Chapter 6

A virosomal therapeutic immunization strategy against cervical cancer and premalignant cervical disease

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Abstract

In this paper we present fusion-active influenza virosomes as a vaccine delivery system capable of efficient induction of CTL, antitumor and humoral responses against encapsulated human papillomavirus type 16 (HPV16) E7 protein. Virosomes are reconstituted viral membranes which do not contain the genetic material of the virus they are derived from. During the reconstitution process protein antigens can be encapsulated in virosomes. Since functionally reconstituted virosomes retain the cell binding and fusion characteristics of the native virus, influenza virosomes deliver their content to the cell cytosol. Here, we studied in vivo induction of immune responses against virosole-encapsulated HPV16 E7 protein in a murine model system. Upon immunization of mice with E7 virosomes the animals developed strong CTL responses. Immunization with virosomes also resulted in E7-specific antibody responses. Tumor challenge experiments demonstrated that immunization with E7 virosomes resulted in prevention of tumor outgrowth in 71% of mice. Thus, virosomes represent an excellent vaccine delivery system for induction of cellular immunity against encapsulated HPV16 E7 and are a promising immunotherapeutic vaccine for treatment of (presursor lesions of) cervical cancer.
nucleocapsid, and subsequent reconstitution of the viral phospholipids and glycoproteins into vesicles [Almeida 1975, Stegmann 1987, Bron 1993]. Properly reconstituted virosomes retain the membrane fusion and cell entry capacity of the native virus. Influenza virus-derived virosomes, therefore, enter cells through receptor-mediated endocytosis after binding to sialic acid residues. The acidic pH within endosomes induces fusion of the virosomal membrane with the membrane of the endosome. This fusion reaction is mediated by the viral spike glycoprotein hemagglutinin (HA) and establishes a continuum between the virosome lumen and the cell cytoplasm, thereby enabling delivery of encapsulated compounds into the cytoplasm of target cells [Schoen 1993, Bron 1994]. Accordingly, virosomal delivery of peptides or proteins to cells thus results in the processing of these antigens for MHC class I presentation [Arkema 2000, Bungener 2002]. Not all virosomes will fuse with the endosomal membrane, some will be degraded to peptides in the endo-lysosomal system of the DC. These peptides will be presented in MHC class II [Bungener 2002].

In the current study we investigated whether administration of virosome-encapsulated recombinant HPV16 E7 induces immunity against this tumor-specific antigen. Our experiments show that fusion-active E7 virosomes induced strong HPV16 E7-specific CTL responses. Moreover, upon delivery of small amounts of virosome-encapsulated E7 mice were protected from the outgrowth of HPV16 E7-expressing tumors.

**Materials and methods**

**Purification of HPV16 E7 protein**

The HPV16 E7 gene was cloned in the BamHI site of pET19b, a plasmid that carries an N-terminal His-Tag sequence (Novagen, Madison WI, USA). The plasmid was transformed into *E. coli* BL21 pLysS and E7 was expressed upon induction with Isopropyl-beta-D-thiogalactopyranoside (IPTG). After overnight culture the bacteria were pelleted and resuspended in binding buffer (Novagen) containing 6 M ureum. E7 was purified using the Novagen purification system according to the Novagen protocol for immobilized metal affinity chromatography, in the presence of 6 M ureum during the whole purification process. The pET19b His-Tag sequence contains 10 consecutive histidine residues and binds to divalent cations (Ni$^{2+}$) immobilized on resin. After unbound proteins were washed away, the target protein was recovered by elution with imidazole. After purification, E7 was dialyzed twice against buffer containing 5 mM Hepes, 20 µM Zn acetate, 5% glycerol followed by dialysis against buffer containing 5 mM Hepes, 20 µM Zn acetate two times. Finally, after a Lowry protein determination [Peterson 1977], E7 was freeze-dried in aliquots for preparation of E7 virosomes.
Virosome-mediated delivery of HPV16 E7 protein \textit{in vivo}

Preparation and characterization of E7 virosomes
Virosomes were prepared from H3N2 subtype influenza virus (A/Panama/2007/99, a gift from Solvay Pharmaceuticals, Weesp, The Netherlands) as previously described [Stegmann 1987, Bron 1993, Bungener 2002]. In short, virus (1.5 µmol of viral membrane phospholipid) was solubilized in 100 mM octa (ethyleneglycol)-n-dodecyl monoether (C\textsubscript{12}E\textsubscript{8}), (Calbiochem, San Diego, CA). The nucleocapsid was removed from the preparation by ultracentrifugation. The supernatant containing the phospholipids and glycoproteins of the influenza virus in C\textsubscript{12}E\textsubscript{8} was added to purified E7 protein to a final concentration of 1 mg E7/ ml. Subsequently, the detergent C\textsubscript{12}E\textsubscript{8} was extracted from the supernatant with BioBeads SM2 (Bio-Rad, Hercules, CA) resulting in formation of virosomes. These virosomes were separated from non-encapsulated E7 by a discontinuous sucrose density gradient (40% and 10% sucrose) and an Optiprep flotation gradient. Finally, the virosomes were dialyzed against buffer containing 5 mM HEPES, 150 mM NaCl and 0.1 mM EDTA (HNE buffer) and sterilized by filtration through a 0.45 µm filter.

The amount of virosomal phospholipid phosphate was determined by phosphate analysis [Böttcher 1961]. Virosomal protein (mainly HA) was determined by the Lowry method [Peterson 1977]. The amount of E7 in the viroosome preparations was determined by encapsulating FITC-labeled E7 (prepared using FITC:E7 in a ratio of 1:5, Molecular Probes). The typical encapsulation efficiency using 1 mg/ ml of E7 during the reconstitution procedure was 0.05 µg E7/ µg of viral protein.

Analysis of purified E7 and E7 in virosomes by SDS PAGE and Western blotting
E7 virosomes were further analyzed on a 12.5% SDS-PAGE gel followed by silver staining using purified E7 as a reference [Blum 1987]. The proteins were blotted on to PVDF membrane (Immobilon-P; Millipore Corp, Bedford, MA, USA) and E7 was detected with a monoclonal mouse anti-HPV16 E7 antibody (Zymed, South San Francisco, CA, USA). After incubation with alkaline phosphatase-linked secondary antibodies the blots were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical).

Determination of fusion characteristics of virosomes
Virosome fusion with erythrocyte ghosts was measured using a lipid mixing assay, based on pyrene excimer fluorescence [Stegmann 1993]. Pyrene PC (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine, 10 mol% with respect to total viral lipid), (Molecular Probes) was co-reconstituted in virosomes. Fusion was continuously monitored at 37°C by measuring the decrease of pyrene excimer fluorescence at an excitation wavelength of 345 nm and an emission wavelength of 480 nm in an AB2 fluorometer (SLM/Aminco, Urbana, IL). First, virosomes and erythrocyte ghosts were incubated for 60 s at neutral pH to allow binding of the virosomal HA to sialic acid
residues present on the erythrocyte membrane. Then, at t= 0 s, fusion was initiated by the addition of 35 µl 0.1 M morpholinoethanesulfonic acid-0.1 M acetic acid (MES/HAc), pretitrated with NaOH to achieve the final desired pH. At t= 210 s, 35 µl of 200 mM C_{12}E_{8} was added to achieve infinite dilution of pyrene PC. The extent of fusion was calculated based on the decrease of pyrene excimer fluorescence at 480 nm, taking the excimer fluorescence of unfused virosomes as the 0% fusion level and the fluorescence after addition of C_{12}E_{8} as the 100% fusion level.

Where indicated, virosomes were fusion-inactivated by incubation at pH 5.0, 37°C for 20 min in the absence of target membranes. The conformation of the influenza virus HA of the H3 subtype is irreversibly changed during this incubation, resulting in fusion-inactivation [Korte 1999]. The pH of 5.0 was achieved by adding a small pre-titrated volume of 0.1 M MES/HAc to the virosome suspension. After fusion-inactivation, the pH of the virosome solution was adjusted to pH 7.4 with a pre-titrated volume of 0.2 M Tris buffer (pH 8.5). Fusion-inactivation of the virosomes was confirmed by fusion measurements.

Cell lines
The TC-1, 13.2 and C3 cell lines were kindly provided by Prof. Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). TC-1 is a cell line generated by transfecting C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus encoding activated c-Ha-ras [Lin 1996]. The 13.2 cell line has been derived from C57Bl/6 embryonic cells transfected with the E1 region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, aa 49-57 (RAHYNIVTF) [Van der Burg 2001]. The C3 cell line has been generated from C57Bl/6 embryonic cells transfected with a plasmid encoding the complete HPV16 genome [Feltkamp 1993].

All cells were cultured in IMDM Glutamax medium (Life Technologies, Paisley, UK) supplemented with 10% FCS (PAA laboratories, Linz, Austria), 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies) and 50 µM β-mercaptoethanol. In addition, medium for TC-1 cells contained non-essential amino acids (Gibco) and 1 mM sodium pyruvate (Gibco).

Mice and immunizations
Specified-pathogen-free female C57BL/6 mice were purchased from Harlan CPB (Zeist, The Netherlands) and immunized at 6-10 weeks of age. The protocol for the animal experiments described in this paper was approved by the Animal Experimentation Ethical Committee of the University of Groningen. For CTL experiments, mice were immunized and boosted two weeks later intraperitoneally (i.p.) with 2.5 µg of E7 in virosomes or empty virosomes admixed with 10 µg of heat-aggregated E7 [Speidel 1997]. As a control, mice were injected with HNE buffer. One week after the booster
immunization, mice were bled under anesthesia, sacrificed and spleens were taken out. Spleen cells were isolated and used in the CTL and tetramer assays. Blood samples were taken to determine the serum antibody titer against HPV16 E7.

For tumor challenge experiments, mice were immunized and boosted (i.p.) once, two weeks later, with 2.5 µg of E7 in virosomes or 2.5 µg of E7 in fusion-inactivated virosomes. A third group of mice was immunized with empty virosomes admixed with 10 µg of heat-aggregated E7. As a control, mice were injected with buffer, empty virosomes or free E7. Mice immunized with empty virosomes received the same amount of viral protein and phospholipids as mice receiving E7 virosomes. Groups of mice receiving E7 in either formulation in the tumor challenge experiments consisted of 7 mice, control groups (buffer and empty virosomes) consisted of 5 mice.

**CTL and tetramer assays**

Spleen cells were stimulated for 6-7 days with irradiated (100 Gy) TC-1 cells at an effector to stimulator ratio of 25:1 in 25 cm² culture flasks, placed upright. Two days before the $^{51}$Cr release assay, 4 U/ml of recombinant human IL-2 (Strathmann Biotech, Hamburg, Germany) was added to the effector cells. CTL activity was determined in a standard 4 h $^{51}$Cr release assay using 13.2 and C3 cells as targets. The target cells were labeled for 1 h with 3.7 MBq $^{51}$Cr per 1x10⁶ cells in 100 µl of medium using $^{51}$Cr-labeled NaCrO₄ (ICN, Costa Mesa, CA). Specific $^{51}$Cr release was calculated according to the following formula: % specific release = 100 x (experimental release – spontaneous release)/ (maximal release – spontaneous release) %. Spontaneous release was determined from target cells incubated without effector cells and maximal release was determined from target cells incubated with medium containing 0.5% Triton X-100. All measurements were performed in triplicate. Spontaneous $^{51}$Cr release was always < 15% and the standard errors of the means of the triplicate determinations were < 10% of the value of the mean.

To analyse the number of CD8+ T cells specific for the E7 peptide we used H-2Db-tetramers containing the E7 epitope RAHYNI VTF produced in the laboratory of Ton Schumacher (Netherlands Cancer Institute, Amsterdam). Spleen cells were washed with FACS buffer (PBS containing 0,5% BSA and 0,02% sodium azide) and stained with FITC-conjugated anti-CD8a (Pharmingen, San Diego, CA) together with PE-conjugated H-2Db-RAHYNI VTF tetramers for 20 minutes at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (ELITE, Coulter). Live cells were selected based on propidium iodide exclusion.

**Antibody response**

HPV 16 E7 immunoglobulin levels were measured by ELISA using recombinant protein fused to glutathione S-transferase; glutathione S-transferase without HPV protein
sequences was used as specificity control and any background response to the latter was subtracted to give the HPV-specific OD as previously described [Sehr 2001].

**Tumor challenge experiments**

Mice were immunized and boosted two weeks later with E7 virosomes, fusion-inactivated E7 virosomes or empty virosomes admixed with 2.5 µg of heat-denatured E7. As controls mice were injected with buffer, empty virosomes or 2.5 µg of free E7 protein. One week after the booster immunization mice were challenged s.c. in the neck with $2 \times 10^6$ TC-1 cells suspended in 200 µl Hanks buffered salt solution. Tumor size was measured with a caliper in two perpendicular directions and tumor volume was calculated using the following formulas: for a sphere: $0.5236 \times \text{diameter of the sphere}^3$, for a cylinder: $0.7854 \times \text{short dimension of the tumor}^2 \times \text{long dimension of the tumor}$.

**Results**

**Characterization of virosomes: HPV16 E7 protein content and fusion activity**

Purified HPV16 E7 protein and influenza virosomes prepared according to the protocol described in Materials and Methods with or without E7 protein were analyzed by SDS PAGE followed by silver staining or Western blotting (Figure 1). The purity of the obtained HPV16 E7 was >95% (Figure 1A, lane 3). On the silver stained gel several influenza proteins were visible in the lane containing virosomes: cleaved HA at about 50 kDa (HA1) and 29 kDa (HA2), the neuraminidase protein at 55 kDa. Furthermore, in the lane loaded with E7 virosomes (lane 1) a clear E7 band was visible at about 25 kDa, corresponding to the Mw of purified E7. The amount of E7 in the E7 virosomes was 0.03 µg/µg of total virosomal protein. For the silver-stained gel and the Western blot 0.25 µg of E7 was loaded on the gel, both for the E7 virosomes and for the purified E7. The silver stained gel and Western blot confirmed the presence of E7 in the E7 virosomes.
The pH-dependent fusion activity of virosomes was determined using a lipid mixing assay with erythrocyte ghosts as target membranes. Previous studies have shown that empty virosomes reconstituted from influenza virus have the same pH-dependent fusion characteristics as the native influenza virus [Stegmann 1993] and that virosomes containing a model protein (OVA) showed similar fusion characteristics as empty virosomes [Bungener 2002]. The optimal pH for fusion of A/Panama influenza virus is pH 5.5. When the fusion activity of E7 virosomes and empty virosomes was monitored at pH 5.5 both preparations showed similar fusion activity (Figure 2), indicating that encapsulation of E7 has no effect on the fusion activity of the virosomes. As expected, at neutral pH no fusion was observed for E7 virosomes.

Figure 1. SDS PAGE followed by silver staining (A) or Western blotting and incubation with mouse anti HPV16 E7 and goat anti mouse AP (B) showing E7 containing virosomes (lane 1), empty virosomes (lane 2) and purified E7 (lane 3).

Figure 2. Fusion activity of E7 virosomes and empty virosomes. Fusion activity of virosomes determined by a pyrene fluorescence excimer quenching assay. Fusion activity of empty virosomes and E7 virosomes are similar at pH 5.5. At neutral pH (7.4) no fusion was observed for E7 virosomes.
Immunization with fusion-active E7 virosomes results in strong E7-specific CTL activity

To characterize the CTL response against HPV16 E7 induced upon immunization with E7 virosomes, mice were immunized and boosted with E7 virosomes containing 2.5 µg of E7. A group of mice was immunized and boosted with the same amount of empty virosomes (based on virosomal phospholipid) admixed with 10 µg of heat-denatured E7. As a control, mice were injected with buffer. The immunization with E7 virosomes resulted in a strong CTL response, with about 50% specific lysis at an effector to target (E:T) ratio of 3 to 80% specific lysis at an E:T ratio of 30 (Figure 3). Immunization of mice with empty virosomes admixed with heat-denatured E7 resulted in a moderate CTL response.

![Figure 3. Induction of strong CTL responses by E7 virosomes.](image)

MHC tetramer analysis using D\(^b\)-RAHYNIIVTF tetramers showed that at the time of CTL assay 19.9+/-11.2% of the CD8\(^+\) cells present in the spleen cell culture of mice immunized with 2.5 µg of E7 in virosomes was tetramer-positive (Figure 4). In contrast, in the mice immunized with empty virosomes admixed with 10 µg of E7 only 2.4+/-1.1% of CD8\(^+\) cells was tetramer-positive.

Earlier immunization experiments performed with OVA virosomes showed that i.p. and i.m. immunization routes result in similar levels of CTL activity [Bungener, in press]. For the CTL and tumor challenge experiments described in this paper mice were immunized i.p. for practical reasons concerning the preparation of virosomes in
sufficiently high concentration for immunization of mice via the i.m. route. However, in a pilot experiment comparing i.p. and i.m. immunization routes for E7 virosomes, these routes of immunization were comparable (data not shown).

![Figure 4](image)

**Figure 4.** Analysis of effector cell population with D\(^1\)-RAHYNIVTF tetramers and anti-CD8. Mice were immunized and boosted as described in Figure 3. For MHC tetramer staining, spleen cells of immunized mice were incubated with D\(^1\)-RAHYNIVTF tetramers after standard restimulation with irradiated TC-1 cells. Results shown are of mice representative for each group.

**Immunization with E7 virosomes results in prevention of tumor outgrowth**

To determine if the strong E7-specific CTL response induced by immunization with E7 virosomes can prevent outgrowth of a HPV16 E7-positive tumor, mice were immunized and boosted with fusion-active E7 virosomes and one week after the booster immunization the animals were challenged s.c. with 2\(\times\)10\(^4\) TC-1 cells. As negative controls mice were injected with buffer. All mice injected with buffer developed tumors by day 14 after tumor challenge. In contrast, in the group of mice immunized with fusion-active E7 virosomes 5 out of 7 mice remained tumor-free for the duration of the experiment (240 days) (Figure 5). One of the mice in the group developed a tumor early in the experiment (day 14). A second animal in this group developed a tumor at day 58, showing a delay in tumor outgrowth compared to mice in the buffer control group. A group of mice was immunized with fusion-active empty virosomes using the same dose (viral phospholipids) as the groups immunized with E7 virosomes. Of the 5 mice in this group, 4 developed a tumor by day 14, the last animal developing a tumor by day 22. As a control, mice were immunized with the same amount of free E7 that was used in immunizations with virosomes (2.5 µg). In this group, in 6 out of 7 mice a tumor was detectable at day 16; the last animal developed a tumor by day 107. Another group of mice was immunized with empty virosomes admixed with 2.5 µg of heat-denatured E7, resulting in tumor-free survival of 2 out of 7 mice and a delay in tumor outgrowth in 3
out of 7 mice. The remaining 2 mice in this group developed tumors at the same time as the control animals.

Figure 5. Effect of immunization with E7 virosomes on the outgrowth of a HPV16 E7-positive tumor. Mice were immunized and boosted two weeks later intraperitoneally with 2.5 µg of E7 in fusion-active virosomes (n=7, open circle), with 2.5 µg of E7 in fusion-inactivated virosomes (n=7, closed triangle), with empty virosomes admixed with 2.5 µg of E7 (n=7, closed square). As a control mice were injected with buffer (n=5, closed circle), with empty virosomes (n=5, open triangle) or with 2.5 µg of free E7 (n=7, open square). One week after the booster immunization mice were injected s.c. with 2x10^6 TC-1 cells and tumor development was monitored. The effect of the immunizations of tumor outgrowth is displayed as the percentage of tumor-free mice (A) and as tumor growth curves for each group (B-G).
In previous studies we showed that in vitro the fusion activity of the virosomes is crucial for delivery of the model protein OVA for MHC class I presentation [Bungener, 2002]. We also showed that, in vivo, fusion-active virosomes induced stronger CTL responses against encapsulated protein (OVA) than fusion-inactivated virosomes. To determine the importance of virosomal fusion activity for the protection of mice against outgrowth of tumors, mice were immunized with fusion-inactivated E7 virosomes in this experiment. Only 1 out of 7 mice in this group remained tumor-free for the duration of the whole tumor challenge experiment, 4 out of 7 mice showed a strong delay in tumor outgrowth compared to the control groups and 2 out of 7 mice showed similar tumor growth characteristics as the control mice.

**Induction of antibody response against virosomal E7**

Antibody levels were determined in serum samples of mice immunized and boosted with fusion-active E7 virosomes via different routes or with empty virosomes admixed with 10 µg of heat-denatured E7. Serum samples were taken at the time of sacrifice. These samples were tested in an ELISA using E7 as the coating antigen, taking four times the average OD_{450} in control samples as a cut-off value (Figure 6). Using this cut-off value, 17 out of 19 mice immunized i.p. with E7 virosomes were positive. In sera of the group of mice immunized i.m. with E7 virosomes 8 out of 9 samples were positive, although 3 of these positive samples were only marginally higher than the cut-off value. Mice immunized s.c. with E7 virosomes showed no E7-specific antibody responses above the cut-off value. When mice were immunized with empty virosomes admixed with free E7 3 out of 3 animals showed an E7-specific antibody response.

![Figure 6](image)

**Figure 6.** Induction of E7-specific antibody response after immunization with E7 virosomes. Anti-E7 antibody titers were determined in serum samples taken at the time of sacrifice of mice immunized twice with fusion-active E7 virosomes via different routes (i.p. closed circles, i.m. open squares or s.c. closed triangles) or mice immunized twice with empty virosomes + 10 µg of heat-denatured E7 (i.p. crosses). As a control mice were injected with buffer (open diamonds). The arrow on the Y axis marks the cut-off value.
Discussion

The data presented here show that immunization of mice with as little as 2.5 µg of recombinant HPV16 E7 in fusion-active influenza virosomes can prevent the outgrowth of an HPV16-transformed tumor in the animals. E7 can be encapsulated efficiently in virosomes without affecting their membrane fusion activity. Upon immunization of mice with E7-containing virosomes a strong induction of E7-specific CTL responses was observed. After one week of in vitro restimulation spleen cells from immunized mice showed a high frequency of E7-specific CD8+ CTLs by staining with D\(^b\)-RAHYNIVTF tetramers. Moreover, using a standard \(^{51}\text{Cr}\)-release assay the induced CTLs were found to be highly cytotoxic. Additionally, i.p. and i.m. immunizations with E7 virosomes induced IgG responses against E7.

The immune response induced by immunization with fusion-active E7 virosomes prevented the outgrowth of the E7-expressing tumor TC-1 in 5 out of 7 mice, whereas immunization with fusion-inactive virosomes only resulted in a delay of tumor growth. This shows that cytoplasmatic delivery of E7 is important in mediating the anti-tumor response. The effect of fusion-inactive E7 virosomes on tumor outgrowth was comparable to the effect of fusion-active empty virosomes admixed with free E7. Both immunizations clearly delay the onset of tumor growth, but do not convey protection. These results are in concordance with the CTL studies where immunization of mice with free protein admixed with empty fusion-active virosomes induced a CTL response that was considerably lower than the response induced by E7 in fusion-active virosomes. The frequency of E7-specific CTLs was almost ten-fold lower, despite the fact that the dose was four times higher and the free E7 was heat-denatured, to make the protein more immunogenic [Speidel 1997].

It is conceivable that upon mixing empty virosomes with E7, some of the protein associates with the virosomes and, upon fusion of the virosomes with the endosomal membrane, escapes to the cytosol. However, for the most part the mechanism by which E7 mixed with empty virosomes, or E7 in fusion-inactive virosomes is introduced into the MHC class I presentation route is probably through cross-presentation. Cross-presentation is a process, employed by professional APC, to shuttle exogenous proteins into the MHC class I pathway [Brode 2004]. Cross-presentation will only lead to efficient cross-priming when additional activation signals are delivered to the APC [Albert 1998, Melief 2003]. Although virosomes can supply such activation signals [Bungener 2002], cross-presentation of E7 from fusion-inactive E7 virosomes or E7 admixed with fusion-active empty virosomes resulted in a weaker anti-tumor response than that achieved by directly introducing E7 into the MHC class I antigen presentation route via fusion-active virosomes. Although, E7 can also be considered as exogenous protein when fusion-active E7 virosomes are used as immunization. The mechanism
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underlying presentation of E7 delivered by fusion-active virosomes could therefore technically also be described as cross-priming.

Besides mediating active delivery of antigens into the cytoplasm of APCs for presentation in MHC class I, virosomes can also act as adjuvants "per se". Culturing peripheral blood mononuclear cells (PBMC) in the presence of empty virosomes and free influenza matrix peptide results in the expansion of peptide-specific CD8+ cells via the induction of a T helper 1 response [Schumacher 2004]. In an earlier study we also noted a significant adjuvant effect of virosomes. In mice, immunization with a mixture of empty virosomes and heat-denatured OVA resulted in the induction of a CTL response. However, this response was not as strong as the response induced by the administration of a lower amount of OVA encapsulated in fusion-active virosomes [Bungener 2004 submitted].

As expected, for the induction of an antibody response against virosome-associated E7, active cytoplasmic delivery is not required as fusion-active and fusion-inactive virosomes are equally capable of inducing an antibody response (data not shown). Humoral responses against E7 are induced probably because some of the E7 protein associates with the outside virosomal membrane during the reconstitution protocol. The route of immunization does appear to have an effect, immunization via the s.c. route inducing no antibodies. This effect was also described for several other immunotherapy and vaccine formulations although the reason for this difference is unclear [Wang 2000, Brayden 2001, Neely 2002, Wunderli 2003]. It may depend on the type of APC involved or on the spreading of the antigen to the draining lymph nodes. Another explanation could be the amount of tissue damage that is inflicted, resulting in different danger signals evoked upon immunization via different routes [Wang 2000].

For the treatment of precursor lesions of cervical cancer an antibody response against E7 is not likely to be an effector mechanism. However, virosomes could not only be applied as a therapeutic vaccine for treatment of existing lesions but also as a prophylactic vaccine to prevent HPV infection. In that case appropriate antigens would be the capsid proteins L1 and/or L2. Clinical trials using virus-like particles (VLP) of the HPV late protein L1 show that VLP are very promising prophylactic vaccines with 100% protection from HPV infection and HPV-induced dysplasia [Jansen 2004]. The fact that virosomes induce both CTL and antibody responses against antigens make them a powerful candidate vaccine against both HPV infection and HPV-induced lesions.

Various peptide- and protein-based immunization strategies have been applied to induce antitumor responses against HPV-transformed tumor cells. A disadvantage of peptide-based immunization protocols is that peptide-based strategies do not incorporate both CTL- and T helper epitopes and are HLA allele restricted [Da Silva 2001, Devaraj 2003]. One way to potentially overcome these drawbacks could be the use of long overlapping peptides [Zwaveling 2002] or proteins. However, proteins are usually poor inducers of CTL responses [Speidel 1997]. It is because of this relatively
low immunogenicity that most HPV protein immunotherapies need the addition of an adjuvant to obtain a good CTL induction. Additionally, these immunotherapies often require immunization protocols with high doses of up to 200 µg of protein to achieve an in vivo effect on the development of HPV expressing tumors [Tarpey 1996, de Bruijn 1998, Hariharan 1998, Gérard 2001, Franconi 2002, Kim 2002]. Upon immunization of mice with fusion-active E7-containing virosomes, the amount of E7 protein required for strong CTL induction and tumor protection is substantially lower. The animals in the study presented here received two consecutive injections of only 2.5 µg of E7 in virosomes.

The only protein-based systems for immunotherapy against cervical cancer, besides fusion-active virosomes, that result in strong CTL responses and tumor protection upon immunization with amounts of E7 protein of 10 µg or less are chimeric HPV or bovine papillomavirus (BPV) VLP. Various chimeric VLP have been described made from L1 protein with or without L2, containing either the whole HPV16 E7 protein [Greenstone 1998], the immunodominant CTL epitope of HPV16 E7 [Peng 1998], the entire amino-terminal part of HPV16 E7 [Schafer 1999], or the immunodominant CTL epitope plus an antibody epitope [Liu 2000]. The common feature of fusion-active virosomes and VLP is their ability to actively introduce E7 antigen in the class I presentation route by binding and penetrate cells in the same way as the infectious viruses these systems are derived from. Furthermore, incubation of DC with either virosomes or HPV VLP results in activation and the expression of costimulatory signals needed for the induction of a CD8+ CTL response [Bungener 2002, Rudolf 2001, Lenz 2001, Fausch 2003]. In three of the VLP studies [Greenestone 1998, Peng 1998, Schafer 1999] mice were protected from tumor development after a single immunization.

For some viral immunizations, pre-existing antibodies are described to be inhibitory. However, for influenza virus, the phenomenon of antibody-dependent enhancement of infection has been described [Tamura 1994]. This means that pre-existing antibodies that bind to the virion enhance uptake of the virus, via Fc-receptor, by professional APC [Takada 2003]. We have preliminary indications that pre-existing antibodies against influenza glycoproteins, indeed, do not hamper but enhance the capacity of virosomes to act as a carrier for the delivery of antigens for CTL induction, an observation that has also been described by others [Zurbriggen 1999, Cusi 2000, Cusi 2001].

In conclusion, virosomes appear to be a very potent antigen delivery system, harboring many of the criteria of ‘the perfect delivery system’, formulated by Morón et al. [Morón 2004]. Virosomes can induce strong specific cellular and humoral immunity against a tumor antigen. Outgrowth of an HPV16 E7-expressing tumor could be prevented in 70% of mice that were immunized with small amounts of recombinant
HPV16 E7 in fusion-active influenza virosomes. Thus, E7 virosomes are a promising immunotherapeutic strategy for treatment of HPV-induced (pre)malignant lesions.

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The picture on the first page of this chapter is a cryo-electronmicroscopy reconstruction of HPV type 1.

*The first two authors have contributed equally to the work described in this chapter.*
References

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Introduction

With 471,000 new cases annually, cervical cancer is the second most common cancer among women worldwide [Parkin 2001]. The primary cause of the disease and its precursor stages, cervical intraepithelial neoplasias (CIN), is infection with a high-risk type of human papillomavirus (HPV) [zur Hausen 2002]. HPV prevalence among cervical cancers is 100% and of the high-risk HPV types, HPV16 is most common with an overall prevalence of almost 60% [Walboomers 1999]. The neoplastic state of infected cells is induced and maintained by the constitutive expression of the viral oncoproteins E6 and E7. These proteins act synergistically. E6 and E7 can both, independently, immortalize cells through their interactions with host cellular proteins involved in cell cycle control, but the oncogenic potential of E7 is greatly enhanced if the malignant phenotype of the infected cell is modulated by E6 [Munger 2002]. The tumor-specific nature of E6 and E7 makes them ideal targets for immunotherapy. Immunotherapeutic strategies against cervical cancer or precursor lesions of cervical cancer would appear to be feasible because the development of HPV-induced neoplasias is controlled immunologically. Indeed, among immunocompromised women these neoplasias occur more frequently [Ferenczy 2003]. Furthermore, the presence of E6- and E7-specific T-helper cells in patients with cleared HPV infections or regressing CIN lesions suggests that the cellular immune response plays a critical role in the resolution of CIN [Welters 2003].

An effective immune response against tumors is predominantly based on a strong induction of CD8+ cytotoxic T lymphocytes (CTL) [Adam 2003]. Such a response in turn requires CD4+ T cell help for the primary differentiation and expansion of effector CTL [Wang 2003], and for the generation of functional memory CTL [Shedlock 2003]. Key orchestrators of T cell responses are properly activated dendritic cells (DC). Development of immunotherapy against cancer should therefore focus on presentation of tumor specific antigens in the context of major histocompatibility complex (MHC) class I and II molecules on the surface of mature DC and trigger the expression of costimulatory molecules on these DC [Melief 2002]. Immunotherapy based on the delivery of antigen by fusion-active influenza virosomes has the potential to achieve these goals. DC incubated in vitro with fusion-active virosomes containing a model antigen efficiently present the encapsulated antigen in both MHC class I and MHC class II [Bungener 2002]. Additionally, in these DC several maturation markers such as CD40, CD69, ICAM-1, B7.1, and B7.2 are upregulated [Bungener 2002]. In vivo, immunization with virosomes results in strong CTL responses against the encapsulated antigen [Arkema 2000, Bungener 2004, in press].

Virosomes are such efficient inducers of CTL responses because they have the capacity to actively deliver their contents in the cytoplasm of cells, including DC. Virosomes are generated by solubilization of virus in a detergent, removal of the viral