Immunotherapy based on influenza virosomes and recombinant Semliki Forest virus

de Mare, Arjan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
CHAPTER 2

VIROSOMES FOR ANTIGEN AND DNA DELIVERY

Toos Daemen
Arjan de Mare
Laura Bungener
Jørgen de Jonge
Anke Huckriede
Jan Wilschut
Abstract

Specific targeting and delivery as well as the display of antigens on the surface of professional antigen-presenting cells (APCs) are key issues in the design and development of new-generation vaccines aimed at the induction of both humoral and cell-mediated immunity. Prophylactic vaccination against infectious diseases in general aims at the induction of humoral immune responses to prevent infection. This humoral immune response is mediated by antibody-producing B cells. On the other hand, therapeutic immunisation against virally-infected cells and tumour cells requires the induction of cytotoxic T lymphocytes (CTL) that can specifically recognise and lyse infected cells or transformed tumour cells. The induction of Major Histocompatibility Complex (MHC) class I restricted CTL activity is optimally achieved by synthesis of antigens within APCs, for example after immunisation with live attenuated virus. However, immunisation with live vaccines bears the risk of causing disease. Therefore, alternative vaccine delivery systems are sought that enable introduction of nonreplicating antigen into the MHC class I presentation pathway. Furthermore, for the induction of effective humoral and cellular responses, MHC class II restricted activation of T helper cells is required. Among other delivery systems, as described in this theme issue of Advanced Drug Delivery Reviews, virosomes seem ideally suited for delivery of antigens into both MHC pathways. In this review we will focus on the use of virosomes as carrier vehicles for the intracellular delivery of protein antigens and DNA, and the induction of a cellular immune response (CTL response) against encapsulated protein antigens and proteins expressed by viroome-associated plasmids.
Background

The virosomal concept

Binding and fusion of membrane-enveloped viruses with cell surfaces are mediated by so-called spike glycoproteins on the viral membrane. Almeida and co-workers [1] were the first to report on the generation of lipid vesicles containing viral spike proteins derived from influenza virus. Using preformed liposomes and hemagglutinin (HA) and neuraminidase (NA), purified from influenza virus, they succeeded to generate membrane vesicles with spike proteins protruding from the vesicle surface. Visualisation of these ‘liposomes’ by electron microscopy revealed that they very much resembled native influenza virus. Consequently, they were named ‘virosomes’. In 1987, our group described a new procedure for the generation of influenza virosomes by reconstitution of virus-like particles solely from viral membrane lipids and proteins [2]. These reconstituted viruses/virosomes enabled biochemical and biophysical analysis of the mechanisms involved in, and conditions required for, virus fusion [3-5]. In addition, the unique features of spike proteins have been exploited in the design of delivery vehicles for protein antigens and DNA.

Ever since the first description of influenza virosomes, virus envelopes have been reconstituted from a diversity of viruses as described below. Yet in the majority of studies, virosomes have been generated from influenza virus.

Influenza virus

Influenza virus, a member of the family of Orthomyxoviridae, is the cause of common flu. Based on the internal proteins, the nucleoprotein (NP) and the matrix protein (M1), three subgroups can be distinguished, influenza A, B and C. Of these influenza A is notorious for its variability. This variability lies in the membrane proteins of the virus, HA and NA. The major antigenic determinants of the virus, eliciting neutralizing antibodies, reside on these membrane proteins. So far, 15 different types of HA and 9 different types of NA have been identified. Most of the HA and NA variants only exist in animals, mainly birds [6]. Influenza virus is a membrane virus containing eight negative strand RNA segments encoding 10 influenza proteins. The lipids of the viral membrane are derived from the host cell during budding of new virus particles. The viral membrane contains about 500 spikes composed of a HA trimer and 100 spikes composed of a NA tetramer and a small number of M2 proteins. The major function of HA is binding of the virus to the target membrane receptor, i.e. sialic acid residues. In addition, the HA spike mediates fusion of the viral and cellular membrane. The role of NA in the influenza life cycle is
not completely unravelled. Yet, NA seems to play a crucial role in the budding of newly formed virus by cleaving sialic acid residues from the cell membrane, thus preventing binding of newly formed virus particles to the membrane of the virus-producing cell. NA may also permit transport of the virus through the mucin layer present in the respiratory tract, enabling the virus to get to the epithelial cells.

*Infection of cells with influenza virus*

Enveloped animal viruses can deliver their genome into the cytoplasm of a host cell by fusing with the plasma membrane or with the endosomal membrane [7]. Paramyxoviruses and herpes viruses are examples of viruses that utilise the first delivery route. Most other viruses, including influenza virus, fuse with the endosomal membrane after being taken up by receptor-mediated endocytosis. As mentioned above, infection of a cell with influenza virus starts by the interaction of the viral HA protein with sialic acid residues on cellular glycoproteins and glycolipids. After binding, the virus is internalised by receptor-mediated endocytosis and ends up in the endosomal/lysosomal system [8]. At the mildly acidic pH in the endosome the HA undergoes a conformational change. A small hydrophobic peptide sequence, buried in the interior of the molecule, becomes exposed and mediates fusion of the viral membrane and the membrane of the endosome [9]. This process delivers the viral RNA into the cytosol and virus replication can start. It is this binding and fusion property of viral spike proteins that can and has been exploited to design carrier systems, such as virosomes, to deliver biological agents into the cytosol of cells.

*Preparation and characterisation of virosomes*

*Introduction*

Virosomes are membranous vesicles that carry viral fusion proteins in their membrane. For influenza virosomes this means that influenza HA is anchored in the virosomal membrane. For efficient cytosolic delivery of encapsulated material, virosomes should be properly reconstituted such that they can bind and fuse to the same extent as the native virus they are derived from.

Our group was the first to develop a method for the preparation of functionally reconstituted fusion-active virosomes using viral membrane lipids and proteins of influenza virus [2]. Viro-
some-like particles containing viral spike proteins have later been generated by several other groups and procedures. These procedures involved for example the insertion of influenza HA and NA into immunostimulating complexes (ISCOMS) [10;11], plain liposomes [1], liposomes containing immunomodulators such as muramyldipeptide [12;13] or liposomes containing cationic lipids (see the section on Preparation techniques for 'DNA-virosomes'). Glück and co-workers [14;15] modified our preparation technique by adding substantial amounts (i.e. 90%) of external lipids to the influenza protein/lipid suspension. These structures are called 'immunopotentiating reconstituted influenza virosomes' or IRIV's. In our hands, however, addition of excess amounts of lipids significantly reduced, and at 75 mol% completely abolished fusion activity.

Sendai virus virosomes have been generated by reconstitution of the Sendai fusion protein (F-protein), with or without the hemagglutinin-neuraminidase protein (HN-protein) in viral lipids [16;17]. Rubella virus virosomes were prepared by incorporating E1 and E2 envelope glycoproteins into liposomes [18] and vesicular stomatitis virus (VSV) virosomes were generated by adding the G-protein of VSV to preformed liposomes [19;20]. In addition, virosomes have been generated based on Epstein-Barr virus [21], human immunodeficiency virus [22], Semliki Forest virus [23], Friend murine leukemia virus [24], herpes simplex virus [25] and Newcastle disease virus [26]. Fusion activity of these virosomes was not always determined. Yet, it should be noted that fusion activity is not essential if virosomes are to be used as vaccines for the induction of antibody responses against the virus they are derived from, as mentioned above.

In the next paragraphs we will focus on the preparation and characterisation of virosomes derived from influenza virus that exhibit the same binding and fusion characteristic as the native virus, and therefore can be used as carriers for cytosolic delivery of encapsulated substances. In addition, several methods for the preparation of DNA-containing virosomes derived from influenza virus and Sendai virus will be described.

**Preparation procedure for fusogenic virosomes**

The majority of methods employed to reconstitute influenza viral membranes, is based on i) detergent solubilisation of the viral membrane, ii) sedimentation of the internal viral proteins and the viral RNA genome by ultracentrifugation and iii) reconstitution of the phospholipids and membrane proteins into a biological membrane by selective removal of the detergent. In 1987, Stegmann et al [2] described a method to produce virosomes with full biological fusion activity. In this study the detergent C_{12}E_{8} was found to be the most suitable for functional reconstitution of HA.
In the past 15 years this procedure has been slightly adapted. Our current procedure (also see Figure 1) has recently been described in detail by Huckriede et al. [27]. In short, influenza virus, replication-inactivated by exposure to formaldehyde or β-propiolactone (by standard procedures also utilized to produce subunit vaccine), is purified on a sucrose-gradient. Typically we start out with 1.5 μmol of viral phospholipid corresponding to 5 mg of viral protein. Next, the virus is pelleted by ultracentrifugation and the pellet is resuspended in 700 μl 100 mM octa(ethylene glycol)-n-dodecyl monoether (C\textsubscript{12}E\textsubscript{8}) and left overnight to allow for complete solubilisation of the viral membrane. The suspension is carefully homogenised and ultracentrifugated to pellet down the viral nucleocapsids. To allow for determination of the fusion activity of the virosomes, a fluorescent probe, pyrene-phosphatidylcholine (pyrene-PC), is added to

Figure 1: Influenza virosome preparation.
Influenza virus (a) is pelleted by ultracentrifugation and the pellet is resuspended in 100 mM C\textsubscript{12}E\textsubscript{8} and left overnight to allow for complete solubilisation of the viral membrane (b). The suspension is carefully homogenised and ultracentrifugated to pellet down the viral nucleocapsids (c). Proteins or peptides can be added to the supernatant (d). Upon detergent extraction using BioBeads the virosomes form (e). Next the virosome suspension is purified by ultracentrifugation on a discontinuous sucrose-gradient to remove non-encapsulated material (f). At the interface of the sucrose layers the virosomes concentrate. After removal of this layer the virosomes can be dialysed against buffer and sterilized by filtration.
the suspension and incorporated in the virosomal membrane. Empty virosomes can then be prepared from the supernatant by detergent extraction using BioBeads. Next, the virosome suspension is purified by ultracentrifugation on a discontinuous sucrose-gradient (40% and 10% of sucrose). At the interface of the sucrose layers the virosomes concentrate. After removal of this layer the virosomes can be dialysed against buffer and sterilized by filtration.

**Antigen loading of virosomes**

For the encapsulation of antigen in virosomes the standard virosole procedure as described above can be employed. The desired amount of antigen is added to the C\textsubscript{12}E\textsubscript{8} solubilised viral membrane suspension, before BioBead treatment (figure 1). Proteins or peptides can be added in a lyophilised form. We for example added 1 mg of NP peptide [28], 3-100 mg of ovalbumine [29] or 1-5 mg of Human papillomavirus type16 E7 protein (L. Bunger et al, manuscript in preparation) to 1.5 μmol of viral phospholipid, without any problems concerning the fusion properties of the loaded virosomes.

**Preparation techniques for 'DNA-virosomes’**

Several methods have been described for the preparation of DNA virosomes containing influenza virus, Sendai virus or VSV fusion proteins. All procedures described so far for influenza virosomes rely on the use of so-called cationic lipids inserted in the virosomal membrane. At present a wide variety of cationic lipids are known, including dioleoyxpropyltrimethylammonium methyl sulphate (DOTAP) and dioleoyldimethylammonium (DODAC). The positively charged head group of the cationic lipid facilitates binding of negatively charged DNA.

Schoen et al [30] developed a method for the preparation of DODAC-containing DNA virosomes based on influenza virus lipids and proteins. In this study the virosomes were well characterised with respect to fusion characteristics, DNA loading efficiency and in vitro transfection efficiency. The virosomes were generated essentially as described above (section 3.2), with the only modification that DODAC, dissolved in C\textsubscript{12}E\textsubscript{8}, was added to the C\textsubscript{12}E\textsubscript{8}-solubilised viral lipids and proteins (0-50 mol% of DODAC). After reconstitution of the viral membranes by removal of the detergent using BioBeads and purification of the virosomes by sucrose density gradient the virosomes were incubated with plasmid DNA for 15 minutes at room temperature. Upon addition of up to 6 μgram of DNA to 20 nmol of 30 mol% DODAC virosomes virtually all DNA associated with the virosomes. Addition of more than 6 μgram of DNA to this amount of virosomes did not result in a higher loading of the virosomes. Although the plasmid was
not encapsulated into the aqueous interior of the virosomes, it was demonstrated that fusion activity of the virosomes was essential for efficient transfection. In the section concerning DNA delivery by virosomes a possible explanation for this observation will be discussed.

Waelti and Glück [31], described a similar method in which they first generated virosomes containing influenza HA trimers and DOTAP to which anti-sense oligonucleotides were added exogenously. This procedure was also used by Correale and co-workers [32], employing a plasmid encoding the parathyroid hormone-related peptide (PTH-rP). Waelti and Glück suggested that the DNA is entrapped within the lumen of the cationic virosomes, yet this is highly unlikely considering the method employed to couple DNA to the virosomes.

For the production of virosomes from other viruses than influenza virus, procedures not involving cationic lipids have been developed as well. In 1997, Ramani and co-workers [33] described a technique to prepare Sendai virosomes containing a plasmid DNA chloramphenicol acetyl transferase (CAT). The Triton X-100 solubilised fraction of Sendai virus was mixed with the plasmid DNA and reconstituted by stepwise removal of detergent using BioBeads. The non-entrapped DNA, adsorbed on the outer surface of the virosomal membrane, was degraded by DNase treatment. The final preparation contained 2-5 μgram of intact DNA encapsulated in 1 mg of virosomes. The DNA associated with the virosomes was shown to be DNase resistant, suggesting that all DNA associated with the virosomes was entrapped within the vesicles.

Kim and Park [34;35] described an alternative method for the preparation of Sendai DNA virosomes. In this procedure the F-protein, synthetic phospholipids, cholesterol and plasmid DNA were dissolved in buffer containing 50 mM octylglycoside. Subsequently, the solution was dialysed against buffer with two changes of medium with BioBeads. For further experiments non-associated DNA was not removed from the virosomes. The DNA-loading efficiency of this procedure was not documented. Similarly, Ponimaskin and colleagues [36] reported on a procedure for the reconstitution of Sendai viral envelopes. Both the fusion and the hemagglutinin-neuraminidase glycoproteins were extracted from purified Sendai virus and reconstituted together with DNA in the presence of cholesterol and synthetic phospholipids.

Yet another procedure to prepare DNA-virosomes based on Sendai virus, (which they named Hemagglutinating virus of Japan (HVJ)), was described by Kaneda and co-workers [37;38]. In this procedure, DNA-liposomes composed of phosphatidylcholine and cholesterol were prepared via vortexing or reverse-phase evaporation. Within one liposome particle 400-600 molecules of plasmid DNA or more than half a million copies of 20-mer oligonucleotides were enclosed. Next, the liposomes were fused with UV-inactivated HVJ to form fusogenic viral liposomes containing DNA. These HVJ-liposomes fused with plasma membranes and delivered
DNA via the cytoplasm to the nucleus of cells as demonstrated using fluorescent oligonucleotides.

Shoji et al [20] recently described a procedure for the coupling of the G-protein of VSV to DNA-liposomes that they then named ‘VSV G-coated virosomes’. DNA liposomes were generated by rehydration of dry films of synthetic phospholipids and cholesterol with buffer containing plasmid DNA. Non-entrapped DNA was not removed from the liposomes. The DNA-liposomes were then mixed with a VSV G protein preparation and incubated for 30 min at pH 5.5 followed by incubation for 15 min at 37°C. The solution was then neutralised to pH 7.5. The VSV G-coated DNA virosomes were purified by sucrose density gradient centrifugation. Remarkably, coupling of the G-protein at acidic pH seemed to be essential for the transfection as virosomes to which the G-protein was coupled at neutral pH did not transfect cells. The authors had no clear explanation for this observation.

**Fusion characteristics of virosomes**

When virosomes are to be used for the delivery of antigen or DNA into the cytosol of cells, fusion activity of the virosomes is of major importance. Depending on the virus they are derived from, properly reconstituted fusogenic virosomes either fuse with the plasma membrane at a neutral pH or with the endosomal pH at an acidic pH. For example, Sendai virus-derived virosomes fuse at neutral pH [33], while influenza virosomes fuse at a pH of 5.5-5.8, the pH range at which influenza HA assumes the fusion-active conformation [39].

Fusion activity of the virosomes can be monitored by using virosomes in which a fluorescent probe such as pyrene-PC is incorporated. When exited at 343 nm pyrene emits fluorescence with a maximum at 377 nm for pyrene monomers and a maximum at 480 nm for pyrene excimers. Eximer fluorescence is directly dependent on the concentration of the pyrene in the membrane. Upon fusion of pyrene-labelled virosomes with a target membrane i.e. liposomes or erythrocyte ghosts, the pyrene-labelled lipids dilute and excimer density decreases. By monitoring the change in excimer fluorescence the fusion process and rate can be monitored online. Fusion of empty virosomes, virosomes containing peptides or entire proteins such as ovalbumin (OVA) [29] and virosomes containing plasmid DNA [30] follow the same kinetics and reach the same level of fusion. Influenza virosomes can be fusion-inactivated by a pre-incubation at low pH, in the absence of target membranes [4]. Thus one can generate fusion-incompetent controls in antigen delivery and immunisation studies. Fusion-inactivation of Sendai virosomes is achieved by heat-treatment of the virosomes for 30 minutes at 56°C [33;40].
Antigen delivery and the induction of cellular immune responses

Introduction

CTLs play an important role in the control of viral infections and tumours. For the differentiation, expansion and memory induction of tumour- or virus-specific CTLs, T helper cells (Th cells) are required. Key orchestrators of T cell responses are properly activated APCs, e.g. dendritic cells (DCs) in particular. Therefore, vaccine delivery systems are sought that will enable introduction of non-replicating antigen into the MHC class I and II presentation pathway and trigger the expression of co-stimulatory molecules on these DCs. An immunisation strategy based on virosomes can achieve these goals. Because of their membrane fusion activity, virosomes can readily deliver encapsulated antigens into the cytosol of APCs (see also Figure 2). In addition, since not all virosomes taken up by an APC will fuse with the endosomal membrane, a fraction of the virosomes will be degraded within the endosomes leading to presentation of antigenic peptides in the context of class II molecules. These antigenic peptides will induce a Th cell response. And finally, uptake of virosomes by DCs induces the maturation and activation of DCs, being a prerequisite for efficient immune activation [29]. An additional advantage of influenza-derived virosomes is that the immune response is likely to be further enhanced by the strong helper activity of the HA [41], which even after fusion of the virosomes with the endosomal membrane remains confined to the lumen of the endosomal/lysosomal system.

By virtue of the fact that reconstituted viral envelopes closely mimic the outer surface of the virus they are derived from, virosomes also represent a very useful system for the induction of antibody responses against the native virus [41-45]. Furthermore, virosomes may also be used to incorporate other unrelated antigens in the virosomal membrane. For example, Glück and co-workers have incorporated hepatitis A virions (HAV) in influenza-derived virosomes and observed a strong stimulation of the HAV-specific antibody response [14]. A virosomal HAV vaccine (Epaxal) is currently on the market.

In the next paragraphs the cytosolic delivery capacity of virosomes, the processing of virosome-encapsulated protein for MHC class I and II presentation and the capacity of virosomes to induce CTLs against encapsulated protein will be presented.

Cytosolic delivery of virosome-encapsulated compounds

Cytoplasmic delivery of influenza virosome-encapsulated material has been conclusively demonstrated using fragment A of diphtheria toxin (DTA) [46]. DTA lacks the B subunit of the holotoxin, which mediates receptor binding and cell entry of the molecule. DTA itself is membrane-
impermeable and therefore non-toxic when added to cells. However, upon cytosolic delivery DTA is toxic by inactivating elongation factor 2, involved in protein synthesis. Fusion-active influenza virosomes have the ability to efficiently deliver encapsulated DTA to the cytosol of cells, as evidenced by complete inhibition of cellular protein synthesis. Agents that raise the endosomal pH block this delivery of DTA. Furthermore, fusion-inactivated virosomes completely lack the ability to deliver encapsulated DTA. When prepared in the presence of cationic
lipids, virosomes are also capable of delivering DNA to the cytoplasm. Again, fusion activity is essential for this DNA delivery since pH-inactivated virosomes are not capable of transfecting cells [30].

The efficiency of membrane fusion between reconstituted Sendai virus envelopes containing only the fusion protein (F-virosomes) and the plasma membrane of mouse teratocarcinoma cells (F9) in culture was determined using an assay based on the relief of self-quenching of a lipid probe incorporated in the F-virosomes [47]. The potential of F-virosomes was also evaluated for targeted cytosolic delivery of lysozyme to F9 cells. Incubation of the loaded F-virosomes with cells led to fusion-mediated delivery, as inferred from the ability of cells to internalise lysozyme in the presence of azide (a potent inhibitor of endocytosis). Recently, this group demonstrated that F-virosomes could even deliver hydrogel nanoparticles of cross-linked polyvinylpyrrolidone (PVP-NP; 35-50 nm in diameter) into the cytosol of human hepatoblastoma cells [48]. Cytosolic delivery of FITC-dextran loaded PVP-NP was visualized by fluorescence microscopy and spectrofluorimetric measurements. Similar to the delivery of lysozyme as discussed above, cytosolic delivery was not inhibited in the presence of azide.

**MHC class I and II presentation of encapsulated antigen**

To demonstrate the antigen delivery capacity of virosomes, we studied the capacity of virosomes to deliver OVA to DCs for MHC class I and II presentation of OVA peptide in vitro. DCs were incubated with OVA encapsulated in either fusion-active or fusion-inactivated virosomes. Subsequently, these DCs were co-cultured with T cells from OT-1 mice which are transgenic for the T cell receptor specific for H-2K\(^{b}\) plus the immunodominant peptide of OVA (SIINFEKL). Stimulation of the OT-1 cells by DCs, as measured by IL-2 release, is a measure for the MHC class I presentation of OVA. For determination of MHC class II presentation by the DCs the CD4\(^{+}\) T cell hybridoma OT4H.1D5 was used, a cell that is specific for I-A\(^{b}\) plus an undefined OVA peptide [29]. Fusion-active OVA virosomes induced MHC class I presentation of the epitope SIINFEKL at an OVA concentration of 10 picomolar, which is 10,000-fold more efficient than presentation of free OVA. Fusion-inactivated OVA-containing virosomes did not deliver the antigens into the MHC class I presentation pathway. In contrast, both fusion-active and fusion-inactive OVA virosomes were able to deliver OVA to DCs for presentation in MHC class II at picomolar concentrations of antigen (equals 0.5 ng OVA/ml).

In agreement with the observations described above, fusogenic Sendai virus virosomes have also been observed to deliver OVA for MHC class I presentation in vitro. EL-4 cells incubated with these virosomes were subsequently lysed by OVA-specific CTLs and could induce pro-
duction of IL-2 by a CD8-OVA T cell hybridoma [49]. However, delivery efficiencies achieved with Sendai virosomes are significantly lower than those seen with influenza virosomes, sensitisation of target cells with Sendai virosomes being obtained only at OVA concentrations of 250 nM.

Virosome-mediated induction of CTL activity in vivo

The capacity of virosomes to deliver antigen to the MHC class I presentation route in vivo and to activate antigen-specific CTLs was first assessed in immunisation experiments in mice, using influenza virosomes containing a synthetic peptide epitope from influenza NP [28]. Two intramuscular immunisations with 0.5 μg of NP peptide in fusion-active virosomes resulted in efficient induction of CTL activity, as measured in a standard 51Cr-release assay using influenza-infected cells as target cells. In contrast, immunisation with fusion-inactive virosomes or liposomes containing NP peptide did not result in CTL induction. Free peptide (100 μg) and empty virosomes were both ineffective [28]. Clearly, in a clinical context, one of the drawbacks of peptide vaccination strategies relates to the problem of MHC restriction due to the MHC (or HLA) polymorphism in the human population. Delivery of entire protein antigens to the cytosol of APCs would circumvent this major problem.

Therefore, we next used virosomes containing OVA, an intact protein that requires processing by proteasomes prior to MHC class I presentation. Immunisation of mice with OVA virosomes followed by a booster with the same preparation resulted in very effective induction of CTLs specific for OVA. Antigen doses as low as 0.7 μg per injection sufficed to induce CTL activity (Bungener et al manuscript submitted). Intramuscular, intraperitoneal and subcutaneous immunisations effectively induced CTLs. Although the booster immunisation potentiated the CTL response, a single immunisation of as little as 1.5 μg of OVA in fusion-active virosomes sufficed to induce CTL activity. Fusion-inactive OVA virosomes were also able to induce CTL responses, yet the percentages of specific lysis were lower than for mice immunised with fusion-active virosomes. This indicates that the fusion activity of the virosomes potentiates, but is not essential for CTL induction in vivo. Immunisation with OVA in virosomes also resulted in a humoral immune response as demonstrated by the presence of anti-OVA IgG in the sera of immunised mice.

Similarly, using the same C₁₂E₈ protocol of virosome preparation, Wijburg and collaborators demonstrated that immunisation of mice with 25 μg of OVA in fusion-active virosomes resulted in effective induction of CTLs against OVA-expressing target cells [50]. Recently, Schumacher and co-workers [51], demonstrated that the in vitro CTL induction from peripheral blood mono-
nuclear cells against an HLA class I restricted peptide of influenza matrix protein or Melan-A/MART-1 could be enhanced by the addition of empty IRIVs.

Immunisation studies aiming at the induction of CTL with protein-antigen loaded virosomes other than influenza-based virosomes have, as far as we know, only been performed with a Sendai-based carrier system. Immunisation of mice with 100 μg of OVA in liposomes fused with Sendai virus resulted in strong CTL responses; immunisation with 25 μg of OVA in these ‘Sendai-virosomes’ was far less effective [49].

DNA delivery

Introduction

Gene therapy requires vectors that are efficient and safe, and simple to prepare. Although viral vectors, in general, very efficiently transfect a variety of cell types, safety issues for most viral vector systems is still a major concern. Accordingly, non-viral techniques for gene transfer that are as efficient as viral vectors are to be developed. DNA vaccines have been developed and numerous clinical studies have been performed. Preclinical studies demonstrated that expression of an antigen or antigens from plasmid DNA may elicit both humoral and cellular immune responses. However, the clinical results using “naked” plasmid DNA are disappointing. Relatively high doses of DNA are needed to elicit minimal responses. Clinical trials using gene gun delivery have been promising, but it is unclear whether this technology will be commercially viable. As a result, there exists a clear need for new vaccine delivery systems for DNA that can be administered at low doses to elicit strong immune responses. In the last 25 years, much effort has been put in the development of lipid-based non-viral gene delivery systems. Numerous formulations of DNA/liposome and DNA/cationic lipid complexes have been, and are being explored for their transfection activity [52]. However, in vivo, these types of carriers still show relatively low transfection activity compared with viral vectors. Combining the potency of viruses to selectively bind and fuse with cell membranes with the capacity of liposomes to entrap DNA or of cationic lipids to bind DNA resulted in the formulation of DNA-containing (cationic) virosomes for gene delivery. The diversity of the preparation procedures employed and the scarcity of information on the amount of DNA associated with the different carriers in the transfection experiments disables a direct comparison of the different carrier systems. Therefore, in the next paragraphs, we will describe some typical studies with respect to transfection characteristics and immune responses using DNA-virosomes.
**DNA delivery by virosomes**

Using DODAC-containing DNA virosomes based on influenza virus lipids and proteins we demonstrated the potential of the influenza virus fusion protein HA to promote uptake and intracellular delivery of viroside-associated plasmid DNA [30]. The transfection activity of the virosomes was dependent on the relative DODAC density in the membrane, the highest activity being obtained with 30 mol % of DODAC. Lower percentages of DODAC presumably resulted in insufficient binding of DNA to the virosomes while with higher percentages aggregate were formed. DNA complexed to the virosomal surface did not affect the fusion activity significantly. The fusogenic property of the virosomes containing cationic lipids was solely HA-mediated, exhibiting kinetics and pH dependency similar to control virosomes. Furthermore, pre-exposure to low pH in the absence of target membranes resulted in fusion-inactivation of the virosomes.

Despite the fact that the plasmid was not encapsulated in the aqueous interior of the virosomes but linked to the cationic lipids, plasmid delivery with cationic virosomes and concomitant expression of reporter gene was dependent on the activity of the fusion protein HA. Fusion-inactivation of the virosomes by exposure of the virosomes to low pH, before complexing with plasmid, abrogated transfection. Furthermore, the presence of NH₄Cl in the medium, preventing acidification of the endosomes and thus fusion of the virosomes with the endosomal membrane, inhibited transfection as well. How can this be explained? The mechanism of DNA-entry into the cytosol is not clear, but might be explained by the fact that HA-mediated fusion is a leaky process enabling the plasmid to escape from the endosomes into the cytoplasm during fusion. Alternatively, cytoplasmic entry could occur as a result of destabilisation of the endosomal membrane following penetration of HA fusion proteins. We are presently studying if transfection can be further improved by the entrapment of DNA within virosomes (De Jonge et al, manuscript in preparation).

Similar studies, using DOTAP-influenza virosomes, demonstrated DNA or oligonucleotide delivery into human tumour cells and human DCs [31;32]. In a murine study, Cusi and colleagues demonstrated that upon intranasal instillation of DNA-loaded DOTAP-IRIVs (vesicles prepared by a mixture of synthetic phospholipids, DOTAP, influenza virus lipids and glycoproteins, exogenously loaded with the mumps virus hemagglutinin gene) DNA was detected in DCs in draining lymph nodes [53].

**In vitro**, as well as **in vivo** studies, demonstrated the potential of DNA-virosomes based on Sendai virus for gene transfection and expression [33;34;40]. **In vitro**, the F/HN-virosomes and HVJ-virosomes, as described above, have been used to transflect for example myocytes,
liver-, kidney-, muscle- and neural cells.

Kim and Park [34] demonstrated that F/HN-Sendai virosomes composed of phosphatidylcholine, cholesterol and phosphatidylserine and the F and/or HN-proteins of Sendai virus could under \textit{in vitro} conditions transform human kidney cells. The transfection efficiencies of these virosomes were approximately two times higher than DOTAP/cholesterol cationic liposomes. Addition of the HN-protein slightly enhanced transfection efficiency, which might be ascribed to improved anchoring of the virosomes to cells. Yet, it has also been suggested that the HN protein plays an active role in the actual fusion process.

In an elegant study by Ramani and co-workers [40] it was shown that upon intravenous injection of Sendai F-virosomes containing a CAT-encoding plasmid, CAT gene expression, both mRNA and protein, was observed predominantly in the liver. Very low activity was observed in lung, kidney and spleen, while CAT expression was not detected in heart, muscle, brain, lymph nodes and skin. Fusion-inactivation of the virosomes by heat treatment (30 min at 56°C) completely abolished transfection activity. Strikingly, RNA expression was observed up to 60 days and protein could be detected up to 120 days after a single injection of virosomes. Liver cell fractionation demonstrated that predominantly the parenchymal cells expressed the transgene. While 2 hr after injection the plasmid DNA was found in the cytosolic fraction of the hepatocytes, after 6 hr the plasmid DNA was found in the nuclear fraction as measured by PCR using CAT-specific primers. Plasmid DNA delivered by heat-treated virosomes could only be detected up to 12 hr after injection in the lysosomal/mitochondrial fraction.

\textit{Immune responses induced upon immunisation with DNA-virosomes}

Up to now, only three studies have been published on the immune responses elicited by the administration of DNA-virosomes. The influenza DOTAP virosomes used in the first study were exogenously loaded with a plasmid DNA encoding the mumps virus hemagglutinin or the mumps virus fusion protein [54]. The authors failed to clarify whether fusogenic DNA-virosomes or DNA-IRIVs (see section above) were used, as for the protocol that was employed to generate the DNA vesicles two references were quoted that describe different methods. Confusingly, both terms were used is this study. Yet, mice primed with an influenza virus vaccine and a mucosal adjuvant followed by intranasal immunisation with these ‘DNA-virosomes’ resulted in a mumps virus–specific serum IgG response and a low but detectable IgA response in the broncho-alveolar cavity. Immunisation of influenza-primed mice with the DNA alone resulted in a slightly lower IgG response but no IgA response. In the next two studies [32;55], DNA expressing the PTH-rP, a protein secreted by prostate and lung carcinoma cells was
coupled to virosomes or IRIVs. Mice immunised and boosted twice intranasally with these ‘DNA-virosomes’ developed a low PTH-rP-specific CTL response. This response could be slightly increased by subcutaneous administration of interleukin-2 for 5 days per week during the entire immunisation/booster protocol (6 weeks). In HLA-A02.01 transgenic mice similar responses were induced suggesting that the vaccine can be employed for immunotherapy of human cancers and metastases overexpressing PTH-rP.

Concluding remarks
Reconstituted viral envelopes (virosomes) appear to be ideally suited for delivery of protein antigens to the cytosol of APCs, and thus for introduction of antigenic peptides into the MHC class I presentation pathway. CTL activity can be induced by immunisation of mice with an antigenic peptide or an entire protein encapsulated in virosomes. The action of influenza virosomes is likely to involve both, delivery of the enclosed antigen to the cytosol of APCs and the powerful helper activity of the virosomal hemagglutinin. It is interesting to note that virosome-mediated delivery of protein antigens would circumvent problems associated with MHC restriction and HLA polymorphism in the human population, since the APC would select its own peptides. A promising potential application of fusogenic virosomes involves the development of strategies for CTL induction against viral infections and cancer through virosome-mediated delivery of virus and/or tumour antigens.

Although the immune responses elicited by DNA-virosomes are moderate, they are promising and warrant further research into the improvement of the loading- and transfection efficiencies of DNA virosomes, to ultimately develop effective DNA-based virosomal vaccines.

Reference list


[26] Kapczynski DR, Tumpey TM. Development of a virosome vaccine for


[38] Dzau VJ, Mann MJ, Morishita R, Kaneda Y. Fusigenic viral liposome for gene


