTH responses to L2E7 were assessed in mice 2 weeks after a second Immunization. Mice were challenged in their right ear with L2E7, while their left ear was injected with buffer. The differential increase in ear swelling was measured with an engineer's micrometer for the following 3 days. Mice immunized with L2E7/Alhydrogel®, and not free L2E7, made a significant DTH response maximal at 24 hours and still present at 48 hours. Mice immunized with L2E7 in Freund's adjuvant gave a greater DTH response than L2E7/Alhydrogel®.

In vitro lymphocyte proliferative responses were measured by taking draining axial and brachial lymph node cells from mice after their second Immunization. Cell proliferation

was assessed by the incorporation of tritiated thymidine at 72 hours. Lymph node cells from mice immunized with L2E7/Alhydrogel® or L2E7 in Freund's adjuvant proliferated to L2E7.

These initial studies show that the inclusion of Alhydrogel® enhances both humoral and cell-mediated arms of the immune response. The enhancement of CMI with this formulation is an interesting observation, as Alhydrogel® is believed to preferentially boost antibody responses.[13] The CMI boosting effect of this Alhydrogel® adjuvanted vaccine formulation may be partly due to the particulate nature of the recombinant L2E7.

Immune Response to L2E7/Alhydrogel®/MPL

Several formulations containing MPL were evaluated and it was found that its simple addition to L2E7/Alhydrogel® preferentially enhanced antigen-specific in vitro T cell proliferative responses, IFN-γ production and in vivo delayed-type hypersensitivity responses.[14]

Reactogenicity of TA-GW Formulations

Rabbits were given four intramuscular Immunizations, at ten day intervals, of 300 μg L2E7/Alhydrogel® or L2E7/Alhydrogel®/MPL. All rabbits immunized with L2E7 produced L2E7-specific serum antibodies. The addition of MPL to L2E7/Alhydrogel® did not alter the systemic reactogenicity of the formulation.[14] There were no clinical signs or injection site reactions attributable to either formulation.

Clinical Development

Overview

L2E7/Alhydrogel® has been evaluated in the following clinical trials:

1. Phase I—A randomized, double-blind, placebo-controlled dose ranging trial in healthy male volunteers;
2. Phase IIa—Two open label phase IIa trials in male patients with new or recurrent/persistent genital warts;
3. Phase IIa—Two pilot studies in patients with nongenital low risk HPV-associated diseases (recurrent respiratory papillomatosis and cutaneous warts).

TA-GW has been developed for use as an immunotherapeutic vaccine for patients with new or recurrent/persistent genital warts. While these populations have been the focus of the clinical development program, TA-GW has also been evaluated in patients with other low risk HPV-associated diseases, namely recurrent respiratory papillomatosis (RRP) and cutaneous plantar warts. Alhydrogel® was chosen as the adjuvant for these initial studies because of its well defined safety profile and established acceptance by regulatory authorities. Table 4.1 summarizes L2E7/Alhydrogel® clinical trials that have been conducted.

Phase I Design

The first administration of TA-GW into man occurred in 1995 with a randomized, double-blind, placebo-controlled, dose-escalation study among 42 healthy adult male volunteers.[15] Subjects with a history of genital warts were excluded. Three intramuscular injections were given into the upper arm, either monthly or by an accelerated schedule totalling 4 weeks. Following vaccination, volunteers were kept under observation for four hours, after which all adverse events were self-recorded in diary cards. Venous samples were taken pre- and postvaccination to evaluate immunogenicity and clinical laboratory safety. Detailed observations were made regularly for 10 weeks and again 7 months following vaccination.

Table 4.1 L2E7/Alhydrogel® Clinical Trials Summary

Phase:	I	IIa	IIa	IIa	IIa
Subjects:	Healthy volunteers	Genital warts new/recurrent	Genital warts new/recurrent	Recurrent respiratory papillomavirus	Cutaneous warts
n:	42	27	30	4	9
Dose (µg):	0 3 30 300	300	300	300	30
Regimen: (weeks)	0,4,8 0,1,4	0,1,4	0,1,4	0,1,4	0,1,4
Design:	randomized double-blind placebo-controlled	open	open	open	open
Follow-up: (weeks)	32	8	26	52	26
Associated therapy:	-	Podophyllo-toxin	Cryotherapy	Surgery	-

Phase II Design

Genital Warts

The first open label phase IIa patient trial was designed to evaluate safety, immunogenicity and preliminary efficacy in males with external anogenital warts.[16] Nineteen subjects with either new or recurrent/persistent warts were given vaccine alone, to evaluate responses using TA-GW as single primary therapy. Another eight patients with new warts could opt for vaccine therapy alone or in combination with conventional podophyllotoxin treatment. Recurrent/persistent warts were defined as those present for greater than one month and a history of previous failed therapy, but not treated in the last two weeks. New warts were defined as lesions present for less than 3 months and no history of previous treatment. Warts were mapped and photographed at regular timepoints, the two maximum perpendicular diameters of each wart were measured and the sum of these products for all warts was defined as the "wart area." The trial protocol included an 8 week follow up after which any subject with persistent warts was offered conventional treatment as deemed appropriate by the physician. While clinic visits were not mandated after this time, the progress of all patients returning to the clinic over the following 6 months were recorded.

Noninvasive sampling for HPV DNA detection was also performed at each visit using a dacron swab, which was rubbed firmly over the warts or the site of previous warts if they had cleared.

The second phase IIa study evaluated TA-GW when used as adjuvant immunotherapy in association with cryotherapy. The trial design was similar to that of the previous study, with the exception that subjects were prospectively followed for 6 months to enable wart recurrence rates to be more accurately assessed. Thirty subjects were enrolled, ten with new warts and 20 with recurrent/persistent warts. Safety and immunogenicity assessments were made throughout. In both studies, 300 μg of TA-GW was given at 0, 1, and 4 weeks; this was the dose and schedule shown to be most immunogenic during the phase I study.

Recurrent Respiratory Papillomatosis

Recurrent respiratory papillomatosis (RRP) is the most common benign tumor of the larynx and is associated with the presence of HPV DNA, usually type 6 or 11. Current treatment is unsatisfactory and requires regular ablative surgery under general anesthesia. A pilot study was conducted enrolling four patients with RRP to assess safety, immunogenicity and clinical outcome; three were HPV6-positive and one HPV11-positive, prior to vaccination. TA-GW was given in association with elective ablative surgery and subjects were followed for 12 months. Accurate clinical outcome assessments were difficult and were mostly necessarily subjective. These included direct and indirect laryngoscopy and detailed voice questionnaires. In addition, intervals between vaccination and subsequent surgical procedures were recorded and compared with historical records.

Cutaneous Warts

A pilot study was conducted in nine patients with persistent cutaneous plantar warts as a result of a clinical observation made during the phase I trial. One of the healthy volunteers was noted to have a recalcitrant plantar wart that began to regress within three weeks after receiving the first 3 μg vaccination of TA-GW. The lesion cleared rapidly and did not recur. There is a historical precedent for the use of immunotherapy to treat cutaneous warts using vaccines prepared from genital or cattle warts.[7,17] During this trial, three 30 μg doses of TA-GW were given without other treatments and subjects were followed for 6 months to assess safety, immunogenicity and change in wart status.

Safety

TA-GW was shown to be very well tolerated in healthy volunteers and wart patients. No serious or immediate reactions were observed. Mild local tenderness was the most frequently reported adverse event, as would be expected following any parenteral vaccination. More severe local tenderness, erythema and induration were reported only infrequently and were maximal 24 hours postvaccination. Among healthy volunteers, systemic events included headache (31%), myalgia (8%), cough (8%) and influenza-like symptoms (3%); all were mild and transient and resolved without treatment. The maximum oral temperature reported was 37.8˚C. The number of injections given did not influence the frequency and severity of adverse events. In addition, no significant clinical laboratory abnormalities were reported.

One subject with genital warts had a transient rise in alanine transaminase (ALT) which followed a reported excessive intake of alcohol. Two subjects with RRP reported lethargy lasting 48 hours following vaccination and one subject with cutaneous warts experienced mild fever and muscle aches for three days following the first injection. Another developed a sore throat and pyrexia requiring antibiotic therapy following the second and third injections.

Immunogenicity

Immunogenicity in Healthy Volunteers

The clinical trial in healthy volunteers is summarized in Table 4.1. Immunogenicity was assessed by measuring L2E7 specific in vitro T cell proliferative responses, production of IFN-γ and IL-5 and serum antibodies. A long term blood sample was obtained from all but four subjects at 32 weeks following their first vaccination.

Vaccination of a healthy volunteer population with L2E7/Alhydrogel® resulted in the following:

1. Both vaccination regimes were effective. However, T cell proliferative responses emerged faster and were marginally greater with the accelerated schedule when compared to the classical schedule. The observed T cell proliferative responses were also long-lived.[15]
2. Peripheral blood mononuclear cells (PBMC) produced both IFN-γ and IL-5 in vitro when cultured with L2E7. The in vitro production of both cytokines was dose dependent, cytokine production increasing with increasing dose of vaccine.[15]
3. Serum titers of anti-L2E7 antibodies were maximal after the third Immunization and persisted for at least 32 weeks. Ten subjects were bled again at 18 months and found to have serum titers of IgG anti-L2E7 antibodies comparable to their levels at 32 weeks.

TA-GW was shown to be clearly immunogenic in healthy male subjects, eliciting both antigen-specific T and B cell responses. Alum-precipitated vaccines have not historically been regarded as good stimulators of T cell-mediated immunity;[18] however, the purified recombinant L2E7 protein spontaneously forms a stable high molecular weight aggregate during the final stage of the purification process. Its particulate nature may contribute to its ability to elicit strong long lasting T cell proliferative and IFN-γ responses.

The immunogenicity of TA-GW demonstrated in healthy male subjects is encouraging, since we believe that the observed rapid T cell proliferation and associated production of IFN-γ are desirable features of a therapeutic vaccine for the treatment of genital warts. The results from this study allowed us to select the 300 μg formulation and accelerated schedule for subsequent genital wart trials.

Immunogenicity in Genital Warts Patients

The immunogenicity of TA-GW in patients with genital warts was determined using the same assays as those used in the healthy volunteer study.

Specific immunogenicity results showed:

1. Prior to vaccination, only one patient exhibited an antigen-specific T cell proliferative response to L2E7. After the first vaccination, 19 of 25 evaluable patients made antigen-specific T cell proliferative responses.
2. Levels of in vitro production of IL-5 by PBMC increased throughout the study, peaking at week 8 for all groups. IFN-γ levels were greatest in the group of patients with new warts receiving both vaccine and podophyllotoxin and peaked at week 4, reaching levels similar to IL-5. IFN-γ levels were comparatively low in the other two groups.
3. Prior to vaccination, there was no detectable serum IgG to HPV6 L2E7. Eight weeks after the final vaccination, all patients had produced serum IgG anti-L2E7 antibodies. Only two patients with persistent warts made weak IgG responses.

In the second phase IIa study, which included a 6 month follow up, 22 patients were shown by PCR to be infected with HPV6, and 7 to have HPV11. Six patients with persistent warts were coinfected with HPV16, and 1 was positive for viral type PAP155. Two patients with newly presenting warts were coinfected with HPV16, one with HPV16 and 33, and one with PAP155.

The specific immunogenicity results from the second phase IIa trial showed:

1. Prior to vaccination, all T cell proliferative responses to L2E7 were low. Following vaccination, 15 of 19 patients with persistent warts made L2E7-specific T cell proliferative responses. Of the 15 patients, 12 made maximal responses after the final vaccination. Seven of 10 patients with newly presenting warts made L2E7-specific T cell proliferative responses.
2. Throughout the study, the level of IL-5 production by PBMC was low. PBMCs from only one patient showed IL-5 above background levels (i.e., >200 pg/ml) and this was prior to vaccination. The level of IFN-γ production was also low prior to vaccination; following the second vaccination PBMC produced significant levels of IFN-γ (>200 pg/ml) in half the patients. The ratio of IFN-γ: IL-5 in vitro was 2:1 prior to vaccination, increasing to 6:1 after the final vaccination.
3. Prior to vaccination, patients with persistent warts had no detectable serum IgG anti-L2E7 antibodies; however, 3 of 10 patients with newly presenting warts had serum IgG anti-L2E7 antibodies present. After vaccination, all patients had IgG anti-L2E7 antibodies, though two subjects with newly presenting warts who had preexisting anti-L2E7 antibodies did not increase their antibody titer. After the final vaccination, 16 of 27 patients had IgG_3 antibodies, 10 patients had IgG_1, 4 patients had IgG_2 and none had IgG_4 antibodies.

Immunogenicity in Other Clinical Indications

RRP patients were vaccinated intramuscularly on days 0, 7 and 28 with 300 μg of TA-GW. They received CO_2 laser surgery at the commencement of the study and then as indicated. PBMC and serum were isolated from blood samples taken at weeks 0, 4, 12 and 24. The results from this trial showed:

1. All four patients completed the three-dose vaccination course; however, follow up visits were not always made at the appointed times.
2. Prior to vaccination, all patients' T cell proliferative responses were low. Only one patient made a weak T cell proliferative response at 24 weeks after the first vaccination.

After the second vaccination, three patients made L2E7-specific T cell proliferative responses.

3. Prior to vaccination, the level of IFN-γ and IL-5 produced in vitro by PBMC was low (<200 pg/ml). After vaccination, the level of cytokines remained low with the exception of one patient whose PBMC produced both IFN-γ and IL-5 at each time point after vaccination. The level of IFN-γ increased over the study period and peaked 24 weeks post-first vaccination. The IL-5 production was variable over the study period, peaking at week 4.
4. Serum samples were collected from all patients recruited to this trial and anti-L2E7 antibody levels were determined by ELISA. Prior to and following the first two vaccinations, no serum IgG anti-L2E7 antibodies were detected. After the final vaccination, all patients had IgG anti-L2E7 antibodies, which remained elevated 24 weeks after vaccination.

All nine patients with recalcitrant cutaneous plantar warts were typed as HPV2a/27/57. In situ hybridization was positive using probes to detect HPV2a and 57 but negative for HPV types 1, 3, 4, 6, 7, 10 and 11. The in situ hybridization did not discriminate between the closely related HPVs 2a/27/ 57.

The specific immunogenicity results showed:

1. Prior to Immunization, PBMC prepared from patients did not proliferate in vitro when cultured with L2E7. After Immunization, PBMC from all but one patient made L2E7-specific in vitro proliferative responses at weeks 4 and/or 8.
2. Prior to Immunization, incubation of patients' lymphocytes with L2E7 did not lead to secretion of significant levels of either IFN-γ or IL-5. After the complete vaccination course, PBMC from three of nine patients produced IFN-γ and five of nine produced IL-5.
3. No patients had detectable pre-Immunization serum IgG antibodies to L2E7. Eight weeks after the first Immunization, five of nine patients had clearly made serum IgG anti-L2E7 antibodies

TA-GW was shown to be immunogenic in all but one patient with cutaneous warts. The patient who did not respond immunologically had a history of diabetes mellitus, suggesting that a limited immune deficit in insulin-dependent diabetes may permit the development of certain infections, including genital warts.[19]

Comparative Immunogenicity Between Infected and Uninfected Subjects

In both phase IIa genital warts clinical trials, 300 μg TA-GW was given at weeks 0, 1 and 4, allowing comparison to be made directly with healthy volunteers who received an identical dosing schedule.

The antibody responses to L2E7 were similar in that all subjects had strong serum IgG anti-L2E7 antibodies after vaccination. IgG subclass antibody responses were also similar, except that IgG4 was not detected in uninfected subjects. Prior to vaccination, T cell proliferative responses were low in all subjects. After vaccination, T cell proliferative responses increased significantly, although proliferative responses in healthy volunteers were markedly higher than those seen in HPV-infected subjects. In vitro stimulation of uninfected volunteers' PBMC with L2E7 produced increasing levels of both IFN-γ and IL-5 over the duration the study. In infected subjects, PBMC produced increasing amounts of IL-5 comparable to the uninfected volunteers, but lower levels of IFN-γ, with some variability between individual patients. Thus, the volunteers showed a trend towards a Th1-type immune response and HPV-infected patients a trend towards a Th2-type immune response following vaccination. This observation raises the possibility that HPV-infected individuals,

who do not readily clear their warts using conventional therapy, may have an impaired or biased immunity to their infecting HPV.

Clinical Efficacy

Genital Warts

Among the total of 16 subjects with persistent/relapsing warts given TA-GW alone, two showed a complete response (CR) and three a partial response (PR) within 8 weeks of vaccination. Of the eight subjects with newly presenting warts given vaccine plus podophyllotoxin, there were three CRs and one PR within 8 weeks. All but one CR occurred between weeks 4 and 8, and no other macroscopic signs associated with regression were observed. It is noteworthy that all five subjects who had a CR remained wart free during follow up without further treatment.

Beyond the planned follow up period of 8 weeks, 12 subjects were lost without confirmation of their wart status. Warts in the remaining ten patients were still present at week 8 and all were given further conventional therapy (range 1-8 treatments, mean 3.5) as deemed appropriate by their physician. Of these, a further eight CRs were reported, giving a total of 13 CRs from the total of 15 subjects followed, and of the original 27 subjects vaccinated. During the follow up period, lasting from 1 to 24 weeks (mean 11.4), it was remarkable that wart recurrence was not reported in any of the 12 subjects with total clearance.

In the second patient study, of 30 subjects given vaccine plus cryotherapy, 12/20 with recurrent/persistent warts and 10/10 with new warts showed a CR. Compliance was excellent, with all but two subjects being followed for the planned 26 weeks following vaccination. During follow up, recurrence was reported in 3/10 new wart and 2/12 recurrent/persistent wart CRs. This rate of wart recurrence is significantly less than that expected using cryotherapy alone in the treatment of genital warts.[20]

Recurrent Respiratory Papillomatosis

The four patients vaccinated were aged from 19 to 58 years (mean 38) with long histories of RRP ranging from 12 to 32 years (mean 20). All underwent direct laryngoscopy under general anesthesia 3 months following vaccination and respiratory papillomata were seen, and ablated, in all. Three of the four required further surgery during the following 12 months; the fourth did not. There were no significant differences in the intervals between operative procedures pre- and postvaccination. Pre- and postvaccination voice questionnaires showed an overall improvement in all subjects, with a marked increase of more than 25% observed in two subjects six months after vaccination. To evaluate clinical efficacy further, future trials would require clearly defined objective outcome assessments such as photographic records or magnetic resonance imaging.

Cutaneous Warts

Nine subjects with a history of persistent cutaneous plantar warts, ranging from 1.5 to 10 years (mean 4.6) were enrolled and vaccinated. Using vaccine alone, three CRs were seen during or shortly after the initial 8 week trial period. Regression was not associated with any discomfort or irritation in or around the warts. All subjects were then followed for six months without further treatment and during this period no wart recurrence was reported. Of the remaining six subjects who failed to respond clinically following vaccination, all were given subsequent cryotherapy and in two cases wart clearance was eventually achieved. These results suggest that Immunization with heterologous HPV proteins may be clinically

efficacious. If so, TA-GW could also exhibit clinical utility in the elimination of cutaneous HPV infection, or indeed HPV16/18 disease.

Summary of Clinical Efficacy

The preliminary clinical efficacy of TA-GW, including the number of subjects showing complete wart clearance and wart recurrence, is summarized in Table 4.2.

During the first phase IIa trial, a high number of subjects were lost to follow up. Compliance was improved significantly in subsequent studies because subjects were aware of the duration of follow up prospectively.

Overall, complete wart clearance was seen in 54% of subjects using TA-GW alone or in association with conventional therapy. The recurrence rate of only 13%, over a follow up period of 11-52 weeks (mean 19), provides very encouraging support for this immunotherapeutic approach.

However, the poor correlation reported between clinical and immune responses requires better understanding. During these trials we have assessed only immune responses to HPV6 in peripheral blood; other potentially useful immune markers such as cutaneous delayed type hypersensitivity and cytotoxic T cell (CTL) responses have not been assessed and should be considered in future trials.

Alternative Vaccine Strategies

TA-GW was designed specifically as an immunotherapeutic vaccine for the treatment of patients with new or recurrent/persistent genital warts. There are currently no other vaccine candidates using this approach; however, interest lies in the development of prophylactic vaccines against low risk HPV types, with vaccine candidates currently under development from both Merck and Medimmune. These focus on the use of a late gene product (L1) from HPV6 or 11 expressed in eukaryotic vectors producing virus-like particles (VLPs). The L1 gene product is the major component of the viral capsid, and L1 VLPs have been shown to elicit protective immunity in the CRPV, canine oral papillomavirus (COPV) and BPV4 models. HPV6 and HPV11 are highly homologous and VLPs from both HPV types can generate crossreacting antibodies to some degree. Such vaccines are still in early clinical development and no data are available as yet. It is expected that any vaccine developed specifically for the prophylaxis of genital warts would necessarily be administered as part of a multivalent combination vaccine, targeted at adolescents. It is noteworthy that L2, the main capsid protein of HPV6 and 11, has been shown to elicit protective antibodies in BPV and CRPV infections;[21] TA-GW, therefore, has the potential to be also used in the prophylaxis of genital wart infections.

Future Development of TA-GW

Despite encouraging preliminary clinical data, we have no plans to conduct further trials using L2E7/Alhydrogel®. The immunogenicity of this formulation, especially in HPV-infected subjects, is suboptimal and we believe that this can be improved through the use of alternative adjuvants.

Future development of TA-GW will be undertaken jointly by Cantab Pharmaceuticals and SmithKline Beecham Biologicals. A novel adjuvant, designated SBAS2, has been formulated with L2E7 protein, to enhance the immunogenicity of the vaccine. The adjuvant contains the immune stimulants monophosphoryl lipid A and QS-21 in an oil-in-water emulsion. Preclinical studies have shown that SBAS2 significantly enhances the immunogenicity of L2E7. A phase I clinical study in HPV-uninfected subjects has been completed and a phase I/II, dose-ranging, safety and immunogenicity study in genital wart patients is ongoing. Controlled phase II efficacy trials in genital wart patients, using

Table 4.2 Summary of the Preliminary Clinical Efficacy of TA-GW

Indication	Treatment	Total n	Lost n (%)	Clearance n (%)	Recurrence n (%)	Follow-up (weeks)
Genital warts -recurrent	TA-GW alone to week 8	16	7 (44%)	9 (56%)	0/9 (0%)	11
Genital warts -new	TA-GW alone to week 8	3	2 (67%)	0 (0%)	N/A	11
Genital warts -new	TA-GW + podophyllotoxin	8	3 (38%)	4 (50%)	0/4 (0%)	11
Genital warts -recurrent	TA-GW + cryotherapy	20	1 (5%)	12 (60%)	2/12 (17%)	26
Genital warts -new	TA-GW + cryotherapy	10	0 (0%)	10 (100%)	3/10 (30%)	26
RRP	TA-GW + surgery	4	0 (0%)	0 (0%)	N/A	52
Cutaneous -recurrent	TA-GW alone	9	0 (0%)	3 (33%)	0/3 (0%)	24
TOTAL		70	13 (19%)	38 (54%)	5/38 (13%)	19

L2E7/SBAS2 alone or in association with conventional treatment, will begin in the near future.

Summary

Today, the major burden in the management of genital warts is centered on the very high rate of wart recurrence following clearance, irrespective of treatment modality employed.[5] TA-GW (L2E7/Alhydrogel®), having been administered to 122 HPV-infected or uninfected subjects, has shown some very encouraging early clinical results, highlighted by an excellent safety profile and low rate of wart recurrence. Despite these positive clinical findings, immune responses have been suboptimal, especially among HPV-infected patients. TA-GW has, therefore, been reformulated to incorporate a novel adjuvant, SBAS2, which has the potential to significantly enhance the vaccine's immunogenicity, and consequently its clinical efficacy. Further clinical trials using TA-GW/SBAS2 are under way.

We conclude that:

1. L2E7/Alhydrogel® is well tolerated in both HPV-infected and uninfected subjects;
2. Initial clinical efficacy is encouraging, highlighted by low rates of wart recurrence;
3. Immunogenicity of L2E7/Alhydrogel® is suboptimal in HPV-infected subjects;
4. No obvious correlation has been seen between clinical and immune responses;
5. SBAS2 has the potential to significantly enhance the immunogenicity and clinical efficacy of TA-GW.

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CHAPTER 5

Anti-Tumor Immunotherapy with Synthetic Peptides Representing Tumor-Associated T-Cell Epitopes:
Implications for Peptide-Based Vaccination of Cervical Cancer

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Alternative approaches for the prevention or treatment of cancer are now emerging. The goal of new treatment modalities is to combine effective anti-tumor activity with a reduction of the side effects to healthy tissues, still constituting a severe complication of conventional cancer therapy (i.e., of surgery, radiotherapy, and chemotherapy). Observations that susceptibility to several cancer types is increased in immunocompromized individuals has led to the assumption that immune responses are able to interfere with tumor development.[1] Immunotherapy aims at increasing the power, and redirecting the specificity, of the patients' immune system to attack the malignant cells without the often severe side effects associated with conventional cancer treatment.

Exploiting the Immune System for Treatment of Cancer

In order to eliminate foreign invaders, including viruses, the immune system is equipped with an innate, antigen-nonspecific arm—usually as a first line of defense—and an acquired, antigen-specific arm. The major advantages of acquired immunity are its specificity and long-term memory. Specific immunity comprises humoral and cellular immune responses. Antibodies are specific for protein conformations rather than primary sequences. As a consequence, they will bind to intact protein structures on foreign invaders, which can result in neutralization of extracellular viruses or bacteria. Antibodies are, therefore, the predominant immune effectors in the initial phases of infection. T lymphocytes, on the other hand, play a major role in the recognition and subsequent elimination of intracellular pathogens or transformed cells.

Effector T cells recognize immunogenic peptides that are presented at the cell surface in the context of major histocompatibility complex (MHC) molecules (human leukocyte antigen (HLA) for humans; H-2 for mice).[2] The vast majority of T cells consists of either

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$CD8^+$ cytotoxic T lymphocytes (CTL) or $CD4^+$ T helper (Th) cells. In general, MHC class I molecules present antigenic peptides to CTL, whereas Th cells recognize peptide epitopes in the context of MHC class II molecules. The latter proteins are mainly found on the cells of the immune system with a specialized antigen presenting function. MHC class I molecules are expressed on the cell surface of virtually all nucleated cells and present peptides processed from cytosolic proteins to CTL. This enables the CTL to screen almost all cells of the body for antigenic peptides that may be presented as a consequence of viral infection or malignant transformation. Therefore, the $CD8^+$ CTL represent a major effector subset of tumor-specific T cells potentially able to reject tumors.

Induction of (Tumor) Antigen-Specific CTL

In order to exploit the full potential of the immune system for the destruction of virus-infected or malignantly transformed cells, it is crucial to understand how the T cell arm of the immune system is activated to recognize and eliminate foreign antigen-expressing cells. Initiation of antigen-specific T lymphocyte responses in vivo usually requires the activation of naive T cells by professional antigen-presenting cells (APC). Several studies have shown that class I-negative syngeneic tumor cells or completely allogeneic tumor cells were effective in inducing CTL-mediated anti-tumor immunity in the context of the host's own MHC class I molecules.[3,4] These experiments indicate that not the injected cells themselves induce T cell-mediated immunity (direct priming), but rather that the professional APC derived from the host bone marrow are capable of capturing antigens from the injected cells for presentation to naive T cells. This form of exogenous antigen presentation has been referred to as "cross-priming."[5] Cross-priming of antigenic determinants by professional APC is generally found to be essential for the induction of T cell immunity, and is implicated in immunity to minor histocompatibility antigens, tumor antigens, viral antigens and self-antigens.[3-6] How the antigen reaches the class I processing pathway and which host bone marrow-derived APC are responsible for cross-priming are subjects of ongoing research. However, the dendritic cells (DC) are the favorite candidates in the process of cross-priming, as these cells are specialized to prime helper and killer T cells in vivo (reviewed by refs. 7, 8). Immature DC have a high capacity for antigen capture and processing. Upon maturation, DC acquire the ability to effectively stimulate T cells. Hallmarks of mature DC include expression of high levels of MHC class I and II molecules, as well as costimulatory molecules (including B7), and production of cytokines such as interleukin (IL)-12. Moreover, cross-priming by DC would provide an explanation for the seemingly contradictory location of induction of an immune response in the lymph nodes and the presence of the antigen in peripheral tissues, such as epithelia. Langerhans cells, the immature DC in the skin, can capture antigen locally, and upon migration to the draining lymph nodes will present these antigens to naive T cells.

Generation of effective CTL responses often requires "help" from $CD4^+$ Th lymphocytes. Dissection of the cellular interactions involved in the induction of CTL by cross-priming revealed that the Th cell and CTL populations must recognize antigen on the same APC.[6,9,10] This requirement for T help in cross-priming of CTL could be explained by a proximity requirement for the efficient delivery by Th of soluble factors such as IL-2.[11] Another possibility is that a cognate interaction between Th and APC is needed to convert the APC to a state that is capable of directly priming naive CTL. For this, CD40 and CD40 ligand (CD154) are attractive candidates (reviewed by refs. 12, 13). The requirement for CD40-CD40 ligand interactions in delivery of T help to CTL is illustrated by the following experiment. In CD4-depleted or class II knockout mice, tumor-specific CTL immunity can no longer be induced via cross-priming. However, in the absence of $CD4^+$ cells, antigen-specific CTL induction could be restored by administration of a CD40-activating monoclonal antibody

(mAb).[14] These data suggest that (antigen-specific) recognition of a T-helper epitope on the professional APC allows interaction of CD40 ligand on the activated T-helper cell with CD40 on the DC, thereby activating the DC to express costimulatory molecules and/ or cytokines (such as IL-12). In turn, the activated DC is enabled to prime naive CTL. Thus, CD40 ligand-CD40 interactions play a pivotal role in the delivery of T cell help for the (cross-)priming of antigen-specific CTL by DC. These and other data indicate that immune intervention schemes aiming at the induction of tumor-specific immunity should ensure full activation of DCs.

To exploit the immune system for cancer therapy optimally, tumor antigens are being identified, i.e., differences between the tumor cells and their healthy counterparts which could be targeted by immune effector cells. Any of the cellular events leading to transformation can potentially give rise to a tumor antigen. A well-defined group of tumor antigens—obviously foreign to the immune system—is encoded by tumor viruses.

HPV and Cervical Carcinoma

Viruses are believed to be involved in the development of approximately 15-20% of human cancers (reviewed by refs. 15, 16). Epstein-Barr virus (EBV) is associated with Burkitt's and Hodgkin's lymphomas and nasopharyngeal carcinoma, hepatitis B virus (HBV) with hepatocellular carcinoma, human T lymphotropic virus 1 with adult T cell leukemia, herpes virus type 8 with Kaposi's sarcoma, and human papillomaviruses (HPV) with skin and anogenital cancers. HPV DNA can be detected in more than 90% of cervical carcinomas, predominantly of the HPV16 and HPV18 genotypes. These types of HPV, as well as other high-risk HPVs, have been implicated in the etiology of cervical cancer.[17] In the majority of cervical tumor cells, early regions 6 and 7 (E6 and E7) are constitutively expressed and are required for maintenance of the transformed state.[18,19] These proteins receive most of the attention in the development of vaccination strategies against cervical cancer. Viral proteins in virus-induced tumors are ideal target structures for tumor-reactive T cells because they are tumor-specific. Much knowledge has been gathered by studying DNA and RNA tumor viruses in mouse models (reviewed by refs. 20, 21). If the immunogenic peptides originate from viral (onco)proteins involved in transformation, these represent attractive immunotherapeutic targets, because evasion of immune recognition through loss of oncoprotein expression would also entail loss of the transformed state.

Immune Surveillance Against HPV-Associated Cervical Carcinoma

Involvement of (T cell-mediated) immune responses in the defense against HPV-induced lesions is suggested by the following observations:

1. HPV infections, cervical intraepithelial neoplasia (CIN) and cervical carcinoma show an increased incidence in immunocompromised patients such as renal transplant recipients or patients infected with HIV (reviewed by refs. 22, 23); this supports the view that cellular immune recognition of HPV is important in the elimination of HPV-infected cells.
2. Seemingly spontaneous regressions of CIN lesions and clearance of concomittant HPV infections have been ascribed to natural immunity against HPV, partly because they are accompanied by extensive cellular infiltrates.[24-26]
3. Some associations of particular HLA alleles with increased susceptibility to or protection from HPV infection and cervical neoplasia have been reported (reviewed by refs. 27, 28) suggesting that these allelic products may present HPV-derived peptides to the immune system. Furthermore, downregulation of MHC class I molecules on cervical carcinoma cells has been described[29,30] which may serve as an immune evasion strategy of these cells.

4. HPV-specific immunity has been demonstrated to be effective in animal models for prevention or, occasionally, treatment of HPV-containing tumors (reviewed by ref. 31). Moreover, indications for natural immunity are now accumulating with the detection of virus-directed immune responses in patients with HPV DNA-positive lesions.

In patients with cervical lesions (CIN and early stage cervical carcinoma), natural CTL responses against HPV16 and HPV18 have been detected that were absent in healthy control donors.[32-34] The frequency of CTL responding to a recombinant vaccinia virus expressing the HPV16 and HPV18 E6/E7 gene products was found to be higher in tumors and lymph nodes compared with that of peripheral blood.[33] These results firmly establish the existence of natural HPV16 E7-specific CTL immunity. In addition, HPV16 E6 and E7-directed Th cells have been identified in patients with abnormal cervical cytology and/or serological responses against HPV16 proteins.[35-37] These HPV16 Th responses were not detectable in individuals without indications of viral infection, indicating that they represent virus-specific Th memory responses primed in vivo. Taken together, it appears that HPV-specific immunity can be raised in response to the virus or expression of viral proteins, and can result in protection against (the development of) tumors. However, in patients with cervical lesions, the HPV-directed immunity has obviously failed in clearing the virus(-infected cells) resulting in persistence of infection with an increased risk of additional oncogenic changes, eventually leading to the development of cancer.

The poor immunogenicity of the HPV-encoded proteins may result from the nonlytic nature of the viral infection.[38] Oncogenic HPV types associated with genital malignancies productively infect only mucosal epithelia such as those lining the cervix. Viral replication and production exploit the normal differentiation of the keratinocytes, resulting in release of virus particles from the shed upper layers of the epithelium. Presumably as a consequence, HPV infection produces little or no local inflammation. Additionally, infected keratinocytes do not have all the features of professional APC. Both the absence of a lytic infection and the lack of virally infected professional APC may explain why HPV infection is initially ignored by the immune system. Failure to eliminate HPV-infected cells may allow oncogenic transformation and tumor development to occur. Upon tissue damage or large overexpression of viral proteins after integration of HPV DNA, the Langerhans cells in the squamous epithelium are alerted to capture viral antigens for transport to the draining lymph nodes and subsequent induction of HPV-directed T cells. However, due to this late arousal of immune effector cells (if at all) and potential immunosuppression accompanying tumor outgrowth, the HPV infected or even transformed cells are likely to outpace the immune system.

In contrast to the natural immunity which may be too late, active immune intervention may provide adequate and effective (T cell-mediated) immunity in time to prevent or treat early HPV-associated neoplasia.

Vaccination Strategies in Animal Models

Since the HPV E6 and E7 oncoproteins constitute tumor-specific antigens for cervical carcinoma, specific active targeting of the immune system towards these antigens is feasible by, for instance, vaccination with peptides.

Protective Immune Responses Induced by Peptide-Based Vaccines

Subcutaneous vaccination with an HPV16 E7-encoded CTL epitope (amino acids 49-57) emulsified in incomplete Freund's adjuvant (IFA) protected mice from a subsequent challenge with a lethal dose of HPV16-transformed syngeneic tumor cells.[39] CTL induced by this Immunization protocol were capable of lysing peptide-pulsed target cells as well as tumor

cells in vitro. Indeed, this naturally processed peptide has been eluted from the presenting H-2D^b molecules.[40] These studies show that selective stimulation of a T cell specificity of choice by vaccination with a peptide derived from an oncogenic protein is effective in inducing protective T cell immunity without the introduction of the entire oncogene.

In addition to several examples of successful prophylactic induction of antiviral and/or anti-tumor immunity with peptides (reviewed by ref. 41), the first indications that peptide vaccination can also result in eradication of preexisting tumor burden have now been reported. In different murine tumor models, treatment of animals bearing established macroscopic tumors with bone marrow-derived dendritic cells (BMDC) pulsed ex vivo with synthetic tumor-associated peptides resulted in sustained tumor regression and tumor-free status in a majority of treated mice (reviewed by ref. 42). Intravenous injection with HPV16 E7 49-57 peptide-pulsed BMDC into mice bearing established HPV16-transformed tumors resulted in tumor regression and long-term immunity.[43] When autologous tumor cells are the only known source of tumor antigens, unfractionated acid-eluted peptides from the tumor can be pulsed onto dendritic cells. Indeed, treatment of HPV16-containing tumors was also possible by vaccination with BMDC pulsed with acid-eluted peptides from this tumor.[44] The latter findings indicate that this strategy can be used to treat tumors with naturally presented peptides. Furthermore, vaccination with a peptide derived from a mutated connexin 37 gap junction protein present on a murine lung carcinoma prevented metastatic spread from a primary tumor that was allowed to develop for 30 days before surgical excision. Peptides either emulsified in IFA or loaded on cells reduced metastatic growth in mice carrying preestablished metastases.[45] This CD8$^+$ T cell-mediated anti-tumor effect resembles a clinical setting of combinational therapy in which debulking of the tumor by surgery or irradiation might be combined with immunotherapeutic clearance of residual tumor cells.

In conclusion, these studies show that vaccination with several peptides can cause protective, or even therapeutic, T cell immunity.

Adverse Effects of Peptide Vaccination

In other models, however, peptides have been reported to downregulate T cell-mediated immune responses. Repeated intraperitoneal administration of high doses of a lymphocytic choriomeningitis virus (LCMV) peptide epitope induced tolerance, in this case useful for the prevention of autoimmune diabetes in a transgenic mouse model.[46,47] Priming versus tolerization of T cells against LCMV depended on peptide dose, route and frequency of peptide administration: Subcutaneous injection of a low dose of LCMV peptide resulted in protection, whereas tolerance was induced by systemic administration of high peptide doses.

Modulation of T cell immune responsiveness was also studied in a tumor model. Adenovirus type 5 (Ad5) E1-transformed cells present at least two CTL epitopes to the immune system, encoded by the E1A and E1B regions.[48,49] CTL clones directed against these peptide epitopes eradicate established tumors in nude mice, showing that these epitopes can mediate tumor regression. In contrast, a single s.c. injection of a low dose of these peptides in IFA (or CFA) into immunocompetent animals—a mode of peptide administration proven to induce protective T cell immunity in several other models—resulted in enhanced tumor outgrowth. This was accompanied by T cell unresponsiveness in vitro to the respective epitopes, indicating that deletion of antigen-specific CTL activity explains the enhanced outgrowth of Ad5 E1 tumor cells.[50,51] Clearly, it is very important to obtain insight into the mechanisms that cause the two Ad5-encoded epitopes to induce functional T cell deletion and enhanced tumor outgrowth, whereas vaccination with other peptide epitopes induces CTL activation associated with protection. Our current data indicate that

not the peptide epitopes per se, but rather the mode of antigen administration determines the outcome of Immunization. For instance, Immunization with irradiated Ad5 E1-transformed tumor cells or with replication-defective adenovirus (both encoding the relevant CTL epitopes) led to protective immunity associated with strong tumor-specific CTL activity.[4,50-52]

Taken together, these data indicate that a delicate balance exists between the induction or elimination of protective T cell responses by peptide vaccination and point to a careful use of peptides for prophylactic or therapeutic approaches.

Finding the Right Formulation

An essential aspect of developing peptide-based vaccines is the identification of adjuvants or delivery systems that facilitate the generation of controlled and reliable T cell-mediated anti-tumor immune responses.

As discussed before, DC are likely to play a crucial role in the induction of T cell immunity and, therefore, they appear a promising vehicle. Antigenic peptides pulsed onto activated dendritic APC provide antigen in combination with costimulatory molecules, and this strategy was shown to be very effective in the induction of anti-tumor T cell responses.[42] In particular, the exquisite ability of DC to induce protective immunity is illustrated by the demonstration that the tolerogenic effect of the Ad5 peptides when administered in IFA was reverted into protective immunity when the peptides were pulsed onto DC. Immunization with these peptide-pulsed DC resulted both in tumor-specific CTL detectable in vitro and in tumor protection in vivo.[53] As an approach to circumvent the limitations of single peptide pulsing, DC can be fed with a target protein of interest to activate both class I and class II-restricted T cell responses.[54-57] For instance, DC pulsed with the recombinant HPV16 E7 protein led to induction of tumor-specific T cells. Moreover, Immunization with these protein-pulsed DC resulted in eradication of a subsequent challenge with HPV16-transformed tumor cells in vivo.[58]

A matter of concern when using in vitro/ex vivo cultured DC as carriers is the labor-intensive isolation of limited amounts of cells. Previously, induction of anti-tumor immunity has been pursued by rendering tumor cells more immunogenic, for instance by transfection or retroviral transduction of genes encoding cytokines or costimulatory molecules (reviewed by refs. 59, 60). However, rather than trying to enhance direct priming through this approach, addressing the DC as professional APC for the induction of anti-tumor immune responses (by cross-priming) is more likely to be generally effective. Alternative ways to deliver the antigen of interest to activated DC in vivo are therefore desirable. These include the administration of longer peptides encompassing the minimal epitope or even of whole proteins in order for the APC to take up and process antigenic peptides in vivo for presentation to both antigen-specific T helpers and CTL. Indeed, the recombinant HPV16 E7 protein emulsified in IFA was capable of inducing protective, $CD8^+$ T cell-mediated immunity against a lethal challenge with HPV16-transformed tumor cells, to the same extent as in vitro isolated DC pulsed with the E7 protein.[58] Alternatively, intracellular routing of an antigen can be manipulated. For instance, a recombinant vaccinia virus encoding HPV16 E7 coupled to a lysosomal protein, LAMP-1, targeted the E7 protein to the endolysosomal compartments of infected cells, thereby favoring MHC class II antigen processing and presentation. Vaccination of mice with this HPV16 E7-LAMP1 vaccinia construct afforded enhanced protection against the outgrowth of E7-expressing tumors compared to a vaccinia virus containing just the E7 gene.[61,62] These results indicate that a more efficient induction of antigen-specific T helper responses resulted in enhanced tumor protection. An additional advantage of vaccination based upon entire antigens, when compared to minimal epitope peptide vaccination, is its wider applicability with respect to

antigen specificity and HLA polymorphism. This may also be achieved by vaccination strategies based on recombinant DNA or viral vectors, such as recombinant adenoviruses or virus-like particles (VLP) expressing whole tumor antigens or multiple defined T cell epitopes in a string-of-beads fashion.

In conclusion, the studies described show that prevention of tumor outgrowth can be achieved in a variety of models, including those for which no tumor antigen has been identified. Nevertheless, therapeutic effects of immune intervention on established tumors have been difficult to demonstrate. Even in those cases where tumor therapy was achieved, the time between onset of tumors and treatment was very limited relative to the situation found in patients with cancer. In general, human cancers are discovered only late after onset of oncogenesis. This allows the tumor a long time interval to acquire both heterogeneity resulting from tumor progression and immune evasive mechanisms. In cases when active induction of anti-tumor immunity is unlikely to be effective due to the advanced stage of the disease or the reduced immune reactiveness of the patient, immunotherapy may be performed by adoptive transfer of ex vivo activated tumor-specific T cells. The notion that T cells can potentiate anti-tumor responses came from animal tumor models, in which adoptive transfer of tumor-specific T cells resulted in eradication of established tumors (reviewed by refs. 20, 63). Moreover, CTL have been reported to exert anti-tumor activity in human beings. For example, ex vivo expanded autologous tumor-infiltrating lymphocytes displayed therapeutic activity in patients with malignant melanoma;[64] and CTL directed against viral proteins have been effective in the control of EBV-associated lymphoma.[65]

In all scenarios, current and future experiments in murine models will yield crucial knowledge for the development of immune intervention strategies.

Extrapolation to Humans: HLA Transgenic Mice and In Vitro Response Inductions

Since administration of antigen can either result in induction or tolerization of T cell responses, it is important to test potential immunotherapeutic protocols extensively before applying them in a clinical setting. To this extent, assessment of peptide binding to MHC molecules in vitro as well as response inductions in vivo in HLA transgenic mice and in vitro with human peripheral blood mononuclear cells (PBMC) (donor or patient-derived) are powerful tools.

A prerequisite for a peptide to represent a T cell epitope is its capacity to bind to MHC molecules. Class I binding affinity has a large impact on the immunogenicity of a CTL epitope.[66-68] Furthermore, stability of peptide-MHC class I complexes correlates with peptide immunogenicity.[69] Taken together, performing MHC binding and dissociation analyses on peptides of interest will yield indications for antigenicity. However, additional studies on in vitro and in vivo peptide immunogenicity are indispensable.

The usefulness of testing vaccines in HLA-transgenic animals depends on their ability to raise physiologically relevant human HLA-restricted T cell responses. For instance, transgenic mouse strains have been developed that express either the entire HLA-A*0201 or a chimeric HLA-A2K^b molecule. The latter chimeric molecule consists of α-1 and α-2 domains encoded by the human HLA-A*0201 gene and an α-3 domain of the murine H-2K^b molecule that permits a natural interaction with murine CD8 molecules. Importantly, Immunization of these transgenic mice with Influenza virus or a recombinant vaccinia virus expressing the hepatitis C virus envelope and core genes yielded HLA-A2-restricted murine CTL that recognized both murine and human targets expressing the viral antigen in combination with HLA-A2.[70-76] Moreover, the epitope specificity of the responding murine CTL was virtually identical to that of human CTL from virus-infected individuals. These data indicate that, in spite of species differences, both the antigen processing and

presentation pathways, as well as the T-cell repertoire, are flexible enough to allow a similar response when the same class I molecule is present.

These results have led to the use of HLA-A2 transgenic mice to assess in vivo immunogenicity of peptides capable of binding to HLA-A2.[66,68,77-79] In general, good concordance was found between the immunogenicity of peptides tested either in vivo in HLA transgenic mice or in vitro in CTL induction experiments with human PBMC, indicating that both approaches are feasible for the identification of potential human T-cell epitopes derived from antigens of choice. However, the CTL obtained were largely tested for their peptide specificity, and only incidentally for their ability to lyse (tumor) cells that naturally process and present these epitopes. Recognition of endogenously processed antigen is obligatory, since only that provides evidence for induction of immunologically "relevant" T cells.

Encouraged by the protective capacity of an HPV16-encoded peptide in mice, the identification of potential human CTL epitopes encoded by HPV16 was undertaken. For five common HLA-A alleles—together covering the HLA allele expression of a majority of the human population—candidate epitopes encoded by HPV16 E6 and E7 capable of binding to these MHC molecules were identified.[80] Of the high affinity HLA-A*0201-binding peptides, three HPV16 E7 peptides were immunogenic both in vivo in HLA-A2K^b transgenic mice and in vitro to PBMC of healthy donors. Human CD8$^+$ CTL clones against these three peptides lysed a human HLA-A*0201$^+$, HPV16$^+$ cervical carcinoma cell line, suggesting these peptides to represent endogenously processed human CTL epitopes of HPV16.[68] However, in vitro CTL induction protocols using healthy donor PBMC as the source for responding T cells have the intrinsic drawback that only a minority of obtained CTL will recognize both the exogenously added synthetic peptide and the endogenously processed antigen.[68,81,82] Factors that can contribute to this include:

1. The reduced requirement for high TCR affinity for the peptide on the induced CTL caused by the abundance of peptides pulsed on the APC used for in vitro restimulation;
2. The lack of efficient antigen processing and presentation of the peptide epitopes concerned; and
3. The limited numbers of specific CTL precursors in the blood of unprimed individuals.

Therefore, additional information can be obtained by employing lymphocytes from primed individuals as a source of responding T cells. Indeed, HLA-A2-restricted CTL responses against two of the HPV16 E7-encoded peptides described above were detected in a number of HLA-A2$^+$ cervical intraepithelial neoplasia and cervical cancer patients with proven HPV16 infections.[32,33] Taken together, these results show that the approaches taken allow insight into the specificity of (natural) immunity directed against HPV. However, they do not allude to the potency of immune intervention strategies against this virus. Therefore, preclinical animal models should be exploited.

In order to directly assess the effects of various HPV16-based vaccination strategies on the outgrowth of HLA-A2K^{b+} HPV16-induced tumors, we are developing an in vivo tumor model in HLA-A2K^b transgenic mice. Preliminary results suggest that vaccination with two HPV16 E7 Cew epitopes combined with a nonHPV-related T helper peptide, PADRE,[83] and emulsified in an IFA-like adjuvant, protects HLA-A2K^b transgenic mice against a subsequent challenge with a lethal dose of syngeneic HPV16 E6, E7 and EJ Ras-transformed cells (unpublished results). With the generation of various HLA class I and II transgenic animals, new tools are now available for analysis of both HLA-restricted CTL and Th responses induced by vaccination of animals against tumor antigens in vivo. Additionally, HPV16 transgenic animals expressing the E7 protein under control of keratinocyte-specific

promoters will become valuable in the study of (breaking) tolerance against tumor antigens. Lastly, SCID mice—deficient in both B cell and T cell immunity—may prove useful for adoptive transfer experiments to determine the effectiveness of human T cells against human target cells in vivo.

In conclusion, the use of both animal models and in vitro response inductions with human cells led to the identification and preclinical application of tumor antigens. The next step is to use appropriately formulated tumor antigens for clinical vaccination strategies aiming at the induction or augmentation of protective or therapeutic T cell-mediated immunity in human beings.

Clinical Trials

The first evidence for prevention of cancer resulting from immune intervention comes from preventive vaccination against HBV. Since the implementation of Taiwan's program of universal hepatitis B vaccination, the incidence of hepatocellular carcinoma in children has declined.[84]

The effectiveness of peptide vaccination for induction of immunity in human beings was first demonstrated in healthy volunteers, with no signs of preceding HBV infection. Injection of a covalently linked construct composed of a lipidated tetanus toxoid helper peptide and an HLA-A2-restricted HBV core CTL epitope appeared to be both safe and capable of inducing an HBV-specific CTL response, as detected by lysis of both peptide-pulsed and HBV-infected HLA-$A2^+$ target cells in vitro.[85]

First Clinical Results of Vaccination with Defined Tumor Antigens

The first results of immunotherapy trials with defined tumor antigens in patients suffering from melanoma or B cell lymphoma have been reported recently. Although not the initial goal of a phase I/II trial, s.c. administration of a peptide representing an HLA-A1-restricted MAGE-3 epitope in PBS resulted, unexpectedly, in tumor regressions in three melanoma patients.[86] However, no evidence was obtained for the presence of anti-MAGE-3 T cell activity in these patients and, therefore, the mechanism of tumor regression remains as yet unexplained. Alternatively, MAGE-1-directed, tumor-specific CTL were detected in another study consisting of three patients with advanced metastatic melanoma that had been vaccinated with autologous APC pulsed with an HLA-A1-binding MAGE-1 peptide.[87] Despite the presence of anti-tumor CTL, these patients did not show major therapeutic responses, possibly due to the advanced stage of the disease. Finally, clinical responses as well as concomittant anti-tumor T cell-mediated immunity after i.v. infusion with autologous DC pulsed in vitro with the patients' tumor-specific idiotype protein have been observed in three patients with follicular B cell lymphoma.[88] These results indicate that tumor regression may have been mediated by tumor-specific T cells that are activated by vaccination with the specific tumor antigen. Currently, DC-based clinical trials are also being applied in patients with a variety of cancers, including melanoma and multiple myeloma.[89]

Outlining Clinical Trials in Cancer Patients

Clinical trials based on vaccination with defined tumor antigens have been and will continue to be performed, initially with (advanced stage) cancer patients. Because of the complicated nature of such studies, the following points require attention.

First, the patient group to be treated must be selected. This can be achieved by genomic typing of patients with regard to HLA types and antigens expressed by the tumor cells using PCR technology. For instance, in an HPV16-based vaccination trial with cervical carcinoma patients, inclusion of 25% of the patients applying was estimated on basis of an occurrence

of 60% HPV16 DNA in cervical carcinoma cells and of 40% HLA-A*0201 in the human population. However, these estimated numbers have not been reached in practice, due to factors such as poor clinical condition or disease progression (manuscript in preparation). This shows that the actual numbers of patients included will be more limited than those selected solely on the basis of HLA type and tumor antigen expression.

Second, animal studies have already demonstrated that therapy of preexisting tumors is far more difficult than preventing outgrowth of a lethal tumor challenge by prophylactic induction of anti-tumor immunity. By analogy, compared to healthy volunteers, it will be more difficult to obtain detectable results by vaccinating patients in whom both natural immunity and conventional cancer treatment have failed. Escape from natural immune reactivity towards the tumor may result from production of paracrine factors by tumor cells[90,91] from poor immunogenicity or even tolerance-inducing capacity of cancer cells,[92,93] and from decreased immunocompetence of cancer patients.[94] Indeed, for patients with cervical lesions, indications have been reported that both antigen presentation by tumor cells and responsiveness of T cells in general can be affected.[29,30,95-97] Immunocompromising factors seem most pronounced in advanced stage cervical carcinoma patients, who showed a marked reduction both in influenza-directed CTL immunity and in proliferative responses against common recall antigens (manuscript in preparation). Nevertheless, in patients with early stage cervical lesions, specific CTL responses directed against influenza virus were only marginally decreased when compared to healthy donors, and occasional HPV16 E7-directed T cell immunity was detected.[32,33] Besides this, tumors which lack sufficient immunogenicity for the induction of T cell immunity can still be sensitive to T cell-mediated lysis,[98,99] indicating that patients might benefit from vaccination inducing vigorous anti-tumor T cell responses. Taken together, active immune intervention should preferably be performed during earlier stages of disease.

Third, assessment of effectiveness of the immunotherapy in patients constitutes a major issue. In vivo parameters can consist of monitoring tumor regression and delayed type hypersensitivity responses to immunizing agents. Alternatively, in vitro T cell reactivity can be measured by antigen-specific proliferation, cytotoxicity, or secretion of cytokines. Determination of the effectiveness of preventive vaccination in populations at risk will be especially important, yet even more difficult to perform, due to the relatively small proportion of infected people that progress to malignancy and the long lag period before transformation and tumor progression. This field requires sufficient attention, since preventive vaccination or combinational therapy are most promising with respect to clinical effectiveness.

HPV-Based Vaccination of Patients with Cervical Carcinoma

In cervical carcinoma patients, several clinical trials have been initiated that aim at inducing or enhancing T cell-mediated immunity against HPV.[100,101] The first three trials are based on Immunization with:

1. An HPV16 E7 fusion protein (Queensland University, Brisbane, Australia);[102]
2. A recombinant vaccinia virus encoding modified forms of HPV16 and 18 E6 and E7 protein sequences (University of Wales, Cardiff, UK); and
3. Two HLA-A*0201-restricted HPV16 E7-encoded peptides administered in combination with a pan-DR-binding T helper epitope (PADRE) emulsified in an IFA-like adjuvant (University Hospital, Leiden, The Netherlands).

A single dose of a recombinant vaccinia virus encoding modified forms of the HPV16 and 18 E6 and E7 protein sequences resulted in a tumor-free status in two patients with cervical carcinoma up to 21 months after vaccination. HPV18 E6/7-specific CTL were detected in one of these patients.[103] However, it is difficult to envisage that these T cells were responsible for tumor regression, since the tumor biopsy sample of this patient did not

contain HPV18 DNA and the E6 and E7 proteins of HPV16 and 18 display little homology. Furthermore, after repeated injection with an HPV16 E7 peptide-based vaccine, two patients have displayed stable disease for over one year. Additionally, local infiltrations of T cells at the site of vaccination were observed, and occasional proliferative responses to the PADRE helper peptide included were detectable after vaccination (manuscript in preparation). In none of these vaccination schemes was severe toxicity of the vaccines observed and, therefore, clinical trials in earlier stages of the disease—where they are more likely to be effective—are now feasible.

Taken together, the first results of these cervical carcinoma and other tumor immunotherapy trials are both encouraging and instructive for further anti-tumor vaccine development.

In several (ongoing) vaccination trials, including our own, clinical effects were observed after vaccination without detectable T cell reactivity against tumor antigens. The source of responding cells, the detection methods used, and the in vitro restimulation protocols followed, may not have been suitable for detection of T cell responses in these settings. Most studies so far have used PBMC, while the effective T cells may reside in the tumors or in the lymph nodes.[33] Moreover, both the immune status and tumor burden of the patients at start of vaccination are important for evaluation of the outcome. These factors do not necessarily hamper prospects for anticancer immunotherapy, but rather stress the importance of designing reliable methods to assess effectiveness of immunotherapy in patients. Future trials in which the given immunotherapy is compared to relevant controls will allow definite conclusions on the effectiveness of T cell-mediated prevention and therapy of malignancies.

Abbreviations

MHC	major histocompatibility complex
HLA	human leukocyte antigen
CTL	cytotoxic T lymphocytes
Th	T helper cells
APC	antigen-presenting cells
DC	dendritic cells
IL	interleukin
EBV	Epstein-Barr virus
HBV	hepatitis B virus
HPV	human papillomaviruses
E6 and E7	early regions 6 and 7
CIN	cervical intraepithelial neoplasia
IFA	incomplete Freund's adjuvant
BMDC	bone marrow-derived dendritic cells
LCMV	lymphocytic choriomeningitis virus
Ad5	adenovirus type 5
VLP	virus-like particles
PBMC	peripheral blood mononuclear cells

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Chapter 6

Recombinant Vaccinia Virus

Stephen Man, E.M.L. Evans, M. Nimako and L.K. Borysiewicz

Cervical cancer is the second most common female malignancy worldwide and remains a clinical problem despite improvements in early detection and therapy. Cervical cancer (and preinvasive CIN3) is strongly associated with infection by human papillomavirus (HPV), particularly types 16 and 18.[1] This association is dependent on two nonstructural viral proteins—E6 and E7—which are constitutively expressed in cervical tumors and maintain the transformed phenotype.[2] Immunologically, these proteins are tumor-specific yet foreign to the host, providing attractive targets for immunotherapy. However, their intranuclear expression focuses on major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) as the major effector mechanism to clear such transformed cells. Unfortunately, little is known about HPV-specific CTL in man, and their role in the immunobiology of this disease. In this chapter, we will review clinical testing of a live vaccinia virus vaccine containing modified HPV16 and 18 E6/E7 proteins and recent research findings using the same construct to detect and understand the role of naturally occurring HPV specific CTL in patients with cervical neoplasia.

Vaccinia Virus

Vaccinia virus was used for the prevention and global eradication of variola virus, the causative agent of smallpox.[3] The paths leading to this result, from Edward Jenner's initial clinical experiments and predictions, have been well chronicled. However, the origin of modern vaccinia strains is obscure. Genetically, vaccinia virus is closely related to but distinct from variola virus, cowpox virus and other naturally occurring members of the poxvirus family.[4] The WHO has agreed to destroy the two remaining frozen samples of variola virus in June 1999 to prevent recurrence of smallpox. However, research into this human poxvirus continues to define mechanisms of pathogenicity in poxvirus-associated diseases which may still become a substantial health care problem, e.g., monkeypox.[5] Vaccinia virus particles are complex in that the 300-400 nm enveloped particles contain the DNA (~200,000 bp) together with viral enzymes such as the RNA polymerase, capping and methylating enzymes and a poly(A) polymerase.[6] The genome encodes a range of viral enzymes and structural proteins, expressed in temporally regulated cascades termed early, intermediate and late phases. Early genes are the first transcribed and these stimulate growth in adjacent cells, protect against host immune responses, allow replication of the virus DNA and transactivate transcription of intermediate genes. The viral replicative cycle is cytoplasmic, and viral genes have vaccinia-specific promotors which in turn regulate these gene cascades, with specific sequences recognized by viral but not host cell factors.[6]

The effectiveness of vaccinia in the smallpox eradication campaign was the consequence of two factors: its physical properties (low cost, heat stability, simple method and visible proof of administration) and ability to induce a wide range of long-lived vaccinia specific

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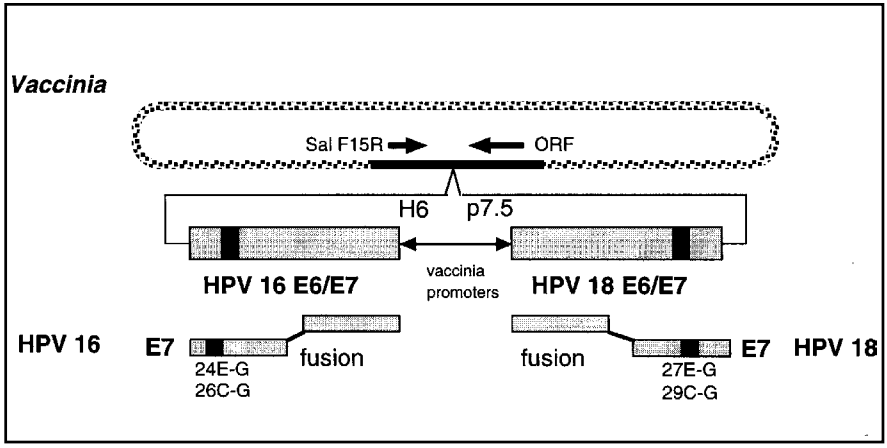

Fig. 6.1 Construction of TA-HPV. HPV16 E6/E7 and HPV 18 E6/E7 fusion genes were inserted into Wyeth strain vaccinia under the control of the H6 and p7.5 vaccinia promoters as described (Boursnell et al, 1996). The HPV E7 genes were modified as indicated to abolish binding to cellular retinoblastoma protein.

responses which crossreact with variola.[3] Unfortunately, although specific protection in animal models has been attributed to the ability to generate both humoral and cellular immunity (especially CTL), the relative roles of these responses in man is less well established.[7] This is further complicated by comparative studies of variola and vaccinia genomes which have revealed arrays of early phase genes interfering with induction of inflammatory and immune responses and modulating the pathogenicity of vaccinia.[8-10]

The ease with which vaccinia can be manipulated genetically and the high levels of expression of inserted genes have made vaccinia a powerful research tool.[6,11] Combining this with the success of vaccinia virus as a live vaccine, it has been widely promulgated as a vector for live recombinant vaccines in man.[12,13] This is supported by the ability of vaccinia recombinants to induce protective immunity, especially CTL, to a variety of inserted genes and host species,[14] including Immunization in the wild to prevent spread of rabies.[15] This has driven use of recombinant poxviruses as live vaccines against infection and for immunotherapy of cancer.

The immunological basis for these prospects has come from experiments, carried out mostly in rodents, where recombinant vaccinia is a potent inducer of antibody and $CD8^+$ cytotoxic T lymphocytes (CTL) to tumor-associated antigens[16] and viral oncogenic proteins.[14,17] The observation that most tumor-specific antigens and oncogenic viral proteins are intracellular, together with the important role of CTL in the clearance of virus-infected cells,[18] encourages current research using vaccinia as a vector for cancer immunotherapy.

Human Papillomavirus

HPV is a frustrating virus to study immunologically for two main reasons:

1. It cannot be grown readily in vitro, and knowledge of immune responses has relied on expression of HPV gene products from recombinant vectors or by transfection;
2. While there are animal papillomavirus models which mimic aspects of human disease, particularly papilloma formation, there is no animal model which can mimic

the progression of HPV induced genital lesions to invasive cancer as observed in man.[19]

Nevertheless, important concepts relevant to immunotherapy of HPV-associated cervical cancer have been established from animal experiments. HPV16 E6 and E7 gene products are immunogenic to rodents, inducing CTL in mice[17,20] and rats.[21] Furthermore, the HPV-specific CTL generated could mediate specific rejection of tumor cells transfected with HPV16 antigens.[20,21] $CD4^+$ T cell-mediated immune responses can also be induced by HPV antigens. HPV16 E7 vaccinia virus recombinants have induced DTH reactions in mice bearing transplanted mouse keratinocytes expressing HPV16 E7 proteins.[22] However, immune responses against HPV gene products can be controlled at many levels, for example the viral antigen dose can be crucial in determining immunity or tolerance.[23]

Full length HPV proteins are not required for protective immunity. In mice, protection from tumor challenge can be achieved after Immunization with a single immunodominant peptide from HPV16 E7,[24] although definition of peptide epitopes from HPV gene products is not straightforward. Peptides predicted from MHC binding motifs[25] are not always those which are immunogenic to CTL.[26,27] Collectively, these studies established that HPV16 E6 and E7 gene products were immunogenic to murine CD4 and CD8 T cells; however, there was little evidence at this time that this was the case in man.[28]

Clinical Trials

Construction of TA-HPV

In order to test whether CTL could be induced against HPV E6 or E7 proteins in vivo, a recombinant viral vaccine (TA-HPV) was developed.[29] This was based on vaccinia virus Wyeth strain, because of its low rate of complications among the various strains used during the smallpox eradication campaign.[30] The lytic life cycle of vaccinia, with cytoplasmic replication, has advantages for delivery of the HPV E6 and E7 genes. These gene products immortalize cells in culture.[31,32] Consequently, there is a risk that introduction of these genes into human cells in vivo could lead to long term expression which could result in cell transformation. Quantification of this risk is extremely difficult. However, since vaccinia is a lytic virus and does not establish persistence or latency,[3] the likelihood that a cell infected with a recombinant expressing E6 and E7 could survive and transform is very small. Furthermore, the expression of the inserted genes is regulated by vaccinia virus promotors, which differ substantially from mammalian promotors and rely on vaccinia-encoded transcription components.[6] It is therefore unlikely that DNA released from vaccinia virus infected cells could be taken up by healthy cells and expressed.

To further reduce these potential risks, the HPV sequences inserted in the vaccinia were also modified. The E7 genes of both HPV16 and 18 were modified by site-directed mutagenesis to the Rb-binding site (Fig. 6.1). Reduced Rb-binding following this modification was demonstrated and cotransformation activity abolished in rat fibroblasts.[29] A further consideration to prevent natural recombination with any HPV strain that may be present at the site of vaccination was the reorientation of E6 and E7 relative to each other and the organization of these two genes as a fusion protein. Although these modifications could have ablated or even created artifactual CTL epitopes, it was considered an appropriate modification, bearing in mind the linear nature of CTL epitopes and the excess of primary structure made available by inclusion of whole proteins rather than subunits of E6/E7. In preclinical testing, Immunization of C56BL/6 mice with TA-HPV induced CTL responses against HPV16 E7.[29]

Phase I/II Trial of TA-HPV in Patients with Advanced/Recurrent Cervical Cancer

The safety and immunogenicity of TA-HPV was investigated in a phase I/II trial of 8 patients with advanced or recurrent cervical cancer. Fifty-eight patients were screened for immunocompetence prior to entry into the study. Assays included total leukocyte count, complement profile, immunoglobulin levels, T-cell subsets and ability to respond to pneumococcal vaccine in vivo. These responses were compared with PHA transformation as the measure most commonly employed to link disseminated vaccinia with immunodeficiency during the smallpox eradication campaign.[3,30] In the light of the problems identified with disseminated vaccinia and a very low CD4 count with HIV infection,[33] stringent criteria (CD4 count >400 μl) were adopted in this population that will have been suppressed by primary and secondary treatment for their disease. The majority tested was excluded on this criterion. For all patients recruited, biopsy samples were HPV16 positive and negative for HPV18, 31 and 33. Vaccination was by multipuncture intradermal scarification through a 20 μl drop of TA-HPV (10^8 pfu/ml). To prevent environmental release of an untried agent, patients were isolated in an Infectious Disease Unit isolation suite for two weeks after vaccination. Subsequent testing revealed that live virus could only be recovered from either the vaccination site or the dressing that contacted it. No infectious virus could be recovered from the environment, and there was no systemic spread. No short- or medium-term complications of vaccination were observed. Furthermore, the inserts were stable by PCR following in vivo passage of the recombinant.[34]

All patients produced detectable anti-vaccinia IgG antibodies after vaccination, confirming successful "take" of the vaccine. While all patients had preexisting HPV16 E6 or E7 antibody responses (Table 6.1), HPV18-specific antibody responses could be detected in patients 1, 3 and 4 after vaccination. Initial attempts to generate HPV-specific CTL used TA-HPV infected cells for secondary in vitro stimulation, but only nonspecific killing was observed (Graham S, unpublished observation). This restimulation protocol was modified to avoid the problems associated with use of vaccinia virus recombinants in this way.[7,35] HPV-specific CTL responses were then measured in 6 patients by stimulation of patient PBMC in vitro using replication deficient recombinant adenoviruses encoding HPV16 E6/E7 and HPV18 E6/E7. However, PBMC from only 3 of the patients (1, 3 and 6) responded to control stimuli such as mitogens, mixed lymphocyte culture and recall antigens, despite pretrial screening for immunocompetence. Only these PBMC samples were deemed evaluable. No HPV-specific CTL were detected from patients 1 and 3; however, HPV-specific CTL could be detected from the PBMC of patient 6 (Table 6.1). These CTL were detected 9 weeks after vaccination, after restimulation with HPV18 E/E7 adenoviruses in vitro (Fig. 6.2). Interestingly, the operationally defined HPV18-specific CTL response was a transient one, as no CTL responses could be detected at 14 or 20 weeks postvaccination. This may reflect a loss of HPV-specific CTL activity and/or an increase in nonspecific lysis of controls. The HPV18-specific CTL detected at week 9 were not present before vaccination, and the tumor biopsy from patient 6 was HPV16 positive and HPV18 negative, suggesting that this response was a result of Immunization with TA-HPV.

While no therapeutic effect can be ascribed to vaccination on the basis of this study (5 of 6 patients died from disease), anecdotally, patient 6 has remained well and tumor free, some 5 years after the study. Clinically, patient 6 is interesting for a number of reasons. Firstly, this patient presented in 1986 and was treated by radical hysterectomy and radiotherapy for FIGO Stage 1b disease. Radiological recurrence was noted in 1991 and there was partial response to further treatment. However, at the time of vaccination (July 1994) a CT scan suggested her recurrent pelvic tumor was still present, although reduced in size following single-agent cisplatin treatment in 1992. Secondly, this patient was initially

Table 6.1

			Prevaccination Responses					Postvaccination Responses			
Patient	**Tumor type**	**FIGO stage**	**HPV type**	**Prev vaccinia**	**Vaccinia abs**	**HPV abs**	**HPV CTL**	**Vaccinia abs**	**Vaccinia abs**	**HPV CTL**	**Clinical Outcome**
1	Adenosquamous	Ib	16	Yes	Yes	Yes	No	Yes	Yes(new HPV18)	No	Died
2	Squamous	IIb	16	No	No	Yes	No	Yes	Yes	No	Died; residual tumor
3	Squamous	Recurrent	16	Yes	Yes	Yes	No	Yes	Yes(new HPV18)	No	Died; renal failure
4	Squamous	Recurrent	16	Yes	Yes	Yes	No	Yes	Yes(new HPV18)	No	Died; intestinal perforation
5	Squamous	Recurrent	16	Yes	Yes	Yes	No	Yes	Yes	No	Died; intestinal perforation
6	Squamous	Recurrent	16	Yes	Yes	Yes	No	Yes	Yes	Yes, HPV 18	Alive; tumor free
7	Squamous	IIIb	16	No	No	Yes	No	Yes	Yes	No	Died; intestinal obstruction
8	Adenosquamous	Recurrent	16	Yes	Yes	Yes	No	Yes	Yes	No	Died; residual tumor

Summary of results from Phase I/II clinical trial with TA-HPV. Both HPV specify antibody (ab) and cytotoxic T lymphocyte (CTL) responses were examined. Previous exposure to vaccinia was assumed if patients had been immunized against smallpox.

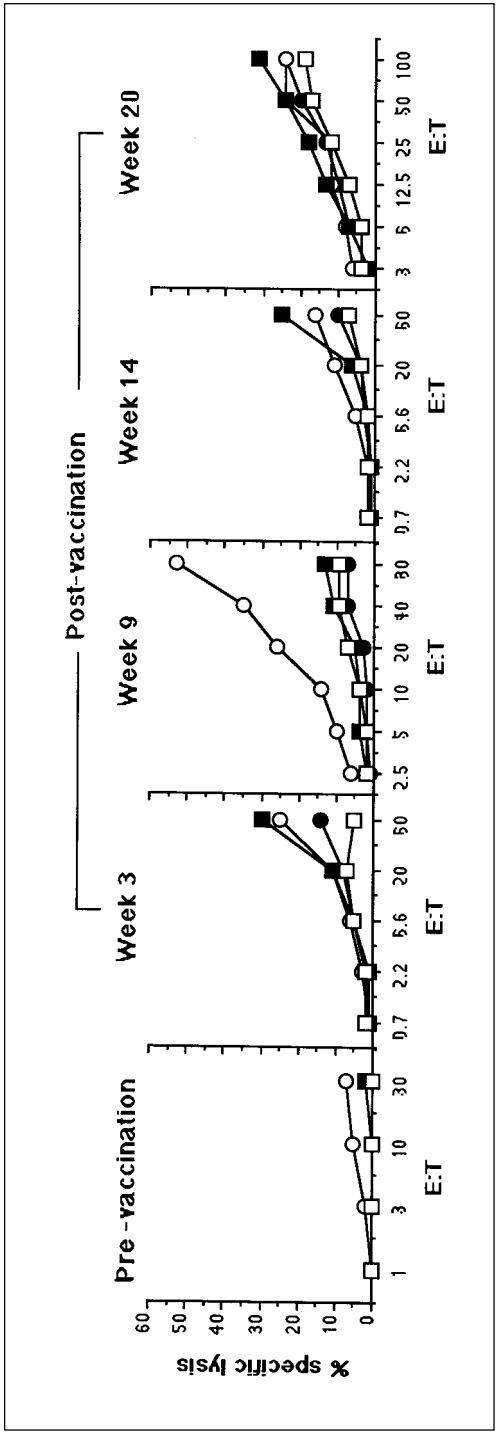

Fig. 6.2. CTL responses following stimulation with RAd102 (HPV18 E6/7) at various time-points pre and post vaccination with TA-HPV. Figure courtesy of Julian Hickling, Cantab Pharmaceuticals. Peripheral blood mononuclear cells were restimulated with irradiated PHA blasts infected with RAd102 on days 0 and 7 before testing in 51Cr cytotoxicity assay on day 14. Autologous B-LCL target cells were: TA-HPV infected (○); uninfected (●); Wyeth (vector control) infected (■); and TA-HPV infected allogeneic (□).

excluded from this study due to a CD4 count below the exclusion limit (<400 CD4 cells/μl); however, ethical permission for vaccination to proceed was granted when the CD4 rose to 320 CD cells/μl. Thirdly, this patient did not produce an anti-HPV antibody response and had preexisting immunity to vaccinia as evidenced by anti-vaccinia antibodies. As an HPV-specific CTL response was detected in this patient, it will be interesting to assess the relationship between cell-mediated responses and these parameters in future studies.

This study, the first European study to use a recombinant viral vaccine in man, suggested that TA-HPV was capable of inducing both antiviral antibody and CTL responses. These results suggested that further studies on dosage and on patients with earlier stages of cervical cancer were warranted.

Immunogenicity of TA-HPV in CIN3 Patients

We have recently completed a further trial involving 12 CIN3 patients. The purpose of this trial was to further investigate the immunogenicity of TA-HPV by delivering two doses of vaccine (8 weeks apart) to an immunocompetent patient group. Again, clinical efficacy was not a primary endpoint of the study, as all vaccinated patients had standard surgical treatment of their CIN3 lesions. At the time of writing, immune evaluation, especially of CTL, was in progress. However, all patients produced anti-vaccinia antibody responses, indicating uniform "take" of the vaccine as observed in the earlier phase I/II study.[34] In contrast to the previous trial with TA-HPV, no anti-HPV antibodies could be detected in this group either pre- or post-vaccination. Preliminary results confirm our observations in CIN3 patients (ref. 36; see below) that HPV E6/E7-specific CTL can be detected in blood prior to Immunization. However, new HPV-specific CTL responses could also be detected after Immunization in 3 further patients who did not have preexisting CTL responses, confirming the single observations made in patient 6.[34] No effect of the additional dose of vaccine or any relationship between prior exposure to vaccinia and detection/development of HPV CTL responses post-vaccination was observed (Man S, in preparation).

Conclusions from Clinical trials

The clinical trials performed with TA-HPV to date have not assessed clinical efficacy but have provided encouraging results relating to immunogenicity, which warrant further evaluation. Further results of immune evaluation following a US-based trial of TA-HPV on 12 patients with late stage cervical cancer are awaited. We are engaged in three further studies to evaluate TA-HPV, with an emphasis on whether it will prove clinically effective:

1. A European trial (coordinated by EORTC) has started and will test 44 cervical cancer patients with early stage disease. Each patient will receive two vaccinations of TA-HPV, one before surgery and one following surgery. Patients will then be monitored for immunological response at a central laboratory to minimize experimental variation. These patients will receive standard treatment for disease, and so this trial can not assess efficacy in the short term. However, it is planned to monitor frequency of disease recurrence in this patient group compared with nonvaccinated controls. Whilst recruitment should be completed by 1999, results from this analysis will not be available for 5-8 years.
2. A small study of CIN3 subjects is being undertaken to evaluate regression in CIN3 in the short-term (<3 months following TA-HPV vaccination. This study will monitor local disease colposcopically and with biopsy, as well as making a fragment of the final excision biopsy available for further immunological evaluation. This will permit evaluation of migration of vaccination induced CTL to sites of disease.
3. A large study of the role of CTL in the natural history of HPV-associated disease is being undertaken in collaboration with the MRC Reproductive Biology Programme

in The Gambia. Although in its early phases, this is of particular importance, as it addresses the issue of the role of CTL in patient populations where an immunotherapeutic approach might offer greatest clinical benefit.

From a human cellular immunologist's point of view, the most important conclusion from the clinical trials to date has been that HPVE6/E7 proteins are capable of inducing cytotoxic T lymphocyte responses in humans. This provides the basis for defining the peptide epitopes recognized by such CTL and for determining the duration of T-cell memory after vaccination. However, current assay systems for measuring CTL responses are crude, requiring large volumes of blood and are labor intensive. Assays need to be refined and simplified if they are to form the basis of surrogate measures in clinical studies. This may require the application of novel technologies such as fluoresceinated MHC-peptide complexes to stain antigen-specific T cells[37] or elispot assays to measure cytokine release.[38] These approaches have increased the sensitivity as well as simplicity of detection of human immune effector cells, although important issues relating to their comparative sensitivity with conventional approaches, as well as their functional specificity, remain to be addressed. Incorporation of these technologies into future clinical trials may allow simultaneous examination of both CD4 and CD8 T cells from limited clinical samples.

We have concentrated on measurement of $CD8^+$ CTL responses; however, this reflects our current expertise and specific interest in the role of these effector cells and does not obviate a role for the $CD4^+$ T-cell. There is increasing evidence that CD4 responses are important in HPV-associated cervical neoplasia.[39-41] Although antibody responses were assessed in the clinical trials, no assays to measure DTH or T-cell proliferation assays against HPV antigens in vitro were performed; however, these will be incorporated into future studies.

Are Recombinant Vaccinia Virus-Based Vaccines the Ideal Vectors for HPV-Associated Cervical Disease?

As described above, the choice of vaccinia as a vector was related to specific features which pragmatically enabled their rapid utilization in clinical studies in man. This choice was based on:

1. The requirement for a large insert size;
2. Lytic replicative cycle and use of virus-specific promotors, limiting expression to cells which already have triggered the lytic replicative cycle;
3. No virus persistence in the host;
4. The documented safety of vaccinia in the smallpox eradication campaign;
5. The lack of animal models of cervical cancer, requiring a rapid implementation of any vector in man.

All of these factors are relative rather than absolute in the choice of vector, and while valid in the first phase of investigations they may be superceded by developments in vector technology.

Safety

The adverse reaction rates for vaccinia viruses in the smallpox vaccination campaign were about 1 in 50,000, and higher for primary as opposed to repeated exposure.[42] Clearly the morbidity and mortality of smallpox meant that this risk was acceptable, although even in the later stages of the eradication campaign the issue of vaccination-induced morbidity was raised.[3] Similar arguments are raised over the use of recombinant vaccinia virus vaccines for cancer immunotherapy; however, in late stage disease the potential benefits outweigh the risks. Nevertheless recombinant vaccinia virus vaccines are composed of live viruses capable of replication and spread, which is of particular concern in immunocompromised individuals and an important issue in those receiving immunosuppresive therapies. Not only is this

suppression pharmacologically induced, but it is often apparent because of the nature of the malignancy; this is the case with cervical cancer [43] and limits recruitment into clinical trials.[34] Two approaches may be adopted, firstly to rigorously screen the patient population (as in our studies, see refs. 34, 43), or secondly to modify the vector to reduce its pathogenicity.

This drive to improve safety has lead to the development of new strains such as modified vaccinia virus Ankara (MVA), NYVAC and avipox viruses. MVA was attenuated by passage in chicken fibroblasts and continues to grow well in these cells, but has lost its ability to replicate in mammalian cells and is apathogenic even in imunodeficient mice.[44,45] Recent studies in mouse models suggest that this vaccinia strain is as effective as replication competent vaccinia in inducing murine CTL responses.[45] The attenuation of this virus is due to multiple deletions resulting in a failure of late stage morphogenesis, which inhibits release of infectious virus from mammalian cells while allowing the expression of viral proteins in the infected cell.[46] Furthermore, MVA was used to vaccinate 120,000 subjects at high risk of side effects from vaccinia without untoward effect.

NYVAC was developed by the deliberate deletion of 18 ORFs in the fully sequenced genome of Copenhagen strain vaccinia.[47] This virus has a debilitated replication phenotype in mammalian cells, yet replicates in chick fibroblasts and, similarly to MVA, has reduced pathogenicity in immunocompromised animals. However, although it has been used in a number of veterinary vaccines, most notably in Japanese B encephalitis in swine and some small trials of human vaccines, it lacks the safety profile established for MVA and other vaccinia strains.[13]

Avipox viruses are restricted in their host range to avian species, yet expression of inserted genes will occur in mammalian cells in the absence of virus replication.[13] Again, this vector has been used in veterinary vaccines in a number of species. HIV vaccination of seronegative subjects using a canarypox vector (with adjuvant recombinant env subunit) has been studied, with an enhanced ability to generate CTL (either $CD4^+$ or $CD8^+$) over subunit alone.[48]

These modifications to conventional vaccinia strains (WR, Wyeth and Copenhagen) have allowed longer expression of antigen in the host and improved their safety profile for immunocompromised subjects. However, this has been achieved at the cost of the full lytic replicative cycle of the virus, with reduced cytopathology in the infected cell. When considering a vector for immunotherapy in man, the nature of the insert often dictates the choice of vector.[29,34] HPV E6 and E7 are potentially oncogenic and, while site-directed mutagenesis can block Rb-binding, it is essential to prevent longterm expression and avoid any persistence of these gene products in the host. These considerations dominated the choice of vector and certainly would not readily permit the use of the less well characterized vaccinia variants described above. It has to be remembered that the choice of vaccinia limits the duration of antigen expression in the host and, while this is necessary to reduce risks to the patient to a minimum, it is undoubtedly suboptimal for efficient antigen presentation. Therefore, the choice of vaccinia is appropriate for these investigations but as other vectors are better characterized and experience of their use in man grows, alternative delivery systems with the required characteristics may be developed.

Role of Prior Antivaccinia Immunity

One of the desirable qualities of vaccinia as a vector is its potent induction of humoral and cellular (including $CD8^+$ CTL) immune responses. In general, these are stronger than those generated using peptide, protein or DNA Immunization. It has been argued that this may be an Achilles heel of vaccinia, as preexisting immunity to vaccinia in those vaccinated against smallpox may limit subsequent responses against recombinant vaccinia virus vaccines.[49] While this has been shown in some animal models,[50,51] leading to development

Table 6.2

CIN3 Subjects	Age	Treatment	HPV type	Sample Timing	CTL specificity
VM	44	Cone biopsy/hstmy	NT	Post-treatment	16
AT	38	LLETZ[a]	NT	Pretreatment	18
GB	47	LLETZ	NT	Pretreatment	
MM	35	LLETZ	16	Pretreatment	16
LF	35	Laser vaporizn	16	Pretreatment	
SB	40	Laser vaporizn	HR	Pretreatment	
VMD	25	Laser vaporizn	HR	Pretreatment	16&18
BJ	28	Laser vaporizn (x2)	16	Pretreatment	18
HF	29	Laser vaporizn	NT	Pretreatment	
SD	37	Laser vaporizn (x2)/hystmy	NT	Pretreatment	16

Naturally occurring HPV-specific CTL responses in CIN3 patients. PBMC samples were obtained from patients with histologically confirmed CIN3. HPV typing was performed by PCR of DNA obtained from archival biopsy material. HR=high risk HPV DNA, either HPV types 16, 18, 31 or 33, NT=not tested due to poor quality of sample. CTL assays on TA-HPV infected targets were performed after 3 weeks of in vitro restimulation with recombinant adenoviruses encoding either HPV16 or HPV18.

of heterologous priming regimes,[52] there is no consensus that this is the case in man. In a study of 18 HIV seronegative individuals immunized with a recombinant HIVgp160-vaccinia, the authors suggested that vaccinia virus vaccines might be more efficacious in vaccinia naive individuals.[49] However, all recombinant vaccinated individuals produced both antibody and T-cell proliferative responses against HIV gp160. Antibody and proliferation responses were reduced on average in the vaccinia primed group compared with the vaccinia naive group, but the sample size of two respective groups (16 naive vs. 2 vaccinia immune) was insufficient to suggest this as a general rule. Results from our recent clinical trials, although limited in the same way by numbers, suggest that previous exposure to vaccinia did not limit the induction of CTL responses by a recombinant vaccinia-based vaccine.

In mice, antivaccinia CTL can be detected directly from the freshly isolated splenocytes of vaccinia immunized animals.[50] Such CTL could limit development of CTL responses after vaccination with recombinant vaccinia virus. However, despite widespread smallpox vaccination, clear-cut antivaccinia CTL responses have been difficult to detect in man.[7,35,53,54] This may be a technical problem, as distinguishing vaccinia-specific CTL from the multiplicity of nonspecific killer cells in PBMC is difficult and is compounded by the lytic replicative cycle of the virus, making in vitro restimulation difficult.

Antigen Processing and Immunogenicity

Examination of the vaccinia genome has identified a number of genes which contribute to its virulence, and their selective deletion may result in the development of safer poxvirus vectors.[10] Interestingly, many of these genes interfere with immune responses against the virus or in the generation of inflammation at the site of disease.[8-10] Early reports on murine responses against influenza A suggested that vaccinia virus could inhibit the processing and presentation of certain viral polypeptides[55,56] via a mechanism unrelated to vaccinia virus-encoded serine protease inhibitors.[57] While there have been no further reports of such inhibition, another concern expressed about using vaccinia virus recombinants expressing

full length gene products is that the viruses may not produce the relevant CTL epitopes, either qualitatively or quantitatively. To overcome this perceived limitation, some have used minigene constructs in which only the epitope of interest is encoded.[58] This removes the rate-limiting step of antigen processing, and should result in more efficient delivery of the peptide to nascent class I molecules in the endoplasmic reticulum. Multiple epitopes could be incorporated in a "string-of-beads" fashion to induce CTL of multiple specificities.[59] In addition to CTL epitopes, epitopes designed to stimulate CD4 T cells could also be incorporated.[60,61] Alternatively, selecting either class I or class II-restricted responses could be accomplished by targeting antigens to different pathways: towards the endoplasmic reticulum for class I responses,[62] ubiquitination for rapid degradation/MHC class I presentation[56] or towards the endosomes for class II responses.[63]

The immunogenicity of vaccinia virus recombinants could also be improved by the incorporation of cytokine genes.[64] In animal models, these promote tumor rejection either through enhancement of antigen processing[65] or through promoting activation or growth of CTL.[66] Induction of CTL could be further enhanced by the inclusion of linked helper epitopes in addition to inclusion of CTL epitopes.[60] Finally, enhanced induction of immune responses by vaccinia could be achieved by maximizing gene expression in the most potent APCs. Recently it has been found that the type of vaccinia virus promoter utilized in the recombinant will determine the levels of antigen expression in dendritic cells and other APCs.[67]

These modifications suggest that the full potential of poxvirus vectors as gene/antigen delivery systems are still to be developed, and therefore this virus will remain a major experimental and clinical delivery system for some time to come.

Use of Recombinant Viral Vectors to Investigate Role of Naturally Occurring HPV-Specific CTL in Cervical Neoplasia

Naturally Occurring HPV-Specific CTL in CIN3 Patients

The methods developed to assess TA-HPV-generated CTL in vaccinated patients have been used to ask a more fundamental question: Are HPV E6/E7-specific CTL induced by naturally infected/transformed cells? It was thought that such responses might be difficult to detect. Despite strong murine immune responses, there was little evidence (with the exception of antibodies) that these proteins were immunogenic in man (discussed in ref. 28). The murine responses observed did not follow natural infection, as HPV is not a pathogen of mice. They were induced by Immunization with transfected cells,[20,21] recombinant vaccinia viruses[26] or peptides.[24] The immunosuppression encountered in cervical cancer patients as a consequence of disease or treatment suggested that these would not be a good population in which to study naturally occurring immune responses.[43,68]

Therefore, we studied 10 patients presenting with colposcopically and histologically confirmed CIN3 (Table 6.2). This group was interesting for several reasons:

1. Unlike cancer patients, neither disease nor treatment results in immunosuppression;
2. It is predicted that 70-80% of CIN3 patients will have evidence of HPV infection;
3. This disease group contains patients who may experience spontaneous regression of their lesions, as well as those who may progress to cancer.[69]

These factors allowed the study of a group with HPV-transformed cells following natural exposure without the potential impact of progressive and invasive malignancy on their immune responses.

Using the adenovirus restimulation protocol, we found HPV-specific CTL responses in most patients tested (Table 6.2), not, however, in control subjects.[36] Recently, these results have been confirmed in studies on Dutch CIN3 patients (Bontkes H, personal

Table 6.3

Target	Shared HLA	5-D10	10-B4	10-B5
MM+TA-HPV	A2, A24, B7, B44	37±1	28±1	35±1
BS+TA-HPV	A2	39±1	3±1	5±1
SM+TA-HPV	A24	2±1	20±3	1±1
MN+TA-HPV	B7	11±1	2±1	39±1
LM+TA-HPV	B44	0±1	0±1	4±2
CaSki	A2, B7	32±1	7±6	50±3
MDA-231	A2	3±1	12±1	15±5

HLA-restriction pattern of HPV specific CTL lines from CIN3 patient MM. Indentification of restricting HLA alleles for recognition of HPV16 E6/E7 by three CTL lines derived from patient MM (Fig. 6.3). These were tested on HLA typed B-LCL lines targets infected with TA-HPV or cervical carcinoma cell lines (CaSki, HPV 16^+) or a breast carcinoma epithelial cell line (MDA-231, $HPV16^-$). The results represent % specific lysis ± SE at an E:T ratio of 20:1.

communication). The adenovirus restimulation protocol was reproducible, as HPV-specific CTL responses were obtained in one patient at multiple time points (Fig. 6.3). Furthermore, propagation of a series of CTL sublines from this patient demonstrated recognition of HPV antigens in the context of multiple HLA alleles and recognition of an HPV16-transformed cervical carcinoma cell line (Table 6.3). This suggests that restimulation with recombinant adenovirus was able to select CTL with multiple specificities and the ability to kill a naturally HPV-transformed cervical keratinocyte. The utility of the recombinant adenovirus approach for restimulation has been confirmed by its ability to detect subdominant EBV antigens.[70]

Higher detection rates of HPV-specific CTL were obtained in this study compared to other published studies.[71,72] This is either a reflection of the patient group studied or a consequence of the use of full length HPV E6/E7 proteins to stimulate CTL, allowing development of CTL recognizing multiple epitopes within E6/E7 proteins and restricted by multiple HLA alleles. Testing of these CTL with a limited number of HPV16 peptides[73] has not revealed any reactivity as yet (Nimako, unpublished observations). Studies in mice suggest that it might be difficult to predict peptide epitopes for HPV-specific CTL,[26,74] and the best approach may be to sequence directly from peptides bound to MHC molecules of HPV-transformed keratinocytes as for other human tumor antigens.[75]

HPV-Specific CTL Infiltrate Sites of Disease

The study described above, in common with many other studies of human CD8 CTL against viruses or tumors, only examined responses in the peripheral blood. If CTL are to be effective in immunotherapy, then they must be able to home to tumor tissue. Therefore we sought to determine whether such CTL were present at sites of disease. We obtained PBMC, lymph node (LN) and tumor samples from patients with advanced cancer undergoing surgery. As controls, similar samples were obtained from patients undergoing hysterectomy for nonmalignant reasons. All samples were tested for CTL responses using two different protocols.

Firstly, a peptide restimulation protocol[71] designed to detect secondary CTL responses in HLA-A*0201^+ patients, was used to examine CTL. Four out of 5 HLA-A*0201 patients (P1-P6) made a detectable CTL response against HPV16 E7 11-20 peptide. No CTL

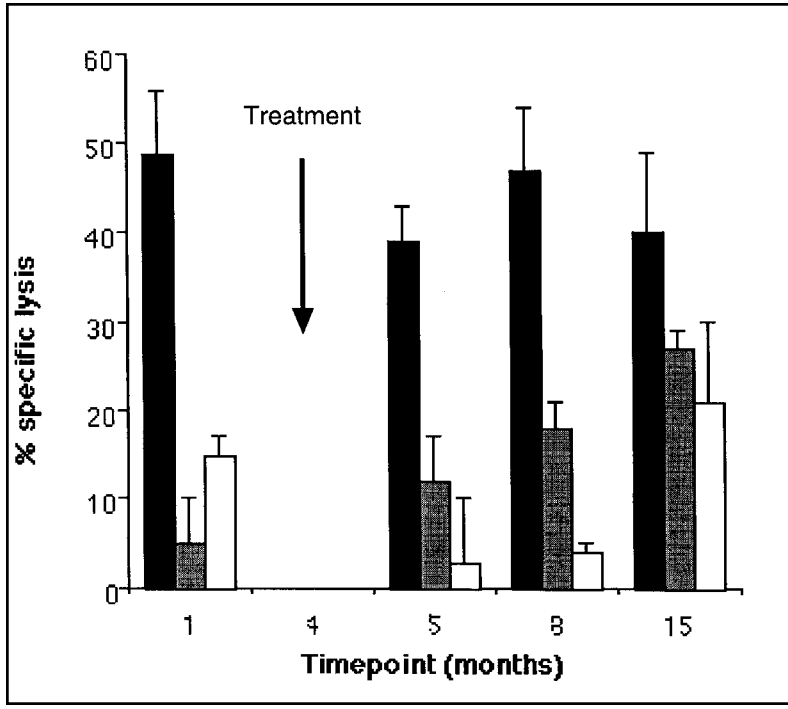

Fig. 6.3. Reproducibility of HPV-specific CTL responses. PBMC from patient MM were taken at various timepoints over 15 months and tested for HPV specific CTL responses after in vitro restimulation with RAd101 (HPV16 E6/E7). Targets tested were autologous B-LCL; infected with TA-HPV (■), infected with Wyeth strain vaccinia (▩), or uninfected (□). Results shown are % specific lysis + SE at an E:T ratio of 60:1.

responses were detected in normal controls (C1-C7) (Fig. 6.4). No responses were made against an HLA-A*0201 binding peptide derived from the malarial parasite *P. falciparum*, while all donors were able to make a memory CTL response against influenza A matrix peptide. These responses are consistent with the observed HPV16 peptide responses being a memory CTL. CTL responses against this peptide were detected in 3 of 4 LN and 1 of 3 TIL.[76] Despite the nonresponsiveness of PBMC, LN, and cervix-infiltrating lymphocytes (CIL) in normal controls, precursor CTL specific for HPV antigens do exist, as primary CTL against either HPV16 $E7^{11-20}$ (Fig. 6.5) and HPV16 $E7^{86-93}$ (Evans E, unpublished observations) can be generated in PBMC from female and male controls. In all cases, recognition of endogenously expressed antigen in the form of TA-HPV targets, as well as HPV16-transformed CaSki cells, was observed (Fig. 6.6). It should be noted that in contrast to other studies using CaSki cells as targets, γ-IFN was not used to enhance MHC class I expression.

Secondly, these findings were extended by quantitation of the HPV-specific responses using limiting dilution analysis (LDA) in the PBMC, LN and TIL of patients. Interestingly, while frequencies in PBMC and LN were low and similar, there was enrichment of CTL recognizing HPV in TIL (Table 6.4). The specificity of these TIL CTL for HPV has been confirmed by subsequent cloning and long term propagation. It was found that 80% of

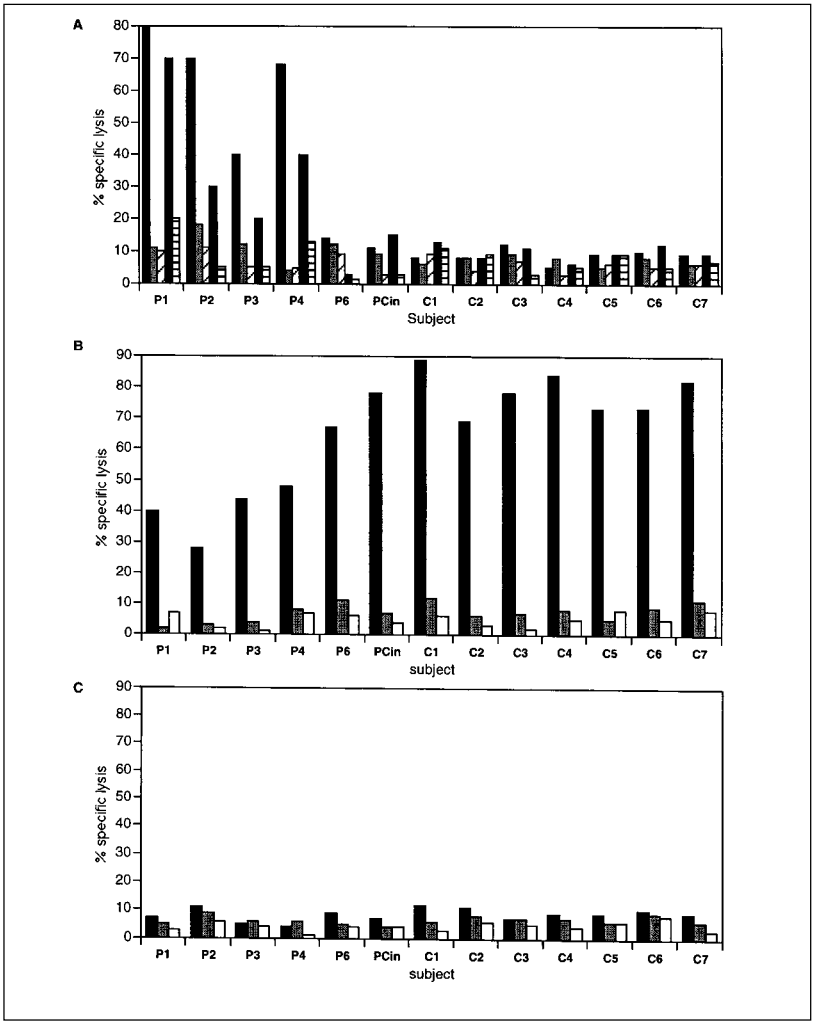

Fig. 6.4. HLA-A*0201-restricted peptide-specific CTL in PBL from patients and controls after 14d culture with (a) HPV-16 E7;[11-20] (b) influenza A M1;[58-66] and (c) Plasmodium falciparum cp36. Results show how representative % specific lysis at an E:T ratio = 100:1 appropriate peptide-pulsed (■) C1R.A2 targets in a chromium release assay, in the presence of K562 cells. Other targets included C1R.A2 cells loaded with an irrelevant HLA-A*0201-restricted peptide (▩), no peptide (□), infected with the recombinant vaccinia virus, TA-HPV (■) or the parental Wyeth vaccinia (▭). All assays are performed at multiple E:T ratios, and a positive result was defined as % specific lysis >10% above that of control targets at 2 or more E:T ratios (SD for all assays was <5% specific lysis).

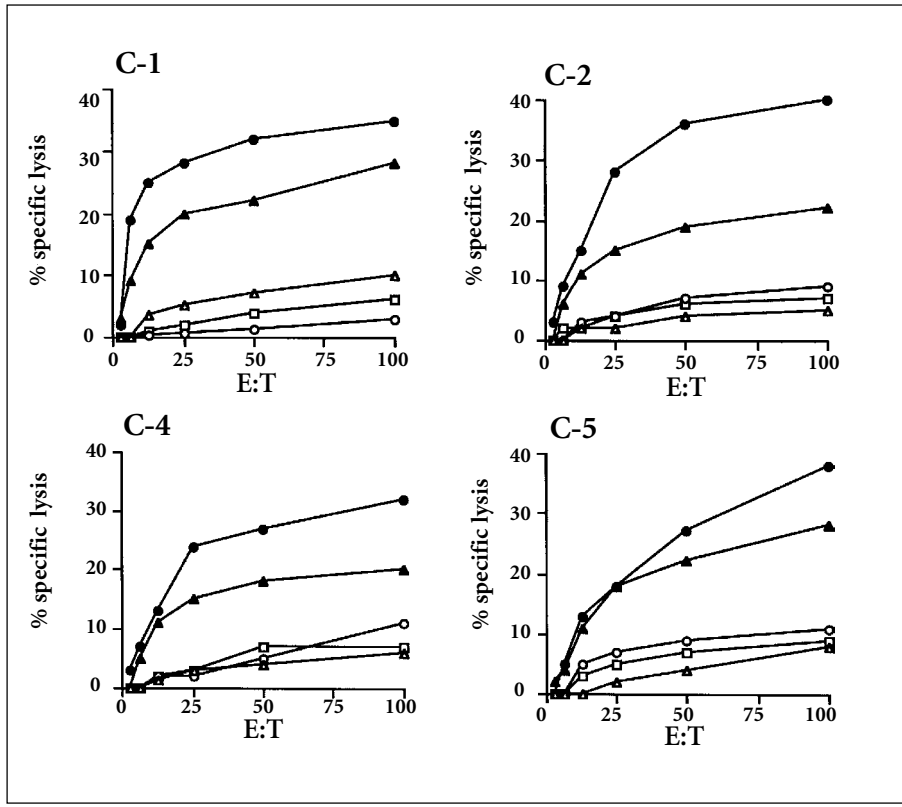

Fig. 6.5. HPV-16 $E7_{11-20}$-specific CTL from peripheral blood of HLA-A*0201+ control subjects. Freshly isolated PBMC were stimulated with $E7_{11-20}$-pulsed dendritic cells and after 35 days in culture, were tested for peptide-specific cytotoxicity in a 51Cr release assay. Targets were C1R.A2 target cells pulsed with the HPV-16 $E7_{11-20}$ peptide (✹), with the Plasmodium falciparum cp36 peptide (□), unpulsed (❍), infected with TA-HPV (▲), or infected with the parental Wyeth strain vaccinia (Δ).

CTL clones generated were able to recognize HPV16 antigens expressed either on TA-HPV infected targets or HPV16-transformed CaSki targets (Evans, manuscript submitted).

Collectively, these studies have identified CTL in both blood and at sites of disease. While their role in this disease is unknown, they provide strong evidence that HPV E6 and E7 proteins can induce natural immunity. A recent study has reemphasized that HPV is a common virus,[77] with the majority of individuals clearing infection naturally. This raises the question of why HPV specific CTL responses were detected only in CIN3 or cervical cancer patients but not in our normal controls. We propose two nonmutually exclusive explanations:

1. Cell-mediated immune responses could be generated in the acute infection but were transient, decreasing in number below the level of detection of current assays after clearance of virus;

Table 6.4

Site	HPV 16/18 E6/E7	Influenza-A
Peripheral blood	1/770 000 - 1/3 000 000	1/556 000 - 1/1 667 000
Lymph node	1/3000 000 - 1/2 500 000	1/333 000 - 1/1 000 000
Tumor	1/43 500 - 1/667 000	1/345 000 - 1/1 250 000

Precursor frequencies of HPV 16/18 E6/E7 (TA-HPV)-specific, and influenza-A-specific CTL at different sites in cervical cancer patients. CTLp frequencies against either TA-HPV infected targets or influenza A infected targets were estimated using limited dilution assays (LDA). The LDA cultures consisted of varying input numbers of lymphocytes together with a fixed number of allogeneic PBMC (pooled from 3 individuals), recombinant interleukin-2 and PHA. No HPV or influenza A specific stimulus was used for the LDA cultures. At the end of 3 weeks culture split well analysis was performed and culture wells were considered positive only if % specific lysis of TA-HPV infected targets was greater than 10% of % specific lysis of control wells (Wyeth infected targets or uninfected targets).

2. No HPV E6/E7-specific CTL developed, due to the inefficient antigen-presenting capabilities of cervical keratinocytes.[78]

In both cases, the observed CTL responses in patients could be explained by the presentation of HPV antigens in transformed tissue, either directly by the tumor cell or via bone marrow-derived antigen-presenting cells.[79] Longterm studies of cohorts of young healthy women as well as patients will be required to address the nature of the induction and persistence of CTL responses to HPV.

Obstacles to CTL-Based Immunotherapy

The ready detection of HPV-specific CTL immediately raises the question of whether these effector T cells are an important mechanism for immune-mediated destruction of tumor cells. This therapeutic optimism has to be tempered with the reality that, while these cells have now been identified, no direct evidence of protection has been provided. Unfortunately, such evidence will require direct clinical experiments in man in the absence of appropriate animal models.[19] Adoptive immunotherapy studies in man are fraught with difficulties, not least being able to grow sufficient CTL in vitro.[80] At present we are engaged in developing this with respect to HPV-specific CTL and then proceeding to examine whether infusion of such cells can result in local reduction of disease. However, these are not the only obstacles to implementing either transferred or induced CTL in treatment for cervical cancer. Others relate to properties of the transformed cell and include HLA downregulation,[81] lack of costimulatory molecules,[82] low antigen expression[23] and production of immunosuppressive cytokines.[83] Yet, despite this reasoning, research remains active in this area because:

1. HLA class I downregulation of at least 1 allele has been shown in 80% of cervical tumors,[81] and although the effect of this on immune function is not known, it has been suggested that this would hamper attempts at CTL-based immunotherapy. This would be more likely when using peptides to induce CTL, as either single or few specificities might be generated. We would argue that previous studies demonstrate that, as at least one allele is expressed by 80% of tumors, this should be sufficient for CTL recognition in the majority of cases. Our laboratory studies suggest that

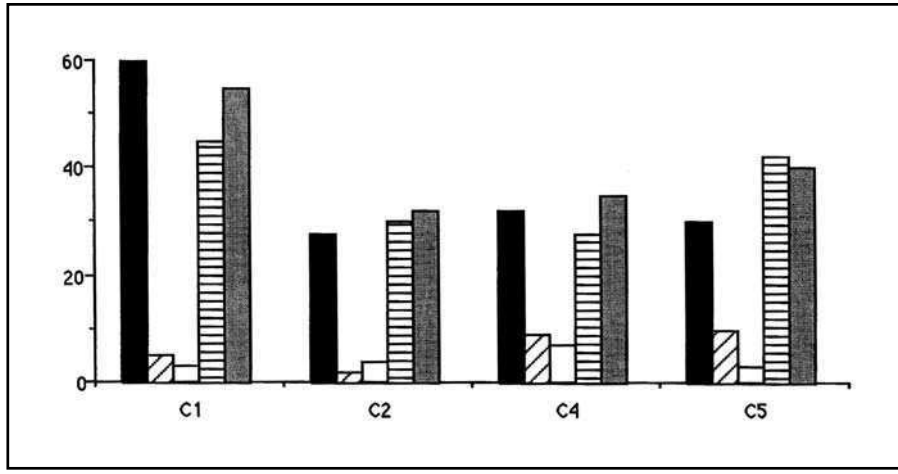

Fig. 6.6. HPV16+ CaSki cervical cancer carcinoma cell line is recognized by HPV16 $E7_{11-20}$ peptide-specific CTL from control subjects. CTL were generated by stimulation of PBMC with HPV16 $E7_{11-20}$ peptide-pulsed dendritic cell populations. CTL activity was measured on day 35 at an E:T ratio of 100:1. Targets were HPV-16 $E7_{11-20}$-pulsed C1R.A2 cells (■), compared with unpulsed CaSki (▤) and $E7_{11-20}$-pulsed CaSki cells (■), in a chromium release assay. Control targets included C1R.A2 cells pulsed with influenza A $M1_{58-66}$ (▨), or left unpulsed (□).

HPV-specific CTL generated after restimulation with full length HPV16 E6/E7 proteins recognize a multitude of MHC class I/HPV peptide combinations. Judicious vaccination and/or selective expansion of CTL in vitro will allow CTL of the correct specificity to be selected. There is also the possibility that downregulation of HLA molecules on tumor cells may result in generation of novel immune responses against the tumor, as has been shown for melanoma.[84]

2. The low levels of HPV antigen expression by HPV transformed cervical keratinocytes may affect priming of CTL responses. However, the epitope density required for effector function of an activated CTL is considerably lower.[85] To date, HPV-specific CTL have been defined using HPV peptide-pulsed target cells or HPV-vaccinia infected target cells. For successful immunotherapy, these same CTL must be able to lyse cervical tumor cells expressing low levels of HPV antigens, and it may be possible to select such CTL using in vitro approaches.[85]
3. The role of cytokines produced by HPV-transformed keratinocytes in regulating immune responses in the cervix is unclear and will require analysis in situ. The potentially immunosuppressive effects of keratinocyte-derived cytokines might be overcome either by local delivery of inflammatory cytokines[65] or simply through sheer numbers of CTL generated by vaccination or adoptive transfer.
4. Success in immunotherapy of other virally encoded tumors such as EBV-related lymphoproliferative disorder.[86,87]

Conclusions

Our studies have demonstrated that HPV-specific CTL can be induced in vivo by vaccination. The clinical effectiveness of this response is being addressed in large scale trials. We have also shown that HPV-specific CTL can occur naturally in patients with

HPV-associated cervical neoplasia. It is not clear whether naturally occurring HPV-specific CTL, or any other type of HPV-specific immune response, plays a role in the natural history of disease. Large scale studies will be required to answer this question; however, this does not obviate a potential role for HPV-specific CTL in the immunotherapy of HPV-associated neoplasia. Whether this potential is realized will depend on more fundamental laboratory studies, particularly those addressing interactions between CTL and naturally HPV-transformed keratinocytes. It is important to remember that while recurrent disease remains a problem in the developed world, 80% of cervical cancer occurs in developing countries, which do not have the economic resources for full availability of surgery and radiotherapy, let alone adoptive immunotherapy. Therefore, these approaches can only hope to impact on the global burden of HPV-associated cervical cancer if immunotherapy is combined with effective prophylactic vaccination.

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CHAPTER 7

Safety and Immunogenicity of HPV16 E7/Algammulin

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The gene products of two early open reading frames of human papillomavirus 16 (HPV16), E6 and E7, are together sufficient to immortalize human keratinocytes in vitro,[1,2] and the open reading frames encoding these viral proteins are preserved in HPV-associated cervical cancers.[3,4] Humoral immune responses to the E7 protein of HPV16 have been observed in 20-50% of patients with cervical cancer,[5-8] confirming that the E7 protein is expressed and can be seen by the immune system. Immunity to E7 protein, induced by Immunization of experimental animals, is able to prevent the growth of transplantable tumors engineered to express HPV16 E7 protein,[9-12] suggesting that an appropriate immune response, induced to E7 protein by Immunization, might similarly control HPV-associated cervical tumors in man. We therefore conducted a phase I/II clinical study of a vaccine based on recombinant HPV16 E7 protein, to establish whether this protein is immunogenic and non-toxic in man, and to estimate a minimal immunogenic dose of E7 protein for future vaccine studies.

Because the safety of many live vectors has yet to be established in man, a single protein vaccine based on Algammulin, a mixture of gamma inulin and "Alhydrogel™", was chosen for this study.

Clinical Study Vaccination

Vaccine Formulation

Preparation and purification of Glutathione-S-tranferase E7 (GST E7) and its combination with Algammulin to produce a vaccine have been described.[13] In brief, E7 protein of HPV16 was produced as a GST fusion protein in *E. coli*, and purified by stepwise urea extractions of the inclusion bodies and anion exchange chromatography. In experiments where sequence-authentic E7 protein was required, the fusion protein was cleaved with thrombin and E7 was purified by preparative SDS-PAGE. For vaccine formulation, GST E7 protein was prepared as a sterile solution containing 100 μg/ml HPV16 E7 protein in 5 mM HEPES, 150 mM NaCl, pH 7.2. The protein content of this solution was >90% GST E7 as judged by SDS PAGE, and the solution, contained <30 ng endotoxin/100 μg protein, and less than 10 pg/100 mg E7 of HPV16 E7 DNA, detected by semiquantitative PCR. E7 was combined with Algammulin[14,15](Batch AG-38) and dispensed in 1 ml sterile preloaded syringes containing 25 mg Algammulin and 5 μg, 25 μg or 100 μg of E7 protein. The product was demonstrated sterile, nonpyrogenic and nontoxic in rabbits, rats and guinea

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pigs, and immunogenic in rats and rabbits, by a pharmaceutical testing company (Pharmatox, NSW, Australia) according to standardized procedures.

Selection of Subjects

Subjects were selected who had otherwise untreatable primary or recurrent biopsy proven cervical cancer. Subjects had normal liver function, no acute renal problem, a Karnofsky score of 70% or better at recruitment, and a life expectancy as judged by the referring physician of at least 6 months. Subjects receiving immunosuppressive drugs or with a history of opportunistic infection were excluded. Subjects gave informed consent to the protocol, which was approved by the Human Ethics committee of the Princess Alexandra Hospital.

Administration of Vaccine

Vaccine was administered at 4 weekly intervals. Initially, 5 μg of E7 was given per immunization. Subsequently, the dose was increased to 25 μg and then 100 μg E7 per dose. Immunization was by subcutaneous injection into the right and left lower abdomen alternately, starting with the left, using a new site for each injection.

Assessment

Laboratory Tests

Routine hematologic immunologic and biochemical parameters were assessed in the diagnostic laboratory. HLA typing was established by routine microcytotoxicity testing, and class II typing confirmed by polymerase chain reaction using sequence-specific primers. Immune competence was assessed by the Multitest CMI, and by measuring the humoral response to a single i.m. dose of 0.5 ml tetanus toxoid (CSL, Melbourne). HPV DNA was sought in sections of formalin fixed and paraffin embedded tissue using DNA extraction protocols, and PCR using consensus and sequence specific primers as previously described.[16]

Papillomavirus-Specific Immunity

Antibody to the E7 protein of HPV16 was measured by 5 methods described in detail elsewhere:[17]

1. ELISA using thrombin-cleaved GST E7 or a series of overlapping 20mer peptides (GF101-109) spanning the 98 amino acids of HPV16 E7[18] as substrate;
2. Immunoblot using SDS-PAGE-separated thrombin-cleaved GST E7 as substrate;
3. Immunoprecipitation from SF9 cells infected with an E7 recombinant baculovirus, followed by detection of precipitated E7 by immunoblot using the HPV16 E7-specific mAb 6D (IP immunoblot);
4. Capture ELISA;[8] or
5. Immunoprecipitation of ^{125}I labeled CaSki cells as a source of radiolabeled E7, as previously described.[19]

T-proliferation assays were carried out essentially as previously described,[18] using 2 x 10^5 peripheral blood lymphocytes per assay, prepared by density gradient centrifugation. Cells were stimulated for 5 days in RPMI 1640 with 2% autologous heat-inactivated serum and purified E7 protein or synthetic peptides GF101-109 spanning the predicted sequence of HPV16 E7. Results are expressed as a stimulation index, obtained by dividing the ^{3}H thymidine incorporation by cells in E7 protein or peptide-stimulated cultures by the incorporation in unstimulated cultures. PPD (CSL Ltd), PHA(Sigma) and tetanus toxoid (CSL Ltd) were used as positive control antigens.

Table 7.1. Clinical status of the trial patients at recruitment

Subject	*Age*	*Diagnosis*	*Prior Therapy*	*Hb (g/l)*	*Creatinine (umol/l)*	*Albumin*	*HLA*	*Karnovsky score*
SW	50	Stage IV squamous carcinoma (SCC) cervix	Radical Radiotherapy (RT)- pelvic recurrence and hydronephrosis	100	0.24	39	A1,A24 B45,B57 DR4,DR7	90
RP	41	Stage II Adenocarcinoma (AdC) cervix 1987	Hysterectomy 1997 Pelvic recurrence 1993 Ileal conduit, RT and Chemotherapy(CT) 1993	121	0.08	39	A11,A28 B35,B51 DR1,DR4	90
LD	26	Stage II AdC of cervix 1990	CT, RT Recurrence in liver and wound 1993 - CT	113	0.12	40	A1,A11 B51,B57 DR2,DR6	100
KB	37	Stage II SCC cervix 1992	Hysterectomy 1992 Recurrence 1993 RT/CT 1993	104	0.13	40	A2, B7,B44 DR2	100
CL	43	Stage II AdC cervix 1992	Hysterectomy 1992 RT 1992, CT 1992 Recurrence 1993	118	0.09	38	A2,A3 B7,B60 DR1,DR4	90

Table 7.2 Assessment of Immune Function

Patient	CD+ve T cells	CD4/CD8 ratio	IgG(g/l) (N:5-15)	Multitest (N:>2mm)	Tet Tox Ab (iU/ml) Pre	Post	HPV DNA	HPV16 E7 antibody at entry
SW	430	1.49	9.8	6	<0.16	<0.16	NK	-ve
RP	1460	0.98	9.8	6	0.19	21.2	NK	-ve
LD	450	1.36	8.4	5	0.70	35.2	NK	+ve
KB	530	2.23	11.6	0	0.21	10.48	NK	-ve
CL	900	0.40	11.0	16	<0.16	<0.16	HPV16	-ve

N: normal values; NK: Not Known. N, normal values; NK, not known; +, positive; -, negative.

Table 7.3 Immunotherapy given and adverse effects

Patient	Immuzations		LFTs		Globulin		Creatinine		ESR		Vaccine	Outcome
	Number	Dose schedule[a]	Pre	Post	Pre	Post	Pre	Post	Pre	Post		
SW	8	5,5,5,100, 100,100, 100,100	N	N	27	34	0.24	0.15	38	64	NIL	Died week 34- cachexia
RP	9	5.5,5,25, 25,25,100 100,100	N	N	35	45	0.08	0.08	32	92	Local	Died week 32- reaction local invasion
LD	9	5.5,5,5,5, 25,25,25, 100,100, 100	N	AP245	38	40	0.12	0.09	58	>100	Local	Died week 36- reaction cachexia
KB	4	5,5,25,100	N	AP232	36	45	0.13	0.46	86	>100	Painful lump	Withdrawn week 14 bilateral ureteric obstruction
CL	4	5,5,5,5	AP136	AP137	44	35	0.09	0.10	84	>100	Nil	Died week 14- arterial thrombosis

a: dose progression in μg—doses given at 4 weekly intervals. AP: Alkaline phosphatase (N: <115)

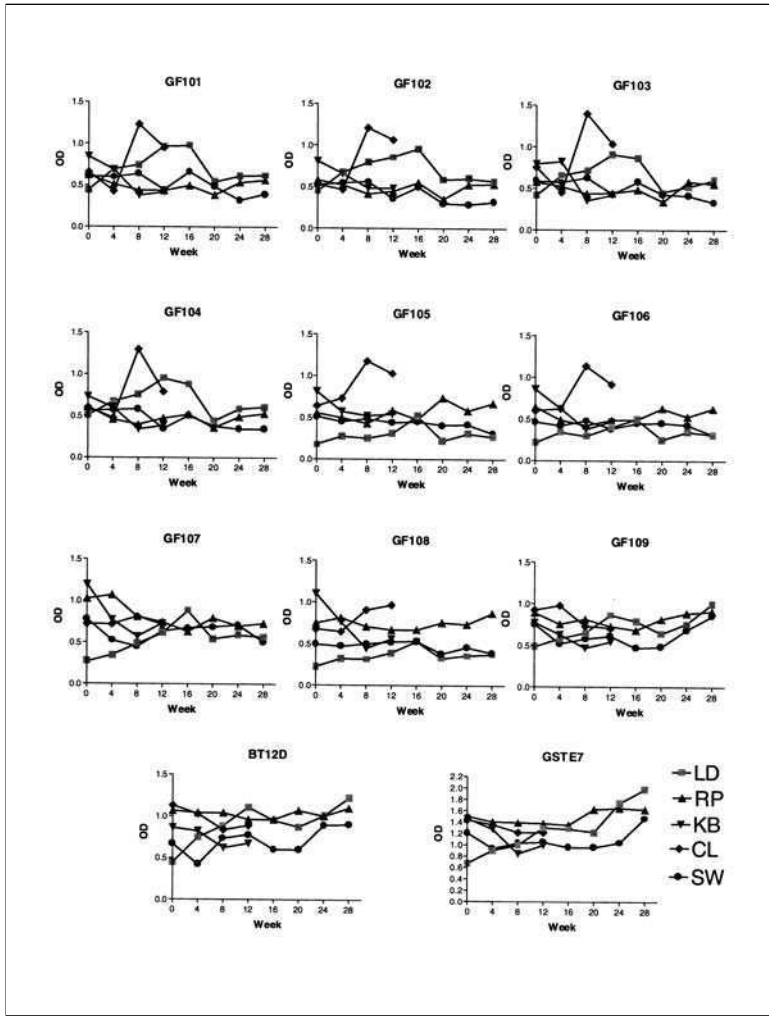

Fig. 7.1. Reactivity of sera with E7 protein and with E7-derived peptides by ELISA. Sera at 1:100 dilution were tested in triplicate prior to Immunization, and at 4 week intervals throughout the Immunization schedule. Substrates include GST-E7, a series of overlapping 20mer peptides from the sequence of HPV16 E7 designated GF101-GF109, and BT12D, a synthetic peptide including 3 B epitopes of the E7 protein.

Cytotoxic T-cells were sought using peripheral blood T-cells as effectors, restimulated in vitro for 8 days with E7 rVV[18] infected autologous EBV transformed B cell lines and 10 U/ml recombinant IL-2. For patients with HLA-A2.1, predicted epitope[20] peptide-pulsed ^{51}Cr labeled PHA blasts were used as targets at effector to target ratios of 10:1 to 100:1. Alternatively, PHA blasts were infected overnight with E7 rVV, labeled, and used as targets.

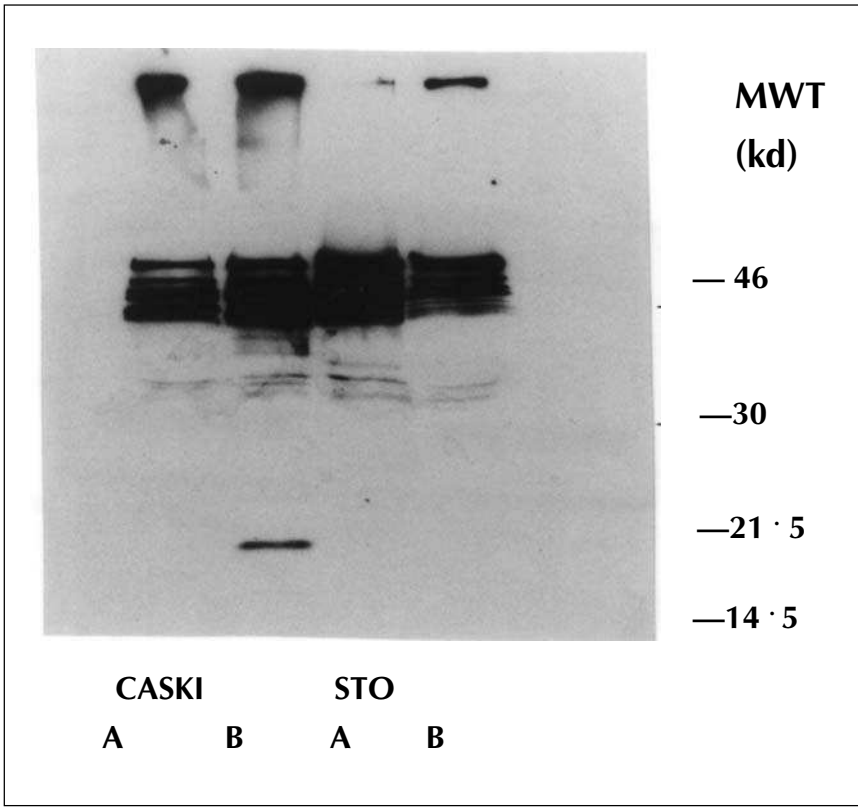

Fig. 7.2. Sera collected from one subject (LD) prior to (A) and following (B) Immunization with HPV16 E7 were used to precipitate protein from radiolabeled CaSki cells (HPV16 E7+ve) and Sto cells (E7–ve fibroblasts). Immune complexes, formed by adding 5μl of serum to radiolabeled cell lysate, were precipitated using Protein A Sepharose, washed extensively, and separated by 12.5% SDS-PAGE. The gel was subjected to autoradiography. E7 protein is identified as a ~20 kDa protein precipitated from CaSki cells only by LD's immune serum.

Results

Subjects Studied

Five patients with cervical cancer were recruited for this study. Each had biopsy-proven disease which had recurred or persisted after definitive treatment (Table 7.1). Each had residual disease at the time of recruitment to the study, but had a Karnofsky performance score of 90% or better. Each subject had received either radiotherapy or chemotherapy or both within 2 years of recruitment, a probable explanation for the lymphopenia (4 subjects) and neutropenia (2 subjects) observed at recruitment (Table 7.2). Immune function was assessed prior to recruitment. Three subjects with measurable antibody to tetanus toxoid responded to a single Immunization with tetanus toxoid with a significiant rise in antibody titre. Two subjects without measurable antibody had no

Table 7.4. T Proliferative Responses to HPV16 E7 GST Protein and Peptides from the E7 Protein

Patient	Time	Tet Tox1	PPD	GST E7	101-103	104-106	107-109	Indvidual peptides[2]
SW	Week -4	<2	<2	<2	<2	<2	<2	
	Week 12	<2	<2	<2	<2	<2	<2	
	Week 22	ND	2.2	<2	<2	<2	<2	
RP	Week -4	<2	4.0	<2	3.7	<2	<2	
	Week 12	6.4	12.3	2.2,3.9	7.2	4.5,5.1	4.4,<2	105,108,109
	Week 22	ND	3.6	2.9	4.0	4.6	4.7	
LD	Week -4	<2	39.3	1.6	<2	7.2	<2	
	Week 12	6.0	48.4	33.4,65.5	5.2	1.3	6.9	
	Week 22	ND	18.4	31	<2	28	29.6	
KB	Week -4	<2	15.6	<2	3.0	2.5	<2	
CL	Week -4	<2	87.0	<2		<2	<2	

1: All results shown as stimulation indices. ND: not determined.
2: Peptides from the overlapping series GF101—GF109 from HPV16 E7 to which stimulation indices of >3.0 were observed.

increase in antibody titre following this single Immunization. Four subjects, including the two with negative antibody titres, had positive DTH recall to at least one of a battery of common antigens (Table 7.2). Tumor from one subject was positive by PCR for HPV16 DNA. For each of the others, amplification by PCR of HPV genes and of cellular β-globin gene from DNA extracted from formalin fixed historic biopsy tissue was unsuccessful. All subjects had normal serum transaminase and alkaline phosphatase activity at recruitment. One patient had known partial ureteric obstruction and one had a functional ileal conduit. Renal function assessed by serum urea and creatinine was in each case stable. The nutritional status of each subject was normal as judged by serum albumin concentration. However, three of the 5 subjects were more than 10% below ideal body weight for their height and age.

Dose Ranging, Toxicity and Safety

Subjects were administered escalating doses of E7 protein, at 4 weekly intervals (Table 7.3). Subjects were asked about a series of injection site and constitutional symptoms at each 2-4 week clinic visit, and blood was routinely monitored for biochemical or hematological abnormalities. Local reactions to the vaccine were observed. Three of five patients developed small (2-3 mm) subcutaneous fibrous lumps at the site of each Immunization during the first two weeks after Immunization which were still present 4-6 weeks later, but diminished in size with time. One patient (LD) developed an erythematous painful swelling at the Immunization site within 12 hours following the first Immunization, which lasted for 5 days and then resolved. Subsequent Immunizations of this patient produced similar but lesser reactions.

Constitutional symptoms reported following Immunization bore no obvious relationship to the Immunizations. One patient had fevers and sweats following one Immunization but had a culture-proven urinary infection; the symptoms settled with antibiotic therapy. Another patient had similar symptoms following one injection without other obvious cause. Serum transaminases remained normal throughout for all subjects. One patient developed a markedly elevated serum creatinine following her third Immunization, which was demonstrated on ultrasound to be due to bilateral ureteric obstruction from an extending pelvic tumor mass; this patient withdrew from the study protocol at this point, but was followed up.

All patients were anemic at some point during the course of the trial. The blood picture was that of the anemia of chronic disease. Progressive anemia necessitated transfusion for three patients; there was no laboratory evidence of autoimmune hemolytic anemia or of DIC in any patient. No patient developed autoantibodies as a result of the Immunizations, although one had a low titre ANA (titre 1/80) prior to treatment which persisted. No local skin abnormalities or tumors developed as a result of the Immunizations during the period of follow-up.

Vaccine Immunogenicity

Reactivity to GST protein was seen in all tested preimmune sera (data not shown). Reactivity to GST-E7 fusion protein was therefore assessed using thrombin-cleaved E7 protein. After repeated Immunization with GST-E7, reactivity to E7 by ELISA increased in 2 of the 5 subjects, both of whom had received 100 μg Immunizations (Fig. 7.1). The composite peptide BT12D, which includes three defined B epitopes from HPV16 E7,[18] and a series of overlapping linear peptides spanning the length of the E7 protein, were used in ELISA to assess the reactivity to linear epitopes from HPV16 E7. One subject only (LD) showed a significant increase in reactivity to BT12D from 0.4 to 1.2 OD units, and to GF109 from 0.48 to 1.01 OD units. As there was significantly increased reactivity with whole E7 protein following Immunization for another subject, which apparently did not map to a linear epitope,

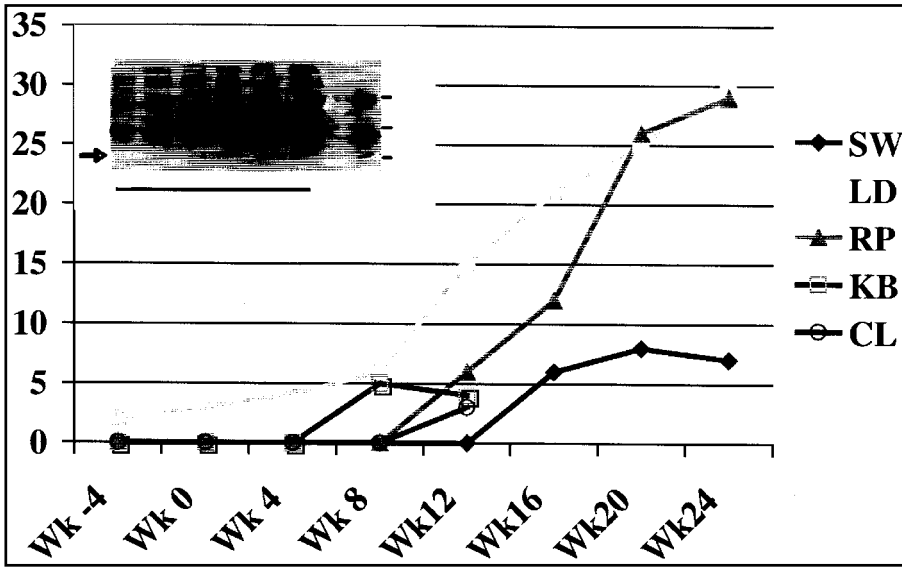

Fig. 7.3. Reactivity of sera with baculovirus recombinant E7 protein by IP immunoblot. Immune complexes, formed by adding 5ml of serum to lysate of SF21 cells infected with E7 recombinant baculovirus, were precipitated using Protein A Sepharose, washed extensively, and separated by 12.5% SDS-PAGE. Protein was transferred to nitrocellulose by immunoblot. E7 was detected using the E7-specific mAb 6D, and quantitated in arbitrary units by densitometry. Inset shows the equivalent immunoblot for one of the five patients—the arrow highlights the E7 reactivity.

it was held possible that reactivity might have been induced to conformational epitopes of E7. To test this, serum from one immunized subject (LD) was tested for ability to precipitate protein from radiolabeled CaSki cells; serum after E7 Immunization, in contrast to serum prior to Immunization, precipitated E7 protein (Fig. 7.2). To quantitate reactivity with conformational epitopes of E7, serum reactivity with E7 protein expressed as a recombinant protein in insect cells was sought by IP immunoblot (Fig. 7.3). New or increased reactivity with E7 in this assay was observed in all 5 subjects after 3 or 4 Immunizations, and the increase in reactivity was most marked in subjects receiving higher doses of E7 protein. The subject (LD) with preexisting antibody to E7 protein developed an increased titre of antibody no more rapidly than the other subjects, suggesting that Immunization produced antibody to epitopes other than those to which tumor-induced E7 antibody is directed. Overall, the IP immunoblot assay proved most sensitive for detection of increased reactivity with E7 protein amongst subjects in this study, with all patients recording positive results after immunization. Two subjects (LD and RP) were positive in all assays after Immunization, including an E7-specific ELISA capture assay, and these subjects gave the most positive results in the immunoprecipitation assay. The other three subjects were negative in all but the IP immunoblot assay after Immunization.

After Immunization, two of three evaluable subjects had peripheral blood T cells that proliferated on exposure to GST E7 protein, and to discrete E7 peptides (Table 7.4). No

reactions at the site of Immunization with a time course suggestive of DTH to E7 were observed in any subject. No cytotoxic T-cell reactivity to a range of algorithm predicted and other potential E7 Tc epitopes was observed at recruitment, and none had developed at week 18 in the 3 subjects tested (data not shown), although one HLA-A2-positive subject demonstrated Tc activity to the A2-restricted 'flu nuclear matrix' peptide at recruitment and on retesting.

Patient Outcome

No patient withdrew from the study as a consequence of vaccine-associated toxicity or adverse reaction. During the course of the study, each of the patients had evidence of progressive disease (Table 7.3). One subject (LD) had an abdominal wound metastasis, which was observed to grow progressively throughout the study, despite increasing titres of antibody to E7. Two patients had imaging evidence of increasing pelvic disease. One developed progressive bilateral ureteric obstruction and was withdrawn from the study at week 14. Four patients died during the period of observation following the study, one at week 14 of progressive femoral arterial and venous occlusion, both of which were present at recruitment, two at week 34 with cachexia, and one at week 32 with progressive sacral plexus neuropathy requiring escalating doses of analgesia for pain control. One patient was lost to follow-up but was subsequently confirmed to have died of progressive disease.

Discussion

This study demonstrates that the E7 protein of HPV16 conjugated to Schistosoma japonicum glutathione S-transferase is immunogenic in man, and that antibody and T helper responses to epitopes of the E7 protein can be produced by such Immunization even in patients with advanced cervical cancer and treatment-associated partial immunosuppression. While HPV16 infection is common, a humoral immune response to the various HPV16 proteins is rarely observed even in fully immunocompetent young patients unless invasive cervical cancer develops,[8,21,22] although cytotoxic T-cells specific for E7 are described in patients with CIN3, the precursor lesion of invasive cervical cancer,[23] and T helper responses are also characteristic of this stage of infection.[24] This present study confirms that a lack of humoral immune response to the E7 protein in some patients with cervical cancer is not due to poor immunogenicity of the E7 protein, but rather to inadequate presentation of the E7 protein to the immune system in the course of natural infection. It should therefore be possible to induce immunotherapeutic responses specific for HPV-associated lesions, given an appropriate Immunization regime.

A previous study of the immunogenicity of whole length E7 protein in patients with cervical cancer utilized a single Immunization with a recombinant vaccinia delivery system,[25] and immune response to E7 delivered by this method was characterized by weak antibody responses and CTL responses in one of 3 evaluable subjects. An optimal tumor-specific immune response would be directed to tumor-specific antigens and might include a DTH and a cytotoxic T-cell response to these antigens. The nature of the immune response to a polypeptide antigen is largely determined by the dose and route and frequency of delivery of the antigen, and the choice of adjuvant. While live vectors are more likely to produce cytotoxic T-cell responses than killed protein vaccines, a single protein vaccine, based on Algammulin, a mixture of gamma inulin and "Alhydrogel™", was chosen for this study because the safety of many live vectors has yet to be established in man, and the immune response to recombinant proteins encoded by vaccinia is compromised in some patients immunized with vaccinia against smallpox.[26] Alum-based adjuvants, the only adjuvants currently licensed for use in man, produce antibody with little or no Tc and DTH response, and the addition of inulin, a product approved for use in man though not as an adjuvant,

has been reported to promote the development of cellular immunity.[27] In the present study there was little evidence of DTH to E7 protein as manifest by the lack of delayed local reactivity to repeated vaccination. There was also no positive evidence of a preexisting or induced cytotoxic T-cell responses to E7 peptides, although the lack of defined Tc epitopes or suitable E7-transduced syngeneic targets for these assays diminishes the significance of this finding.

One patient had preexisting antibody to E7 at recruitment but nevertheless required three Immunizations before any enhancement of this antibody to E7 was demonstrated, suggesting that the natural immune response might be directed against different epitopes of the E7 protein from the vaccine-induced response. This is in keeping with the observation that the immune responses to intracellular E7 in mice and man[28] tend to be directed against the C-terminal epitopes, while Immunization-induced antibodies recognize a number of other, non-C-terminal epitopes.[29] The dose ranging studies suggest that to achieve a substantial antibody response to E7, 25 μg would be a minimum dose and 100 μg, which was not associated with any more adverse reaction, would be better. The smaller dose was used initially in the expectation of inducing DTH or Tc responses to E7, and as there is no expected correlation of antibody titre with disease protection the dose response curve should simply be used as an indicator that E7 protein can be expected to behave as an immunogen in man similarly to other protein antigens.

We conclude that it is rational to conduct further studies of administration of E7 protein as immunotherapy for cervical cancer, and that further studies should be conducted with adjuvanting or delivery systems thought likely to induce tumor lytic DTH or cytotoxic T-cell responses.

Acknowledgments

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CHAPTER 8

DNA Vaccines for Papillomavirus Infections

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The Three Waves of Vaccinology

The major advances in approaches in vaccinology can be subdivided into three "waves", each resulting from breakthrough technologies. The initial wave employed newly developed cell culture methods to produce attenuated live viruses and killed viruses for use as vaccines. The next wave began with the advent of recombinant DNA technology and protein methods which allowed for the production of recombinant proteins or viral subunits for use as vaccines. The invention of novel techniques for delivering biologically active DNAs into living tissues of a host permitted the third wave in which eukaryotic expression vectors encoding relevant antigens serve directly as vaccines. The papillomavirus field was not prepared to ride the first wave, has benefited from technology applied during the second wave and is poised to be a leader in the advancement of the third wave.

The First Wave: Live, Live Attenuated, and Killed Virus Vaccines

Live virus vaccines are propagated in vitro, i.e., in cell culture, as live virus particles that contain intact and biologically active viral genomes. Live vaccines against mumps, measles, rubella and smallpox induce prolonged intracellular expression of viral proteins and production of virions. They are extremely effective and have revolutionized the practice of medicine. However, live vaccines also cause significant complications in a small number of vaccine recipients. In an attempt to overcome the complications of live vaccination, killed virus vaccines were developed by inactivating (killing) live virus with chemical treatments. Killed vaccines were safer because of their inability to express viral gene products and infectious virus in vivo, i.e., in the whole animal, and they still induced protective immunity—to viral capsid proteins. However, in the case of papillomavirus infection, neither live attenuated nor killed viral vaccines are ever likely to be used because the usual course of the infection is benign (and therefore does not warrant the risk of a live vaccine) and also because large quantities of HPV cannot be propagated in vitro or in animals.

The Second Wave: Recombinant Subunit Vaccines

The remarkable efficacy of anti-viral vaccination stimulated scientific exploration of the mechanisms involved and led to the discovery that protection also could be achieved with individual viral protein subunits, if those subunits contained appropriate B and/or T cell epitopes. Major research efforts have been increasingly devoted to the development and

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production of new vaccines to prevent and treat infectious and malignant diseases. This is largely due to a 1986 change in a United States Federal law that now protects American vaccine manufacturers from all liability not related to manufacturing error. The first subunit vaccine licensed in the United States was against hepatitis B virus (HBV) infection. Although the original HBV vaccine used surface antigen isolated from human blood of HBV carriers, the newer, equally effective, HBV vaccine utilizes a recombinant form of the surface antigen, which eliminates safety concerns associated with the use of human blood products. The vast majority of new vaccines will undoubtedly be recombinant subunit vaccines. The subunits may be administered as recombinant proteins, recombinant DNAs, or organisms containing recombinant nucleic acid. Papillomavirus like particles (VLPs) that are synthesized in vitro using recombinant, self-assembling, capsid proteins are a special type of protein subunit vaccine (see chapter 3).[1-3] VLPs offer the nearest recombinant approximation to a killed papillomavirus vaccine available for mass production. In animal models VLPs can induce high titers of virus neutralizing antibodies and confer protection against challenge with live papillomavirus; similar antibodies have also been identified in humans and non-human primates.[4-9] (see comprehensive discussion in chapter 3). Available data suggest that VLP vaccines will provide effective prophylaxis against initial HPV infection, and clinical trials using HPV VLP vaccines are currently in progress.

Vaccinia viral vectors expressing recombinant antigens, e.g., papillomavirus E6 or E7 proteins, may also be considered a special type of subunit vaccine.[10-17] Another specialized subunit vaccine uses an ex vivo approach in which nontumorigenic murine cell lines are engineered to express a gene product and then used as a vaccine. An advantage of these systems is that they can induce cell-mediated immunity using cannonical immunologic pathways, much as do live viruses (see chapter 6).[10-17] The use of live recombinant vectors as vaccines, however, poses some of the risks associated with the use of attenuated live viruses. Certainly the use of live viruses would be potentially more dangerous in cancer patients who may be functionally immunosuppressed. In addition, the use of any infectious recombinant virus or bacteria as a vector for administering other antigens is problematic in that an immune response can and does develop against the vector itself. These problems make the use of papillomavirus vaccinia virus recombinants unlikely except for cervical cancer (see chapter 6).

The Third Wave: Nucleic Acid Vaccination

One of the most important developments in vaccinology and one that has the potential to again revolutionize the field, is the use of nucleic acids as subunit vaccines. The vaccination method is simple: DNA sequences encoding a full-length protein or antigenic epitope/epitopes are cloned into a eukaryotic expression vector—a recombinant bacterial plasmid—and inoculated in naked form (i.e., no associated proteins) directly into an animal. Alternatively, synthetic mRNA transcripts can be used.[18] Intracellular expression of a plasmid (or RNA) vaccine occurs using normal host cell machinery. The antigen is then processed and presented to the host immune system via classical cellular pathways. This immunization process has been described as DNA vaccination, genetic immunization, polynucleotide immunization, and others. Since mRNA may be used as well as DNA for vaccination purposes,[18-22] the term DNA vaccination does not encompass the entire spectrum of possibilities. Genetic immunization is a more general term, but may lead to an erroneous fear of genomic manipulation in the nonscientific community. The "nucleic acid" nomenclature was decided upon by vote at the 1994 World Health Organization sponsored international meeting on nucleic acid vaccines and this term will be used in this chapter to describe the general approach. In addition, since most studies thus far have used DNA, we also will use "DNA vaccination" when appropriate.

Nucleic acid vaccines have several highly attractive features. First, vaccine construction is easy and rapid using standard recombinant methods. Since immune responses rarely develop against the plasmid DNA, standardized and optimized vectors can be developed for general use. Second, nucleic acid vaccines have had enormous success in animal models (see Tables 8.1 and 8.2). They can induce strong, specific and persistent humoral and cell-mediated immunity in many different model systems.[36,37,50,69,89,90,121,149,159,165,168,173,184,202,237-242] Not only can DNA vaccines protect against viral and other infections (see Table 8.1), they also can cause established tumors to regress. [243,244] Third, vaccine modifications are easy to introduce. Modifications can increase immunogenicity, eliminate potential pathogenicity, or alter the nature of an immune response, e.g., by mutation, truncation, fusion or rearrangement of the vaccine gene/antigen. Fourth, nucleic acid vaccination can be performed with whole genome expression libraries, bypassing the prerequisite of knowing the appropriate antigens a priori and providing a novel means to identify previously unknown antigenic epitopes.[245-247] Fifth, nucleic acid vaccines easily can be combined to generate a single multivalent vaccine.[155,166,248-250] Alternatively or in addition, DNA vaccines can be combined with cytokine or co-stimulatory genes to improve immunogenicity and/or modify the nature of an immune response.[35,40,62,63,68,104,106,109,116,117,156,169,170,209,221,225,227,228,234,251-262] Sixth, DNA-mediated immunization can circumvent haplotype-linked nonresponsiveness and may induce cell-mediated immunity in hosts that are traditionally less responsive such as alcoholics and neonates.[40,50,63,187,191,206,263] Seventh, nucleic acid vaccines are eminently suitable for worldwide use because they are inexpensive to produce and purify and because they are highly stable and should not require refrigeration. Lastly, and possibly most importantly, the malleability of nucleic acid vaccination suggests the possibility of producing a collection of vaccine and immune-modifier constructs that can be purposefully combined to produce "designer vaccines". Various combinations (for a given virus or tumor) could be tailored to the patient's genotype, state of immunocompetency and stage of disease.

Nucleic Acid Vaccination

Nucleic acid vaccination is one of the most rapidly expanding areas of vaccinology, and there are several resources available for the interested reader, including a site on the world wide web (Whalen RG (1997): http://www.genweb.com/Dnavax/dnavax.html.) and numerous review articles compiled as special edition publications (Current Topics in Microbiology and Immunology, Volume 226, 1998; Springer Seminars in Immunopathology, Volume 19, 1997; Vaccine, Volume 15, 1997; and Annals of the New York Academy of Sciences, Volume 772, 1995). The earliest demonstration that nucleic acids could induce biological responses when delivered directly into living tissue in vivo came in the 1960s when Yoshei Ito and colleagues showed that naked DNA isolated from rabbit papillomas could induce new papillomas following intracutaneous inoculation into naive rabbits.[264,265] Moreover, since the complete life cycle of the virus—cottontail rabbit papillomavirus (CRPV)—could be recapitulated, it was apparent that the full repertoire of viral genes was expressed. Analogous results have also been obtained using recombinant CRPV genomes.[266,267]

About 30 years later (1990), it was discovered that myocytes could be transduced in vivo with eukaryotic expression vectors and that transduction also resulted in protein synthesis.[268] In 1992, Tang et al demonstrated that DNA inoculation by "particle bombardment" could induce immune responses.[241] Particle bombardment is a method in which tiny (1-9 mm) gold particles are coated with plasmid DNA, loaded into a gene gun, and delivered ("shot") directly into tissues, most often skin, by entrainment in a high pressured stream of helium. It was soon shown that these immune responses generated in this way could be protective in animal models of infectious disease.[143,159,165,269] By 1993 Ulmer

Table 8.1 Nucleic Acid Vaccines for the Treatment of Viral Infections

Virus	Animal Model	Clinical Response	Immune Response	Refs.
Bovine Respiratory Syncytial Virus	Calf	Protection	↑ γδ T-cells	23
Bovine Viral Diarrhea Virus	Mouse	NI*	Ab**	24
Cytomegalovirus (human)	Mouse	NI	Ab	25
Cytomegalovirus (murine)	Mouse	↓ virus; protection?	Ab, CTL^{+}	26
Dengue Type 2 Virus	Mouse	NI	Ab	27
Ebola Virus	Guinea pig	Protection	Ab, CMI^{++}	28
Encephalitus Virus (Japanese)	Mouse	Protection	Ab	30
Encephalitis Virus (St. Louis)	Mouse	Protection	Ab	30
Encephalitus Virus (Russian spring and summer and Central European)	Mouse	Protection	Ab	31

Virus	Animal Model	Clinical Response	Immune Response	Refs.
Encephalomyo-carditis Virus	Mouse	Protection	Ab	32
Foot and Mouth Virus	Swine	Protection	Ab	33
Hepatitis B Virus, human	Mouse	Protection	Ab, CTL, CMI	34-51
	Transgenic Mouse	↓ virus	Ab	40,52
	Rat, Rabbit, Swine	NI	Ab	42
	Non-human Primate	Protection	Ab	42,46,53,54
Hepatitis B, duck	Duck, duckling	Protection	Ab	55
Hepatitis C Virus	Mouse	Protection	Ab, CTL	47,56-65
Hepatitus E	Mouse	NI	Ab	66,67
Herpesvirus, Bovine	Mouse	NI	Ab,CMI	68-70
	Cow	↓ virus	Ab	69

Virus	Animal Model	Clinical Response	Immune Response	Refs.
Herpesvirus, Equine	Mouse	Protection	Ab, DTH***	71
Herpes Simplex Virus 1	Mouse	Protection	Ab, DTH, CTL	72-77
Herpes Simplex Virus 2	Mouse	Protection;↓ virus	Ab, CTL	78-82
	Guinea pig	Protection	Ab, CTL	81,83
HTLV-1	Mouse, Rat Rabbit	NI	Ab	84,85
Immunodeficiency Virus, Feline	Cat	Protection?	Ab, CMI	86-88
Immunodeficiency Virus, Human, type I	Mouse	NI	Ab, Proliferation, CTL, DTH	46,82,85-118
	Guinea pig Rabbit	NI	Ab, CTL	99,108
	Non-human Primate	Protection,↓ virus	Ab, Proliferation, CTL, DTH	46,90,92,94,96,108,119-126

Virus	Animal Model	Clinical Response	Immune Response	Refs.
Immunodeficiency Virus, Human type 2	Mouse	NI	Ab, CMI	127
Immunodeficiency Virus, Simian	Rhesus macaque	↓ virus	Ab, CTL	128-132
Infectious Hematopoietic Necrosis Virus	Rainbow Trout	Protection	Ab	133
Influenza Virus	Mouse	Protection	Ab, CTL	134-175
	Chicken, Ferret, Swine, Rabbit	Protection;↓ virus	Ab, CMI	159,160,163,176-181
	Non-Human Primate	NI	Ab	149,176
Lymphocytic Choriomeningitis Virus	Mouse	Protection,↓ virus	Ab, CTL	182-187
Measles Virus	Mouse	NI	Ab, CTL	188-192
	Rabbit	NI	Ab	192

Virus	Animal Model	Clinical Response	Immune Response	Refs.
Murine Leukemia Virus (Cas-Br-M)	Mouse	Protection	CTL	193
Newcastle Disease Virus	Chicken	Protection	Ab	194
Papillomavirus	Rabbit	Protection	Ab, Proliferation	195-198
Pseudorabies Virus (Aujeszky's Disease)	Swine	Protection	Ab, Proliferation	199,200
Rabies Virus	Mouse	Protection	Ab, CTL	201-211
Rotavirus, murine	Mouse	Protection	Ab, CTL	212-215
Rotavirus, Bovine	Mouse	NI	CMI, No Ab	216

NI*= Not indicated in the abstract; Ab**= Antibody response in at least one of the abstracts; DTH***= Delayed-type hypersensitivity response in at least one of the abstracts; CTL^{+}= cytotoxic T-lymphocyte response in at least one of the absrtracts; CMI^{++}= cell-mediated immunity other than CTL response in at least one of the abstracts; ↓ virus indicates either decreased viral titer, increased clearance of virus, decreased viral shedding, decreased viral load, or decreassed viral replication.

Table 8.2 Nucleic acid vaccines for the treatment of cancer

Cancer	Antigen	Reference
Cervical	E6 and E7 epitopes in human clinical trial	217
Colon	CEA (DNA and mRNA); CEA co-expressed with B7-1 or GM-CSF; B-gal as model tumor antigen	22, 218-222
Hepatocellular	p53	223
Lymphoma	Idiotypic fragments; idiotype-tetanus toxin fusion; coinjection with IL-2 and GM-CSF; idiotype-GM-CSF fusion; idiotype-IL-1 beta fusion	224-230
Melanoma	MART-1; B-galMAGE-1 and MAGE-3 alone and co-expressed with GM-CSF or B7-1	231-235

For general review, see ref. 236.

et al[242] had demonstrated that intramuscular administration of plasmid DNA encoding influenza A nucleoprotein could induce cytotoxic T-lymphocyte responses and even protect against lethal challenge with a heterologous strain of influenza virus. As discussed above, the immune responses obtained with nucleic acid vaccination are long-lived, with antibody and cytotoxic T-lymphocyte activity persisting in mice for years.

Immunobiology of DNA Vaccination

Immune Responses to Papillomavirus Infection

Appreciation of the mechanisms underlying DNA vaccination requires some understanding of the process by which the immune system naturally mounts an effective response to viral infection.[270] In papillomavirus infection, the virus infects undifferentiated keratinocytes but production of infectious viral particles occurs only in differentiated keratinocytes. Figure 8.1 shows a proposed mechanism for the spontaneous immune response which may occur during papillomavirus infection. Regardless of its state of differentiation, an infected keratinocyte will express papillomavirus proteins. Most of these proteins are used to produce additional infectious virus, but some of the protein molecules are (recognized as abnormal and) ubiquitinated and thus targeted for degradation in the proteasome. Viral peptides in the proteasome are transported by the TAP transporter to the endoplasmic reticulum where they bind to the peptide cleft of MHC class I molecules and are eventually expressed in this

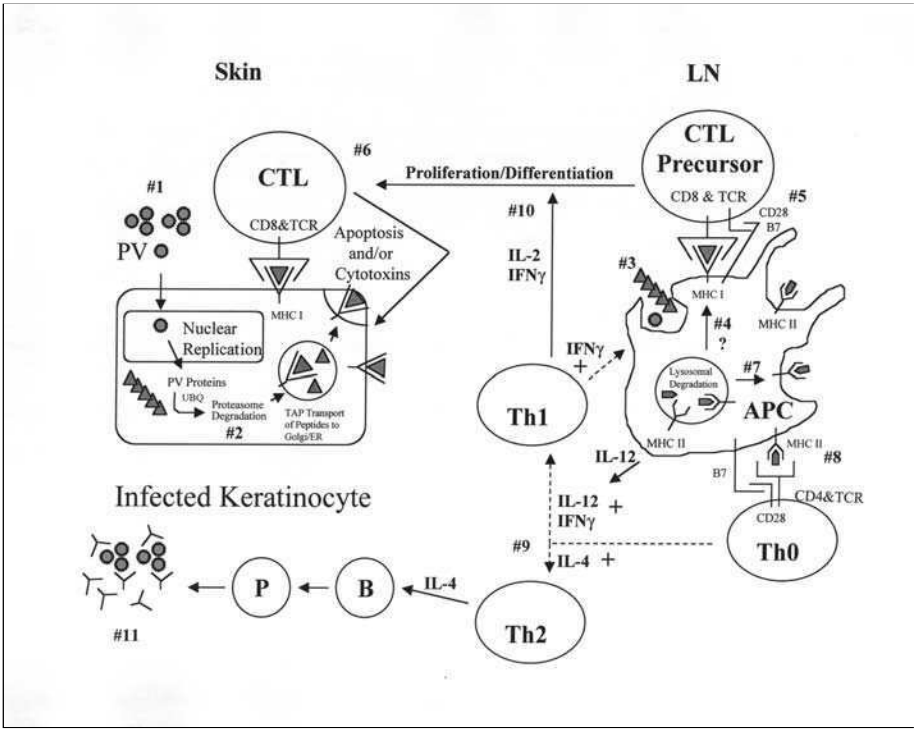

Fig. 8.1. Immune Response Against Papillomavirus Infection. Figure 8.1 shows a proposed mechanism for the spontaneous immune response which may occur during papillomavirus infection: (#1) infectious papillomavirus infects susceptible undifferentiated keratinocytes; (#2) papillomavirus proteins are expressed intracellularly and some of these proteins become ubiquitinated, leading to degradation in the proteasome; peptides generated in the proteasome are transported by the TAP transporter to the endoplasmic reticulum where they become associated with MHC class I molecules and are eventually expressed in this context on the cell surface; (#3) papillomavirions or papillomavirus proteins (synthesized and released by infected keratinocytes) are engulfed by professional antigen-presenting cells and degraded in the lysosomal compartment; (#4) some of the peptides bind to the major groove of MHC I molecules and are recognized by cytotoxic T-cell precursors via CD8 and the TCR; costimulation via B7 and CD28 interaction activates the precursor cell and stimulates proliferation and differentiation into a CTL; (#6) CTLs are able to recognize, bind to, and destroy cells which are infected with papillomavirus (those expressing PV peptides in the context of MHC I) by inducing apoptosis and/or through the production of cytotoxins; the CTL is able to release and attack additional infected cells; (#7) papillomavirus proteins degraded in the lysosome become associated with MHC class II molecules and are expressed on the surface of the APC; (#8) T-helper cell precursors (Th0) are able to recognize the papillomavirus peptide in the context of MHC II and also receive costimulation by B7, inducing proliferation; (#9) Th0 cells that are exposed to IL-12 and IFNγ differentiate into T-inflammatory (Th1) cells whereas Th0 cells exposed to IL-4 differentiate into T-helper (Th2) cells; (#10) Th1 cells produce IL-2 and IFNγ which promotes the CTL response by aiding in proliferation and differentiation of CTLs; (#11) Th2 cells produce IL-4 and provide B-cell help which leads to differentiation into plasma cells and anti-papillomavirus antibody production which will aid in clearance of virus.

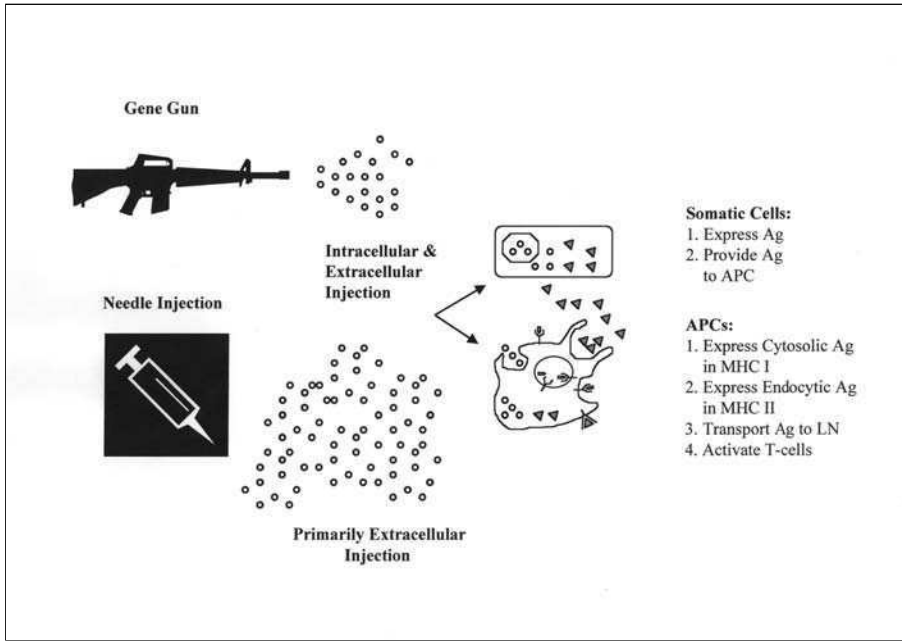

Fig. 8.2. Immunobiology of DNA Vaccination. Figure 8.2 illustrates mechanisms involved in generation of an immune response following vaccination of DNA by gene gun or needle injection. Both methods administer plasmid DNA into intracellular and extracellular compartments, but more DNA is required using needle injection (for equivalent immune responses), possibly because gene gun administration allows more direct intracellular access. At the site of vaccination, both somatic cells and APCs are transfected. Somatic cells express plasmid-encoded antigens, often for long periods of time, and are able to act as a reservoir of antigen for the APCs. APCs are able to engulf plasmid DNA and protein/peptide antigen (produced by the somatic cells) as well as express the plasmid encoded antigen independently. This allows for antigen presentation in the context of both MHC I and MHC II. APCs are particularly well suited to antigen presentation as they have the necessary costimulatory molecules required to stimulate activation and differentiation of T-lymphocyte precursors. APCs migrate to regional LN where the immune response is initiated. Since both MHC I and MHC II pathways are stimulated, DNA vaccination usually results in both cellular and humoral immunity.

context on the cell surface. This is the means by which the host's cellular immune system will be able to recognize a papillomavirus-infected cell.

One of the first steps in the process of recognizing the papillomavirus infection involves uptake and presentation of virus-derived proteins by professional antigen presenting cells (i.e., Langerhans cells, dermal dendritic cells, and macrophages). Papillomavirus proteins and intact virions are synthesized by and released from infected keratinocytes. Some of the virions and viral proteins are engulfed during routine immunosurveillance by professional antigen presenting cells (APCs) where they are degraded in either the lysosomal compartment or in the proteasome. The peptides derived from the proteasome follow the same pathway described above and are eventually expressed on the APCs surface in the context of MHC class I. Peptides which are produced via lysosomal degradation bind to the peptide binding cleft of MHC class II molecules and are also presented on the APC's surface.

APCs, expressing viral peptides in the context of both MHC I and MHC II, migrate to a regional lymph node and present the viral peptide antigens to T-lymphocytes. APCs are particularly well suited to antigen presentation as they express 1) both MHC class I and MHC class II molecules binding viral peptides, and 2) large amounts of the costimulatory molecules which are required to stimulate activation and differentiation of naive T-lymphocytes.

T lymphocytes can be divided into two subpopulations, one with CD8 and the other with CD4 molecules on the cell's surface. Each of these populations contributes to the overall immune response in very different ways. The T-cell receptors on $CD8^+$ cytotoxic T lymphocyte (CTL) precursors recognize the complex of viral peptides bound to MHC I molecules whereas the T-cell receptors on naïve $CD4^+$ T-lymphocytes recognize peptides within the context of MHC II molecules. Importantly, simultaneous binding of costimulatory molecules such as B7 (on APCs) to CD28 (on T cells) activates the precursor cells and stimulates their differentiation into mature effector cells.

Following antigen presentation, activated CTLs return to the circulation and the skin where they are able to recognize, bind to, and destroy papillomavirus-infected cells (those keratinocytes expressing papillomavirus peptides in the context of MHC I). Cellular destruction probably occurs by the induction of apoptosis and/or through the production of cytotoxins. One CTL is able to destroy many infected cells. Because CTLs are able to selectively eliminate infected cells, this form of cell-mediated immunity is probably the most effective means of eradicating established papillomavirus infection.

$CD4^+$ T-helper cell precursors (Th0) differentiate into T-inflammatory (Th1) or T-helper (Th2) cells in response to APC antigen presentation. The pathway of differentiation depends on a variety of factors, one of the most important of which is the cytokines to which they are exposed. Th0 cells that are exposed to antigen in the presence of IL-12 and IFNg differentiate into T-inflammatory (Th1) cells whereas, Th0 cells exposed to antigen in the presence of IL-4 differentiate into T-helper (Th2) cells. In reality, it is likely that both subtypes are produced to some extent, but that one subtype becomes dominant over time with the development of a dominant cytokine milieu. For example, in response to papillomavirus infection, natural killer (NK) cells produce IFNγ and activated APCs produce IL-12. This cytokine profile drives Th0 cells into the Th1 pathway and inhibits differentiation in the Th2 direction. Th1 cells secrete a cytokine profile that further promotes differentiation toward the Th1 and away from the Th2 pathway.

Both Th1 and Th2 cells may contribute to complete elimination of papillomavirus infection. Th1 cells produce cytokines, including IL-2 and IFNγ, that promote proliferation and differentiation of CTLs. Thus, an increased number of papillomavirus-specific CTLs become available for elimination of papillomavirus infected cells. Th2 cells produce IL-4 and provide B-cell help, leading to B-cell differentiation into plasma cells and anti-papillomavirus antibody production. A strong humoral response aids in clearance of papillomavirus virus particles and can probably prevent infection of additional keratinocytes. Although a humoral response will not be able to eliminate cells that are already infected with papillomavirus, it will undoubtedly complement a cell-mediated CTL response in established infection.

Immune Responses to DNA Vaccination

The immunobiology and mechanisms operating in DNA vaccination are currently under intense investigation. Figure 8.2 summarizes the mechanisms currently believed to be involved in the generation of an immune response following vaccination with plasmid DNA. Gene gun particle bombardment and needle injection are both effective in eliciting potent humoral and cell-mediated immune responses that can protect against viral infections. As

will be discussed in a later section, the amount of DNA required for equivalent responses is higher for needle injection than gene gun delivery. This may be because gene gun administration delivers more DNA directly into the cytosol or nucleus, increasing intracellular expression and leading to a better MHC class I directed CTL response. Both somatic cells and APCs are transduced at the site of vaccination, but the immune response is initiated entirely by the APCs, as elegantly demonstrated in recent experiments. Somatic cells express plasmid-encoded antigens, often for extended periods of time, and they are able to act as a reservoir of antigen for the APCs.

The by now famous "van Gogh experiment" of Johnston and colleagues provided early evidence that immune responses against DNA vaccination were induced by circulating cells (e.g., data presented at IBC's Fifth International Conference on Vaccine Technologies Novel Developments and Approaches, Feb 9-11, 1998; Washington, DC). In this experiment, mouse ears were immunized intracutaneously with plasmid DNA using a gene gun. Within seconds the inoculated sites were surgically removed. The highly enlightening result was that the mice treated in this way still developed a small but definite antibody response, strongly implicating the involvement of transduced circulating cells and/or the less likely possibility of rapid transport of DNA to lymph nodes through lymphatics. An extension of this work was performed by Klinman et al [271] who vaccinated mice via gene gun inoculation and then surgically excised the vaccination sites at several time points after the vaccination. The excised skin was then grafted onto syngeneic mice that had not received the vaccine and the immune responses of both groups were monitored. The results of these experiments confirmed that migratory cells present at the vaccination site were responsible for initiating the immune response. A series of experiments performed by Condon et al[235] have shown that dendritic cells at the site of gene gun DNA vaccination are transduced and that these same APCs migrate within twenty-four hours to regional lymph nodes where they continue to express antigen. These data confirm that a mechanism exists by which APCs can facilitate the immune response.

The experiments described above illustrate the importance of circulating APCs. However, they do not address the issues of whether the expression of DNA by somatic cells plays an important role in the induction of an immune response or whether somatic cells can be converted into APCs. To test the hypothesis that only bone marrow-derived cells were able to induce an immune response, experiments utilizing mouse chimeras were performed. [82,140,167,170,272] Chimeric mice were generated by transplantation of bone marrow from donors with a single MHC haplotype (H2b or H2d) to recipients with both haplotypes (i.e., H2bxd). The resulting chimeras had somatic cells with both haplotypes; T lymphocytes capable of recognizing H2b-resticted and H2d-restricted epitopes; and bone marrow derived cells (including APCs) with a single haplotype (H2b or H2d, depending on which marrow they received). DNA vaccinations utilized a plasmid encoding an antigen that contained H2b-restricted epitopes and H2d-restricted epitopes. Chimeras that received the H2b bone marrow recognized the H2b-restricted epitope only, and those with the H2d bone marrow recognized the H2d-restricted epitope only. This indicates that the immune response was induced only by bone marrow-derived cells, because if somatic cells (with both haplotypes) had been able to stimulate a T-cell response, both epitopes would have been recognized. [167]

Doe et al[82] obtained similar results using a scid mouse system. Immunodeficient H2b or H2d scid mice (having APCs, but no T-lymphocytes) were engrafted with either spleen cells alone or spleen cells plus bone marrow from H2bxd immunocompetent mice. Plasmids encoding antigens with H2b or H2d restricted epitopes were inoculated into the chimeric mice. Mice engrafted with H2bxd spleen cells (containing T-lymphocytes able to recognize both H2b and H2d restricted epitopes, but containing few APCs) only recognized

H2b or H2d restricted epitopes, depending on the background of the scid mouse. This suggested that the recipient (scid) mouse APCs were responsible for induction of immunity. However, when both spleen cells (as the T-cell source) and bone marrow (containing both H2bxd APCs and T-cell precursors) were engrafted, both epitopes were recognized, even if the engraftment occurred weeks after DNA inoculation. This indicated first, that APCs from the donor marrow were able to induce an immune response, and second, that previously transduced cells were able to transfer antigen to the APCs effectively even after weeks. These results were confirmed and extended by Fu et al.[140] This group transplanted H2bxd myoblasts that were stably expressing antigen into H2bxd chimeric mice with H2b or H2d bone marrow derived cells. The CTL response was still restricted to the MHC haplotype of the donor bone marrow. This confirmed the importance of antigen presenting cells in inducing immunity and showed that myocytes could act as a source of antigen for APCs and that direct transduction of APCs with antigen DNA is not a prerequisite for an immune response.

The experiments described above still do not address whether it is possible to convert somatic cells into APCs. Keratinocytes and myocytes can be stimulated by cytokines such as IFNγ and IL-4 to express MHC class II and costimulatory molecules.[273-276] This suggests that under appropriate conditions, it might be possible for somatic cells to stimulate an immune response. On the other hand, when DNAs encoding GM-CSF, IL-12, or B7-2 were coexpressed with antigen in the chimeric mouse system, transduced muscle cells were not converted into APCs.[170] It is possible that the conditions required to convert a somatic cell into an APC were not met (i.e., a different combination of cytokines or different costimulatory molecules were required) because a myoblast cell line transfected with MHC II is capable of antigen presentation in vitro.[276] In addition, certain pathologic conditions are associated with keratinocyte expression of MHC class II molecules.[277-287] Since CD28 deficient mice are unable to generate humoral or cell-mediated immune responses following DNA vaccination, it is clear that costimulation also is important in mediating the response to DNA vaccines.[261] Therefore, care must be used in attempting to convert somatic cells into APCs by direct transduction of MHC class II genes without costimulation because of the potential to induce tolerance.[273,288] Further studies will be necessary to determine if somatic cells can act as efficient APCs when given appropriate stimulation.

In summary, at present, it is thought that the immune response generated by DNA vaccination is by APC presentation of antigen in the context of both MHC class I and II. This leads to cell-mediated and humoral immunity. Somatic cells do not appear to be capable of inducing an immune response on their own. However, somatic cells can transfer antigen to APCs for effective induction of immunity. Somatic cells at the site of vaccination may act as the primary source of expressed antigen, and may be responsible for the potent effect of DNA vaccination because they are able to act as a relatively constant source of antigen for extended periods of time.

Immune Response to DNA Vaccination Against Papillomavirus

Papillomavirus is notoriously effective in evading the natural surveillance mechanisms of the immune system, presumably at several levels. DNA vaccination has the potential to overcome many of the subversive tactics of papillomaviruses. DNA vaccination mimicks an actual papillomavirus infection in many regards. Vaccine DNA is expressed intracellularly, using the host cell's machinery in much the same way as the papillomavirus genome. Plasmid encoded viral proteins will therefore be processed and presented in a similar fashion to viral proteins in naturally infected cells, giving the immune system the best possible chance to recognize infected cells. One reason for the lack of a strong immune response against papillomavirus may be the relatively low level of expression of viral proteins, especially in

undifferentiated cells. The proteins that are expressed in undifferentiated cells are early proteins, meaning that CTLs recognizing the much more abundant late protein-derived antigens will not recognize the undifferentiated cells. Thus, the more differentiated keratinocyte, producing most of the viral protein, may be recognized by the immune system while the undifferentiated cells are left undetected and thus continue the infectious process. DNA vaccination with early papillomavirus proteins could increase the number of CTLs able to recognize and eliminate undifferentiated cells. In addition, cervical carcinoma cells which often produce only early proteins could also be detected by inducing an immune response against these proteins.

Another potentially immunoevasive feature of papillomavirus infection is the limitation of the infection to keratinocytes. Since APCs are never directly infected by papillomavirus, the predominant pathway of immune induction is not the expression of viral peptides in MHC class I of the APC. Limitation of this pathway leads to a poor CTL response and ineffective elimination of established papillomavirus infection. Since APCs can be transduced directly in DNA vaccination, particularly by the gene gun method, a much more vigorous CTL response would be expected, leading to a better chance for elimination of the virus. Finally, the eradication of papillomavirus infection may require both cell mediated and humoral immunity, a relatively common feature of the DNA vaccination technique.

Variables that Affect Vaccine Efficacy

Because the field of nucleic acid vaccination is so new, the basic principles that account for how these vaccines work are only partially understood. For example, the mechanisms that result in uptake of intact, functional plasmid DNA are still enigmatic. In addition, several parameters must be chosen for each experiment, including the expression vector, the vaccine gene, the delivery system and the optional use of adjuvants. Because of the large number of potential combinations of variables and the unique nature of each model system, however, general principles to guide the choice of a specific set of parameters are not firmly established. Despite these uncertainties, several highly effective DNA vaccines have been developed experimentally and a number of clinical trials are currently underway. At least eight institutions are investigating the efficacy of DNA vaccination against influenza, hepatitis B (Powderject Vaccines, Inc., Middleton, WI), malaria, HIV (Apollon Inc., Malvern, PA), high grade cervical dysplasia (Pangaea Pharmaceuticals Inc., Cambridge, MA) and several malignancies, (Vical Inc., San Diego, CA)[252] (see also http://www.genweb.com/Dnavax/dnavax.html for continuous updates). Currently, most human protocols are in Phase I and no large scale Phase III trials have yet been reported. The most important parameters in the development of a nucleic acid vaccine are discussed below.

The Vaccine Vector

Expression vectors that induce high in vivo levels of antigen are usually advantageous for nucleic acid vaccination, presumably because more antigen stimulates better immunity. Strong promiscuous promoters—such as the cytomegalovirus (CMV) immediate early promoter/enhancer and the Rous sarcoma virus (RSV) LTR—are generally preferred. When compared directly, CMV promoters induce significantly higher levels of antigen expression[289-291] and most DNA vaccine vectors now carry a CMV promoter. Some studies have shown that expression also is facilitated by including an intron, e.g., simian virus 40 (SV40) intron A.[143,291,292] Similarly, various termination/polyadenylation sites have been utilized.

The quantity of antigen, and hence the strength of an immune response, also can be increased by increasing the dose of a vaccine. Indeed, immune responses have frequently been enhanced by vaccinating with larger doses of nucleic acid, particularly when the goal

was to induce humoral immunity.[38,53,121,138,162,204,269,293] In certain situations, however, high levels of antigen appear to be more detrimental than beneficial, e.g., if a protein is toxic or otherwise limits the normal functions of the cells that express it. For example, nucleic acid-induced immunity to rabies virus glycoprotein B was not increased when expression of the protein was enhanced, presumably because of cytotoxicity.[201] These results suggest that in similar situations, it could be better to drive protein expression using a weaker promoter, such as the SV40 promoter, and/or to decrease the dose of nucleic acid vaccine.

Furthermore, if the goal of vaccination is to induce cytotoxic T-cell responses, high doses of vaccine may not be advantageous. Several groups have induced strong CTL and protective responses by vaccination with nanogram quantities of DNA.[173,204,269] In fact, vaccination with too much DNA may decrease CTL responses. For example, mice vaccinated with HIV gp160 DNA delivered by gene gun showed a reduced CTL response when more than 40 ng of DNA was used. (data cited in ref. 254) The dose of DNA and thus the amount of antigen available to APCs may directly or indirectly influence the development of CTLs in a way yet to be elucidated. Regardless of the operational mechanisms, titrating the dose of a nucleic acid vaccine may greatly improve the attainment of desired immunologic outcomes. Boosting regimens may also be important in eliciting optimal immune responses and seem to be more important for humoral immunity than for cell-mediated immunity (where a single inoculation is often sufficient).[100,294,295] In some cases, however, boosting seems to have no beneficial effect,[188,190] so optimal boosting regimens also have yet to be established.

The Vaccine Gene/Antigen

The sequence of the nucleic acid and the structure and function of the antigen it encodes affects the immune response. Repeated, low-dose intradermal or intramuscular injections of secreted and cytosolic protein antigens do not recapitulate the immune response induced by DNA vaccination of the same antigens, implying an independent adjuvant effect by the plasmid DNA.[296] Other studies have shown that the mammalian immune system recognizes pattern motifs in bacterial DNA that occur only rarely in eukaryotic DNA, e.g, two 5′ purines, an unmethylated CpG motif, and two 3′ pyrimidines, and responds to them by producing cytokines, e.g., IL-12, TNF-α, IL-6, IFN-α/β, IFN-γ, activating polyclonal B cells and producing antibody responses to neighboring foreign proteins.[297-304] Methylation or DNase treatment of the CpG motifs prevents the adjuvant effect[298,304] and use of bacterial DNA that does not contain the CpG motifs does not produce the adjuvant effect or stimulate cytokine release.[300] Roman et al reported that these so-called immunostimulatory sequences (ISS) could serve as adjuvants for immunization with protein subunit vaccines.[301] Moreover, the strength of the immune response and the preference for Th1 immunity depended on the exact sequence of the ISS. These results imply that the immunogenicity of a nucleic acid vaccine also may be augmented by co-administration with ISS oligonucleotides or by modification of the vaccine to incorporate ISS in to the plasmid vector. Short oligos containing these sequence motifs now have been used to promote a similar Th1-predominant immune response.[305,306] These exciting results are somewhat tempered, however, by the findings of Schwartz, D.A. et al who showed that ISS also can cause detrimental effects.[307] Treatment of mouse lungs with bacterial DNA and ISS resulted not only in a rapid inflammatory response, accompanied by the production of several cytokines and chemokines, but also in lung damage resembling cystic fibrosis (CF). A very similar effect was induced with sputum from CF patients, implying that bacterial DNA may contribute to parenchymal damage during pulmonary infection. Work is in progress to optimize and understand the mechanism of action of these motifs as well as to develop motifs which stimulate useful responses but not the harmful responses.[308] Several nucleic acid vaccination studies are currently incorporating ISS into their protocols. Once

the effects are better characterized, additional and more specific immunostimulatory sequences (ISS) may easily be incorporated into plasmid vectors to stimulate stronger specific immune responses.

Although not a feature specific to DNA vaccination, the amino acid sequence of an antigen is also important in elicitation of the immune response. First, it is clear that MHC I and MHC II molecules recognize and bind to specific amino acid sequence motifs and that different MHC haplotypes bind to different protein peptides. This means that any given antigen will have a variable number of recognizable epitopes based on its own sequence and upon the MHC (HLA) haplotype of the host. MHC restricted epitopes in any given antigen may be 1) predicted with computer programs, 2) predicted through the use of animal models and 3) determined by analysis of the T lymphocytes from patients that have been exposed to the antigen. These methods have been used to predict antigenic epitopes in papillomavirus E2, E4, E6, E7, and L1 proteins.[309-328] It is probable that a combination of the most promising epitopes will be used in a DNA vaccine clinical trial in the near future.[249,329,330]

The structure and function of the antigen also plays a role in the immune response generated against it. For example, self-assembling capsid proteins such as the papillomavirus L1 protein have a unique structure and function that provides certain advantages in stimulating an immune response. The self-assembling nature of L1 makes it a unique antigen for DNA vaccination in that it may serve to generate VLPs as well as intracellular peptide antigens, leading to enhanced humoral and cellular immunity. In contrast, proteins with toxic intracellular properties may not be as antigenic because they kill the cell before it has a chance to produce enough antigen to stimulate an immune response. It may be possible to overcome the detrimental properties of these toxic antigens by using a less efficient promoter, less DNA, and/or by targeting the antigen for almost immediate degradation, or by using relevant epitopes within the antigen rather than the entire protein. Finally, antigens may have structures that target them to specific intracellular compartments or make them secreted, membrane-bound, or cytoplasmic proteins and this may have an impact on the type of immune response generated. As will be discussed in the next section, the purposeful intracellular targeting of nucleotide encoded antigens represents another parameter that is capable of significantly influencing the immunological response to an antigen.

Intracellular Targeting of the Antigen

The intracellular destination of an antigen, e.g., whether it is a secreted, membrane-bound, cytosolic, or nuclear protein is another antigen-associated variable in nucleic acid vaccination. Genes encoding secreted proteins have generally been preferred for the induction of humoral immunity since extracellular proteins normally induce primarily humoral immunity via the exogenous pathway. Gene products that are not normally secreted can be genetically modified to do so, simply by engineering a secretory signal sequence at the 5' end of the gene. Although DNA encoding secreted antigen tends to promote higher antibody titers (predominantly IgG1),[64,331] several investigators have shown that DNA encoding secreted or non-secreted forms of the same antigen induce similar cell-mediated responses (Th1 and CTL) as well as strong humoral responses.[35,45,64,201,331,332] This parameter might be easily modified to predispose the immune response toward a humoral or cell-mediated direction.

One feature that determines the type of immune response induced by DNA vaccination is the degradation pathway into which an antigen is directed, as discussed previously in the immunobiology section. Exogenous (engulfed) antigen is classically processed via lysosomes and endosomes for presentation to $CD4^+$ T-lymphocytes in the context of MHC class II (see Immunobiology section). The lysosomal-associated membrane protein LAMP-1 is a

transmembrane protein involved in lysosomal sorting of endogenously synthesized receptor proteins.[333] Fusion of the DNA encoding LAMP-1 to DNA encoding other antigens has the potential to target those antigens to the lysosome where processing by the exogenous pathway may be enhanced. Wu et al[334] have shown that this intracellular targeting strategy works for papillomavirus E7 protein (which will be discussed in detail in the progress section of this chapter). In theory, this targeting strategy should lead to more efficient MHC class II antigen presentation and increased Th1 or Th2 responses against any antigen.

The second major intracellular degradation pathway is through ubiquitination and trafficking to the proteasome (see Immunobiology section).[245,270] Peptides produced by proteasomal degradation ultimately bind to MHC class I molecules and travel to the cell surface where they are presented to $CD8^+$ T cells. A nucleic acid vaccination strategy that elegantly capitalized on this mechanism was recently described by Rodriguez et al who engineered the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) gene to be translated in frame with a ubiquitin monomer.[185] Immunization of mice with this gene induced strong CTL responses presumably due to rapid entry into the MHC class I pathway, which left insufficient antigen for interaction with B cells. Moreover, mice immunized with the modified NP gene and challenged with LCMV were markedly protected, whereas control mice vaccinated with a plasmid encoding native NP were only minimally protected compared to controls that did not receive the NP gene. Thus, fusion of DNA encoding ubiquitin with DNA encoding other antigens appears to be a promising way to increase CTL responses against any antigen and should be considered when that type of immunity is advantageous.

Delivery Systems

Delivery vehicles comprise another arena with the potential to direct an immune response and permit new routes to be utilized. It is likely that the amount of DNA used, the mechanism by which DNA is transduced into the host cells, and the immunologic microenvironment at the site of injection all play a role in the outcome of the vaccination. Some studies have shown that the delivery device affects the induction of a Th-1 vs. Th-2 type of cellular immunity.[145,168] Feltaquate et al[168] found that a Th-1 type of cellular immunity was preferentially induced by needle injection of DNA (either intramuscularly or intradermally), whereas a Th-2 type of response was most prominent when a gene gun was used in the same tissues. This might suggest that needle injection could be more effective for infections that are eradicated via the Th1 pathway (e.g., *Leishmania*) and gene gun inoculation for infections whose clearance utilizes the Th2 pathway (e.g., *pneumococcus*). Part of the apparent bias toward Th1 responses with needle injection as compared to gene gun inoculation may be due to the larger amounts of DNA used in the needle injection method which may result in an adjuvant effect that predisposes toward the Th1 pathway. Alternatively, most of the needle injection experiments demonstrating Th1 predominance have been administered intramuscularly where a Th1 bias may be due to the more prolonged antigen expression that is seen in muscle vs. skin. In support of this idea, others have shown that a humoral immune response could be shifted from IgG1 to IgG2a (isotypes indicative of a Th2 or Th1 pattern of response, respectively) by increasing the immunizing dose of DNA using a gene gun. Other studies, however, fail to support the idea that Th1 responses are favored by high doses of nucleic acid vaccines because strong CTL responses (i.e., Th1) also can be induced using nanogram quantities of DNA.[173,204] More investigation is necessary to determine the mechanism by which these apparently contradictory results are occurring. However, once the fundamental principles have been established, it is expected that modifications in the method and route of DNA delivery will help direct the outcome of the vaccination.

Because many infections will probably require mucosal immunity, another area under extensive investigation is DNA immunization of mucosal surfaces. Studies in progress will determine whether immune responses can be induced by direct inoculation of oral and genital mucosa with DNA-coated gold particles using a gene gun (Russ Smestad, Powderject, Inc., Middleton, WI, personal communication). Other investigations are underway to optimize delivery of DNA to nasal,[73,97,101,103,111,190,256,291,335,336] gastrointestinal,[337,338] and respiratory[339-341] mucosae. Cationic lipids, commonly used for transfection in vitro, are now also being applied in several in vivo models for the mucosal delivery of DNAs.[40,43,190,256,291,336,340-346] Encapsulation of nucleic acid vaccines for nonmucosal inoculation could prove beneficial by protecting the otherwise naked DNA (or RNA) from the shearing and degradative forces it encounters on its way to the cell nucleus. A promising encapsulation technology for this purpose is being developed by Pangaea, Pharmaceuticals Inc., Cambridge MA.[217]

Another related approach to obtaining mucosal or non-mucosal immunity with nucleic acid vaccines is to incorporate them into live bacteria with an appropriate tissue specificity, using standard transformation methods. The feasibility of this approach has been demonstrated using *Shigella* transformed with a plasmid encoding β-galactosidase,[347,348] a highly immunogenic protein. Mice vaccinated intranasally with the recombinant *Shigella* developed β-gal specific antibody and cellular proliferative responses. This method, which is one step removed from using the naked plasmid itself, should greatly increase the efficiency of DNA delivery and provide a simple methodology for developing oral and other vaccines. Other bacterial vectors, including *Salmonella*, also will be useful.[349-352] VLPs with the potential to penetrate gastric mucosa (e.g., rotavirus and Norwalk virus VLPs) are also being evaluated as potential carriers for nucleic acid vaccines.[338]

Host Factors

Host variability contributes another level of complexity. Much of the work with nucleic acid vaccination has been performed with inbred mouse populations that have syngeneic immune systems. It has long been known that there is variability in the immune response against DNA vaccines between different inbred mouse strains. This variability is compounded when outbred animal models are required, but outbred animals are more representative of the human situation. A second feature of the host which is relevant to nucleic acid vaccine design is the age of the host.[263,353] Immature hosts are often more responsive to DNA vaccination than adults, probably because of higher levels of expression of DNA in young animals.[54,136,206,254,353] In fact, it is possible that immunodeficiencies or predispositions toward T2 responses, secondary to immaturity of the host, may be overcome through DNA vaccination.[77,154,187,191,354] This feature is of particular relevance when one considers the fact that prophylactic human vaccines are often administered at a young age. It might even be advantageous in certain circumstances to select young animal model systems for the initial evaluation of nucleic acid vaccination.

Augmentation of Immune Responses

Recent studies suggest that cytokine genes can augment the effects of DNA vaccination. Several studies have shown that granulocyte-macrophage colony stimulating factor (GM-CSF), for example, augments the ability of APCs to express antigen and costimulatory molecules.[355,356] Immunization with peptide-pulsed dendritic cells is greatly enhanced by adding GM-CSF to the culture medium prior to inoculation.[357] Furthermore, intradermal injection of recombinant GM-CSF protein into human skin substantially increased the number of Langerhans cells at the site of injection.[358] Thus, strategies to augment immunity induced by nucleic acids include coinjections of the vaccine gene with a GM-CSF

gene or injection of a GM-CSF-antigen fusion gene.[259,260] These strategies have yielded promising results in vaccines for tumors[221,227,228,234,253] as well as for viruses.[32,62,258]

Another cytokine, interleukin 12 (IL-12) has the capacity to boost anti-tumor immune responses in vitro and in vivo.[359-361] Its presence stimulates proliferation and cytolytic activity in a variety of tumor infiltrating lymphocyte (TIL) lines.[361-363] For example, specific $CD8^+$ T cell cytolytic activity against the tumor line mastocytoma P815 was enhanced 10-fold in the presence of IL-12.[363,364] Furthermore, tumor-specific cytotoxic activity of TILs isolated from a number of human cancers was greatly augmented by in vitro culture in IL-12-containing media.[361] Tahara et al showed that IL-12 transfection of mouse MCA207 sarcoma tumor cells rendered them capable of protecting mice against MCA2076 tumor challenge.[361] As with GM-CSF, coadministration of IL-12 enhanced both antiviral[104,109,116,127,169,256] and anti-tumor[255] immunity induced by DNA vaccination. Hence, coinoculation of a cytokine expression vector together with a nucleic acid vaccine or fusion of the two may be used to enhance immune responses to nucleic acid vaccines.

Safety Concerns Regarding Nucleic Acid Vaccination

While adverse effects of nucleic acid vaccination have not been observed in animal studies generally, there are potential safety hazards.[365-372] An obvious concern is that a vaccine gene, derived from a pathogenic organism, could induce a pathogenic effect on its own. For example, the high risk HPV E6 or E7 gene could disrupt normal functions of the cellular tumor suppressor gene p53 or pRb, respectively,[373] and thereby potentially induce tumors. This possibility can and should be circumvented by mutating and functionally inactivating the vaccine gene. Indeed, the most effective nucleic acid vaccines will probably encode a series of immunogenic epitopes and lack all pathogenic activities associated with the target gene.

Another concern is that integration of the plasmid into cellular genes could induce tumorigenesis.[371] However, PCR analysis using methods that could detect 1/150,000 nuclei failed to detect integration events in muscle tissues of mice that had been experimentally inoculated with plasmid DNA. This was calculated to be three orders of magnitude less than the spontaneous mutation rate.[374] Since the most likely mechanism of integration is through homologous recombination, known sequences in the human genome should be avoided. Regulatory elements should be of minimal size and derived, if possible, from eukaryotes other than humans. If cytokine or chemokine genes are to be used, it may be possible to substitute for the human gene a homologue from another species, although this also poses the risk of potentiating immune responses to the (heterologous) cytokine itself. Another possibility for preventing recombination is to alter codon usage to minimize the extent of nucleic acid homology while preserving amino acid homology. Alternatively, the cytokine mRNA (or even cytokine protein) could be substituted for the cytokine gene. Finally, the limited success of gene therapy vectors that are specifically designed for integration into cellular DNA strongly suggests that unintentional integration will not be a major problem.

Several outcomes can be envisaged as a consequence of insertional mutagenesis of a cellular gene by nucleic acid vaccine sequence. First, the integration could disrupt an essential gene, which would lead to cell death. Alternatively, the effect could be silent, if the integration disrupted one copy of a gene and the other copy was functional. If the disruption occurred in a tumor suppressor gene, the immediate effect would be silent although long-term the cell might be predisposed to the development of cancer. Still, since cellular mutations occur constantly (without vaccination), and since tumorigenesis is a multistep process, the effects of such an event would most likely be minimal. Integration might cause genetic instability which, if sufficiently massive, would almost certainly lead directly to cell death. Chromosomal rearrangements could also induce the expression of

novel antigens, e.g., by fusing two unrelated genes. Cells that expressed those antigens could be eliminated by normal host immunologic surveillance mechanisms. In addition, cells with vaccine-induced mutations might still express the vaccine gene and thus be destroyed by immunologic responses induced by the vaccination itself.

Another area of concern is the possible induction of hyperimmune responses to a nucleic acid vaccine, as can occur with tetanus vaccination. Responses could be directed to the antigens encoded by the vaccine or to the nucleic acid sequences of which it is made. Should a severe reaction occur, the cells expressing the vaccine antigen and/or containing the vaccine should be surgically removed. Excision of a skin site would obviously be simpler and less traumatic than excision of a muscle site. Future studies would then identify the epitope or DNA sequences responsible for this effect, so that it/they could be eliminated from the vaccine, or at least from vaccine preparations for an identifiable subset of high risk patients.

DNA vaccination could have a potential to induce autoimmunity or immunologic tolerance. Tolerance has been induced in neonates of some species[375] but not in others, e.g., intramuscular injection of subimmunogenic doses of DNA vaccines did not induce tolerance in young non-human primates.[295] A study by Mor et al showed that intramuscular delivery of DNA vaccines did not stimulate the production of antimuscle antibodies or the development of myositis.[376] Furthermore, repeated DNA vaccination did not affect the development of nascent autoimmunity in lupus-prone mice, suggesting that the DNA vaccines did not initiate or accelerate the development of systemic autoimmunity.

Nucleic Acid Vaccines for Papillomavirus: Recent Progress

Cottontail Rabbit Papillomavirus Model System

Nucleic acid vaccines against papillomaviruses have been studied in rabbits and mice. The cottontail rabbit papillomavirus (CRPV)-rabbit system is an established small animal model of virus-induced papillomatosis.[377,378] (see chapter 10). Rabbit papillomas form three to six weeks after experimental CRPV infection. The clinical course of papilloma formation and growth during the first four months is dramatic as illustrated in Figure 8.3. After about one year, rabbit papillomas frequently progress to squamous cell carcinomas, closely paralleling disease progression in high-risk HPV-associated lesions in humans. The CPRV-rabbit model has been used to explore nucleic acid vaccination as a means of preventing viral infection (Table 8.3). Two laboratories, including ours, have immunized rabbits with CMV-driven DNA expression vectors encoding the CRPV L1 major capsid protein, via an intramuscular[196] or intracutaneous[198] route. Both vaccines induced high titered L1-specific antibody responses, and antisera from L1 DNA-immunized rabbits also neutralized infection by CRPV. Most importantly, the L1 DNA vaccines induced virtually complete protection against papilloma formation following CRPV challenge.[196,198] L1 DNA vaccination also induced L1-specific cellular proliferative responses,[198] suggesting that an L1 vaccine could be useful for immunotherapy.

Our laboratory recently reported that intracutaneous vaccination of rabbits with a CMV-driven DNA expression vector encoding the full length E6 protein also provided significant protection against CRPV infection.[195] None of the E6 DNA-vaccinated rabbits developed detectable antibodies to E6 protein, but half of them (3/6 rabbits) exhibited significant cellular proliferative responses ($p = 0.03$) and, of these, two were completely protected (0/18 sites formed papillomas). The remaining four E6-vaccinated rabbits were partially protected, as evidenced by: 1) a retarded onset of papilloma formation, 2) a reduced frequency of papilloma formation, and 3) a slower rate of growth, compared to control rabbits (that developed large papillomas at 36/36 challenge sites within five weeks).

Table 8.3 Prophylactic DNA vaccination in the CRPV-rabbit model

Formulation Vaccine Gene	DNA Delivery		Immune Response		Protection Against Papilloma				
						Partial Protection [d]			
	Tissue	Device	Humoral [a]	Cellular [b]	Complete [c] Protection	Onset	Frequency	Size	Ref.
L1	Muscle	Needle	100% [e]	NI	80% [f]	NI [g]	NI	↓	196
L1	Skin	Gene gun	100%	80%	80%	NI	↓	↓	198
E6	Skin	Gene gun	None [h]	50%	33%	↓	↓	↓	195
E6	Muscle	Needle	None	+ [i]	None	None	NI	None [j]	376
E1	Muscle	Needle	None	+	None	None	NI	None	376
E2	Muscle	Needle	None	+	None	None	NI	↓	376
E7	Muscle	Needle	None	+	None	None	NI	None	376

[a]Determined prior to CRPV challenge by ELISA (195, 196, 198) or Western blot (376).
[b]Determined prior to CRPV challenge by cellular proliferation assays.
[c]Rabbits that did not form any papilloma.
[d]Partial protection means a retarded onset and/or lower frequency of papilloma formation and/or smaller papilloma size.
[e]Percentages indicate the percentage of rabbits that exhibited the characteristic.
[f]In addition, one rabbit (1/5) formed a papilloma that subsequently regressed.
[g]NI = no information reported.
[h]None indicates that the characteristic was not detected.
[i]A plus sign means that an unknown percentage of rabbits exhibited the characteristic.
[j]Papillomas in these rabbits grew *larger* than controls and they were *not* protected.

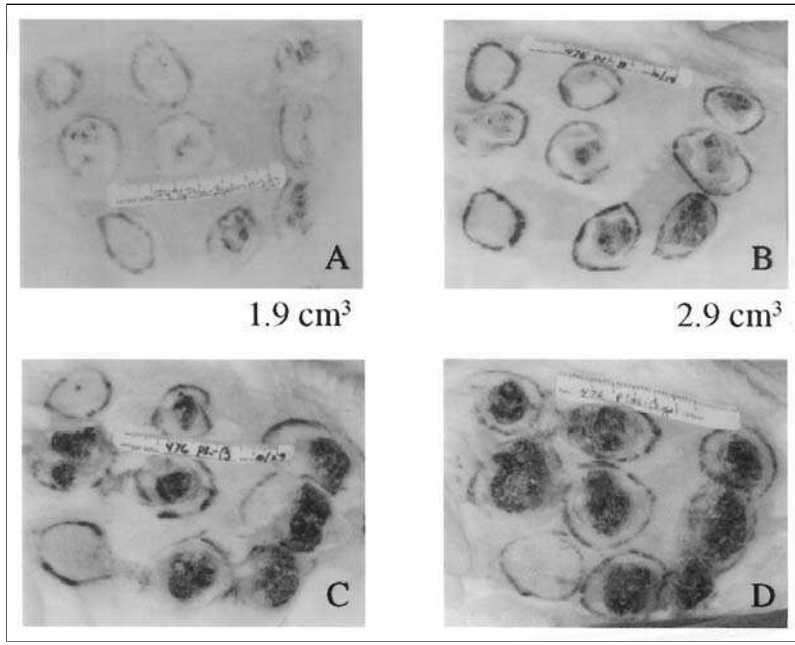

Fig. 8.3. The Clinical Course of Papilloma Formation and Growth in the CRPV-Rabbit Model. A New Zealand white rabbit was inoculated with control DNA vaccine and then infected with CRPV virus at nine sites. Photographs in panels A, B, C, D were

Recently, we have greatly augmented rabbit protection by using a cytokine gene in combination with our E6 DNA vaccine (unpublished results).

The laboratories of Neil Christensen and John Kreider have used an intramuscular route to vaccinate rabbits with another CMV-driven E6 expression vector. Several of their E6-vaccinated rabbits developed E6-specific cellular proliferative responses, but none developed E6-specific antibodies, a result that agrees with our study. However, in contrast to our results, their E6-vaccinated rabbits were not protected and formed papillomas that were larger than the controls[379] (and Neil Christensen, personal communication). One explanation for the discrepancies between these results and ours could be the DNA dose. Our group used low doses of a E6 DNA vaccine compared to the Christensen group. Higher doses of DNA are known to decrease CTL responses in some systems, and thus the Christensen group may have induced a level of antigen that exceeded the appropriate level for an effective prophylactic response. Vaccination site and method of delivery also clearly affect the type and degree of immune responses. Therefore, a second possibility for the discrepancy is that the generation of immunity against E6 was due to a difference in the site of vaccination, i.e., skin vs. muscle, or the method of vaccine delivery, i.e., gene gun vs. needle.

Christensen's experiment also contained additional experimental groups of rabbits that were injected with vaccines encoding the CRPV E1, E2 or E7 gene. Some rabbits developed cellular proliferative (but not antibody) responses to the protein encoded by the vaccine. None of the rabbits was completely protected from CRPV challenge, although it was noted that papilloma volumes in the E2 DNA-vaccinated rabbits were smaller than controls. Much

work remains to be done in this system to determine the optimal choice of gene products and vaccination conditions to obtain DNA-induced prophylaxis of and, ultimately, treatment regimens for papillomavirus infections.

Mouse Model Systems

The second animal host in which papillomavirus DNA vaccination studies are being performed is the laboratory mouse. Mice are ideal for studying the immunological basis of an immune response because of the advanced knowledge the mouse immune system, the availability of many laboratory reagents and the large number of inbred mouse strains. However, mice are not susceptible to infection by known papillomaviruses, so they are challenged instead by transplantation of tumor cell lines engineered to express papillomavirus antigens. While the challenge is artificial, it does provide the ability to evaluate immunological responses to vaccines expressing human papillomavirus antigens.

The laboratories of Drew Pardoll and T.C. Wu have shown that naked DNA may be just as effective in inducing immunity as their previously described recombinant vaccinia virus.[380] These investigators inoculated C57/BL6 mice with 1) Sig/16E7/LAMP-1 (a DNA vector encoding a fusion between sequences encoding the lysosomal-associated membrane protein 1 (LAMP-1) and the HPV16 E7 protein), 2) a wild type HPV16 E7 expression vector, or 3) control plasmid DNA. The mice received a single injection of DNA, delivered either intramuscularly or intracutaneously. An impressive eighty percent of the Sig/16E7/LAMP-1 DNA-vaccinated mice (4/5) remained tumor free one month after challenge, whereas all other mice developed tumors within two weeks[380] (and T.C. Wu, personal communication). In addition, only Sig/16E7/LAMP-1 DNA-vaccinated mice developed E7-specific CTL and antibody responses. These results highlight the way in which experimental animal studies facilitate the optimization of vaccination parameters for a particular antigen or type of immune response.

Human Clinical Trials

The first clinical trial to test the safety and efficacy of DNA vaccination for papillomavirus infection has recently begun at the Brigham and Women's Hospital, Boston, MA. This trial uses technology developed by Pangaea Pharmaceuticals Inc. for the delivery of DNA vaccines. The goal of the trial is to treat HPV16-associated high grade squamous epithelial lesions (HSIL) using a DNA vaccine that will incorporate several HPV16-specific CTL epitope-encoding plasmids into biospheres that will be inoculated intramuscularly or subcutaneously. A proprietary method of targeting these antigenic epitopes into intracellular compartments will also be utilized in an effort to maximize the immune response (Robert Urban, Pangaea Inc., personal communication).

In summary, DNA vaccines based on the papillomavirus early and late genes have already induced prophylactic immunity against challenge in the CRPV-rabbit model of viral infection. The HPV16 E7 gene also has been shown to induce (in mice) partial protection against challenge with E7-expressing tumor cells. Future studies will undoubtedly be expanded to explore the induction of mucosal immunity and to test the ability of DNA or mRNA vaccines to induce therapeutic immunity against preestablished papillomavirus infections of varying stages of duration and clinical severity.

Future Possibilities and Promising Advances

VLPs as a DNA Vaccine

Several studies have demonstrated that self-assembling protein particles, e.g., hepatitis B surface antigen (HBsAg), induce strong prophylactic immunity when used for vaccination.

Furthermore, recombinant chimeric particles composed of a self-assembling protein fused to additional heterologous epitopes can induce strong and protective immunity to the fusion partner antigens, e.g., HBsAg fused to HIV or hepatitis C antigens.[46,47,381] Similar experiments using protein chimeras between papillomavirus VLPs and the papillomavirus E7 protein, i.e., a L1-E7 fusion protein, induce immune CTL responses to both members of the chimeric antigen.[382,383] DNA vaccines encoding the CRPV L1 gene also function as highly protective immunogens, inducing conformational and neutralizing antibodies as well as cellular immunity.[196,198] These results indicate that chimeric DNA vaccine genes composed of L1 coding sequences fused to coding sequences of heterologous antigens could serve as an excellent vehicle to induce/enhance immunity to heterologous fusion partners.

Vaccination Augmentation Through Intracellular Targeting

As discussed earlier in this chapter, intracellular targeting of antigen to the lysosome for degradation via fusion with LAMP-1 has the potential to augment the immune response to that antigen. Wu et al vaccinated mice with a vaccinia virus that had been engineered to express a chimeric protein from a gene composed of the HPV16 E7 gene inserted between DNA fragments for the N-terminal signal peptide and the transmembrane and cytoplasmic tail of LAMP-1.[334] The vaccinated mice developed not only enhanced MHC-II presentation of E7 to $CD4^+$ T cells, but also enhanced priming of MHC-I restricted $CD8^+$ cytotoxic T cells (presumably secondary to augmented $CD4^+$ T cell help). Moreover, immunization with the LAMP-1-E7 expression vector prevented the growth of an E7-expressing tumor and even cured mice with small established tumors.[385] This group has also vaccinated mice with a plasmid DNA encoding the Sig/HPV16 E7/LAMP-1 gene in subsequent experiments and produced results similar to those achieved with the recombinant vaccinia virus (see the section on mouse model systems).[380] These experiments suggest that a promising way to augment the effect of DNA vaccination is through the use of chimeric plasmids, encoding LAMP-1 targeting sequences linked to sequences encoding the antigen.

Nucleic Acid Vaccination with Expression Libraries

One of the most promising new developments in nucleic acid vaccination is the use of entire expression libraries.[245-247] Animals are vaccinated with a library that expresses all the antigenic epitopes encoded by an organism. The vaccinated animals are then challenged by experimental infection and monitored for the desired outcome (prophylaxis, therapy, immune response). Those animals that exhibit the desired response are then screened to identify the antigens to which they have mounted effective immune responses. Once relevant epitopes have been identified, they can be combined to create an effective multivalent vaccine. The advantage of this method is that it allows the relevant epitopes for a given outcome to be determined directly rather than by trial and error of individual proteins. This method is particularly valuable for developing vaccines against organisms for which the antigenic epitopes have not yet been identified. This could apply to complex organisms such as *Mycobacterium pulmonis*[245] but also to much simpler organisms such as papillomavirus, since numerous papillomavirus-specific B- and T-cell epitopes are still unknown.

Designer Vaccines for Papillomavirus Infections

To conclude this chapter, we offer a discussion of nucleic acid "designer vaccines" for the prevention and treatment of papillomavirus infections. Designer vaccines will be tailored to induce a specific type of immune response, for a certain stage of disease, in a particular individual. Nucleic acid vaccinology offers the best mechanism to produce designer vaccines, and HPV is an ideal candidate for this approach. This is because HPVs

comprise a large number of virus (sub)types, e.g., over 20 different HPVs in the genital tract; induce a heterogeneous array of clinical disease manifestations; and and elicit variable host immune responses, depending on the individual.

We envision a collection of papillomavirus vaccine components, each encoding a unique set of viral epitopes, that can be combined in different ways to individualize a therapeutic or prophylactic vaccine. A special advantage of the cocktail approach is that individualized vaccines can be quickly assembled to target (or exclude) specific antigens and/or antigens from new viral types. Vaccines will be tailored to match a particular viral type(s) in a particular stage of disease (i.e., active infection vs. latent infection vs. malignancy or even prophylaxis). Components to treat benign, productive papillomas will induce cellular immunity (for the elimination of infected cells) and humoral immunity (for the clearance of viral particles). Components to induce a CTL response will undoubtedly encode linear epitopes of the early proteins E1, E2, E4, E6 and E7. Components to induce neutralizing antibodies will encode conformational epitopes of the L1 and L2 capsid proteins, in particulate and/or secreted forms. The L1/L2 components also would be particularly useful for prophylaxis. Vaccines to treat latently infected cells will contain components that are specifically expressed during latency. For example, Maran et al showed that laryngeal tissue latently infected with HPV11 expressed E1 and E2 transcripts but not E6 or E7 transcripts,[384] suggesting that HPV11 latency in the larynx could best be treated by vaccines against HPV11 E1 and E2 epitopes. Vaccines to prevent or treat premalignant and malignant disease will encode E6 and E7 epitopes, that are consistently expressed in those lesions.

Designer vaccines will also be tailored to the individual vaccinee according to his or her genetic background. HLA-restricted epitopes from each HPV type-specific protein will be matched to the patient's HLA type. Other individual needs also can be accommodated by this approach, e.g., the patient's state of immunocompetence. For example, different cytokine genes will be used as part of a vaccine cocktail to induce or augment an immune response. The choice of cytokine genes may be determined by an individual's profile of natural cytokine production and/or her ability to produce cytokines following DNA vaccination. The designer approach may also permit effective vaccination of patients with specific immunodeficiencies (who are usually anergic to standard vaccine protocols) through the use of cytokine genes that can complement the deficiency.

Recent advances in biomedical research strongly suggest that nucleic acid designer vaccines will come into widespread use in the near future. DNA cocktails are already easy to prepare. Knowledge regarding the role of individual papillomavirus proteins during various stages of the virus life cycle, and of host genotype-specific response patterns, will provide powerful tools to guide the choice of viral epitopes. The combination of appropriate epitopes in the context of appropriate adjuvants will create optimal vaccines to induce strong, long-lasting immune responses to each of the stages of papillomavirus infection.

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CHAPTER 9

Experimental Vaccine Strategies for Cancer Immunotherapy

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The ideal cancer therapy for advanced cervical cancers should have the potency to eradicate systemic tumor in multiple sites in the body, as well as the specificity to discriminate between neoplastic and nonneoplastic cells. In both of these respects, immunotherapy is an attractive approach. The immune system comprises a number of cell types which, when activated, are extremely efficient at killing target cells. Most important, the immune system is highly specific. The two arms of the immune system (B cells and T cells), each possess vast arrays of clonally distributed antigen receptors. The tremendous diversity of these receptors enables the system to recognize foreign antigens and to discriminate self from nonself.

Importance of Cell-Mediated Immune Responses in Controlling Both 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 refs. 1-3). First, the prevalence of HPV-related diseases is increased in transplant recipients, patients receiving immunosuppressive therapy, pregnant women, and patients infected with the human immunodeficiency virus (HIV).[4] 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.[5,6] Third, infiltrating $CD4^+$ (T helper cells) and $CD8^+$ (cytotoxic/suppressor T cells) T cells have been observed in spontaneously regressing warts, and warts in patients who are receiving immunosuppressive therapy often disappear when this treatment is discontinued.

The cell-mediated immune system is equipped with multiple effector mechanisms capable of eradicating tumors, and most of these anti-tumor immune responses are regulated by T cells. Activated T cells may function directly as effector cells, providing anti-tumor immunity through the lysis of tumor cells or through the release of cytokines capable of interfering with the propagation of tumor. Anti-tumor immune responses can also be achieved through the influence of T cell-derived cytokines on other components of the immune system. Cytokines released by helper T cells can alter the nature of the ensuing immune response through their effects on T-cell activation. Also, the function of "nonspecific" effector cells such as macrophages, natural killer (NK) cells and granulocytes are critically regulated by T cell-derived factors. In addition to their regulatory function, T cells also possess the ability to recognize tumor-specific antigens. Tumor-specific antigens (such as HPV16 E6 and E7) serve as targets which T cells can use to distinguish neoplastic from

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nonneoplastic tissues. Therefore, T-cell-mediated immunity is the most crucial component of antigen-specific anti-tumor immunity.

Antigen Processing and Presentation: Relevance for T-Cell Recognition of Specific Antigens

It is now clear that at least two distinct pathways exist for the processing of antigens recognized by T cells. $CD8^+$ cytolytic T lymphocytes (CTL) recognize antigens that are presented on MHC class I molecules. MHC class I molecules are expressed on most cells of the body and carry with them peptide fragments of endogenously synthesized proteins. These peptide fragments are transported from the cytoplasm into the endoplasmic reticulum, where they complex with newly assembled class I molecules on their way to the cell surface.[7] In this way, $CD8^+$ CTL are capable of identifying novel foreign antigens derived from the cell interior. In contrast, $CD4^+$ T helper cells identify peptide antigens that are presented on MHC class II molecules predominantly expressed on specialized antigen-presenting cells (APCs) such as macrophages, dendritic cells, and activated B cells. For $CD4^+$ T cells to recognize complex antigenic proteins, the exogenous antigens must first be engulfed by APCs and delivered to low pH endosomal and lysosomal compartments containing proteases, where they are degraded into peptide fragments. The peptide fragments are further sent to the compartments of peptide loading where they bind with MHC class II molecules and are presented to $CD4^+$ T helper cells.[7] Tumor-specific antigens, when efficiently presented by antigen presenting cells to both $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, are capable of inducing potent T cell mediated immunity—the most crucial component of anti-tumor immunity. Therefore, the ideal cancer vaccine would enhance both CD8+ cytotoxic T cell and $CD4^+$ helper T-cell responses by delivering a tumor-specific antigen into both the MHC class I and class II pathways of antigen presentation.

Dendritic Cell as a Central Player for Tumor Vaccine Development

Dendritic cells (DCs) are the most potent professional antigen-presenting cells (APCs) that are specialized to prime helper and killer T cells in vivo (for review see refs. 8-10). Dendritic cells can stimulate T cells because of their high levels of MHC class I and class II molecules, costimulatory molecules like B7, and adhesion molecules like ICAM-1 and ICAM-3 and LFA-3. To effectively present antigens, DCs perform a series of coordinated tasks.[11] Immature DCs develop from hematopoietic progenitors and are strategically located at body surfaces and in interstitial spaces of most tissues. There, DCs are equipped to capture antigens and to produce large numbers of immunogenic MHC-peptide complexes. In the presence of maturation-inducing stimuli such as inflammatory cytokines or stimulation via CD40,[12] DCs upregulate adhesion and costimulatory molecules to become more potent, terminally differentiated, stimulators of T-cell immunity. At the same time, numerous intracellular MHC class II compartments seem to discharge MHC class II-peptide complexes to the cell surface where they can be unusually long lived.[13,14] DCs also migrate to secondary lymphoid organs to select and stimulate rare antigen-specific T cells.[15] Thus, vaccine strategies employing dendritic cells to enhance T-cell-mediated immunity against tumor have become extremely important.

In this chapter, we will review current strategies that focus on using dendritic cells in the development of tumor vaccines. We will first discuss strategies that use dendritic cells via ex vivo methods for the development of cancer vaccines. We will then focus on strategies that expand functional dendritic cells in vivo. A particular emphasis will be placed on GM-CSF and Flt3 ligand (Flt3-L) which have recently proven to be important factors in the in vitro as well as in vivo generation of dendritic cells. Finally, the increased understanding of intracellular sorting pathways for antigen presentation creates the potential to modify the

antigen delivery pathways to enhance vaccine potency. We will describe how researchers use this information to design tumor vaccines to enhance both MHC class I and class II presentation. Although some of these strategies are not developed using HPV antigens, all of these approaches that we cover in this chapter are applicable to the development of HPV vaccines.

Manipulation of the Dendritic Cells Via Ex Vivo Methods

Generation of Dendritic Cells Ex Vivo

For years it has remained difficult to generate large numbers of DCs because little was known about DC maturation and the lineage specific markers which define their cellular differentiation state. Recently, new advances have been made in understanding not only the origin of DCs and their antigen uptake mechanisms, but also the signals that stimulate their migration and maturation into immunostimulatory antigen-presenting cells (for recent review see refs. 14, 16).

These advances in DC biology have contributed to the development of several different methods to generate large numbers of active DCs in vitro. The methods have been derived from the realization that the cytokines, granulocyte macrophage-colony stimulating factor (GM-CSF) and Flt-3 ligand, are critical factors in inducing the differentiation of primitive hematopoietic precursors in DCs as a lineage distinct from macrophages and granulocytes.[16-21] DCs can be generated from either proliferating $CD34^+$ or from nonproliferating $CD14^+$ progenitors. $CD34^+$ cells are present in bone marrow, in cord blood, and in adult blood, and each can serve as a source of progenitors for DCs. Under GM-CSF and TNF-α, $CD34^+$ hematopoietic progenitors develop into functional DCs.[22-29] Similarly, $CD14^+$ blood monocytes can differentiate into functionally mature dendritic cells under the function of GM-CSF and IL-4 cytokines.[30-32] Recently, Flt3-L has been noted to have a growth-stimulatory effect on dendritic cells.[33,34] Flt3-L in cooperation with TGF-β1 potentiates in vitro development of dendritic cells and allows single cell dendritic cell cluster formation under serum-free conditions.[35] More recently, morphologically equivalent DCs have been generated in serum free media. This method avoids the undesirable clinical risks of allergic reaction or simulation of immune responses against unintended antigens.[36] The DCs derived from culturing hematopoietic progenitors appear to have similar APC function as that of purified mature DCs. Therefore, DCs generated ex vivo provide a source of professional APCs for use in experimental immunotherapeutics.

Immortalized DC lines have recently become available. Paglia et al generated an immortalized murine DC line utilizing a retrovirally delivered env-myc fusion gene into primary murine splenic cultures. The cloned DCs exhibited the ability to process and present antigen in vitro as well as to sensitize T lymphocytes in vivo.[37] Recently, Shen et al transduced GM-CSF into murine bone marrow cultures followed by supertransfection with myc and raf oncogenes. They successfully generated an immortalized clone which displayed dendritic morphology, dendritic cell-specific markers, and high levels of MHC class I and II molecules and costimulatory molecules.[38] These immortalized DCs represent a very important reagent for testing various tumor vaccine strategies that use DCs as adjuvants.

Vaccine Strategies Using the Dendritic Cells Generated Ex Vivo

The availability of large quantities of active DCs generated ex vivo has created the opportunity to test various vaccine strategies employing DCs for immunotherapy. There are several vaccine strategies using DCs prepared with known tumor specific antigens such as HPV16 E6/E7 or with undefined tumor antigens. Vaccine strategies using the DCs generated ex vivo can be classified as the following:

1. Pulsing the DC with peptides/proteins
2. Transducing genes encoding tumor specific antigens (TSAs) into the DC through naked DNA or viral vectors;
3. DCs armed with the full antigenic spectrum of tumor cells (for those tumors with uncharacterized tumor specific antigens).

The various vaccine strategies are summarized in Figure 9.1. Of these vaccine strategies, some have been used in the design of HPV vaccines, while others potentially can be applied to the future development of HPV vaccines.

Dendritic Cells Pulsed with Peptides/Proteins

Peptide-based vaccines for cervical cancers are discussed in chapter 5. Here, we mainly focus on recent development on vaccine strategies using dendritic cells pulsed with peptides/proteins. Presentation of peptides derived from tumor-specific antigens to the immune system by DCs is a promising method of circumventing tumor-mediated immunosuppression. Dendritic cells pulsed with tumor-specific T-cell epitopes can generate protective anti-tumor T cell-mediated immunity. Treatment of tumors with peptide-pulsed DCs has resulted in sustained tumor regression in several different tumor models (for review see refs. 10, 39). Furthermore, peptide-pulsed DCs have been used in patients with prostate cancers and found to be effective in generating cell-mediated immune responses.[40] Several studies using DCs pulsed with HPV-specific peptides have been reported.[41,42] For example, Mayordomo et al demonstrated in murine tumor models that bone marrow (BM)-derived DC pulsed ex vivo with synthetic HPV16 E7 peptide (aa 49-57) serve as an effective anti-tumor vaccine, protecting animals against an otherwise lethal tumor challenge.[41] The identification of MHC class I and class II epitopes of HPV proteins can facilitate the application of DC pulsed with HPV peptides. Several studies have defined murine[43-46] and human[47,48] T helper cell epitopes and murine[49-51] and human[52-54] CTL epitopes on HPV proteins. The defined epitopes then can be used in this ex vivo DC peptide pulsing strategy for vaccination against HPV associated neoplasms.

DCs pulsed with proteins can also generate effective anti-tumor immunity.[55,56] For example, Hsu et al investigated the ability of autologous dendritic cells pulsed ex vivo with tumor-specific idiotype protein to stimulate host anti-tumor immunity. They have observed that all their patients develop measurable anti-tumor cellular immune response. Furthermore, significant clinical responses have been observed in some of their patients.[56]

Dendritic Cells Transduced with Genes Coding for Tumor Specific Antigens

The MHC restriction of peptide-based vaccines may be bypassed with approaches that directly transduce genes coding for tumor specific antigens (TSAs) inside the DCs so that the peptides can be presented by any given patient's HLA molecules. DNA-based vaccines (for further review see chapter 8) have an advantage over peptide vaccines in that their MHC-peptide complexes are expressed for longer periods of time on the surface of the DCs, which may be vital given recent data on the relatively short half life of MHC I-peptide complexes on DCs.[14] Gene transfer into DCs can be accomplished by a variety of methods involving either naked DNA or the use of viral vectors.

Dendritic Cells Transfected with Naked DNA

The use of naked DNA for gene transfer into DCs ex vivo has the clear advantages of purity, simplicity of preparation, and stability. These advantages support an enthusiastic interest in the pursuit of vaccine development employing DCs transfected with naked DNA. Methods to transfer naked DNA into mammalian cells include:

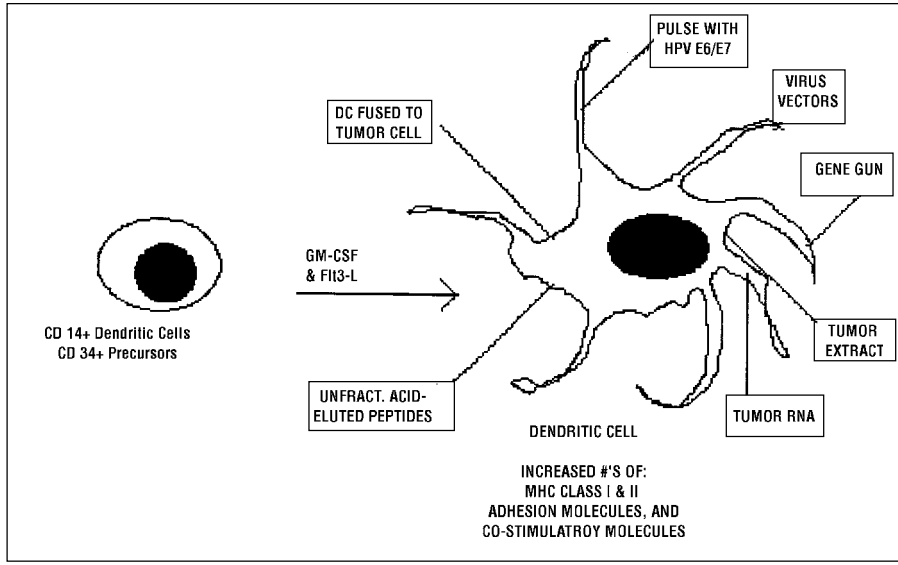

Fig. 9.1. Cartoon drawing depicting the various methods of manipulating the DC generated ex vivo with cytokines such as GM-CSF and Flt3-L. These vaccine designs are discussed in the first part of the chapter.

1. Coprecipitation with calcium phosphate;
2. The use of electroporation, which exposes cells to rapid pulses of high voltage current, thereby providing a physically induced opening in the cell membrane for entry of DNA;
3. Direct introduction of DNA into cells by microinjection;
4. Encapsidation of DNA into liposomes; and
5. Particle-mediated transfer of DNA by gene gun.

The major limitation to naked DNA gene transfer into the DCs is poor transfection efficiency by various physical methods.[57] However, several groups have improved the poor transfection efficiency in DCs. Rosse et al have used the cationic lipid DOTAP to form a complex with the DNA to enhance the efficiency of transfecting naked DNA into the DCs. The DOTAP-complexed DNA-transfected DCs induced a strong CTL response, which was superior to the CTL response induced by similarly transfected macrophages.[58] Tuting et al have used particle mediated transfer of genes encoding tumor-specific antigens to generate DCs expressing tumor peptide-MHC complexes.[59] Bone marrow-derived DCs were transfected with plasmid DNA encoding HPV16 E7 ex vivo, and they then applied the transfected DCs as a vaccine in mice. The method is unique in that the plasmid DNA was precipitated onto gold particles which were loaded into a helium pulse gun (gene gun) and used to bombard DCs evenly spread onto the bottom of a prewetted well. Not only did the vaccine successfully generate an antigen-specific CTL response in vivo, but it also promoted the rejection of a subsequent, normally lethal challenge with an HPV16-transformed tumor cell line.[59] The gene gun approach has also been used to transduce DCs in vivo. For example, Condon et al showed that cutaneous genetic Immunization with naked DNA coated onto gold particles resulted in potent, antigen-specific, CTL-mediated protective tumor immunity.[60] This method of Immunization relies on the uptake of antigen by tissue-resident

DCs, either through direct transfection of the DC or indirectly via the reprocessing of antigens released from alternate transfected cells.[60] (For more information on DNA vaccines, see chapter 8 in this book).

Dendritic Cells Transduced with Recombinant Adenovirus Vectors

The efficiency of gene transfer by the recombinant adenovirus (Ad) vector has been shown to be superior to the naked plasmid DNA transfection approach.[57,61] Wan et al showed that up to 90% of the murine bone marrow-derived DCs could be infected with an Ad vector expressing the beta-galactosidase (β-gal) gene.[62] The physiologic and phenotypic characteristics of the DCs were unchanged when infected with the Ad vector.[62] The efficiency of Ad-mediated gene transfer into DCs can be further enhanced by cationic liposomes.[61] In one study, the expression of the gene transduced was detectable in the spleen for at least 3 days following intravenous injection of Ad-transduced DCs.[62] The efficiency of Ad-infected DCs in eliciting a specific CTL response was reported to be superior to that of a DC infected with recombinant vaccinia virus or pulsed with peptide or protein.[63] The benefits of vaccine strategies using DCs transduced with genes via Ad vectors were clearly demonstrated in several studies using several model antigens including the β-gal,[62,64,65] chicken ovalbumin,[63] and the human MART-1/Melan-A melanoma antigen.[66] These studies have shown that antigen-specific CTL activities and protective anti-tumor immunity were generated in mice vaccinated with DCs transduced with genes via Ad vectors. Furthermore, Ad-mediated gene transfer into DCs generated a therapeutic response to preestablished tumors.[62,64]

When Ad-mediated gene transfer into DCs is compared to direct Immunization of mice with recombinant Ad, the former avoids the disadvantage of stimulating the production of neutralizing antibodies which is almost always seen in direct Immunization with virus vectors.[63] Direct Immunization of mice with recombinant adenovirus resulted in the induction of high titers of neutralizing Abs, which precluded a boost of a CTL response after repeated inoculations. However, repeated injections of virus-infected DCs induced only low titers of neutralizing antibodies.[63] In addition, the presence of neutralizing Abs specific for the virus did not affect the usefulness of infected DCs, as repeated applications of virus-infected DCs boosted the CTL response even in mice previously infected with the recombinant vector.[63]

Dendritic Cells Transduced with Recombinant Retroviruses

The major limitation of adenoviral vectors for gene transfer approaches is that the transfected DNA does not integrate into the genome of the transduced cell and thus expression is lost after one to four weeks. Retroviruses, on the other hand, can infect and integrate into the host genome, a critical requirement for efficient gene transfer and for the establishment of stable expression. Highly efficient retroviral vectors have recently become available for gene transfer.[67] The use of these high efficiency retroviral vectors to carry TSA genes into dendritic cells has a number of specific advantages. The transduction is rapid and selection is unnecessary. In addition, the absence of selection avoids the potential problem that selected subclones may display an altered physiology as compared to the original dendritic cell population. Several studies have shown that gene transfer in murine and human DCs using retroviruses is a feasible strategy, reporting between 11.5-72% of the DCs expressing the specific genes transferred.[68-71] In addition, retrovirally transduced DCs maintain their functional properties, stimulating allogeneic T cells with similar efficiency as nontransduced DCs.[71,72] More recently, retrovirally transduced DCs have been shown to be capable of generating antigen-specifc CTL activities and protective anti-tumor immunity in murine models.[69,70] For example, Specht et al reported that lac-Z-transduced DCs

significantly reduced the number of lung metastases using the β-gal-expressing murine model tumor, CT26.CL25.[70] In addition, the β-gal-specific CTL activities generated in mice vaccinated with β-gal-transduced DCs were significantly higher than CTL activities generated from mice immunized with DCs pulsed with β-gal peptide.[70]

There are several potential adverse consequences of retroviral infection. There is the theoretical concern that retroviral integration may lead to insertional mutagenesis because the virus integrates into random sites in the host's genome, and may lead to:

1. The activation of silent host genes such as protooncogenes;
2. The inactivation of tumor suppressor genes;
3. The activation of other latent viruses integrated into the dendritic cell's genome;
4. Transformation of the defective viral vector from replication incompetent to replication competent by recombination with host gene sequences.

Therefore, recombinant retroviruses should be used with caution in the design of human cancer vaccines.

Dendritic Cells Transduced with Recombinant Vaccinia Virus

Vaccinia virus, in the family of poxviruses, can also be used to mediate the transfer of genes into DCs. This gene transfer strategy offers several appealing features including high efficiency of infection and high levels of recombinant gene expression. Infection with recombinant vaccinia and the expression of the desired gene product occurs quickly, minimizing the period of dendritic cell culture that is required before Immunization. Furthermore, the vaccinia genome has abundant "room" for accommodating large genetic insertions. More recently, DCs transduced with recombinant vaccinia virus have been shown to be capable of generating antigen-specific CTL activities.[73,74] Specifically, Bronte el al demonstrated that murine DCs infected with a lac-Z recombinant vaccinia virus express β-gal and generate a β-gal-specific CTL response.[73] They also showed that dendritic cells express β-gal only under the control of the early promoters of vaccinia, even though late promoters were intrinsically more active in cell types other than dendritic cells.[73] Kim et al also showed that human DCs infected with a recombinant poxvirus encoding the melanoma-associated antigen gene (MART-1) induced MART-1-specific CTL responses in melanoma patients.[74] These data highlight the potential of DCs transduced with recombinant vaccinia virus for use in vaccine development.

The availability of replication-deficient recombinant poxviruses such as canarypox virus has generated a great opportunity for using recombinant canarypox virus as a vector for gene transfer into dendritic cells. First, these constructs should be extremely safe, as productive viral replication is restricted to avian species.[75] Second, T cell responses against vaccinia antigens do not significantly crossreact with canarypox antigens,[76] obviating the concern that such preexisting immunity would preclude Immunization with dendritic cells infected with canarypox virus.

In summary, when genetically modified DCs expressing tumor-specific antigens were used as vaccines, anti-tumor killer cells were generated and anti-tumor effects were observed. The specific concern related to HPV vaccines is the oncogenicity of HPV E6 and E7 proteins. Transducing DCs with the genes encoding HPV oncogenic proteins E6/E7 raises a legitimate safety concern. Detailed biochemical knowledge of the E6 and E7 proteins has led to the identification of a number of mutations in the molecules that abrogate the function of transformation.[77-81] The vectors employed in human clinical trials should use minimally mutated E6 and E7 genes, which would preserve as many potential epitopes as possible while diminishing oncogenic activity.

Dendritic Cells Armed with the Full Antigenic Spectrum of Tumor Cells

Even with the promise that DCs pulsed with peptides/proteins and gene-transduced with TSAs have shown in tumor models, there are several limitations. The main limitation is that, for most cancers, the TSAs and T-cell epitopes have not yet been characterized. An evolving goal of tumor immunology is to develop vaccines that will enable the body's immune system to recognize the whole spectrum of TSAs. Even though in cases in which a TSA is known, such as HPV-associated neoplasms, it is reasonable to pursue vaccine strategies that arm the DCs with the full antigenic spectrum of tumor cells because the full antigenic spectrum may prove to be more immunogenic than simply using one or two TSAs. Several strategies have been developed using the DCs generated ex vivo. They can be classified as the following:

1. DCs pulsed with unfractionated acid-eluted peptides;
2. DCs pulsed with tumor extracts;
3. DCs fused with tumor cells, and
4. DCs transduced with tumor-derived RNA.

Dendritic Cells Pulsed with Unfractionated Acid-Eluted Peptides

DCs pulsed with unfractionated acid-eluted tumor peptides are a plausible option for bypassing the need for identifying tumor specific antigens (TSAs). A modified acid elution method has been used to isolate epitopes from class I complexes expressed at the cell surface of viable cells.[82] Recently, Zitvogel et al used DCs pulsed with unfractionated acid-eluted tumor peptides to treat mice bearing spontaneous, established tumors. They showed that the adoptive transfer of unfractionated tumor peptide-pulsed DCs dramatically suppressed the growth of weakly immunogenic tumors in MCA205 (H2b) and TS/A (H2d) tumor models.[83] They also observed a marked upregulation of IL-4 and IFN-γ production in tumor-bearing mice immunized with DCs pulsed with tumor-eluted peptides.[83] Thus, DCs pulsed with acid-eluted peptides derived from autologous tumors represent a potential approach to treat established, weakly immunogenic tumors.

Dendritic Cells Pulsed with Tumor Extracts

Vaccination with tumor extracts is another approach that has been recently described as a method for circumventing the need to identify TSAs. The technique involves sonicating tumor cells, combining the tumor extracts with the cationic lipid DOTAP, and adding the mixture to DCs generated ex vivo.[84] Immunization of mice with DC pulsed with tumor extracts has been proven to generate tumor-specific CTL responses in poorly immunogenic murine tumor models.[84-86] Ashley et al also showed that DC tumor extract-pulsed vaccines prolonged the survival in mice with preestablished tumors to the same extent as GM-CSF gene-modified tumor vaccines.[84] The DC-tumor extract-pulsed vaccine has also been shown to actually induce a cure in at least 40% of the animals injected with the murine bladder tumor (MBT-2)[85] and to cause a significant reduction in lung metastases in the B16 melanoma lung metastasis model.[85,86] In addition to tumor extracts, irradiated tumor cells can also be mixed with DCs and used as vaccines. In one study, dendritic cells mixed with irradiated tumor cells inhibited the growth of primary breast cancer in a murine breast carcinoma (4T1) model.[87] However, the potential concern in using the DC-pulsed tumor extracts or mixed with irradiated tumor cells in humans is the fear that they may contain some normal antigens and that they may therefore induce autoimmunity.[84]

Dendritic Cells Fused with Tumor Cells

Another vaccine strategy that avoids the need to identify specific tumor antigens is to use DCs fused with tumor cells as vaccines. In the past, when DCs were not readily available,

activated B cells had been fused with tumor cells and used as tumor vaccines; Guo et al have successfully fused BERH-2 rat hepatocellular carcinoma cells with activated B cells and used the hybrid cell as a vaccine in syngeneic rats, which resulted in substantial protective and therapeutic responses.[88] Recently, it has been shown that murine hybrid cell lines produced by the fusion of DCs and a tumor cell preserve the DC phenotypes.[89,90] For example, Gong et al have fused DCs with murine MC38 adenocarcinoma cells. The fusion cells were positive for major histocompatibility (MHC) class I and II, costimulatory molecules and intercellular cell adhesion molecule-1 (ICAM-1). The fusion cells stimulated naive T cells in the primary mixed lymphocyte reaction (MLR) and induced MC38 tumor-specific CTLs in vivo. Furthermore, Immunization with the fusion cells induces the rejection of established metastases.[90] These findings suggest that the fusion of DCs and tumor cells potentially can be used in the control of tumors.

Dendritic Cells Pulsed with Tumor-Derived RNA

DCs pulsed with tumor-derived RNA have recently emerged as an effective way to induce CTL and tumor immunity. DCs pulsed with in vitro synthesized chicken ovalbumin (OVA) RNA were more effective than OVA peptide-pulsed DCs in stimulating primary, OVA-specific CTL responses in vitro.[91] In another report, mice vaccinated with DCs pulsed with B-16 total RNA generated specific cytotoxic T lymphocytes against B-16 tumor cells.[84] In addition, these mice revealed a dramatic reduction in lung metastases in a highly metastatic, B-16/F10.9 tumor model.[91] Furthermore, using the B-16 murine melanoma as a model for CNS tumor, Ashley et al demonstrated that DCs pulsed with unfractionated B-16 total RNA can lead to prolonged survival in mice with tumor cells placed in the CNS before the initiation of vaccine therapy.[84]

DCs pulsed with tumor-derived RNA have several advantages over the strategies using DCs pulsed with unfractionated tumor-derived peptides/proteins or using DCs fused with tumor. Both DCs pulsed with unfractionated tumor-derived peptides/proteins and DCs fused with tumor require substantial amounts of viable tumor cells or tumor tissue. This requirement can be difficult for patients with very small amounts of tumor. The tumor-derived RNA-pulsed DC-based vaccines are potentially suitable for patients bearing very small, possibly microscopic, tumors. Since cDNA can be easily generated from a very small amount of tumor cells and RNA can be further transcribed in vitro from cDNA cloned in a bacterial plasmid, it is possible to amplify the antigenic content through RNA from a small number of tumor cells. Boczkowski et al have shown that RNA generated using this approach can be highly effective in sensitizing DCs against tumors.[91] Furthermore, tumor-specific RNA can be enriched by subtractive hybridization with RNA from normal tissue, and transfected RNA may serve as a continuous source for the generation of antigenic peptides.[91] All these features have made DCs pulsed with tumor-derived RNA a desirable approach in tumor vaccine development.

Manipulation of the Dendritic Cells Via In Vivo Methods

All of the methods discussed thus far involved ex vivo methods to enrich and prime DCs with tumor antigens before giving these DCs as vaccines to hosts. This section will highlight vaccine strategies that increase the numbers and function of DCs in vivo. As discussed previously, DCs can be generated from hematopoietic progenitors in the setting of various cytokines, mainly GM-CSF and Flt-3 ligand. Instead of generating potent DCs ex vivo and injecting the cells back into a patient as a cancer vaccine, a different strategical approach is to use a vaccine with GM-CSF or Flt3 ligand cytokines to expand and prime DCs in vivo. Such vaccine strategies can be classified as the following:

1. Autologous GM-CSF transduced cell-based vaccines;
2. Allogeneic GM-CSF transduced cell-based vaccines;
3. Bystander GM-CSF-releasing microspheres or cells;
4. Chimeric GM-CSF-peptide/protein molecules; and
5. Strategies related to Flt-3 ligand.

These vaccine strategies are summarized in Figure 9.2. Of these vaccine strategies, many have provided great stepping stones to the development of HPV vaccines.

Vaccine Strategies Related to GM-CSF

GM-CSF as the Most Potent Cytokine in Cytokine-Transduced Cell-Based Vaccines

The initial development of cytokines for cancer immunotherapy involved systemic administration of pharmacologic doses of recombinant cytokines.[92] However, this approach failed to account for a major principle in lymphokine physiology, namely, that lymphokines maintain the specificity of immunologic responses partially through their paracrine function. In the past several years, there has been intense interest in the study of immune responses generated by tumor cells engineered to secrete various cytokines. This strategy seeks to locally alter the immunological environment of the tumor cell so as to either enhance the antigen presentation of tumor-specific antigens to the immune system or to enhance the activation of tumor-specific lymphocytes. The important concept underlying the use of cytokine gene-transduced tumor cells is that the cytokine is produced at very high concentrations local to the injected tumor cells. Systemic concentrations are generally quite low. This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines. Numerous reports have analyzed biological effects of injections of tumors transduced with multiple different cytokine genes (for review see ref. 93).

Because of the wide range of local inflammatory and vaccine effects of cytokine-secreting tumors as well as other parameters affecting experimental outcome, it is critical that these cytokines be compared for efficacy. The first study that directly compared the efficacy of multiple cytokines and other genes in murine tumor models used a highly transmissible replication-defective retroviral vector. This study demonstrated that, in a number of poorly and moderately immunogenic tumors, Immunization with GM-CSF-transduced tumors produced the greatest degree of systemic immunity.[67] Immunity was dependent on both $CD4^+$ and $CD8^+$ T cells. The potency of GM-CSF's effect locally may relate to its unique ability in promoting the differentiation of hematopoietic precursors into dendritic cells.

Clinical Trial Using Autologous GM-CSF Transduced Cell-Based Vaccines

The success of autologous GM-CSF-transduced tumor vaccines in the murine model has prompted the design of several clinical trials of human patients with renal cell carcinoma,[94] sarcoma[95] and melanoma.[95,96] A phase I clinical trial was performed at Johns Hopkins Medical Institutions to evaluate for the safety and the induction of immune responses of a GM-CSF-transduced renal cell carcinoma vaccine in patients with metastatic renal cell carcinoma (RCC). Patients with stage IIIb and IV RCC were treated in a randomized, double-blind, dose escalation study with equivalent doses of autologous, irradiated RCC vaccine cells with or without ex vivo human GM-CSF gene transfer. The replication-defective retroviral vector MFG was used for GM-CSF gene transfer.[67] In all fully evaluable patients, no dose-limiting toxicities were encountered. Furthermore, no evidence of autoimmune disease was observed. Biopsies of intradermal sites of

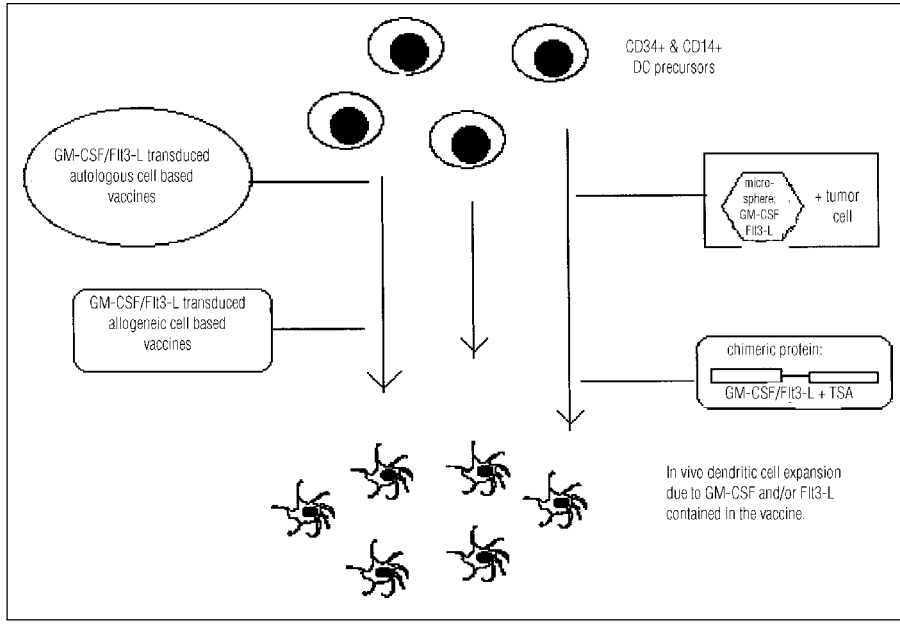

Fig. 9.2. Cartoon drawing depicting the various methods of vaccine design that potentially expand the DC in vivo.

injection with GM-CSF gene-transduced vaccines revealed distinctive macrophage, dendritic cell, eosinophil, neutrophil, and T cell infiltrates similar to those observed in preclinical models.[94] More interestingly, a significant clinical response was observed in a patient treated with GM-CSF gene-transduced vaccine who displayed the largest delayed-type hypersensitivity conversion. No replication-competent retrovirus was detected in vaccinated patients. This phase I study demonstrated the feasibility, safety, and bioactivity of an autologous GM-CSF gene-transduced tumor vaccine for RCC patients.[94]

Although the autologous GM-CSF-transduced cell-based vaccines have generated tremendous interest, there are several limitations of autologous tumor cell-based vaccines.[93] The limitations mainly are due to its labor intensity, in that the generation of new vaccine for each patient is time consuming and technically challenging. Furthermore, the yield from in vitro cell expansion is often insufficient. Each preparation of the vaccine requires the expansion of a tumor explant into a homogeneous culture, followed by GM-CSF gene transfer, cell selection, cell expansion, assays for GM-CSF secretion level, and irradiation before the vaccine can be used. There is also a critical relationship between the quantity of GM-CSF produced by a vaccinating cell population and the potency of the systemic anti-tumor immune response.[93] All of these limitations have prompted the generation of new vaccine strategies to avoid the technical difficulties previously encountered.

Allogeneic GM-CSF-Transduced Cell-Based Vaccines

The use of allogeneic vaccines can decrease the labor intensity and variability of vaccine production. With this strategy, a single standardized GM-CSF-transduced cell line or mixture of GM-CSF-transduced cell lines is used to generate the vaccine. Critical to any chance of success with a transduced allogeneic tumor vaccine is that the allogeneic vaccines

share antigens with the patient's tumor.[93] This is not a problem for most HPV-associated cervical cancers, since they have common tumor-specific antigens, E6 and E7. Recently, it has been shown that the professional antigen-presenting cells of the host, rather than the vaccinating tumor cells themselves, prime the T-cell arm of the immune response.[97] This implies that the vaccinating cells used as the source of tumor antigens do not have to be major histocompatibility complex (MHC)-compatible with the host for successful priming of an anti-tumor immune response. Indeed, allogeneic tumor cells have been shown to generate protective anti-tumor immunity in immunized mice.[98] Currently, a phase I clinical trial evaluating an allogeneic, GM-CSF-secreting tumor vaccine in patients with advanced pancreatic adenocarcinoma is in progress (Jaffee, personal communication).

Bystander GM-CSF-Releasing Microspheres or Cells

Another possible solution to the difficulties surrounding in vitro cell culture and individualized cytokine gene transfer is to bypass the tumor cell itself. The appreciation of GM-CSF's paracrine physiology and potent anti-tumor effect, and the success that polymer-controlled drug delivery systems have had on improving certain drug therapies,[99] has led to the design of cell-sized polymer microspheres containing GM-CSF.[100] The idea behind the design is that the microspheres would degrade over time, releasing a continued, controlled supply of GM-CSF in the vicinity of tumor cells. Pettit et al have described the preparation and characterization of a controlled release formulation of GM-CSF encapsulated in microspheres.[101] They reported that steady release of GM-CSF was achieved over a period of time without a significant "burst" of protein from the microspheres, and GM-CSF released from the microspheres was found to be biologically active and physically intact by bioassay and chromatographic analysis.[101] Golumbek et al have used high doses of GM-CSF encapsulated in cell-sized gelatin-chondroitin sulfate microspheres to mix with irradiated tumor cells for vaccination.[102] They found that GM-CSF initially released from the microspheres attracted and activated inflammatory cells, which in turn produced extracellular enzymes that degraded the collagen/chondroitin sulfate microsphere matrix. The rapid degradation resulted in increased GM-CSF delivery, which amplified the process. When B-16/F10 melanoma cells were used in C57BL/6 mice, the microsphere containing GM-CSF admixed with irradiated tumor cells resulted in the same specific cytokine effect as that induced by GM-CSF-transduced tumor cells.[102]

Another approach is to use bystander GM-CSF-releasing cells mixed with irradiated tumor cells. Several studies have used cytokine-secreting fibroblasts admixed with tumor cells in animal models[103,104] and even in human cancer patients.[105] Fibroblasts are easily accessible via skin biopsies. In addition, the high GM-CSF-secreting allogeneic/syngeneic fibroblasts can be selected before mixing with irradiated tumor cells in order to achieve a therapeutic level of GM-CSF. The relative efficacy of GM-CSF-transduced allogeneic versus syngeneic fibroblasts admixed with tumor cells has been recently examined in mice by Aruga et al.[106] They have shown that the syngeneic fibroblasts were superior to allogeneic fibroblasts as a vehicle to deliver GM-CSF in tumor vaccines. The use of allogeneic fibroblasts was associated with suppressed immune responses when used in high fibroblast:tumor cell ratios. Therefore, allogenic GM-CSF-transduced fibroblasts may not be suitable in clinical settings.[106]

Chimeric GM-CSF-Proteins

Another vaccine strategy related to GM-CSF is to use GM-CSF fused to a tumor-specific antigen such as E6/E7. If dendritic and/or other GM-CSF responsive cells are the critical APCs in generating enhanced immune responses, then linkage of the antigen to each GM-CSF molecule in the chimeric vaccine may enhance the targeting of antigen into

the endocytic compartment of these cells after the molecule has bound to the GM-CSF receptor. Tao et al have shown that the immunogenicity of idiotype (Id) (the variable region of the immunoglobulin molecule expressed on malignant B cells) can be dramatically increased by fusing tumor Id to GM-CSF. The chimeric Id-GM-CSF was capable of inducing idiotype-specific antibodies without other carrier proteins or adjuvants and protecting recipient animals from challenge with an otherwise lethal dose of tumor cells.[107] Another study has been carried out using the HPV16 E6/E7 as a model antigen to fuse to GM-CSF. When a chimeric murine GM-CSF-E7 protein was designed and used to immunize mice, it was shown that the chimeric protein preserved murine GM-CSF function and at the same time the E7 antigenicity was maintained. GM-CSF-E7 generated the highest E7-specific antibody responses compared to controls (Wu, personal observation). These studies indicate that GM-CSF linked to an antigen can significantly enhance the immunogenicity of the antigen, and suggest a novel strategy to enhance the potency of recombinant vaccines. A potential setback to this strategy was, however, observed when mice were hyperimmunized with idiotype-GM-CSF.[108] The vaccinated mice developed neutralizing anti-GM-CSF antibodies. However, the neutralizing response can be avoided if additional immunizations are performed with only the idiotype protein.[108]

Vaccine Strategies Related to Flt3 Ligand

Flt3 Ligand and Development of Dendritic Cells In Vivo

Flt3 ligand (Flt3-L) has emerged as an important molecule for the development of tumor vaccines that augment the function and quantity of dendritic cells in vivo. Flt3 (Fms-like tyrosine kinase 3), a murine tyrosine kinase receptor, was first described in 1991[109,110] and found to be a member of the same family of receptors as c-Kit and c-Fms receptors—the type III receptor kinase family (for review see ref. 16). Flt3 is expressed in lymphohematopoietic tissues, placenta, brain, and a high proportion of leukemia and lymphoma cell lines.[21] In hematopoietic tissues, expression is restricted to the CD34-positive progenitors, including cells with the capacity for long term reconstitution of irradiated hosts. Flt3 has been used to identify and subsequently clone the corresponding ligand, Flt3-L.[111,112] Flt3-L is a member of a small family of growth factors that stimulate the proliferation of hematopoietic cells. The predominant form of Flt3-L is synthesized as a transmembrane protein from which the soluble form is generated, presumably by proteolytic cleavage.[113] These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells. Flt3-L alone has a weak stimulatory effect on in vitro progenitor cell colony growth but has additive or synergistic effects when combined with GM-CSF, granulocyte-CSF, IL-3, IL-11, IL-12, erythropoietin, stem cell factor and other cytokines.[114-116] Recently, Flt3-L has been noted to possess a growth-stimulatory effect on dendritic cells and to be capable of generating large numbers of DCs in vivo.[33,34] It has also been shown that Flt3-L, in cooperation with transforming growth factor β1, potentiates in vitro development of Langerhans-type dendritic cells.[27,35] Because of the association of Flt3-L with the development of dendritic cells, several studies have aimed at developing vaccines using Flt3-L or tumor cells expressing Flt3-L for anti-tumor immunity.

Vaccine Strategies Using the Flt3 Ligand

There are several successful studies using Flt3-L and/or tumor cells expressing Flt3-L in controlling murine tumors. For example, Lynch et al have reported an effective treatment of murine fibrosarcoma by Flt3-L therapy.[117] They showed that Flt3-L treatment not only induced complete tumor regression in a significant proportion of mice, but also decreased

tumor growth rate in the remaining mice.[117] Similarly, Chen et al used a murine breast cancer model and found that Flt3-L had anti-tumor properties.[115] More recently, Esche et al have demonstrated that Flt3-L administration inhibits tumor growth in murine melanoma and lymphoma models.[118] They observed an increase of DCs within these challenged tumor sites.[118] These data are consistent with the hypothesis that the generation of an effective immune response to the tumor may be related to the augmented generation of DCs in the vaccinated animal by Flt3-L treatment.

All of these exciting reports indicate that vaccine strategies that enhance the function and quantity of dendritic cells in vivo may lead to effective control of tumor and can potentially be applied to human trials. It is conceivable that all of the vaccine strategies related to GM-CSF can be applied to the newly identified cytokine Flt3-L. Furthermore, the combination of both GM-CSF and Flt3-L in tumor vaccine design potentially can generate the highest number of DCs and the most potent antiumor immunity. We can foresee similar strategies that will be applied to HPV-associated tumor models and possibly patients with cervical cancers in the future.

Modification of Antigen Presentation Pathways for the Design of Cancer Vaccines

Tumor-specific antigens, when efficiently presented by antigen-presenting cells to both $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, are capable of inducing potent T cell-mediated immunity—the most crucial component of anti-tumor immunity. Therefore, the ideal cancer vaccine would enhance both $CD8^+$ cytotoxic T cell and $CD4^+$ helper T cell responses by delivering a tumor-specific antigen into MHC class I and class II pathways of antigen presentation. There are now several methods available to modify exogenous proteins so that they are more efficiently presented in the MHC class I pathway. There are also strategies to enhance MHC class II presentation of cytoplasmic proteins such as HPV16 E6/E7.

Modified Exogenous Antigens to Present in the MHC Class I Pathway

In general, the administration of soluble exogenous protein does not generate CTL responses; however, a growing number of modified exogenous protein antigens have been shown to prime MHC-class I-restricted CTL responses (for review see ref. 119). For example, association of exogenous antigens with some adjuvants, such as immune-stimulating complexes (ISCOMs),[120] liposomes,[43] saponin,[121-123] or squalene,[124] enhances their immunogenicity for CTL precursors. Tindle et al have reported successful generation of CTL activities using exogenous E7 peptides mixed with ISCAR (immunostimulatory carrier) adjuvants.[125] Several reports have described CTL priming by injection of large, particulate antigens such as recombinant bacteria,[126] crude cell lysates,[127] denatured aggregates[128] and antigen-coupled beads.[129] Modification of proteins with lipid conjugation converts them into potent inducers for priming CTL activities.[130] CTL responses are efficiently primed by the injection of protein antigens either complexed with or fused with heat shock proteins.[127,131] Virus-like particles (VLPs) have been shown to prime CTL responses[132] (see chapter 3 for more information about HPV VLPs). These strategies for modified exogenous antigens can be specifically used with the dendritic cell. All these methods for modifying exogenous proteins can be applied to HPV E6/E7 oncogenic proteins to generate an effective DC vaccine that should enhance E6/E7-specific T cell-mediated immunity.

The exact mechanisms by which modified exogenous antigens are presented through the MHC class I pathway (cross-priming) remains to be elucidated. There is not a general consensus as to the cellular mechanism of antigen transfer in these experiments. Initial studies suggested that class I presentation of exogenous antigens occurs by regurgitation of peptides generated in the phagosomal compartment to the cell exterior followed by binding

to empty MHC class I molecules.[133] Another set of studies suggested a phagosome to cytosol transfer of antigens with ultimate cytosolic processing and TAP-dependent transport of peptides into the ER for binding to nascent MHC class I molecules.[134] The various proposed pathways for antigen presentation were distinguishable in that the first model proposed a TAP-independent pathway for class I processing of exogenous antigens, whereas the later model proposed a pathway which was TAP dependent. It is possible that both cellular mechanisms are accurate but are dependent on the exact modification and type of exogenous antigens used.

There are several advantages of using DCs in conjunction with modified exogenous tumor antigens. First, modified exogenous antigens potentially can be presented on both MHC class I and class II molecules, resulting in the activation of both $CD4^+$ and $CD8^+$ T cells. Second, such an approach can avoid the risks of introducing a viable infective viral agent into the DCs. The possibility of oncogenic transformation can not be ignored when using a viral vector carrying HPV E6/E7 genes. Finally, as mentioned above, exogenous full length antigens can be processed and presented as numerous different epitopes that are specific to that individual's DC MHC molecules, therefore avoiding the disadvantage of MHC restriction.

Enhancement of MHC Class II Presentation of Cytoplasmic Proteins by Viral Vectors

The cytoplasmic proteins that are encoded by the genes transduced by viral vectors are, in general, presented through the MHC class I pathway. The MHC class II presentation of cytoplasmic proteins such as E6 and E7 is very insufficient. Therefore, strategies that will enhance the MHC class II presentation of these cytoplasmic proteins in APCs potentially will be useful for vaccine design.

It is now clear that MHC class II-restricted $CD4^+$ T cells are critical cells in the generation of effective anti-tumor immunity. $CD4^+$ T cells are the major helper T cell phenotype whose predominant function is to generate cytokines which regulate essentially all other functions of the immune response. $CD4^+$ T cells have also been shown to have cytotoxic capacity in a number of systems, including a response to fragments of the HIV gp160 protein.[135] $CD4^+$ cells have also been shown to be of great importance in the immune response against a number of different solid malignancies in mice[136,137] and in humans.[138,139] Several mouse tumors that were transfected with syngeneic MHC class II genes have become very effective vaccines against subsequent challenge with wild type class II negative tumors.[140-142] It is postulated that tumor cells transfected with syngeneic MHC class II genes could present endogenously synthesized tumor peptides in the context of MHC class II molecules, thereby directly presenting antigen to $CD4^+$ Th cells.[140] Furthermore, $CD4^+$ cells appear to be crucial memory cells in the T cell arm of the immune response. For these reasons, there has been increased interest in developing strategies that will most effectively activate $CD4^+$ MHC class II-restricted cells against a given specific antigen.[143]

The increased understanding of the intracellular pathways for antigen presentation creates the potential for designing novel strategies to enhance vaccine potency. A molecular approach that would directly route an antigen into the MHC class II processing and presentation pathway might enhance its presentation to MHC class II-restricted $CD4^+$ T cells. In an attempt to enhance MHC class II antigen processing, Wu et al have previously linked the sorting signals of the lysosome associated membrane protein (LAMP-1) to the cytoplasmic/nuclear HPV16 E7 antigen, creating a chimera, Sig/E7/LAMP-1. They found that the expression of this chimera in vitro and in vivo with a recombinant vaccinia vector targeted E7 to endosomal and lysosomal compartments and enhanced MHC class II presentation to $CD4^+$ T cells compared to vaccinia expressing wild type E7. They tested

these recombinant vaccinia for in vivo protection against an E6/E7 expressing tumor, TC-1. TC-1 was derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV16 E6 and E7 and c-Ha-ras oncogenes. All mice vaccinated with wild type E7 vaccinia showed progressive tumor growth when challenged with a tumorigenic dose of TC-1 tumor cells; in contrast, 80% of mice vaccinated with the chimeric Sig/E7/LAMP-1 vaccinia remained tumor free three months after tumor injection. Furthermore, treatment with the Sig/E7/LAMP-1 vaccinia vaccine cured mice with small established TC-1 tumors, while the wild type E7 vaccinia showed no effect on this established tumor burden.[144] These findings point out the therapeutic limitations of recombinant vaccinia expressing unmodified tumor antigens. Further, they demonstrate that modifications which reroute a cytosolic tumor antigen to the endosomal/lysosomal compartment can profoundly improve the in vivo therapeutic potency of recombinant vaccines. Another system that successfully applies the LAMP-1 strategy to enhance MHC class II presentation is the HIV envelope protein.[145,146] More recently, the LAMP-1 strategy has been successfully applied to HPV DNA vaccines (Ji, personal communication) and RNA-transfected dendritic cells (Gilboa et al personal communication) to enhance vaccine potency.

The success of the LAMP-1 targeting strategy suggests that continuous exploration of antigen presentation pathways with intracellular targeting strategies may lead to the development of better immunotherapeutic strategies and vaccines. One of the potential targeting strategies to enhance MHC class II antigen presentation is the use of the intracellular sorting signals of the MHC class II-associated invariant chain (Ii). The Ii chain is thought to act as a chaperone that assists the MHC class II molecule during folding, assembly, and transport (for review see ref. 7). The intracellular sorting signals responsible for targeting these molecules to MHC class II pathways have been determined (for review see ref. 147). Furthermore, endogenously synthesized ovalbumin or hen egg lysozyme has been shown to be efficiently presented as peptide-MHC class II complexes when they are expressed as fusion proteins with the endosomal targeting signal located at the N-terminal cytoplasmic portion of the Ii chain.[148] Therefore, the chimeric molecules that contain the endosomal targeting signals of the Ii molecule potentially can reroute endogenously synthesized proteins such as HPV E6 and E7 to enter the MHC class II presentation pathway.

Another potential targeting strategy to enhance MHC class II antigen presentation is to use the intracellular sorting signals of the HLA-DM molecule. The nonpolymorphic human class II molecule HLA-DM has been found to play a key role in antigen presentation by MHC class II molecules (for review see ref. 149). HLA-DM and its murine equivalent H2-M are located intracellularly and are absent from the cell surface. In a murine system, H2-M was transported to an endosomal compartment in the absence of invariant chain. A tyrosine-based targeting motif in the cytoplasmic tail of H2-M beta was responsible for the endosomal location.[150] Mutation of the tyrosine residue to alanine resulted in redistribution of hybrid molecules to the cell surface.[151] It will be interesting to generate chimeric molecules similar to the E7/LAMP-1 construct to see if such a strategy will enhance the MHC class II presentation of cytoplasmic protein. All these strategies can be further investigated in vitro using the dendritic cells.

Summary

Dendritic cells have become a central player for tumor vaccine development because of their ability to activate both $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. The advances in DC biology have made it possible to generate large numbers of active DCs in vivo and ex vivo using key cytokines such as GM-CSF and Flt3-L. Impressive progress has been made in the development of vaccine strategies that employ dendritic cells. Our increased

understanding of intracellular pathways for antigen presentation has created the possibility of designing novel methods to enhance vaccine potency. All of these vaccine strategies, related to dendritic cells and modification of antigenic presentation pathways, potentially can be applied to the development of HPV vaccines. We predict that by the turn of the century, several additional HPV vaccines will be available based on the strategies described in this chapter and it is probably fair to say that some of these vaccines will be soon used for the prevention and treatment of HPV infections and HPV-related malignancies in patients.

Acknowledgments

This review is not intended to be an encyclopedic one and I apologize to the authors not cited. Additional references on this and related topics can be found in Hart[10] (1997), Schuler[152] (1997), Cella[9] (1997), and Lyman[16] (1998). I would like to thank Drs. Elizabeth M. Jaffee, Ralph H. Hruban, Matthew L. Kashima, Keerti V. Shah, Robert J. Kurman and Drew Pardoll for helpful discussions and critical review of the manuscript.

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Chapter 10

Live Recombinant Vaccine Vectors for HPV Antigens Associated with Infection and Malignancy

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Classical vaccines for polio, small pox, and influenza use attenuated viruses to induce immunity in diseases where first time exposure normally elicits protective immunity against a second infection with the same or closely related agent. These vaccines are successful because the diseases in human hosts are caused by the primary infections. However, viral adaptations to host immunity have led some viruses to evolve strategies to resist host immune elimination, leading to either chronic infection or latent infection that eventually causes major pathology. Traditional vaccine approaches which mimic the primary infection are successful in preventing these pathologies in some cases, such as the hepatitis surface antigen (HBsAg) vaccine.[1] An example of a failure to prevent a latent infection is the live chicken pox vaccine, which is largely protective against an acute clinically significant infection and reduces but does not prevent the occurrence of herpes zoster, which is the reemergence of the virus after a latent infection in nerve ganglia.[2] The challenge for modern vaccines is to circumvent the viral adaptations to allow host mechanisms to either prevent the latent viral phase or to eliminate the virally infected cells. The increased knowledge of vaccines, combined with the increased knowledge of the life cycle of viral pathogens, is leading to clinical trials of new vaccine approaches. An identification of genes expressed during the latent phase of viral infections has made them ideal targets for vaccines. Unfortunately, no clear theoretical basis has been established that would suggest that vaccine approaches could alter the immune response in a way that could lead to the elimination of the latently infected cell.

Human papilloma viruses (HPVs) are viruses that inhabit the squamous epithelium of the mucocutaneous surface. Acute infections cause either minor subclinical disease or chronic infections such as warts. Severe life threatening disease occurs when latent infection leads to malignant disease caused by a subset of papilloma viruses. Cervical HPV infections are largely immunologically quiescent.[3] However, from the studies of HPV-related diseases in immunocompromised HIV patients, both men[4,5] and women,[6-9] it is clear that the immune system is effective in eliminating HPV-induced transformed cells in healthy people. Nevertheless, normal immune responses do not protect a significant percentage of women against incidence of cervical cancer that occurs after papilloma infection with a number of different serotypes, possibly due to a lack of local APCs[3] or from loss or mutation of immunologically important target molecules such as major histocompatibility antigens (MHC) class I.[10]

Vaccines for Human Papillomavirus Infection and Anogenital Disease, edited by Robert W. Tindle.

The cellular mechanisms of protection against HPV infections are not well understood. One approach in analyzing the types of immunity against papilloma viruses has been described by Tindle.[11] He describes the life cycle of the virus in terms of where the virus is localized and which viral proteins are expressed by the infected cell during each phase of infection. Because the cellular targets of infection and the viral proteins that are expressed differ during the life cycle, the ability of infected cells of the host to act as targets will vary and thus the immune response will also vary. As mentioned before, the goal for immunologists interested in the disease is to change the immune response in individuals with inadequate immune responses to HPV, either qualitatively or quantitatively, so as to prevent or treat HPV associated disease. Prevention would be to generate an immune state that could block a primary infection before a latent phase can be established. Potentially, a humoral response or a cellular response in the mucosal tissues could prevent such a latent phase. Treatment requires the development of an effective cytotoxic immune response against viral antigens expressed during the latent phase.

Viruses other than papilloma viruses, and intracellular bacteria, are clearly able to generate potent immune responses that can dramatically alter the natural history of those viral diseases in exposed individuals. A research direction in recent years has been to harness these other immunologically potent vectors to deliver a papilloma viral antigen to a susceptible host in order to generate a protective or therapeutic immune response. The use of the pox virus in this way is discussed in detail elsewhere in this volume. We will discuss other potential vaccine vectors. While some of these have not been used to deliver papilloma antigens, we will describe their use in a variety of other diseases and try to draw from these experiments those lessons that may be applicable to developing a vaccine against papilloma viruses.

Adenovirus

The adenovirus has several well recognized advantages as a vaccine delivery agent. First, it has a safety record proven after the vaccination of millions of military recruits using a live oral vaccine.[12] Second, its molecular biology is well developed science, although by no means complete.[13-17] Third, the methods for viral production have been established as an economical and technically feasible approach using a lyophilized live vaccine. Fourth, it has been used extensively as a vehicle to deliver recombinant proteins with much resultant knowledge in host-vector interaction.[18] Fifth, adenovirus vectors can infect a wide range of cells, including professional antigen-presenting cells, without requiring host cell replication as do retroviral vectors.[18] Sixth, there is a limited cytopathic effect from viral infection allowing relatively long recombinant protein expression.[19] We will discuss in the following sections:

1. Adenovirus life cycle and immunity;
2. Adenovirus as a vaccine to generate humoral immunity; and
3. Adenovirus to generate cellular immunity as measured against tumor targets.

Adenovirus Life Cycle and Pathogenicity

Adenoviruses can infect a wide range of cells. Recent work on the molecular requirements for viral entry have identified necessary host proteins for some serotypes, including the CAR protein which binds to viral fiber protein[20] and the integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ which bind to the viral penton base protein.[21,22] While both of these proteins are widely expressed on many cell types, some cells, notably resting T cells, may not express them.[23] Entry into the cell then involves receptor-mediated endocytosis. Although the virus access to the cell has been well studied, its pathway to the nucleus and the regulation of that process is not well understood.[24,25] The transcription of adenovirus gene products has been divided into

early and late phases separated by the onset of DNA replication, about 8 hours after infection. There are 6 early regions, defined by 6 transcripts giving rise to at least 30 proteins and one late transcript arising from one promoter, the major late promoter, MLP.[26,27] Discussion of the viral gene products is beyond the scope of this review except to make a few points relating to the variations in viral constructs used for vectors. Many of the adenoviral vectors used to create vaccines have deletions of either the E1 or E3 regions. The E1 region is necessary for DNA replication while the E3 region is not. Thus, vaccines with E1 deletions will not replicate, which makes a safer vector. However, nonreplicating virus may also be potentially less effective.[28] The E1 region is also required to initiate DNA synthesis and the shut down of cellular functions. Deletions of the E3 region would be replication competent and potentially more immunogenic, as will be discussed later. The overall size of inserts into the adenovirus genome is limited by the requirement that the virus can only package efficiently a DNA that is up to 105% of the wild type size of about 36 kb. Thus, with both E1 and E3 deletions, the adenovirus can incorporate no more than 7.5 kb of foreign DNA. The E2 region has important functions for viral replication and transcription modulation. Mutations in E2 have shown a role in decreasing late protein production. E4 deletions have been used to a limited extent as well.[29,30]

As a disease in humans, adenovirus can infect a wide range of tissues causing pneumonia, pharyngitis, conjunctivitis, gastroenteritis, and cystitis. Forty-seven serotypes of human adenoviruses have been described. The serotypes differ in their pathogenicity. For example, serotypes 40 and 41 produce gastroenteritis, while 2 and 5 do not. Many infections with adenoviruses do not cause noticeable disease but are identified by serological conversion. The serotypes that have been used in military vaccinations against the respiratory diseases they cause are Ad4 and Ad7. Ad2 and Ad5 have been preferred for use in recombinant constructs because they are very nonpathogenic.[26] The host range for most adenoviral serotypes is limited, which makes animal models for replicating viruses difficult. Ginsberg and Prince have used the cotton rat as a model to investigate the molecular and cellular pathogenesis of adenoviral pneumonia.[19] In that species, Ad 5 replicates and causes a disease similar to that in humans, although viral replication is not as robust as in humans. When large numbers of the virus are delivered intranasally, there is an early infection of bronchiolar epithelial cells, accompanied by a diffuse infiltration by monocyte-macrophages, neutrophils and lymphocytes, mostly CD4 T cells and NK cells with a few B cells, peaking at about 2-3 days. This is followed by a late phase beginning about 5 days after infection that peaks at 7 days where there is a peribronchiolar and perivascular infiltrate of lymphocytes, which are primarily CD8 T cells. Interestingly, the infected cells do not die in great numbers, similar to the lack of cytopathic effect in vitro. Viral replication is not needed to produce pneumonia. Viral replication can be detected in alveolar macrophages and macrophage/monocytes in the hilar lymph nodes of infected rats, suggesting that viral products of the early genes at least will be expressed in antigen-presenting cells.

The genes of the E3 region have been found to affect the host-virus interaction. Deletion of the E3 region causes a pneumonia more severe than wild type Ad5.[31] It was found that gp19, coded within the E3 region, is responsible for this effect and that gp19 causes a downregulation of the class I expression in infected cells.[32-34] This suggests that the immune response to the infection is a direct cause of the pneumonia. Similarly, the deletion of the E3 gene coding a 14.7 kDa protein causes an increase in the PMNs that infiltrate early in infection. The 14.7 kDa protein has been found to protect infected cells from TNF lysis.[35,36] Antibodies to TNF-α reduce the early inflammation by 50-75 percent. Thus, deletion of the E3 region appears to cause a more potent inflammatory response to viral infection. Whether this has a large effect on the effectiveness as a vaccine is a complex issue that has not been well studied. In a chimpanzee model, a comparison was made between

$E3^{+/-}$ vaccines in a complex protocol using three sequential vaccinations with different serotype replicating Ad vaccines against HBsAg.[37] While it is difficult to make conclusions based on four animals, the suggestion was made that the presence of E3 prolonged viral expression, thus leading to higher anti-HBsAg antibody titers.

This observation is consistent with the demonstration in other animal models, using replication deficient viral infection, that expression of foreign proteins is limited temporally by the immune system. This has been amply demonstrated in models of gene therapy where adenoviruses have been used in attempts to stably express foreign genes in host cells.[38] The immune response against the virally expressed proteins is responsible for the elimination of the cells that express the proteins. From a vaccine point of view, this suggests that a potent cytotoxic response does occur after primary vaccination and that these responses will eliminate to a large extent the cells that express these antigens. It would be very interesting to know if it would be possible to develop methods that have both a potent initial immune response against the virus and its recombinant antigens, but that also provide a second viral variant that would remain in APCs for an extended period to prolong the period of protection.

Use of Adenoviral Vectors to Induce Antiviral Humoral and Cytotoxic Immunity

Adenoviral vectors have been used to induce antiviral immune responses against many viruses including hepatitis B,[39] HIV,[40] SIV,[41] herpes simplex virus,[42] rabies,[43,44] respiratory syncytial virus,[45] hepatitis C[46] and many others. In most of these studies, the efficacy of the vaccine has been measured as neutralizing antibody titers. Many different animal models were used, depending on the virus, including monkeys, dogs, mice, cotton rats, rabbits, etc. Interestingly, little effort has been made to use adenoviral vectors against papilloma viruses. Generalizing the studies against other viruses to papilloma virus targets in man is certainly not possible, but some general lessons may be learned.

A hepatitis B vaccine exists, but as an injectable vaccine it is too expensive for worldwide applicability. Adenoviral vectors expressing HBsAg were among the first serious efforts to develop a clinical vaccine and they have been tested in hamsters,[47] dogs,[39] and chimpanzees.[37,40] High titers of high affinity antibodies have been found in the dog model. In the chimpanzee experiment, two chimps were immunized using an oral vaccine with a boost using a second serotype. Protection was obtained with one animal and partial protection in the second. Remarkably, the anti-HBsAg titers were higher and more prolonged in viruses where the E3 region was intact, suggesting that persistence of the virus may be associated with a better response.[37] More complex vaccines have been developed that express more than one hepatitis B protein. When tested initially in human volunteers, neutralizing antibodies were not generated using an Ad7 E3-deleted vaccine expressing HBsAg, although only a primary vaccination was given.[48] A similar approach using alternating serotypes for booster vaccination was needed for complete protection from RSV infection in a ferret model.[45] A general strategy with Ad vaccines learned from these experiments is that multiple vaccinations with different serotypes will be necessary for some antigens and, as pointed out earlier, E3 deletions may be detrimental.

This approach was modified for the production of neutralizing antibodies against HIV in a chimpanzee model.[40] Here the investigators used a combination approach, termed prime-boost,[49] with an initial vaccination(s) with Ad-HIV-1 MNgp120 with one or more different adenovirus serotypes, and boosting with HIV-1 SF2gp120 protein. The animals were challenged with a low dose of HIV followed by a high dose one year later without a subsequent boost. They found protection in three of five animals vaccinated. Again, with the small number of animals, it is hard to reach conclusions as to the contribution of

boosting with different serotype viruses or subunit proteins, but potentially one can achieve protective neutralizing antibodies against some strains of HIV.

Cytotoxic responses contribute to protective immunity against papilloma viral infection. Adenoviral vectors are clearly able to generate cytotoxic responses against heterologous viral antigens.[43,50,51] EBV can cause lymphocyte proliferative disease in man and other primates unless a cytotoxic immune response suppresses the disease. A replication-competent adenovirus expressing the EBV envelope glycoprotein gp340/220 could induce protective immunity against a live EBV viral challenge in the cotton top tamarin, whereas unvaccinated animals developed severe lymphadenopathy.[52]

Use of Adenoviral Vectors to Generate Mucosal Immunity

For a virus that enters the body via a sexually transmitted route, such as those papilloma viruses that cause cervical cancer, developing a protective mucosal immunity will be critical in protection against a primary infection. Such an immune response may be measured by mucosal antibody levels or by mucosal cytotoxic responses. The experience with adenoviral vectors in generating such responses is limited. Primary herpes simplex virus infections occur at mucous membranes, and adenovirus vectors have been used to deliver HSV antigens to generate protective immunity. Initially, it was shown that a single intraperitoneal (i.p.) injection of an adenovirus expressing glycoprotein B from HSV-1 could protect against a lethal challenge of HSV given i.p.[53] It was subsequently shown that, comparing i.p. to intranasal injection of adenovirus, the i.p. injections raised higher anti-CMV IgG but less mucosal IgA and intranasal (i.n.) injection was more potent in protection from i.n. challenge of a lethal dose of HSV.[54] This suggests that adenoviral vectors can be potent inducers of mucosal immunity when given i.n. but not i.p.[51,54,55]

Use of Adenoviral Vectors to Induce Cytotoxic Responses Against Tumor Cells Expressing Foreign Antigens

As described earlier, papilloma-induced tumors necessarily express viral proteins, including E6 and E7. Thus, the development of models where cytotoxic immunity can be generated against tumors expressing known tumor antigens can test the possibility of immunological elimination of papilloma-induced cancers. No perfect model exists for these tumors. The ideal model would not have the E6 and E7 expressed as an autologous gene, but instead it would be expressed on a slow growing population of cells prior to the delivery of the vaccine, to mimic a human clinical situation where vaccines are used in a therapeutic way. A model where the vaccine is given to an animal which has never been exposed to the antigen, and where latent virus proteins are not expressed, is not a realistic model. The closest model has been developed using a transgenic E6 and E7 with a keratin promoter.[56] The skin from these animals is prone to develop squamous carcinoma, and the transfer of skin expressing transgenic E6 and E7 to another mouse without those genes mimics some of the biology of human HPV infection and carcinogenesis. A potential problem with this model is that there may not be E7 epitopes that can be presented by the H2q haplotype of these mice.[57] Vaccination protocols have been tested in this model, but not yet with adenoviral vectors. We will discuss below other models where adenoviral vectors have been successfully tested against tumor targets.

A replication-defective adenovirus was used to vaccinate mice against bacterial β-galactosidase.[58] A tumor model was used wherein the mouse colocarcinoma cell line CT26 expressed *E. coli* β-galacatosidase. Tumor cells were given intravenously, the mice were vaccinated 3 days later and lung metastases were quantitated after 14 days. The investigators were able to show that a single vaccination of 10^8 infectious units was partially therapeutic (metastases going from >200 to 75) and that the effect was improved

by the administration of IL-2 (metastases <10). They argued that when a nonreplicating virus was used, the number of infectious particles given is very important, since lower doses of virus had little effect. In a second model, a nonreplicating adenovirus expressing a human colon carcinoma antigen in the mouse could produce a therapeutic effect when given to a mouse preinjected with a tumor expressing the human protein.[59] These experiments suggest that when a tumor expresses a foreign antigen and when there exists a low tumor burden, then adenoviral vectors can be effective.

Because dendritic cells are the most potent professional APCs, another vaccination approach has been to express antigens on dendritic cells and use them as a vaccine. In one set of experiments, Brossart et al found that dendritic cells injected i.p. developed a stronger cytotoxic response when the dendritic cells were infected with an adenovirus expressing the antigen than when the dendritic cells took up either the peptide or the protein.[60] Furthermore, they found that the animal developed a weaker antiviral antibody response, which allowed repeated inoculations of the virally infected dendritic cells. Similarly, Song et al found that vaccination with dendritic cells infected with a replication-defective adenovirus expressing β-galactosidase could develop a potent cytotoxic response and slow the growth of preexisting tumors expressing the antigen.[58] With a very slow growing tumor, as in the human situation, and the likely presence of a preexisting antiviral immune response, vaccination with dendritic cells may offer the greatest likelihood of maximizing a cytotoxic immune response with adenoviral vectors.

Future Goals for Applying Adenoviral Vaccines to Papilloma Virus Infections and Malignancies

A vaccine directed against the primary infection by papilloma virus would have to be extremely safe for both individuals receiving the vaccine and those who may be exposed to them. Concern has been raised about the safety of even replication-incompetent recombinant adenoviral vaccines.[61] Therefore, it is unlikely that the first clinical trials of recombinant adenoviral vectors against papilloma virus would be used in this setting, even though as discussed earlier, the adenoviral vectors have been extremely effective in generating a protective response against viral challenge, particularly with intranasal injection to create a mucosal immune response. Instead, it is more likely that it will be used for patients who already have a life threatening illness for whom the risks of the virus are outweighed by the potential benefit of the vaccine. As such, the E6 and E7 genes of the cancer-causing papilloma viruses are likely candidates for therapeutic trials. For these antigens, the small insert size of adenoviruses is not a drawback. The most difficult problem remains efficacy. Clearly, formidable hurdles remain to be overcome before a reasonable expectation of therapeutic benefit will be achieved. Cervical carcinoma cells have evolved protective adaptations that may render them poor targets for immune attack.[10] In mouse models, even with foreign antigens that are reasonably strong antigens such as β-galactosidase and chicken ovalbumin, significant tumors cannot be eliminated by vaccination with adenoviral vectors at the current time. A number of approaches to augment the response, such as cytokine augmentation, need to be explored.[62,63] Multiple vaccinations with different vaccines of complementing strengths, such as dendritic cells, different serotypes, vectors with targeting capabilities or other vectors also need to be tested.

Comparison of Adenoviral Vectors with Other Vaccine Approaches

A meaningful comparison between adenoviral vectors and other viral vectors is difficult to test because of the biological differences between different species and viral targets. Given that acknowledged caveat, some comparisons have been made. Gonin et al have compared an adenoviral (Ad2 using the MLP) and poxvirus (Nyvac strain of vaccinia)

vaccine expressing the same antigen, gD, against a pseudorabies virus challenge.[64] When they used i.m. injections of each, they found that both vaccines could be protective at high doses and that the adenovirus was more effective in generating an anti-gD antibody response. When they used i.n. injections, the adenoviral virus protected at the higher doses, while they could not obtain high enough titers of the poxvirus for similar doses intranasally. They also found that the poxvirus did not elicit a humoral response i.n. Xiang et al have compared a nonreplicating adenovirus, and a poxvirus expressing the rabies virus glycoprotein which is currently being used in wildlife protection in Europe,[65] in a mouse model.[43] The adenoviral vector could protect from a 10 LD_{50} challenge even after one subcutaneous injection of 2 x 10^4 iu, while the poxvirus was only partially protective. Another issue when comparing poxvirus with adenoviral vectors is that of toxicity. Work from Morgan and associates suggests that a protective vaccinia vaccine was significantly morbid in the cotton top tamarin model of EBV infection, whereas an attenuated strain of vaccinia was not protective. This is in contrast to the adenoviral vaccine which was protective and did not cause morbidity.[52]

A comparison has been made between a DNA vaccine and an adenoviral vector in a mouse measles model.[66] The measles virus nucleocapsid protein was expressed using a CMV promoter in a plasmid vector and antibodies were made after i.m. injection of the DNA. However, when the mice were challenged intracranially, protection was not obtained, in contrast to a protective vaccination with an adenoviral vector. Again, this difference could be due to many factors relating to the model and details of the experimental protocols.

A truly fair test of any two vaccine approaches would be to use an optimal virus, route, and dosing schedule for each virus and to compare them in an appropriate model. Even then, caution should be exercised in extrapolating to other targets and other species.

An Introduction to Bacteria-Based HPV Vaccines

Recent advances in genetic engineering now permit a more modern approach to the development of recombinant vaccines, particularly bacterial organisms. Intracellular bacteria are being exploited as vaccine vectors because of their ability to generate a strong T-cell immunity, which is important in resistance to viral infections and in the eradication of tumor cells.[67-70] Bacterial vaccine vectors may have an added advantage over viral vectors because of their ability to produce toxins which could serve as an adjuvant in generating effective innate immunity and cytokine release.[70] In addition, because of the existence of a wide range of antibiotics, bacteria-based vaccines are considered safer to use in humans, and therefore are more likely to be approved by regulatory organizations. To date there are no effective recombinant bacteria-based vaccines for HPV infection or HPV-associated malignancies. However, a number of laboratories are working hard to develop an effective recombinant bacterial vaccine targeted mainly against the E6 and E7 proteins, which are expressed on most HPV-associated tumors. Candidates for bacterial vaccine vectors for this approach principally include intracellular organisms such as *Listeria* monocytogenes (Lm),[71-74] *Salmonella* sp.[75-77] and BCG.[78-80] To a lesser extent *Shigella* sp.[81] and *Echerichia*[82] are currently being evaluated by some groups as vectors for heterologous antigens. For the scope of this review, only *Listeria*, *Salmonella* and BCG are discussed below.

Overview of Biological Properties and Infection Cycle of Bacterial Vectors

Listeria Monocytogenes

Listeria monocytogenes (Lm) is a β-hemolytic gram positive, facultative intracellular bacterium. It is a food-borne human and animal pathogen that causes mild gastrointestinal

upset in healthy hosts but may also cause serious infections in immunocompromised individuals and neonates.[83] This organism has been used extensively for decades to study cell-mediated immunity.[84] Lm infects an array of cells, including antigen-presenting cells like macrophages. Lm is engulfed by the host cell by phagocytosis and takes up temporary residence in phagolysosomes, where the majority of the bacteria are killed.[84] Unlike other intracellular organisms, a fraction of the bacteria escape into the cytoplasm of the host cell by disrupting the phagosomal membrane, mainly through the action of the hemolysin, listeriolysin O (LLO).[85] The bacteria replicate in the cytoplasm and use actin filaments to move to the periphery of the cell, where they form pseudopod-like structures that are recognized and internalized by adjacent cells.[85] The cycle is subsequently repeated without the bacterium coming into contact with the extracellular compartment. In this way humoral immunity (neutralizing antibodies) has a very small role to play in resistance to *Listerial* infections.[84,85] Peptides derived from Lm in the phagolysosome can be presented via the MHC class II pathway, which subsequently expands $CD4^+$ cells with Th1-type properties. In the cytosolic compartment, peptides derived from Lm are presented via the MHC class I pathway, which induces strong $CD8^+$ T-cell responses. In addition, antigens derived from Lm can be presented via nonclassical pathways to Lm-specific $CD8^+$ T cells restricted by MHC class Ib molecules H2-M3[86] and CD1.[87]

Listeria has received increasing attention as a vaccine vector because of these unusual characteristics and immunological properties. It was first proposed that Lm could be an effective recombinant vaccine vector when the induction of a cellular immune response to a passenger β-galactosidase antigen by a recombinant *Listeria monocytogenes* was demonstrated.[88] Since then, a lot has been achieved, and recombinant Lm expressing HPV and cottontail rabbit papilloma virus (CRPV) antigens have been developed.[89-90]

HPV is naturally a mucosal disease and, therefore, the ability of any vaccine against HPV infection or associated tumors to induce a strong mucosal response is highly desirable. In the gut, Lm principally invades lymphocytes and M cells in the Peyer's patches (PP).[91] Even though mice are highly resistant to intragastric Lm infection, small doses have been shown to increase intraepithelial lymphocytes, antibody titer and TCR $\gamma\delta^+$ T lymphocytes which secrete IFN-γ.[92] In one study. it was demonstrated that neutrophil depletion, by administration of an anti-granulocyte monoclonal antibody, significantly impaired resistance to oral challenge with Lm.[93] 60% of these neutrophil-depleted mice died after intragastric administration, with as few as 4×10^4 cfu Lm, whereas inoculation with 4×10^8 cfu resulted in the death of only 20% of the control mice.[93] Therefore, neutrophils appear to play an important role in local mucosal defense against extracellular or *Listeria*-infected cells, as well as clearing Lm in the bloodstream and thus protecting against invasion of organs.[92-94]

Salmonella sp.

Salmonella is one of the most diverse species of bacterial organisms. There are over 1000 serotypes based on the variation of surface polysaccharide antigens. The organism causes gastroentritis in humans and may cause severe systemic disease in immunocompromised individuals.[95] Principally, *Salmonella* infect following ingestion of contaminated food. In the gut, the bacteria invade the PP, where they transcytose the M cells. In addition, *Salmonella* have been shown to invade cells of epithelial origin that line the gastric mucosa, but there was no evidence that these cells serve as antigen-presenting cells.[96] *Salmonella* are also phagocytosed by macrophages in the PP, after which they disseminate via the mesenteric lymph nodes to become a systemic disease. These properties have enabled *Salmonella* to induce a broad based cellular, humoral and mucosal immunity.

The role of $CD4^+$ and $CD8^+$ cells in Salmonellosis is still under investigation. *Salmonella typhimurium* like *Listeria* has been shown to induce a Th1-type immune response,[97,98] even when delivered orally.[98,99] In addition IFN-γ, which is a major Th1-type cytokine, is important in inducing immunity to *S. typhimurium* infection through the activation of macrophages.[100,101] *Salmonella*, unlike *Listeria* and *Shigella*, does not escape the endosomal compartment; however, it has been demonstrated that antigens derived from *Salmonella* can be processed via the MHC class I pathway with the generation of antigen-specific $CD8^+$ T cells.[77,102-107]

The humoral arm of mucosal immunity to *Salmonella* has been extensively studied.[96] It has long been established that plasma cells from PP are capable of secreting IgA into the gut lumen, while cells from peripheral blood and lymph nodes tend to colonize the spleen and give rise to predominantly IgG antibodies.[108-110] Recombinant *Salmonella* expressing foreign protein has been demonstrated to generate IgA responses to the expressed proteins.[108,110] In one study it was shown that Th2-derived IL-10, together with IL-6 produced by macrophages, provides important signals for the development of mucosal IgA and serum IgG responses in the absence of preferential expansion of Th2 cells producing IL-4 and IL-5.[111]

BCG

BCG, baccilus Calmette-Guerin, is a human tuberculosis vaccine derived from a strain of *Mycobacterium bovis* that has been attenuated by 230 serial passages in vitro.[112] *Mycobacteria* are intracellular organisms which like *Listeria* and *Salmonella* invade macrophages. They cause a variety of systemic diseases in immunocompromised individuals affecting virtually all organs in the body.[113] Unlike *Listeria* and *Salmonella*, *Mycobacteria* are transmitted as aerosolized droplets via the respiratory route. *Mycobacteria* are phagocytosed by resident nasal or alveolar macrophages. Macrophages kill all or most of the bacteria in immunocompetent individuals; however, in some instances the bacteria are able to evade the bactericidal mechanisms of macrophages and remain dormant for years. Many research groups have studied survival and death of *Mycobacteria* in macrophages with differing results, which could be due to diverse macrophage populations in relation to their stage of differentiation and activation.[114] $CD4^+$ T cells are important in resistance to mycobacterial infection, as evidenced by increased susceptibility in AIDS patients.[115] In addition, mycobacterial infections, including BCG, like *Listeria* and *Salmonella* induce a predominantly Th1-type immune response.[116-118] There is strong evidence that peptides derived from intracellular *Mycobacteria* can be presented directly to a $CD8^+$ T cell-population via the MHC class I pathway.[119,120] Furthermore, only live *Mycobacteria* are efficient in facilitating antigen processing via the MHC class I pathway in murine macrophages.[121] However, the role of $CD8^+$ T cells in resistance to mycobacterial infection is not yet fully established in humans. Even though much of the focus of studies on protective immunity is on the cellular immune response to *Mycobacteria*, humoral responses to the bacterium have been described. In particular, strong IgM-specific responses have been shown by ELISA in sera from patients with the lepromatous form of *M. leprae*.[122] In addition, Lagranderie et al demonstrated IgA antigen-specific responses in guinea pigs after oral Immunization with recombinant BCG expressing β-galactosidase.[123]

Mycobacteria are one of the most studied bacterial species, which has resulted in a wide body of knowledge of their interactions with the human immune system and, therefore, the potential use of attenuated forms as vaccine vectors. In addition, the completion of the whole genome seqeunce of *M. tuberculosis*[124] will improve our understanding of the basic biology of this bacterial species and their virulence factors.

Induction of Host Immunity to Virally-Derived Proteins and Tumor Antigens

In order to successfully construct a live recombinant bacterial vaccine for effective resistance to viral infection or eradication of tumor cells, the following criteria have to be met.

First, the transgene has to be maintained and expressed, and the gene product should be secreted outside the bacterial cell wall for potent CTL responses. It has been demonstrated that Lm secreted proteins LLO and p60 are target antigens for T cells in *listerial* infections, and they tend to induce strong cellular immunity and cytokine production of the Th1-type. This suggests that secreted Lm proteins may be the most important antigens for inducing $CD8^+$ T cell activity.[125,126] In the case of live recombinant *Salmonella* and BCG, antigen secretion does not appear to be required in order to be effectively presented to cytotoxic T lymphocytes.[102,104,119]

Second, the immunogenicity of the recombinant strain should not be compromised or should at least be comparable to the wild type strain.

Third, the genetic makeup of the recombinant strain should not be significantly altered in such a way that it becomes hazardous to the host cell (altered characteristics, transformation etc.).

Recombinant Listeria Monocytogenes as a Vaccine Vector for Viral and Tumor Antigens

The ability of Lm to express foreign antigens was first demonstrated by Schafer et al.[88] Subsequently, recombinant Lm expressing and secreting a fusion protein of listeriolysin O and NP (LLO-NP) was shown to induce significant NP-specific cytotoxic activity[127] and protect mice against challenge with i.n. Immunization with influenza virus.[72] Other investigators have also explored the possibility of using Lm as a heterologous antigen expression system and were able to develop a recombinant Lm that expressed lymphocytic choriomeningitis virus NP.[128,129] They demonstrated that Immunization of mice with the recombinant vaccine conferred protection against challenge with a virulent strain of LCMV and that protective antiviral immunity was due to $CD8^+$ T cells. Similarly, Frankel and associates demonstrated that recombinant Lm expressing HIV-1 Gag protein could induce cell-mediated immunity, with the production of CTL capable of killing syngeneic tumor cells infected with vaccinia vectors carrying the HIV-1 gag gene.[71]

A murine tumor model system that used influenza (NP) as a model tumor antigen[73] was first employed to test the efficacy of Lm as a cancer vaccine. In this system recombinant Lm that expresses NP from influenza strain A/PR8/34 was used. Lm-NP had previously been demonstrated to present the K^d-restricted NP epitope in vitro and to induce NP-specific CTL in vivo.[127] Two MHC class I^+/II^- murine tumor lines, RENCA and CT26, were transduced with the NP gene and express the NP protein as a tumor antigen. These cell lines were as tumorigenic as the parent cell lines. Moreover, NP-specific CTLs could not be detected in mice that had RENCA-NP or CT26-NP tumors, suggesting that the expressed NP on the tumors behaves very similarly to naturally occurring tumor antigens. Tumors were established subcutaneously and the vaccine was delivered intraperitoneally.

In this model system, it was demonstrated that Lm-NP could protect mice against 100x the tumoricidal dose of RENCA-NP or CT26-NP tumor challenge. 100% protection was observed in the RENCA-NP model, while more than 50% protection was observed in the CT26-NP model. Furthermore, Lm-NP was shown to cause regression of established tumors. Nine out of ten mice with RENCA-NP tumor had complete and lasting remission, while in the CT26-NP model there was significant slowing of the tumor growth to a near

standstill. The anti-tumor effect of Lm-NP was demonstrated not to be antibody mediated but rather induced by a T cell-dependent immunity.

This system was further extended to test the efficacy of the vaccine orally.[130] This study confirmed that Lm-NP when introduced orally was an effective cancer immunotherapeutic that can cure 50-60% of mice with established RENCA-NP and CT26-NP tumors and significantly limit the growth of B16F10-NP melanoma. In addition, attenuated Lm has been used effectively as an oral vaccine carrier to trigger a long lasting immune response against an aggressive mouse fibrosarcoma which expresses β-galactosidase as a tumor-associated antigen.[131]

Using a model much closer to HPV, the CRPV model, Jensen and associates studied the efficacy of recombinant Lm expressing CRPV E1 protein (E1-rLm) as a prophylactic vaccine for CRPV and CRPV DNA-induced papillomas .[89] This study showed that E1-rLm Immunization promotes papilloma regression in about 77% of rabbits and those that respond to E1-rLm vaccination remained papilloma free for at least five months. However, some rabbits had transient, partial or no regression of papillomas. They also demonstrated that the protection observed was cell mediated and E1-specific antibody was not involved.

In our laboratory we have successfully constructed recombinant *Listeria* monocytogenes-expressing and secreting E6/E7 proteins. Our recombinant vaccine constructs have been demonstrated to contain E6 and E7 genes in their chromosome and the gene products expressed and secreted. In addition the vaccines have been characterized and found to have a growth rate and virulence that are comparable to a wild type strain. We have developed a feasible system of producing recombinant *Listeria* monocytogenes that secretes tumor viral antigens, and we are in the process of exploring the potential of the system as a cancer vaccine for E6 and E7-expressing mouse tumors (Zubair, Gunn, Pan and Paterson, manuscript in preparation).

The potential of recombinant Lm as a therapeutic vaccine vector for targeting HPV-associated malignancies lies in the ability of Lm to induce strong CD4 and CD8 cellular responses, in addition to its ability to induce the array of cytokine responses that skews cellular immunity towards a Th1-type response. The successful development of recombinant Lm expressing influenza nucleoprotein (Lm-NP) and the demonstration of such a vaccine in conferring complete protection and significant regression of established murine tumor lines generated the idea of developing recombinant Lm as a cancer vaccine.[73,130] However, one of the drawbacks with the Lm-NP system is that the antigen (NP) is not an endogenous tumor antigen. Thus, the use of E6 and E7 as passenger antigens, which are virally derived as well as endogenous tumor antigens, not only has application to human disease but allows us to test the Lm approach in a more relevant model.

Recombinant Salmonella sp. as a Vaccine Vector for Viral and Tumor Antigens

In spite of the variable immunogenicity of recombinant *Salmonella* vectors, there is a general consensus that *Salmonella* vectors are capable of eliciting protective humoral, mucosal and systemic responses against viral antigens. It has been shown that recombinant *Salmonella* vaccines expressing viral antigens could induce a protective humoral response against influenza virus[132] and herpes simplex type 1 virus[133] in laboratory animals. In addition, Wu et al demonstrated the induction of mucosal and systemic responses against human immunodeficiency virus type 1 (HIV-1) glycoprotein 120 (gp120) in mice after an oral dose of recombinant *Salmonella* vaccine which expresses HIV gp120 protein.[134] Following a similar approach, using recombinant *Salmonella* expressing SIV capsid protein, another group demonstrated the induction of SIV capsid-specific CTL and mucosal secretory IgA in mice after Immunization with the recombinant vaccine.[135]

A recombinant attenuated *Salmonella* vector vaccine that expresses HPV16 virus-like particles (VLPs) has been developed and used to generate mucosal and systemic neutralizing antibodies in mice against the HPV L1 major capsid protein.[136] This was the first demonstration that HPV16 VLPs can self-assemble in prokaryotes. Using the ability of *Salmonella* to induce mucosal immunity, this approach could provide a most effective prophylactic vaccine against HPV infection in the genital tract. Induction of an antibody response in mice against HPV16 E6 and E7 after Immunization with recombinant *aroA*$^{-}$ *Salmonella* strains expressing E6 and E7 has also been demonstrated.[76] Taken together, these studies demonstrate the potential of *Salmonella* as a vaccine vector for both prophylaxis against HPV infection and therapy for HPV-associated malignancies.

A great deal of knowledge of mucosal immunity has been acquired through the studies of recombinant *Salmonella*. However, the role of recombinant *Salmonella* as a cancer therapeutic against epithelial tumors has only been explored by a few investigators. Pawelek et al developed an attenuated auxotrophic recombinant strain of *Salmonella* that preferentially accumulated in both human and murine tumor tissues.[137] They demonstrated melanoma regression after Immunization of melanoma-bearing mice with this recombinant vaccine expressing HSV thymidine kinase and a prodrug, gancicyclovir. This study demonstrated that attenuated *Salmonella* would be useful both for inherent anti-tumor activity and delivery of therapeutic proteins to cancer cells in vivo.[137]

In another approach, attenuated *Salmonella typhimurium* (*aroA*, *aroD*) strains were constructed that directed the expression of hepatitis B core antigen particles (HBcAg) or the fusion protein of HBcAg and HPV16 E7. These were put under the control of an in vivo inducible nirB promoter. Mice were immunized orally or intravenously, and humoral and cellular E7 and HBcAg-mediated responses were monitored. The strain expressing the HBcAg-E7 fusion protein induced anti-E7 humoral IgG and IgA responses in the intestines of orally immunized mice.[138] This provides evidence that bacterial vaccine vectors, particularly *Salmonella*, can induce mucosal immunity against HPV-derived antigens.

Recombinant BCG as a Vaccine Vector for Viral and Tumor Antigens

Recombinant DNA was first introduced in *Mycobacteria* by a protoplast transformation procedure, and subsequently by a high-efficiency electrotransformation protocol that gave more than a thousand transformants per microgram of plasmid DNA in a suitable host.[139] However, *Mycobacteria* are notorious for discarding foreign plasmids. A number of shuttle plasmids with the ability to replicate independently or integrate into the mycobacterial chromosome have been developed. In an approach that utilizes nonintegrating plasmids, shuttle plasmids contain a gene that is essential for the survival of the host *Mycobacterium*. In one study it was demonstrated that β-galactosidase protein could be expressed and detected well in *Mycobacteria*.[140] In this study, a shuttle plasmid was constructed with both a mycobacterial and an *E. coli* replication origin to allow for replication in both hosts, and a kanamycin-resistant gene that could be expressed in both *E. coli* and *Mycobacteria*. This method provides a high copy number of the shuttle plasmid, but has the disadvantage of instability in transformants, especially when the transgene affects the growth rate of the host bacterium. In contrast, approaches that use integrating shuttle plasmids have the advantage of stability of the transformants but the efficiency of the system is very low.[141]

Recombinant BCG expressing HIV-1 and SIV polypeptides have been developed and shown to induce humoral and cellular immune responses in murine models.[78,142] Moreover, DNA sequences coding for HIV-1 Gag, Pol, Env, and Nef were fused to the mycobacterial heat shock protein hsp70 promoter, and these recombinant constructs were injected in mice to induce both humoral and cellular responses.[78,139,142]

BCG has been proven to have inherent immunostimulatory properties, and this has been exploited in the therapy of superficial bladder cancer with varying success[143,144] and as an adjuvant in the treatment of melanoma.[145] Large and frequent doses are required to induce a strong immune response to eradicate tumors, and occasionally this may lead to a disseminated mycobacteriosis. The possibilities of genetically manipulating *Mycobacterium* allowed investigators to explore the possibility of modifying or potentiating the inherent immunostimulatory properties of *Mycobacterium* by the secretion of mammalian cytokines.[146] Recombinant BCG strains secreting functional IL-2, IL-4, IL-6, GM-CSF and IFN-γ have been generated and characterized.[146] These recombinant cytokine-secreting BCG strains have demonstrated a more potent induction of cell-mediated immune response than the wild type BCG strain. The most profound effect was induced by IL-2, GM-CSF and IFN-γ-secreting BCG strains that exhibited an enhanced antigen-specific T-cell response following in vitro stimulation with purified protein derivative (PPD). In addition, when administered intravenously in mice to induce CTL, no adverse effects were observed.[146] Therefore, the use of recombinant BCG that can secrete cytokine gene products could offer improved cancer immunotherapy with BCG.

Safety Issues and Limitations of Bacteria-Based Recombinant Vaccines

Safety is a great concern when using live recombinant vaccines. A good candidate recombinant vaccine vector should have very minimal complications in humans. One of the drawbacks for using viral vectors is the concern of their transforming properties and high risk of mutagenesis, in addition to infectious pathology. On the other hand, bacterial vectors have minimal frequency of these properties, though they do retain the ability to cause serious systemic diseases. BCG has a good safety record and has been used as a safe vaccine against TB but it still is not free of complications. Side effects of BCG vaccination have been correlated with the amount of dead BCG cells in the vaccine preparations.[147] *Salmonella* causes minor gastroenteritis but can cause serious systemic disease even in healthy individuals. There is a serious safety issue about *Listeria* in humans with impaired immune systems, such as immunosuppressed adults, newborn infants and pregnant women. The most likely individuals for vaccination with live recombinant vaccines are cancer patients with advanced disease whose immune systems may have been damaged by prior chemotherapy or radiation treatment. Unfortunately, most if not all Live recombinant vaccine vectors are potentially hazardous to immunocompromised individuals. Use of attenuated strains, even though they have variable efficacy, might be a good option with minimal side effects, especially to immunocompromised patients.

HPV-associated malignancies are unique for constitutively expressing defined tumor antigens E6 and E7, unlike most malignancies whose antigens are difficult to identify or may be constantly mutating as the tumor evolves. However, even HPV-associated malignancies like cervical cancer are causally associated with a number of HPV serotypes (16, 18, 31, 33, 52) depending on the geographic region.[148] Therefore, for a live recombinant vaccine expressing E6 and E7 proteins to be effectively used worldwide, a cocktail of recombinant strains expressing E6 and E7 proteins derived from the common HPV serotypes appears to be indicated.

For a live recombinant vaccine to be effective against evolving epitopes during tumor growth, the vaccine should be able to induce epitope spreading. This is an immunological phenomenon in which an immune response initially induced to specific epitopes eventually spreads to other epitopes not originally recognized. So far this phenomenon has not been described after Immunization with recombinant bacterial vaccines. However, it has been demonstrated that the administration of antibodies to CTLA-4 during therapy with

Lm-NP may induce epitope spreading from NP to endogenous antigens (Weiskirch, Paterson, unpublished data).

Another important practical limitation to using live recombinant vaccines is prior exposure to the vaccine vectors. It has been shown that there is reduced efficacy of live recombinant vaccines if the individual has had a prior infection with the live vaccine vector or has been deliberately exposed by a prior vaccination. Unfortunately, most of the good candidate vaccine vectors are common pathogens with subclinical symptoms. Therefore, the infection rate or the incidence is often difficult to determine due to underreporting.

It is very clear there are still many hurdles to be overcome to optimize the efficacy of live recombinant bacterial vectors for use in humans. However, the system has proven effective against viral and tumor-derived antigens in experimental animals, with few practical limitations. To explore the potential of the bacterial vectors as vaccines for HPV and HPV-associated malignancies yet remains.

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