Therapeutic Immunization strategies against cervical cancer

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Chapter 1

General introduction
Scope of the study described in this thesis

Cervical cancer is the second most common cancer among women worldwide, with about 470000 new cases and 233000 deaths each year [Parkin 2001]. This form of cancer develops from non-invasive premalignant lesions called cervical intraepithelial neoplasia (CIN lesions). Cervical cancer is caused by high-risk types of human papillomavirus (HPV). Two of the HPV early proteins (E6 and E7) are constitutively expressed in cervical cancer cells. The immune system plays an important role in the control of HPV infections and in the regression of CIN lesions [Welters 2003]. These factors make cervical cancer and particularly CIN lesions a possible target for immunotherapy. The research described in this thesis focuses on two immunization approaches which could potentially be used as immunotherapeutic strategies against CIN lesions and cervical cancer.

This chapter presents a short introduction on HPV and cervical cancer. Subsequently, the immune system is described and more specifically the cellular immune system which is the part of the immune system that mediates killing of tumor cells. This is followed by a section on different immunization strategies and an introduction to the two strategies used in this thesis for induction of cellular immune responses. The two immunization strategies described here are the recombinant Semliki Forest virus (rSFV) expression system and protein antigen-containing influenza virosomes. An outline of the rest of the thesis concludes this chapter.

Human papillomavirus and cervical cancer

Human papillomaviruses (HPV) are double-stranded deoxyribonucleic acid (DNA) viruses belonging to a subfamily of the *Papovaviridae* family. Papillomavirus particles consist of a circular DNA genome of about 8000 base pairs surrounded by an icosahedral protein capsid of 45-55 nm in diameter. The HPV genome is divided into an early region, encoding genes required for DNA replication and cellular transformation (E1, E2, E4, E5, E6 and E7) and a late region, encoding the two viral capsid proteins L1 and L2. Over 100 types of HPV have been described so far and the subfamily encompasses high-risk and low-risk HPV types. Low-risk HPV cause warts whereas high-risk HPV are implicated in a number of cancers, including cancers of the anogenital tract and some head and neck cancers [zur Hausen 2002]. Cervical cancer is the clearest example of a cancer caused by high-risk HPV. Two of the early proteins of high-risk HPV, E6 and E7, interact with the tumor suppressor proteins p53 and retinoblastoma (pRB), respectively. Interaction of E6 and E7 with these cell cycle regulation proteins can cause immortalization of cells and continuous expression of E6 and E7 is required for the maintenance of tumor cells.
In all cervical cancers HPV DNA can be detected [Walboomers 1999]. HPV16 is the most prominent high-risk type accounting for about 60% of cases. HPV18, HPV31, HPV33 and HPV45 are examples of other high-risk HPV involved in cervical cancer [Muñoz 2003, Longworth 2004]. Cervical cancer accounts for 10% of all cancer-related deaths among women with 470000 new cases and 233000 deaths each year as indicated above [Parkin 2001, Einstein 2002]. It develops from non-invasive premalignant CIN lesions graded from CIN I to CIN III depending on the severity of dysplasia [Bekkers 2004].

The immune system plays an important role in clearance of HPV infections and in spontaneous regression of CIN lesions [Welters 2003]. HPV-induced (pre)malignant lesions occur more frequently in immunocompromised women, like organ recipients or HIV-infected individuals, than in immunocompetent individuals, indicating the influence of the immune system on the control of HPV [Bradford 1990, Ferenczy 2003]. The tumor-specific nature of E6 and E7 expression and the fact that the cellular immune system plays an important role in the control of HPV indicates that an immunotherapy against CIN lesions and cervical cancer could be feasible.

There are two types of immunization possible in the case of HPV infection and HPV-associated cervical cancer: prophylactic and therapeutic immunization [reviewed in Roden 2004]. Prophylactic vaccines aim at preventing infection with HPV by inducing antibody responses against the virus. Prophylactic vaccines to prevent HPV infection focus on the two late, structural, proteins of HPV, L1 and L2, which form the capsid of the virus. When HPV L1 protein is produced in animal, insect, yeast or bacterial cells, virus-like particles (VLP) resembling the HPV capsid are spontaneously formed [Kirnbauer 1992]. HPV16 L1 VLP have been tested in large clinical trials and are able to induce antibody responses against HPV16 and prevent HPV16 infections [Koutsky 2002, Fife 2004]. Therapeutic immunization on the other hand, aims at the treatment of existing lesions by inducing specific cell-mediated immunity. The studies described in this thesis focus on development of a therapeutic immunization strategy against HPV16-induced cervical cancer and CIN lesions.

The immune system

The immune system protects the host by non-specific and specific mechanisms. Physical barriers and non-specific mechanisms form the first line of defense against pathogens and tumors. This first, non-specific line of defense is called innate immunity [Beutler 2004]. Innate immune reactions include activation of complement, secretion of cytokines (type I interferons), activation of natural killer (NK) cells and phagocytosis by antigen-presenting cells (APC). Innate immunity is induced by recognition of common features present on pathogens but absent on host tissue. These features are recognized by pattern recognition receptors, including toll-like receptors (TLR). TLR are
able to detect bacteria and viruses, and promote activation of the immune system [Boehme 2004]. APC link innate immunity to the specific adaptive immunity by presenting phagocytosed antigens to T and B lymphocytes which represent the most important cells of the specific or cellular immune system [Chain 2003, Reis e Sousa 2004].

Cellular immune responses are required for effective elimination of virus-infected cells and tumor cells. The effector population of the cellular immune response consists of CD8⁺ cytotoxic T lymphocytes (CTL). T cell receptors (TCR) on CD8⁺ T cells recognize peptides presented in class I major histocompatibility (MHC) molecules which are present on almost every cell type. Activation of CD8⁺ T cells to effector T cells only takes place when MHC class I-peptide complexes are presented to CD8⁺ T cells by activated APC which also express costimulatory molecules. Dendritic cells (DC) are the most potent APC. Effective priming of CD8⁺ T cells results in their differentiation to CTL which can lyse target cells presenting the virus- or tumor-derived peptides on MHC class I molecules. There are two pathways via which DC can process antigens for presentation in MHC class I. These pathways, the conventional and the cross-presentation pathway are outlined in the next sections of this chapter.

On the other hand, the immune response against pathogens also relies on antibody responses against the infectious agent to fight infection and prevent reinfection [Zinkernagel 2004]. This humoral immune response is mediated by antibody-producing B cells, which are activated by interaction with soluble antigen and primed CD4⁺ T helper cells.

Prophylactic vaccination against HPV usually aims at induction of antibody responses. Therapeutic immunization approaches against tumor antigens, like the high-risk HPV proteins E6 and E7, are primarily aimed at induction of powerful CTL responses.

Conventional antigen presentation by DC

The cells that mediate the activation of T cells are APC, which can be DC, macrophages, monocytes or B cells. DC are the only APC that are able to activate naïve T cells and are therefore considered to be the most potent professional APC [Banchereau 1998, Théry 2001]. Immature DC readily take up antigens via phagocytosis or macropinocytosis but they are inefficient T cell stimulators. Mature DC have high surface expression of MHC class I and II and costimulatory molecules and are therefore very efficient in stimulating T cells. Maturation of DC is triggered by viral products (dsRNA), bacterial products (lipopolysaccharide (LPS)) or CD4⁺ helper T cells (CD40-CD40 ligand interactions).

DC process antigen in different ways (Figure 1) [Bhardwaj 2001]. Proteins that are present in the cytosol of the cell are degraded to peptides by the proteasome. These
peptides are transported into the endoplasmic reticulum (ER) via the transporter of antigen processing (TAP), which is a heterodimer of TAP1 and 2 subunits. TAP is associated with MHC class I-β2-microglobulin dimers via the peptide-loading complex consisting of tapasin, calreticulin and ERp57.

![Diagram of antigen presentation by APC to CD8⁺ T cells (left) and CD4⁺ T cells (right).](image1)

**Figure 1 Antigen presentation by APC to CD8⁺ T cells (left) and CD4⁺ T cells (right).**

Cytosolic proteins (endogenous proteins) are degraded into peptides by the proteasome and transported into the ER. In the ER, peptides become associated with newly generated MHC class I molecules and the MHC class I-peptide complexes are transported via the Golgi network to the cell surface where they stimulate CD8⁺ T cells. Exogenous proteins enter the APC via endocytosis or phagocytosis. Newly synthesized MHC class II molecules associate with invariant chain molecules in the ER. Upon fusion of the compartment containing MHC II molecules with endo-lysosomes containing antigenic peptides, the invariant chain is proteolytically cleaved. Part of the invariant chain, CLIP, is left in the peptide binding groove of MHC II until its exchange for antigenic peptide. The resulting MHC class II-peptide complexes are transported to the cell surface.

In the ER, peptides are further trimmed to a length of 8-9 amino acid residues by an ER aminopeptidase which results in mature MHC class I binding peptides which, in conjunction with MHC class I molecules, are transported to the surface of the cell. CD8⁺
T cells encounter these MHC class I-peptide complexes and become sensitized to the antigen. As mentioned above, proteins have to be present in the cytosol in order to enter this route of antigen presentation. Consequently, proteins synthesized by the DC are a prominent source of peptides presented on MHC class I molecules. However, antigens that are delivered actively into the cytosol also have access to this presentation pathway.

Cross-presentation of antigens by DC

In addition to the conventional antigen presentation pathways, DC and macrophages have a special capacity to process exogenous antigens for MHC class I presentation. This process is called cross-presentation of antigen and results in cross-priming of CD8+ T cells [reviewed by Zinkernagel 2002, Melief 2003, Norbury 2003, Brode 2004]. Exogenous antigen may be in the form of free soluble protein, protein aggregates or apoptotic bodies of cells dying via apoptosis. For cross-presentation to take place, exogenous antigen taken up by endocytosis or phagocytosis needs to colocalize with MHC class I molecules. This process is still under investigation but a mechanism has been described by which antigens reach MHC class I molecules (Figure 2). This mechanism involves transport of antigen or antigenic peptides directly from the endosome to the cytoplasm. Antigen released in this way will be degraded by proteasomes and end up in the classical MHC class I presentation pathway [Rodriguez 1999]. Dextran molecules of 3 and 10 kDa can reach the cytosol efficiently via this mechanism, 40 kDa dextrans are translocated less efficiently and 200 kDa molecules do not escape from the endosomes [Rodriguez 1999].

Recent evidence indicates that cross-presentation might take place after transient fusion of phagosomes with the ER after which ER membrane components, notably the protein-translocation channel Sec61, would mediate protein escape to the cytosol [Houde 2004, Guermonprez 2004]. After this translocation, proteins have access to the ubiquitin/proteasome complex on the cytoplasmic side of the phagosomes resulting in formation of antigenic peptides. These peptides can be transported back to the ER or phagosome lumen by TAP molecules followed by loading onto MHC class I molecules. Cross-presentation is an important component of the immune response against infectious diseases with uptake of infected apoptotic cells being a primary source of antigen [den Haan 2001].
Figure 2 Cross-presentation of antigen by DC. Exogenous proteins enter the DC via endocytosis or phagocytosis. Upon escape from the endosome (the mechanism of this escape is not yet unraveled completely, see text), cytosolic proteins are degraded into peptides by the proteasome and transported into the endoplasmic reticulum (ER). In the ER, peptides become associated with newly generated MHC class I molecules and the MHC class I-peptide complexes are transported via the Golgi network to the cell surface where they stimulate CD8\(^+\) T cells.

Role of CD4\(^+\) T cells and costimulation

For the generation of an effective CTL response, the presentation of peptide in the context of MHC class I is not sufficient. The presence of CD4\(^+\) helper T cells that secrete cytokines promoting expansion and maturation of the CTL is equally essential. Furthermore, CD4\(^+\) helper T cells can mediate complete maturation of DC through interaction of CD40 ligand on the T cell with CD40 on the DC [Heath 1999]. Induction of CD4\(^+\) T helper cells is mediated by presentation of peptides of the antigen in the context of MHC class II molecules (Figure 1). These peptides are derived from exogenous proteins taken up by APC. The proteins end up in the endo-lysosomal pathway and are degraded to peptides. These peptides associate with MHC class II
molecules and are subsequently transported to the cell surface. MHC class II molecules are only present on APC, such as DC, macrophages and B cells [Ramachandra 1999].

Finally, costimulation of T cells by costimulatory molecules present on the APC is required for the induction of an effective CTL response. These costimulatory molecules, like B7-1, B7-2 and CD40, interact with their counterparts on the T cell (CD28 or CD40L). This second signal to the T cell results in their activation [Grewal 1996, Schultze 1996].

**Immunization strategies**

Various immunization strategies have been developed for induction of CTL and antibody responses [Morón 2004]. Immunization with protein alone is relatively ineffective in inducing a strong immune response. Therefore, protein antigens are frequently admixed with an adjuvant prior to immunization. Examples of adjuvants for immunization are Freund’s adjuvant (FA), aluminum hydroxide (alum), CpG DNA, MF59, liposomes, bacterial toxins (E. coli heat-labile enterotoxin (LT), V. cholerae toxin (CT), the B subunits of LT or CT), immune complexes, immunestimulating complexes (ISCOMs) or virosomes [Verweij 1998, Bungener 2002a, Bonifaz 2002, Podda 2003]. Some of these adjuvants are particularly effective for induction of antibody responses against admixed antigens (FA); others can also elicit CTL responses (virosomes, ISCOMs).

Since DC have the unique ability to effectively prime immune responses, immunization strategies based on DC are being explored extensively. Immunization with DC loaded with peptides, proteins or cell lysates or infected with viral vectors, DNA or ribonucleic acid (RNA) have been demonstrated to elicit antigen-specific immune responses [Banchereau 2001, Gunzer 2001]. However, a major drawback with DC as vaccines is that for each patient a new vaccine containing the patient’s own DC would have to be generated since the DC would have to match the human leukocyte antigen (HLA) type of the patient.

Genetic immunization with naked DNA or RNA, encoding protein antigens, has been shown to be effective in inducing CTL and antibody responses [Restifo 2000, Shedlock 2000]. Another approach for delivery of DNA or RNA is the use of viral vectors. These viral vectors encode the protein of interest on their DNA or RNA genomes. Viral DNA or RNA delivery systems can be very effective in induction of immune responses [Bonnet 2000, Hewson 2000, Lundstrom 2003]. The recombinant Semliki Forest virus (rSFV) vector system is an example of a viral RNA delivery system.
Recombinant Semliki Forest virus vector system

Semliki Forest virus
Semliki Forest virus (SFV) belongs to the Alphavirus genus of the Togaviridae family. Alphaviruses are small (70 nm) enveloped viruses containing single-stranded positive-sense RNA [Rayner 2002]. The viral RNA is complexed with 240 copies of the capsid protein forming a nucleocapsid, which in turn is surrounded by a lipid bilayer containing 80 protein spikes. In SFV, each spike consists of two transmembrane glycoproteins, E1 and E2 and a small protein called E3. Alphavirus RNA has a positive polarity enabling the genomic RNA to initiate an infection when introduced into the cytoplasm of a cell. Furthermore, since the RNA encodes its own replicase, it is self-replicating.

Alphaviruses have several characteristics making them attractive candidates for development of replication-deficient viral vectors for application in humans: (i) alphaviruses induce high-level expression of encoded proteins which is important in induction of immune responses, (ii) expression of the antigen is transient, a characteristic particularly important in vectors encoding oncogenic proteins such as HPV E6 and E7, (iii) since most alphaviruses are not endemic in humans, no pre-existing immunity, which could limit vaccine efficacy, exists, (iv) alphaviruses induce apoptosis upon infection of cells which may be very beneficial for induction of immune responses via cross-presentation as discussed above [Restifo 2000b, Huckriede 2004].

rSFV expression system
The rSFV expression system was constructed from a full-length cDNA copy of the viral genome. This cDNA has been cloned in bacterial plasmids including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed in vitro [Liljeström 1991]. The sequence encoding non-structural viral proteins is present on a plasmid containing a multiple cloning site for insertion of the transgene of interest [Berglund 1993]. The structural genes of the virus are encoded on a second plasmid, the helper plasmid. Recombinant viral particles are produced by electroporation of RNA transcribed from both plasmids into cells. Introduction of these RNAs into cells suffices to initiate replication and a full infection cycle, resulting in virus formation. Since the helper RNA does not contain a packaging signal, the resulting recombinant viral particles contain only the RNA encoding the non-structural proteins and the transgene.

The absence of a packaging signal on the RNA encoding structural genes results in a safe system with recombinant virus able to infect cells once without generation of new infectious particles (Helper 1 rSFV system). Safety of the rSFV system was further improved by inserting a mutation in the furin cleavage site of p62, the precursor of the E2 and E3 membrane proteins. This mutation blocks maturation of p62 into E3 and E2, resulting in viral particles that are non-infectious (Helper 2 rSFV system). The viral particles can be made infectious by treatment with protease. After insertion of this
mutation, infectivity was not detected when $2 \times 10^{10}$ particles were used in a plaque assay [Berglund 1993].

![Diagram of rSFV production and infection](image)

**Figure 3 Production of rSFV particles and infection of a cell by rSFV.** Recombinant and helper RNA are electroporated into baby hamster kidney (BHK) cells (1). Replicase on the recombinant RNA molecule ensures replication of both RNA molecules (2). RNAs are transcribed and capsid proteins and glycoproteins are produced (3). Recombinant RNA is packaged into the capsid (4) and viral particles are produced (5). After binding to a receptor (6), rSFV enters a cell via receptor-mediated endocytosis (7). Inside the endosome the virus particle fuses with the endosomal membrane upon lowering of the pH (8) thereby releasing the capsid containing the recombinant RNA into the cytosol (9). The positive-strand RNA is transcribed and the produced replicase ensures replication of the RNA (10). The replicated RNA is transcribed resulting in high production of the recombinant protein (11).
Upon infection of a cell with rSFV the RNA will be replicated and high levels of the encoded transgene protein will be produced. After 48-72 h of protein expression infected cells die by apoptosis resulting in apoptotic bodies containing high levels of the transgene protein [Glasgow 1998, Murphy 2000].

Tissue distribution of rSFV upon immunization
Studies into the distribution and possible pathological effects of rSFV vectors in mice and chickens revealed that transgene RNA persisted at the intramuscular site of injection for up to 7 days in mice and 1 day in chickens [Morris-Downes 2001]. In the draining lymph nodes of mice transgene RNA was present for up to 1 day, in the spleen for up to 16 hours. No transgene or SFV RNA was present in liver or brain or other muscles of the mice injected with rSFV. Similarly, in a study investigating the tissue distribution and induction of CTL responses after intramuscular, intravenous and subcutaneous injection of rSFV in mice, transgene RNA had disappeared 10 days after the injection [Colmonero 2001]. These studies confirm the transient expression pattern of rSFV encoded transgenes and therefore the excellent safety profile of rSFV as a possible oncogene-expressing vaccine.

Immunization studies with rSFV
Recombinant SFV has been used for immunization against a number of antigens in several animal models. In mice, immunization with rSFV encoding influenza nucleoprotein (NP) induced CTL and antibody responses [Zhou 1994, 1995]. The induced CTL responses lasted for more than a year and mice immunized with rSFV encoding influenza NP or hemagglutinin (HA) were protected against influenza challenge [Berglund 1999]. Mice immunized with rSFV encoding a protein of human immunodeficiency virus-1 clade A (HIVA antigen) showed antigen-specific CTL and T cells producing interferon γ (IFNγ) [Hanke 2003]. Immunization of macaques with rSFV encoding simian immunodeficiency virus (SIV) glycoprotein 160 (gp160) resulted in lower virus loads in plasma and protection from lethal infection upon challenge with SIV [Mossman 1996]. When macaques were immunized with rSFV encoding the gp160 of HIV-1 IIIB, antibody and proliferative T cell responses were detected and upon virus challenge vaccinated monkeys had lower virus loads than control animals [Berglund 1997]. Mice with experimental autoimmune encephalomyelitis that were treated intranasally with rSFV encoding interleukin 10 (IL-10) showed a decrease in disease symptoms [Jerusalmi 2003]. Immunization of mice with rSFV expressing louping ill virus antigens provided better protection against louping ill virus challenge than vaccination with naked DNA encoding the same antigens or the commercially available formaldehyde-inactivated virus preparation [Fleeton 2000].

Other antigens expressed by rSFV include segment A of infectious pancreatic necrosis virus to produce virus-like particles (VLP) of this virus [McKenna 2001], the
alpha 2B-adrenergic receptor for biochemical studies of this receptor [Sen 2003] and Bcl-2 to study the mechanism of rSFV–mediated apoptosis [Lundstrom 1997]. The rSFV vector system was also used to express murine and human granulocyte-macrophage colony-stimulating factor (GM-CSF) in murine and human tumor cell lines with the production of autologous cancer vaccines as the ultimate goal [Withoff 2001]. Studies in mice with intraperitoneally injected rSFV encoding murine GM-CSF indicated that the produced GM-CSF resulted in activation of peritoneal macrophages [Klimp 2001]. Treatment of mice bearing an ovarian tumor with rSFV encoding GM-CSF resulted in a two-week delay in tumor growth [Klimp 2001].

A number of rSFV applications will be tested in clinical trials in the near future. The European Vaccine Effort Against HIV/AIDS (EuroVac) is developing rSFV vectors encoding HIV-1 subtype C gag, pol, nef and env genes for use in human clinical trials [see www.eurovac.net]. For rSFV encoding IL-12 a phase I/II clinical trial for glioblastoma multiforme and a phase I clinical trial using rSFVIL-12 in patients with stage III or IV metastasizing melanoma or renal cell cancer is proposed [Ren 2003].

Virosomes

Virosomes are reconstituted viral envelopes that can be derived from different enveloped viruses. In 1975, Almeida et al. first described virosomes as liposomes with influenza virus hemagglutinin (HA) and neuraminidase (NA) spikes on their surface [Almeida 1975]. Virosomes of different viral origin have been described. Influenza virus is used most often to produce virosomes but virosomes made of Sendai virus [Tomasi 1981, 1982, Kim 2002], Epstein-Barr virus [Grimaldi 1995], HIV [Cornet 1990], Sindbis [Scheule 1986], Semliki Forest virus [Helenius 1977], Friend murine leukaemia virus [Schneider 1983], Herpes simplex virus [Johnson 1984] and Newcastle disease virus [Kapczynski 2003] have been described.

The entry properties of the virus used to prepare virosomes are important for virosomes. The ability of a virus to enter a cell and deliver its contents, the genome, into the cytosol can be utilized by virosomes to deliver encapsulated substances into the cytoplasm of cells.

Entry of enveloped viruses into cells

Enveloped viruses are able to enter cells and deliver their genome into the cytoplasm via two different fusion pathways: plasma membrane fusion and acid-induced fusion from within endosomes [reviewed in Smith 2004]. Influenza virus is an enveloped negative-strand RNA virus belonging to the family Orthomyxoviridae. Entry of the virus into its host cells and delivery of the viral genome to the cell cytosol is mediated by hemagglutinin (HA), the major viral envelope glycoprotein. First, influenza virus HA binds to sialic acid residues on the surface of cells. Subsequently, the virus is
internalized via receptor-mediated endocytosis [Matlin 1981]. The mildly acidic pH in the endosome triggers a conformational change in HA such that it achieves its fusion-active state. This results in fusion of the viral membrane with the endosomal membrane and thereby release of the viral nucleocapsid into the cytosol of the cell [Skehel 2000]. Sendai virus is an example of a virus that uses the plasma membrane fusion pathway [Ghosh 2000].

Virosome preparation
Virosomes can be prepared by solubilization of influenza virus with a detergent followed by removal of the nucleocapsid and reconstitution of the membrane containing the viral glycoproteins [Stegmann 1987, 1993, Bron 1993]. This method of virosoome preparation results in virosomes which have a membrane glycoprotein density comparable to that of the native virus. These reconstituted virosomes retain the cell entry and membrane fusion characteristics of the virus they are derived from. As a consequence, foreign substances encapsulated within the lumen of virosomes are effectively delivered to the cytosol of target cells. This is demonstrated by delivery of a membrane-impermeable toxin, the subunit A of diphtheria toxin (DTA), to the cytosol of cells, resulting in complete inhibition of cellular protein synthesis [Bron 1994]. In the case of encapsulated protein antigens, this implies that virosomes in principle have the capacity to deliver exogenous antigens to the MHC class I presentation pathway of APC, and thus represent a promising system for priming of CTL activity. Since it is unlikely that all virosomes entering a cell will fuse with the endosomal membrane some will continue in the endo-lysosomal pathway resulting in degradation of the proteins to peptides. These peptides will be presented in the context of MHC class II molecules. Fusogenic influenza virosomes are thus expected to deliver encapsulated protein antigen for presentation in both MHC class I and II resulting in optimal activation of the immune system.

Alternative methods of virosoome preparation
Several alternative methods for virosoome preparation have been described. Virosomes prepared according to the protocol of Berna Biotech are called immunopotentiating reconstituted influenza virosomes (IRIV). IRIV are prepared by adding viral membrane glycoproteins to excess amounts of phospholipids resulting in liposomes with viral proteins in their membrane [Glück 1992a, b]. These IRIV have about 10-fold lower membrane glycoprotein densities because of the large amount of excess lipids added during reconstitution. In our hands adding excess amounts of lipid (phosphatidylcholine (PC)) during virosoome reconstitution has negative effects on the fusion activity of the virosomes. Addition of 25 mol% extra PC during reconstitution had no effect on fusion activity, adding 50 mol% extra PC results in a two-fold decrease in fusion activity. At 75 mol% extra PC the fusion activity is completely abolished [Huckriede, unpublished
observations]. These results indicate that preparation of influenza virosomes with extra PC at the concentrations described for IRIV (90 mol%) will most likely not result in fusion-active virosomes.

Figure 4 Mechanism of delivery of protein antigens for MHC class I and II presentation in APC by fusogenic influenza virosomes. Virosomes bind to sialic acid receptors and are taken up by receptor-mediated endocytosis. Fusion of the virosomal membrane with the endosomal membrane results in delivery of encapsulated antigen to the cytosol and thus to the MHC class I route. Degradation of the virosome in the endo-lysosomal pathway results in presentation of virosome-derived peptides on MHC class II molecules.

Another way to prepare virosomes is by fusion of liposomes containing foreign substances with inactivated viral particles [Nakanishi 1998, 2000]. This method has been described for Sendai virosomes and results in a membrane glycoprotein density that is about twofold lower than the native virus since the liposomes used are of the same size as the viral particles [Nakanishi 1998]. These virosomes, in contrast to influenza virosomes prepared according to the protocol described in the section above, do contain the viral RNA. Sendai virosomes fuse with the plasma membrane of cells and are thus able to deliver substances that were encapsulated in the liposomes to the cytosol of cells. An advantage to this method is the relatively easy preparation of virosomes containing foreign substances since these substances can first be encapsulated into the liposomes.

Virosome applications
A number of virosomal vaccines are registered for use in humans. Epaxal® was licensed in 1996 and Inflexal® V has been on the market for several years, both vaccines are produced by Berna Biotech. Epaxal® is a Hepatitis A vaccine consisting of
formalin-inactivated hepatitis A virions coupled to IRIV, this vaccine was tested in healthy volunteers [Glück 1992a, Loutan 1994, Holzer 1996, Cryz 1998]. The influenza IRIV vaccine Inflexal® is a trivalent vaccine comprised of IRIV of the three influenza strains recommended by the World Health Organisation (WHO) [Mischler 2002]. Inflexal® was tested in healthy volunteers, elderly nursing home patients [Glück 1994], children [Kanra 2004] and cystic fibrosis patients [Schaad 2000]. In children Inflexal® is more immunogenic than the split influenza vaccine Fluarix® [Kanra 2004]. Solvay Pharmaceuticals has recently obtained European marketing approval for Invivac®, a trivalent virosomal influenza vaccine.

Nasalflu® was another registered Berna Biotech influenza vaccine containing IRIV and the E. coli heat labile enterotoxin (HLT). This intranasal vaccine was on the market in Italy and Switzerland when, in the first vaccination season, an unusual number of vaccinated individuals experienced adverse effects. Affected individuals suffered from temporal Bell’s palsy, most likely due to the HLT present in the vaccine. These cases resulted in withdrawal of the vaccine from the market and a study was conducted to investigate the connection between temporal Bell’s palsy and vaccination with Nasalflu® [Mutsch 2004, Couch 2004]. This study indeed suggested a strong association between Nasalflu® vaccination and occurrence of temporal Bell’s palsy, the relative risk of Bell’s palsy was 19 times higher in vaccinated individuals than in controls [Mutsch 2004]. Nasalflu® is no longer in clinical use.

Several virosomal vaccines have been tested in clinical trials, but are not yet registered for use in humans. A fivefold combined IRIV vaccine against hepatitis A and B, diphtheria, tetanus and influenza A/B was administered to healthy volunteers to test the possibility of IRIV as carriers for multiple antigens [Mengiardi 1995]. However, four weeks after vaccination the seroconversion for Hepatitis A in this study was decreased to 65% versus 100% for a non-combined vaccine.

Some applications for virosomes are still in the research phase, these applications are summarized in Table 1. Plasmid DNA or antisense oligonucleotides can be complexed into virosomes containing cationic lipids like N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP) or dioleoyldimethylammonium chloride (DODAC) in their membranes. Such virosomes have been used successfully to infect cells in vitro and in vivo [references mentioned in Table 1]. Another application for DNA virosomes is the in vivo induction of CTL and antibody responses against the encoded antigen. Virosomes are also used as vaccines for efficient induction of antibody responses against the virus they are derived from. Both influenza virosomes and IRIV have been used in this way to generate humoral immune responses against influenza virus [Huckriede 2003a, b, Glück 1994, Conne 1997]. Other applications for virosomes include encapsulation and subsequent delivery of LPS, enzymes or cytostatics [references mentioned in Table 1]. Induction of CTL and antibody responses against protein and peptide antigens encapsulated in virosomes is another possibility...
[Bungener, in press, Chapter 5 and 6, references mentioned in Table 1]. Obviously, virosomes can also be used as model systems for study of virus-membrane interactions [Schoen 1997, Chams 1999, Günther-Ausborn 2000].

Table 1. Applications of virosomes.

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<th>Type of virosomes</th>
<th>Target</th>
<th>Reference</th>
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<td></td>
<td><em>In vivo</em> induction of antibody response against influenza antigens and encapsulated protein antigens</td>
<td>Huckriede 2003a, b, Bungener, Chapter 6</td>
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<td></td>
<td><em>In vitro</em> delivery of OVA for MHC class I and II presentation</td>
<td>Bungener 2002b</td>
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<td><em>In vitro</em> targeting of virosomes to ovarian cancer cells using Fab' fragments</td>
<td>Mastrobattista 2001</td>
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<td></td>
<td><em>In vitro</em> delivery of DTA and LPS</td>
<td>Bron 1994, Dijkstra 1996</td>
</tr>
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<td>Fusogenic influenza virosomes containing cationic lipids</td>
<td><em>In vitro</em> delivery of DNA</td>
<td>Schoen 1999</td>
</tr>
<tr>
<td>IRIV</td>
<td><em>In vitro</em> priming of CTL against influenza matrix, Melan-A and Hepatitis C peptides</td>
<td>Schumacher 2004, Hunziker 2002</td>
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<td></td>
<td><em>In vivo</em> antibody and low CTL response to RSV F protein + HLT</td>
<td>Cusi 2002</td>
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<td><em>In vitro</em> delivery of doxorubicin via her-2/neu targeting + <em>in vivo</em> tumor treatment</td>
<td>Waelti, 2002</td>
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<td><em>In vivo</em> antibody response to malaria SPf66 peptide after pre-immunization with influenza IRIVs</td>
<td>Pöltl-Frank 1999</td>
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<td>Immunization with DNA IRIVs</td>
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<td><em>In vitro</em> delivery of DTA</td>
<td>Uchida 1979, Mizuguchi 1996b, Watabe 1999</td>
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<td><em>In vitro</em> delivery of nanoparticles</td>
<td>Jana 2002</td>
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<td><em>In vitro</em> delivery of lysozyme</td>
<td>Bagai 1994</td>
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<td><em>In vitro</em> delivery of doxorubicin</td>
<td>Cho 2001</td>
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<td><em>In vitro</em> and <em>in vivo</em> CTL and antibody induction against OVA</td>
<td>Hayashi 1999, Nakanishi 2000, Kunisawa 2001</td>
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<td>Liposomes with VSV-G protein</td>
<td><em>In vitro</em> delivery of DNA</td>
<td>Shoji 2004</td>
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<td>Newcastle disease virosomes</td>
<td><em>In vivo</em> antibody response</td>
<td>Kapczynski 2003</td>
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Outline of this thesis

The next two chapters of this thesis [Chapter 2 and 3] focus on the use of rSFV for immunization and the mechanism of immune response induction by this vector. Chapter 2 describes the construction and characterization of rSFV encoding the early proteins E6 and E7 of HPV16 followed by immunization and tumor challenge studies with this rSFVE6E7 virus in mice. The induction of CTL responses against HPV16 E6 and E7 in mice by immunization with rSFVE6E7 turned out to be quite effective. In mice immunized with rSFVE6E7 particles, 40% was protected from tumor challenge with tumor cells expressing the HPV16 antigens E6 and E7. Next, the mechanism of rSFV-mediated induction of CTL responses was investigated [Chapter 3]. Infection studies with murine and human DC revealed that direct infection of DC is very inefficient. Further infection and immunization experiments were performed using three different rSFV constructs encoding the influenza virus nucleoprotein (NP) as a model antigen: a standard NP construct, a construct encoding enhanced NP resulting in higher production of the protein and a construct encoding an unstable NP variant. The induction of CTL activity against NP was most efficient in mice that received the rSFV construct with enhanced NP. Mice that received a construct encoding an unstable variant of NP had the lowest levels of NP-positive T cells. These results point towards a mechanism of cross-priming for rSFV-mediated CTL induction.

The second part of this thesis [Chapter 4 to 6] focuses on induction of immune responses against protein antigens delivered by influenza virosomes. First, we studied delivery of the model antigen ovalbumin (OVA) to antigen-presenting cells, especially dendritic cells (DC) in vitro [Chapter 4]. Murine DC were incubated with fusion-active or fusion-inactivated OVA virosomes and MHC class I and II presentation of OVA peptides by DC was monitored. OVA virosomes were capable of inducing MHC class I and II presentation of OVA peptide at very low concentrations of antigen. The induction of MHC class I presentation of OVA peptides was dependent on the fusion-activity of the virosomes, in contrast to the induction of MHC class II presentation of OVA peptide. Next, the immune response to OVA virosomes was studied in mice [Chapter 5]. Immunization with as little as 0.75 µg of virosomal OVA turned out to be sufficient for induction of a powerful CTL response. All of the immunization routes tested were effective, intramuscular and intraperitoneal immunizations being slightly superior to subcutaneous injection. Finally, immunization and tumor challenge studies were performed in mice with influenza virosomes containing the HPV16 early protein E7 [Chapter 6]. For these experiments E7 was produced in E. coli and purified. Influenza virosomes containing the purified E7 protein were injected into mice and CTL and antibody responses to the protein were measured. Immunization with E7 virosomes resulted in strong HPV16 E7-specific CTL responses. In addition, most mice immunized with E7 virosomes mounted an E7-specific IgG response. The induced E7-specific CTL
were capable of protecting 70% of mice against a tumor challenge with HPV16 E6 and E7-expressing tumor cells.

In Chapter 7 the two immunization strategies are compared and their potential application for therapeutic vaccination against CIN lesions and cervical cancer is discussed. Chapter 8 contains a summary of this thesis.
References


