TARGETED LIPOSOMES FOR CYTOSOLIC DRUG DELIVERY TO TUMOR CELLS

ENRICO MASTROBATTISTA

TARGETED LIPOSOMES FOR CYTOSOLIC DRUG DELIVERY TO TUMOR CELLS

Doelgerichte liposomen voor cytosolaire afgifte van farmaca aan kankercellen
(met samenvattinging in het Nederlands)

PROEFSCHRIFT

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the real voyage
of discovery
consists
not
in seeking
new landscapes,
but
in having
new eyes

Marcel Proust, 1871-1922

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PREFACE

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

INTRODUCTION

One of the persisting key research issues in the design of drug delivery systems is how to achieve significant cytoplasmic delivery of drug molecules. Many small drug molecules are readily taken up into cells by passive diffusion across cell membranes, but a growing number of pharmacologically active agents derived from the emerging field of biotechnology such as proteins, peptides and nucleic acids cannot easily pass biological membranes due to their unfavorable physicochemical characteristics. Therefore, these biotherapeutics rely on delivery systems that allow translocation over cellular membranes.

Many different drug delivery systems have been investigated for cytosolic drug delivery [1-5]. From these drug delivery systems liposomes have attracted considerable attention [4,6-8]. Liposomes are spherical vesicles, consisting of one (unilamellar) or more (multilamellar) concentric bilayers enclosing one or more aqueous compartments. Both the aqueous spaces and the lipidic bilayers of liposomes can accommodate drug molecules allowing entrapment of both hydrophilic and hydrophobic compounds. The composition of the lipid bilayers can be manipulated to influence the physicochemical characteristics of the liposomes (*e.g.* surface charge, sensitivity to pH changes, and bilayer rigidity) [9,10].

For selective drug delivery, liposomes have been targeted to specific cell types by conjugating ligands to the surface of liposomes that recognize specific molecules on target cells [11-16]. The most widely used molecules for targeting liposomes have been monoclonal antibodies as these molecules often have high affinity and specificity for the cell surface antigens against which they have been raised. As an example, antibody-targeted liposomes, also referred to as immunoliposomes, have been used for the selective drug delivery to cancer cells by targeting to tumor-associated antigens [17-20]. However, specific binding of liposomes to target cells is not enough. After target cell binding, the entrapped drugs must be able to reach the cellular interior, often the cytoplasm of target cells. Previous work from our group described in two theses [21,22] has demonstrated that immunoliposomes directed against cell-surface epitopes that were preferentially expressed by certain types of carcinoma cells specifically bound to ovarian carcinoma (OVCAR-3) cells both *in vitro* and *in vivo.* However, despite efficient tumor cell binding, no therapeutic advantages were observed *in vivo* when anticancer agents were entrapped in these immunoliposomes. The reason for lack of therapeutic benefit is most likely due to inefficient intracellular delivery of immunoliposome-entrapped drug after tumor cell binding. Clearly –as exemplified with our own findings– there is a strong need for drug delivery systems that guarantee efficient delivery of drugs from cell-bound immunoliposomes into the cytoplasm of target cells.

Cytosolic delivery of immunoliposome-entrapped drugs may be obtained in different ways as illustrated in Figure 1. Two of these potential routes, extracellular release of drugs from cell-surface bound immunoliposomes and selective transfer of lipophilic drugs, are only applicable to drug molecules which intrinsically are able to cross cell membranes. Another route, which is

independent of the capacity of entrapped drug to cross cell membranes and therefore applicable to a broader spectrum of drugs, is fusion of the liposomal membrane with the plasma membrane of target cells after binding of the immunoliposomes to specific receptors on the cell surface. However, fusion between plasma membranes and liposomal membranes is very unlikely to happen spontaneously and therefore relies on molecules that can disrupt or penetrate the plasma membrane. Incorporation of viral fusion proteins that allow fusion of liposomes at neutral pH with the plasma membranes of target cells seems to be an attractive option [23,24] but appears to be difficult to realize in a targeted context [25-27]. Moreover, a major impediment of direct plasma membrane penetration is that it may harness the integrity of the plasma membrane thereby disturbing the electrochemical potential generated by the asymmetric distribution of ions across the plasma membrane. Perforation of endosomal membranes to obtain cytosolic drug delivery is thought to be less injurious to cells and hence will be less cytotoxic than perforating the plasma membrane.

Immunoliposomes may also deliver their drug contents into the cytosol of target cells via the route of receptor-mediated endocytosis [28-31]. Receptor-mediated endocytosis is a process utilized by cells for the uptake of specific substances such as iron, lipids, proteins and peptides [32,33]. Internalization is initiated by binding of the ligand to its receptor, which provokes receptor

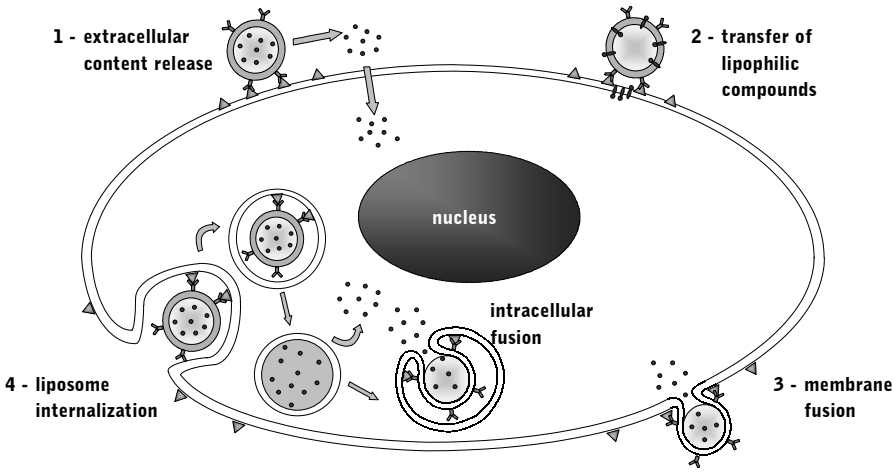

Figure 1 — Schematic representation of potential ways by which targeted immunoliposomes can achieve cytosolic drug delivery. 1. Extracellular release of drug, followed by subsequent active or passive transport into the cell. 2. Selective transfer of lipophilic compounds from the liposomal bilayer to the plasma membrane. 3. Fusion of the liposomal membrane with the plasma membrane. 4. Receptor-mediated endocytosis of immunoliposomes with subsequent cytosolic release of liberated drug molecules by passive diffusion out of the endosomes or via low pH-induced endosomal membrane destabilization or fusion.

clustering and subsequent activation of signal transduction pathways that induce endocytosis of the receptor with its bound ligand by coated pits. The cargo (*i.e.* the receptor-bound ligand) is, dependent on the receptor type, processed via different pathways in different intracellular organelles. The cargo may be recycled (*e.g.* transferrin), degraded (*e.g.* LDL and growth factors), transported to the secretory pathway (*e.g.* bacterial toxins), or, in a pathway specific for polarized cells, transcytosed to the contralateral side (*e.g.* immunoglobulins). Many reports have demonstrated that immunoliposomes are taken up by receptor-mediated endocytosis provided that the receptor allows internalization and that liposomes are small enough (<150 nm) [34-40]. Unfortunately, following receptor-mediated endocytosis of the immunoliposome-drug-receptor complex, the complex enters into an acidic endosomal compartment and eventually into an acidic lysosomal compartment where immunoliposomes and often also the entrapped drugs will be degraded [37,41-43]. Therefore, mechanisms that allow endosomal escape of liposome-entrapped drugs into the cytoplasm are often required for effective drug delivery. As illustrated in Figure 1, low-pH induced membrane fusion between internalized liposomes and endosomes is an attractive option to achieve cytosolic drug delivery after receptor-mediated endocytosis.

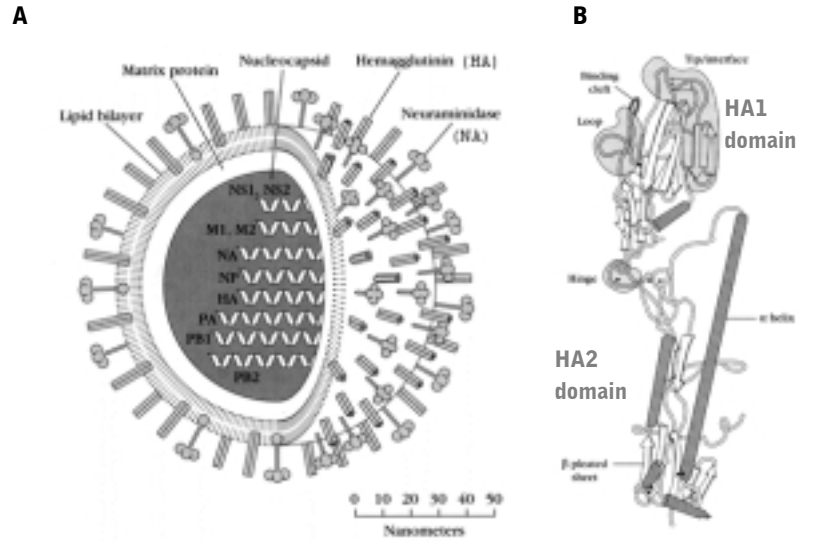

Figure 2, panel A — Schematic representation of a cross-section through an influenza virus A particle. The influenza virus particle consists of a lipid envelope containing the viral spike proteins hemagglutinin (HA) and neuraminidase (NA). Homotrimers of HA form one rod-like spike. The matrix protein covers the internal layer of the lipid envelope, which engulfs the nucleocapsids of the virion. **Panel B —** schematic representation of the quaternary structure of influenza virus HA monomer at neutral pH. The protein is composed of two disulfide-linked subunits, HA1 and HA2. HA1 forms the head of the spike and contains the sialic acid binding pocket. HA2, which forms the stem of the spike, contains the fusion peptide domain at its N-terminus. This peptide domain is thought to play an important role in the membrane fusion mechanism.

ENDOSOMAL ESCAPE

In developing immunoliposomes that are able to fuse with endosomal membranes, much can be learned from viruses. Several enveloped viruses enter cells by a process of receptor-mediated endocytosis, routing the viral particles into the endosomes and eventually lysosomes [44-46]. The low pH within the endosomes triggers the fusion of viral envelopes with the endosomal membrane, thereby releasing the viral nucleocapsids into the cytoplasm of host cells. Both the adhesion of virus particles to the host cell membrane, which triggers internalization, and the low-pH induced fusion reaction are mediated by viral spike glycoproteins [47,48]. One of the best-characterized viral fusion proteins is undoubtedly the influenza virus hemagglutinin (HA) (Figure 2) [49-52]. Trimers of HA make up the rod-like spikes, which abundantly cover the viral envelope (approximately 500 spikes per virus particle). A HA monomer, whose quaternary structure has been elucidated by X-ray crystallography [53], is built up of two disulfide-linked subunits, HA1 and HA2 (Figure 2). HA1 forms the head of the spike and contains the sialic acid binding pocket and most of the major antigenic determinants. HA2 forms the membrane-anchored stem region that contains a stretch of 20 hydrophobic amino acids (the so-called fusion peptide) at its N-terminal end, which is buried

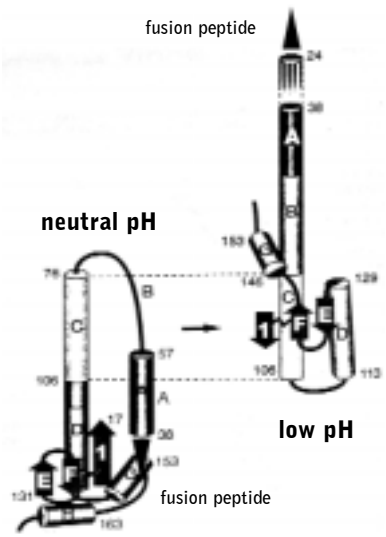

Figure 3 — Schematic representation of the structural changes in HA2 induced at acidic pH. Equivalent regions of HA2 at neutral and acidic pH are indicated with the same letter. Regions of α-helix are indicated by cylinders, and β-structure is shown as arrows. The first β-strand of HA1, which is disulphide-linked to β-strand F of HA2, is labeled 1. Structures are aligned on the C region, which is unaffected by the conformational change. The location of the N-terminal fusion peptide domain is indicated with a black triangle. Adapted from Skehel et al. [55].

within the trimers approximately 3.5 nm from the viral membrane. Upon exposure to low pH within the endosomes, a conformational change occurs in the HA-protein causing dissociation of the globular heads and transposition of the fusion peptide (Figure 3) [54-57]. The fusion peptide comes in close proximity of the target membrane and, due to its hydrophobicity, will insert into the target membrane [58,59]. The fusion peptide plays an important role in the fusion process, as it is the part of the protein that destabilizes the target membrane and presumably also the viral membrane. Although much research has been performed on the fusion mechanism of influenza virus, the exact mechanisms underlying the fusion process are still not completely understood. Synthetic analogs of the N-terminal fusogenic peptide of the influenza virus HA2 subunit have been shown to destabilize and/or fuse membranes of liposomes exclusively at low pH [60-63]. They are hydrophobic peptides rich in glycine and alanine residues alternated with several acidic amino acids. Upon exposure to low pH the acidic amino acids become protonated with loss of repulsive forces between the negatively charged side groups (Figure 4). This allows the fusogenic peptides to adopt an α-helix where the smaller hydrophobic glycine residues are all located at one side of the helix and the larger hydrophobic residues on the other side (Figure 4). This so-called glyasymmetry seems to be important for oblique or "sided" insertion of peptides into membranes [64]. It is thought that oblique insertion of fusion peptides into the membrane increases the negative curvature strain of the lipid bilayer and facilitates local bilayer rearrangements [65]. Most of the synthetic analogs of the influenza virus fusion peptide were able to destabilize bilayers at low pH causing leakage of liposome-entrapped solutes. In addition, some of them were able to induce lipid

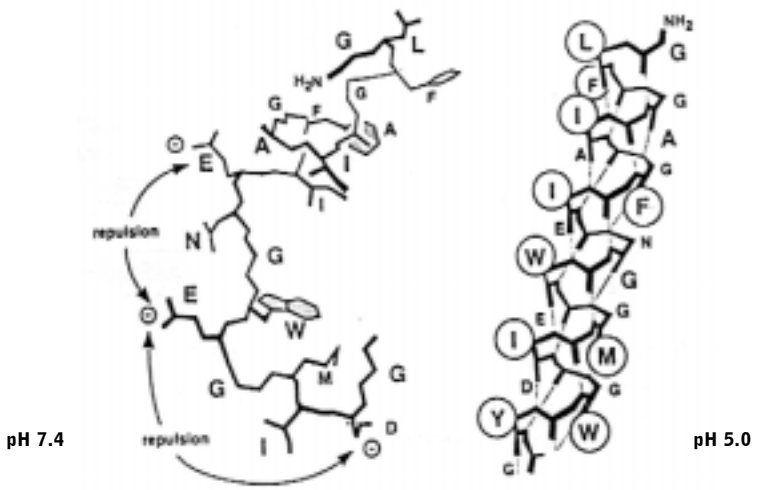

Figure 4 — Conformation of the N-terminal fusogenic peptide of influenza virus hemagglutinin subunit HA2 at pH 7 and pH 5. At pH 7 the glutamic acid and aspartic acid side chains are negatively charged. This electrostatic repulsion is thought to prevent α-helix formation. At pH 5, the fusogenic peptide is thought to adopt an α-helical conformation as determined by CD-measurements. Within the α-helix, glycine residues are located at one side of the helix whereas the larger, more hydrophobic amino acids are located at the opposite side. Adapted from Oberhauser et al. [69].

mixing and/or aqueous contents mixing between liposomes, which is indicative for fusion [66-68]. Moreover, synthetic analogs of the influenza virus fusion peptide have been successfully used to enhance the endosomal escape of polylyisine/DNA complexes [62,69-71] and DNA from liposomes [72]. However, it should be mentioned that in no case the efficiency of intracellular delivery achieved with these synthetic peptides equals the efficiency of naturally evolved translocation or fusion mechanisms of viruses. Nevertheless, synthetic pH-dependent fusion peptides offer an attractive opportunity to enhance cytosolic delivery of drugs by facilitating endosomal escape of compounds following their endocytic uptake.

AIMS AND OUTLINE OF THE THESIS

The principal aim of the research described in this thesis is to develop targeted liposome systems able to mediate cytosolic drug delivery into tumor cells. The research is focused on both antibody-mediated targeting of liposomes to tumor-associated cell surface receptors that results in receptor-mediated endocytosis of the immunoliposomes and on the subsequent cytosolic delivery of their contents via endosomal escape mechanisms. In particular, fusion mechanisms are exploited to realize endosomal escape. The molecules delivered via this approach (DTA, plasmid DNA) are representative molecules of the growing class of biotherapeutics that have poor membrane permeability characteristics.

A detailed overview of the literature dealing with immunoliposome-mediated targeting of anticancer agents to tumor cells is given in **chapter 1**. Besides summarizing the current status of the *in vivo* application of immunoliposomes in tumor models, new developments on the utilization of immunoliposomes for the treatment of cancer are highlighted.

Chapter 2 deals with antibody-mediated targeting of liposomes to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells. Specific attention is given to the issue of internalization and the impact on drug delivery to sites of inflammation.

The **appendix to chapter 2** extends the internalizing receptor-targeting concept to ovarian carcinoma cells, as the latter cells are the main target cells in this thesis. Several receptors expressed by OVCAR-3 cells are evaluated regarding their usefulness for inducing internalization of immunoliposomes.

In chapters 3 and 4 the influenza virus HA protein is central to the development of antibody-targeted fusogenic liposomes. Reconstituted envelopes of influenza virus particles (virosomes) containing HA have been successfully used for the delivery of proteins and genes into the cytosol of cells. However, as cellular uptake of these virosomes is mediated by the interaction of HA with sialic acid residues, which are ubiquitous on the surface of many cell types, specific targeting of influenza virosomes has been problematic. In **chapter 3** a 2-step strategy is followed to target or re-direct influenza virosomes towards ovarian carcinoma cells. The first step involves incorporation of the hydrophilic polymer poly(ethylene glycol) (PEG) conjugated to phospholipids into the

membrane of influenza virosomes. The surface-exposed PEG layer should prevent HA from interacting with sialic acid residues on target cell membranes. Second, the PEG-layer is meant to function as a spacer to which specific antibodies can be conjugated for targeting purposes.
As the conventional method of influenza virosome preparation, which is used in chapter 3, does not allow efficient encapsulation of hydrophilic drug molecules into the virosomes a different method to prepare HA-containing liposomes (*i.e.* virosomes) compatible with high encapsulation efficiencies is explored in **chapter 4**.
The use of the dimeric fusion peptide (diINF-7) to enhance endosomal escape of the bacterial toxin fragment DTA after receptor-mediated endocytosis of DTA-containing immunoliposomes is investigated in **chapter 5.** diINF-7 is a synthetic analogue of the N-terminal fusion peptide domain of the influenza virus. To ascertain effective concentrations of this fusion peptide in the endosomes the diINF-7 peptides are co-encapsulated with DTA into liposomes targeted towards an internalizing receptor on OVCAR-3 cells.
Chapter 6 is devoted to the issue of targeted delivery of plasmid DNA to ovarian cancer cells. The non-viral gene carrier poly(2-(dimethylethylamino)ethyl methacrylate) (p(DMAEMA)), which has been developed in our department, has been shown to efficiently condense plasmid DNA into small positively charged particles. *In vitro* these so-called polyplexes are internalized by cells due to electrostatic interactions with cell membranes, which eventually results in gene expression. p(DMAEMA)-based polyplexes have two major disadvantages when used for the delivery of genes to tumor cells. First, they are difficult to target to specific cell types due to the predominant electrostatic interaction with cell membranes, even if specific ligands have been attached. Second, intraperitoneal administration of polyplexes in mice bearing OVCAR-3 cells in the peritoneal cavity resulted in loss of transfection activity. Evidence has been collected pointing to inactivation by polyanions such as hyaluronic acid present in the intraperitoneal tumor ascitic fluid. Attempts are made to solve these problems by coating polyplexes with anionic lipids using a detergent-removal method followed by conjugation of a tumor-specific antibody to achieve tumor cell targeting.
The last chapter of this thesis, **chapter 7**, provides a summary and general discussion of the results. Suggestions for future research are given as well.

REFERENCE LIST

1 Gharat, L., Taneja, R., Weerapreeyakul, N., Rege, B., Polli, J., and Chikhale, P.J. (2001): **Targeted drug delivery systems 6: Intracellular bioreductive activation, uptake and transport of an anticancer drug delivery system across intestinal Caco-2 cell monolayers**. *Int.J.Pharm.*, 219:1-10.

2 Rapoport, N.Y., Herron, J.N., Pitt, W.G., and Pitina, L. (1999): **Micellar delivery of doxorubicin and its paramagnetic analog, ruboxyl, to HL-60 cells: effect of micelle structure and ultrasound on the intracellular drug uptake**. *J.Control.Release*, 58:153-162.

3 Duncan, R., Rejmanova, P., Kopecek, J., and Lloyd, J.B. (1981): **Pinocytic uptake and intracellular degradation of N-(2-hydroxypropyl)methacrylamide copolymers. A potential drug delivery system**. *Biochim.Biophys.Acta*, 678:143-150.

4 Gerasimov, O.V., Boomer, J.A., Qualls, M.M., and Thompson, D.H. (1999): **Cytosolic drug delivery using pH- and light-sensitive liposomes**. *Adv.Drug.Del.Rev.*, 38:317-338.

5 van de Wetering, P., Cherng, J.Y., Talsma, H., Crommelin, D.J., and Hennink, W.E. (1998): **2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents**. *J.Control.Release*, 53:145-153.

6 Maruyama, K. (2000): **In vivo targeting by liposomes**. *Biol.Pharm.Bull.*, 23:791-799.

7 Langner, M. and Kral, T.E. (1999): **Liposome-based drug delivery systems**. *Pol.J.Pharmacol.*, 51:211-222.

8 Storm, G. and Crommelin, D.J.A. (1998): **Liposomes: quo vadis?** *Pharm.Sci.Tech.Today*, 1:19-31.

9 Vemuri, S. and Rhodes, C.T. (1995): **Preparation and characterization of liposomes as therapeutic delivery systems: a review**. *Pharm.Acta Helv.*, 70:95-111.

10 Gregoriadis, G. (1995): **Engineering liposomes for drug delivery: progress and problems**. *Trends.Biotechnol.*, 13:527-537.

11 Huang, A., Huang, L., and Kennel, S.J. (1980): **Monoclonal antibody covalently coupled with fatty acid. A reagent for in vitro liposome targeting**. *J.Biol.Chem.*, 255:8015-8018.

12 Martin, F.J., Hubbell, W.L., and Papahadjopoulos, D. (1981): **Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds**. *Biochemistry*, 20:4229-4238.

13 Martin, F.J. and Papahadjopoulos, D. (1982): **Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting**. *J.Biol.Chem.*, 257:286-288.

14 Sullivan, S.M., Connor, J., and Huang, L. (1986): **Immunoliposomes: preparation, properties, and applications**. *Med.Res.Rev.*, 6:171-195.

15 Forssen, E. and Willis, M. (1998): **Ligand-targeted liposomes**. *Adv.Drug.Del.Rev.*, 29:249-271.

16 Allen, T.M., Hansen, C.B., and Stuart, D.D. (1998): **Targeted sterically stabilized liposomal drug delivery.** In: *Medical Applications of Liposomes*, edited by D.D. Lasic, et al, pp. 297-323. Elsevier, Amsterdam.

17 Park, J.W., Hong, K., Kirpotin, D.B., Meyer, O., Papahadjopoulos, D., and Benz, C.C. (1997): **Anti-HER2 immunoliposomes for targeted therapy of human tumors**. *Cancer Lett.*, 118:153-160.

18 Lopes de Menezes, D.E., Pilarski, L.M., and Allen, T.M. (1998): **In vitro and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma**. *Cancer Res.* , 58:3320-3330.

19 Koning, G.A., Gorter, A., Scherphof, G., and Kamps, J.A.A.M. (1999): **Antiproliferative effect of immunoliposomes containing 5-fluorodeoxyuridine-dipalmitate on colon cancer cells**. *Br.J.Cancer*, 80:1718-1725.

20 Tseng, Y.L., Hong, R.L., Tao, M.H., and Chang, F.H. (1999): **Sterically stabilized anti-idiotype immunoliposomes improve the therapeutic efficacy of doxorubicin in a murine B-cell lymphoma model**. *Int.J.Cancer*, 80:723-730.

21 Nässander, U. K. **Liposomes, immunoliposomes, and ovarian carcinoma.** Thesis. 1991. Utrecht University.

22 Vingerhoeds, M. H. **Immunoliposomes against ovarian carcinoma: from targeting to antitumor activity.** Thesis. 1995. Utrecht University.

23 Mizuguchi, H., Nakanishi, M., Nakanishi, T., Nakagawa, T., Nakagawa, S., and Mayumi, T. (1996): **Application of fusogenic liposomes containing fragment A of diphtheria toxin to cancer therapy**. *Br.J.Cancer*, 73:472-476.

24 Ponimaskin, E., Bareesel, K.K., Markgraf, K., Reszka, R., Lehmann, K., Gelderblom, H.R., Gawaz, M., and Schmidt, M.F. (2000): **Sendai virosomes revisited: reconstitution with exogenous lipids leads to potent vehicles for gene transfer**. *Virology*, 269:391-403.

25 Dzau, V.J., Mann, M.J., Morishita, R., and Kaneda, Y. (1996): **Fusigenic viral liposome for gene therapy in cardiovascular diseases**. *Proc.Natl.Acad.Sci.U.S.A.*, 93:11421-11425.

26 Kaneda, Y., Saeki, Y., and Morishita, R. (1999): **Gene therapy using HVJ-liposomes: the best of both worlds?** *Mol.Med.Today*, 5:298-303.

27 Kaneda, Y. (2000): **Virosomes: evolution of the liposome as a targeted drug delivery system**. *Adv.Drug.Del.Rev.*, 43:197-205.

28 Mastrobattista, E., Storm, G., van Bloois, L., Reszka, R., Bloemen, P.G., Crommelin, D.J., and Henricks, P.A. (1999): **Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells**. *Biochim.Biophys.Acta*, 1419:353-363.

29 Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. (1997): **Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro***. *Biochemistry*, 36:66-75.

30 Huwyler, J., Wu, D., and Pardridge, W.M. (1996): **Brain drug delivery of small molecules using immunoliposomes**. *Proc.Natl.Acad.Sci.U.S.A.*, 93:14164-14169.

31 Heath, T.D., Montgomery, J.A., Piper, J.R., and Papahadjopoulos, D. (1983): **Antibody-targeted liposomes: increase in specific toxicity of methotrexate-gamma-aspartate**. *Proc.Natl.Acad.Sci.U.S.A.*, 80:1377-1381.

32 Goldstein, J.L., Anderson, R.G., and Brown, M.S. (1979): **Coated pits, coated vesicles, and receptor-mediated endocytosis**. *Nature*, 279:679-685.

33 Okamoto, C.T. (1998): **Endocytosis and transcytosis**. *Adv.Drug.Del.Rev.*, 29:215-228.

34 Suzuki, H., Zelphati, O., Hildebrand, G., and Leserman, L. (1991): **CD4 and CD7 molecules as targets for drug delivery from antibody bearing liposomes**. *Exp.Cell Res.*, 193:112-119.

35 Matthay, K.K., Abai, A.M., Cobb, S., Hong, K., Papahadjopoulos, D., and Straubinger, R.M. (1989): **Role of ligand in antibody-directed endocytosis of liposomes by human T- leukemia cells**. *Cancer Res.*, 49:4879-4886.

36 Berinstein, N., Matthay, K.K., Papahadjopoulos, D., Levy, R., and Sikic, B.I. (1987): **Antibody-directed targeting of liposomes to human cell lines: role of binding and internalization on growth inhibition**. *Cancer Res.*, 47:5954-5959.

37 Derksen, J.T., Morselt, H.W., and Scherphof, G.L. (1987): **Processing of different liposome markers after *in vitro* uptake of immunoglobulin-coated liposomes by rat liver macrophages**. *Biochim.Biophys.Acta*, 931:33-40.

38 Leserman, L.D., Weinstein, J.N., Blumenthal, R., and Terry, W.D. (1980): **Receptor-mediated endocytosis of antibody-opsonized liposomes by tumor cells**. *Proc.Natl.Acad.Sci.U.S.A.*, 77:4089-4093.

39 Lopes de Menezes, D.E., Pilarski, L.M., Belch, A.R., and Allen, T.M. (2000): **Selective targeting of immunoliposomal doxorubicin against human multiple myeloma *in vitro* and ex vivo**. *Biochim.Biophys.Acta*, 1466:205-220.

40 Lundberg, B.B., Griffiths, G., and Hansen, H.J. (2000): **Specific binding of sterically stabilized anti-B-cell immunoliposomes and cytotoxicity of entrapped doxorubicin**. *Int.J.Pharm.*, 205:101-108.

41 Daleke, D.L., Hong, K., and Papahadjopoulos, D. (1990): **Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay**. *Biochim.Biophys.Acta*, 1024:352-366.

42 Straubinger, R.M., Papahadjopoulos, D., and Hong, K.L. (1990): **Endocytosis and intracellular fate of liposomes using pyranine as a probe**. *Biochemistry*, 29:4929-4939.

43 Yoshimura, T., Shono, M., Imai, K., and Hong, K. (1995): **Kinetic analysis of endocytosis and intracellular fate of liposomes in single macrophages**. *J.Biochem.(Tokyo)*., 117:34-41.

44 Hoekstra, D. and Kok, J.W. (1989): **Entry mechanisms of enveloped viruses. Implications for fusion of intracellular membranes**. *Biosci.Rep.*, 9:273-305.

45 Marsh, M. (1984): **The entry of enveloped viruses into cells by endocytosis**. *Biochem.J.*, 218:1-10.

46 Helenius, A. and Marsh, M. (1982): **Endocytosis of enveloped animal viruses**. *Ciba.Found.Symp.*, 59-76.

47 White, J.M. (1992): **Membrane fusion**. *Science*, 258:917-924.

48 White, J.M. (1990): **Viral and cellular membrane fusion proteins**. *Annu.Rev.Physiol.*, 52:675-697:675-697.

49 Ramalho, S. and de, L. (1998): **The influenza virus hemagglutinin: a model protein in the study of membrane fusion**. *Biochim.Biophys.Acta*, 1376:147-154.

50 Webster, R.G. and Rott, R. (1987): **Influenza virus A pathogenicity: the pivotal role of hemagglutinin**. *Cell*, 50:665-666.

51 Wiley, D.C. and Skehel, J.J. (1987): **The structure and function of the hemagglutinin membrane glycoprotein of influenza virus**. *Annu.Rev.Biochem.*, 56:365-394.

52 Ward, C.W. (1981): **Structure of the influenza virus hemagglutinin**. *Curr.Top.Microbiol.Immunol.*, 94-95:1-74.

53 Wilson, I.A., Skehel, J.J., and Wiley, D.C. (1981): **Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution**. *Nature*, 289:366-373.

54 Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A., and Wiley, D.C. (1982): **Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion**. *Proc.Natl.Acad.Sci.U.S.A.*, 79:968-972.

55 Skehel, J.J. and Wiley, D.C. (1995): **Influenza viruses and cell membranes**. *Am.J.Respir.Crit.Care Med.*, 152:S13-5.

56 Sato, S.B., Kawasaki, K., and Ohnishi, S. (1983): **Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments**. *Proc.Natl.Acad.Sci.U.S.A.*, 80:3153-3157.

57 Doms, R.W., Helenius, A., and White, J. (1985): **Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change**. *J.Biol.Chem.*, 260:2973-2981.

58 Harter, C., James, P., Bachi, T., Semenza, G., and Brunner, J. (1989): **Hydrophobic binding of the ectodomain of influenza hemagglutinin to membranes occurs through the "fusion peptide"**. *J.Biol.Chem.*, 264:6459-6464.

59 Stegmann, T., Delfino, J.M., Richards, F.M., and Helenius, A. (1991): **The HA2 subunit of influenza hemagglutinin inserts into the target membrane prior to fusion**. *J.Biol.Chem.*, 266:18404-18410.

60 Düzgünes, N. and Gambale, F. (1988): **Membrane action of synthetic N-terminal peptides of influenza virus hemagglutinin and its mutants**. *FEBS Lett.*, 227:110-114.

61 Clague, M.J., Knutson, J.R., Blumenthal, R., and Herrmann, A. (1991): **Interaction of influenza hemagglutinin amino-terminal peptide with phospholipid vesicles: a fluorescence study**. *Biochemistry*, 30:5491-5497.

62 Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M.L. (1992): **Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle**. *Proc.Natl.Acad.Sci.U.S.A.*, 89:7934-7938.

63 Düzgünes, N. (1993): **Synthetic peptides as probes of function of viral envelope proteins**. *Methods Enzymol.*, 221:82-95:82-95.

64 Fujii, G. (1999): **To fuse or not to fuse: the effects of electrostatic interactions, hydrophobic forces, and structural amphiphilicity on protein-mediated membrane destabilization**. *Adv.Drug.Del.Rev.*, 38:257-277.

65 Siegel, D.P. (1999): **The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion**. *Biophys.J.*, 76:291-313.

66 Matsumoto, T. (1999): **Membrane destabilizing activity of influenza virus hemagglutinin-based synthetic peptide: implications of critical glycine residue in fusion peptide**. *Biophys.Chem.*, 79:153-162.

67 Steinhauer, D.A., Wharton, S.A., Skehel, J.J., and Wiley, D.C. (1995): **Studies of the membrane fusion activities of fusion peptide mutants of influenza virus hemagglutinin**. *J.Virol.*, 69:6643-6651.

68 Wharton, S.A., Martin, S.R., Ruigrok, R.W., Skehel, J.J., and Wiley, D.C. (1988): **Membrane fusion by peptide analogues of influenza virus haemagglutinin**. *J.Gen.Virol.*, 69 (Pt 8):1847-1857.

69 Oberhauser, B., Plank, C., and Wagner, E. (1995): **Enhancing endosomal exit of nucleic acids using pH-sensitive viral fusion peptides.** In: *Delivery strategies for antisense oligonucleotide therapeutics***,** edited by S. Akhtar, pp. 247-266. CRC Press, Boca Raton.

70 Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D.T., and Birnstiel, M.L. (1992): **Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes**. *Proc.Natl.Acad.Sci.U.S.A.*, 89:6099-6103.

71 Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994): **The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems**. *J.Biol.Chem.*, 269:12918-12924.

72 Baru, M., Nahum, O., Jaaro, H., Sha'anani, J., and Nur, I. (1998): **Lysosome-disrupting peptide increases the efficiency of in-vivo gene transfer by liposome-encapsulated DNA**. *J.Drug Target.*, 6:191-199.

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IMMUNOLIPOSOMES FOR THE TARGETED DELIVERY OF ANTITUMOR DRUGS

IMMUNOLIPOSOMES FOR THE TARGETED DELIVERY OF ANTITUMOR DRUGS

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SUMMARY

This review presents an overview of the field of immunoliposome-mediated targeting of anticancer agents. First, problems that are encountered when immunoliposomes are used for systemic anticancer drug delivery are discussed, as well as potential solutions. Second, an update is given of the *in vivo* results obtained with immunoliposomes in tumor models. Finally, new developments on the utilization of immunoliposomes for the treatment of cancer are highlighted.

INTRODUCTION

An attractive strategy to enhance the therapeutic index of anticancer drugs is to specifically deliver these agents to tumor cells, thereby keeping them away from non-malignant cells sensitive to the toxic effects of the drug. This would allow more effective treatments achieved with doses that are better tolerated. Among the colloidal drug carrier systems proposed for site-specific drug delivery, liposomes have attracted considerable attention [1-6]. Liposomes consist of one or more concentric phospholipid bilayers, each enclosing an aqueous compartment. A large variety of therapeutically active molecules (*e.g.* antitumor drugs, oligonucleotides, DNA, enzymes, peptides and hormones) have been successfully incorporated in liposomes. Especially in the field of cancer chemotherapy, much effort has been invested to realize site-specific drug delivery with liposomal systems. Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells [6]. Several types of ligands have been used for this purpose, including antibodies, antibody fragments [7-10], vitamins [11], glycoproteins [12,13], peptides (RGD-sequences) [14,15], and oligonucleotide aptamers [16]. This review will mainly focus on the use of antibodies and antibody-fragments to actively target liposomes, as this is the type of targeting ligand mostly used.

The first report on antibody-targeted liposomes came from Torchilin et al. about two decades ago [17]. These antibody-targeted liposomes (further referred to as immunoliposomes) were shown to be able to specifically bind to the antigen that is expressed on the target cells. Since then, several coupling techniques have been described for conjugating antibodies or their fragments to liposomes, each with their own advantages and drawbacks [9,18,19]. Many *in vitro* experiments have demonstrated highly specific binding of immunoliposomes to target cells. However, despite the excellent targeting properties *in vitro*, successful results on targeting of immunoliposomes in tumor models are scarce up to now.

In this review we aim to present the current status regarding the application of immunoliposomes for anticancer drug delivery *in vivo.* First, problems will be listed that are encountered when immunoliposomes are used for systemic anticancer drug delivery and potential solutions will be discussed. Second, an update will be given on the *in vivo* results obtained with immunoliposomes in animal models. Finally, special emphasis will be placed on highlighting new developments regarding the utilization of immunoliposomes for the treatment of cancer.

IMMUNOLIPOSOMES IN VIVO: MANY RIVERS TO CROSS

Accessibility of tumor cells is a critical issue when immunoliposomes are to be targeted to tumors. Unlike some types of tumors (*e.g.* hematological malignancies and tumors confined within a body cavity), many tumors are located at sites that are less accessible by intravenously (*i.v.*) injected liposomes. The process of targeted drug delivery with immunoliposomes can be roughly divided into two phases: *the transport phase*, in which the immunoliposomes travel from the site of administration (often *i.v.*) to the target cells, and *the effector phase* that includes the specific binding of immunoliposomes to the target cells and the subsequent delivery of entrapped drugs. This section deals with the physiological and anatomical barriers to cross when immunoliposomes are administered *i.v.* and will point out potential solutions that have been investigated to enable immunoliposomes to fulfill their task.

BARRIERS ENCOUNTERED IN THE TRANSPORT PHASE

Stability of immunoliposomes in the circulation

Directly after *i.v.* administration, immunoliposomes are exposed to a variety of factors that may compromise the integrity of the liposomal carrier. For example, adsorption of serum proteins to the liposome surface can induce premature leakage of entrapped compounds and liposome aggregation upon injection into the bloodstream [20-22]. Liposome aggregates will be rapidly cleared from the circulation by liver and spleen phagocytes and/or embolize lung capillaries. Furthermore, it has been demonstrated that naturally occurring antibodies (class IgM and IgG) with reactivity against the headgroup of phospholipids are present in serum of rabbits [23] and men [24]. Such naturally occurring polyclonal antibodies were able to activate complement via the alternative pathway, resulting in complement-mediated lysis of liposomes.

Clearance of immunoliposomes from the circulation

Perhaps the most important barrier limiting the usefulness of immunoliposomes for targeted drug delivery has been the rapid recognition and removal from the blood by cells of the mononuclear phagocyte system (MPS), particularly the macrophages in liver and spleen [25,26]. In addition, the presence of whole antibodies conjugated to the liposomal surface makes immunoliposomes highly susceptible to Fc-receptor-mediated phagocytosis and, as a result, even more prone to rapid clearance [25,27]. The Fc-receptor family, which is expressed by different cells of the MPS, binds the constant region (Fc) of antibodies resulting in internalization of antibody-opsonized complexes (*e.g.* bacteria) [28]. Similarly, immunoliposomes bearing whole antibodies are cleared rapidly due to exposed Fc parts [25,27,29].

The advent of so-called long-circulating liposomes produced by coating the liposome surface with the polymer polyethylene glycol (PEG), has revived interest in targeted drug delivery [30,31]. The PEG-coating has a dual effect. It sterically stabilizes the liposomal membrane by decreasing interactions with destabilizing and opsonic factors *in vivo*. As a consequence, PEG-coated liposomes show longer circulation times and reduced uptake by the MPS, relative to conventional liposomes. Although the exact mechanism behind the MPS avoidance phenomenon is still under discussion, it is thought that stabilization occurs by the formation of a highly hydrated shield of polymer molecules around the liposome which sterically inhibits both electrostatic and hydrophobic interactions of serum components with the liposomal bilayer [32,33]. Therefore, PEG-liposomes are often referred to as sterically stabilized liposomes.

There are different methods available for coupling antibodies to PEG-liposomes [58]. The pros and cons were recently discussed [9]. For attaching antibodies to the surface of PEG-liposomes, two main strategies have been followed: those in which the ligand is coupled directly to the liposome bilayer (Figure 1A) and those in which the ligand is attached to the terminal end of PEG (Figure 1B,C,D) [8,10,34]. The latter strategy yields protein present at the surface of the PEG coating. Indeed, it has been shown that the clearance rate of PEG-immunoliposomes is dependent on the antibody density at the liposome surface [8,10,35]. At low antibody density (below about 50 µg mAb/µmol PL), the PEG-immunoliposomes are cleared at rates only slightly more rapid than those

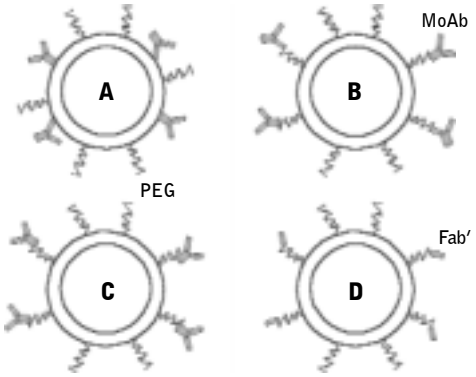

Figure 1 — Overview of the currently used types of PEG-immunoliposomes. The main difference is the position of the antibodies on the PEG-liposomes; they can be attached either directly to the liposomal bilayer (A) or to the distal end of the PEG-chains (B, C and D). In most coupling methods antibodies are randomly derivatized for attachment to functionalized groups on the liposome. This results in a random orientation of the antibody molecules on the liposomal bilayer (A) or on the PEG-chains (B), thereby resulting in exposition of the Fc-portion of at least part of the attached antibodies. One method has been described in which the antibody molecules are specifically attached via their Fc-portions to a hydrazide-group at the terminal end of the PEG-chains (C), resulting in correctly orientated antibodies at the PEG-chains and diminished Fc-exposition [36]. The presence of the Fc-portion can be avoided by using Fab'-fragments, which can be attached to the terminal end of PEG-chains, for example by exploiting the free thiol-group that is created after reducing $F(ab')_2$-fragments into Fab' (D).

seen for antibody-free PEG-liposomes. At higher antibody density (>100 μg mAb/ μmol PL), the clearance becomes very rapid with half-lives of only a few minutes [8]. Likely, clearance is mediated by the exposed Fc-region of the whole antibodies conjugated to the PEG-terminal ends.
An interesting observation came from Maruyama et al. who investigated whether the use of Fab'-fragments instead of whole antibodies makes a difference in terms of pharmacokinetics and biodistribution of PEG-immunoliposomes [10,36]. PEG-immunoliposomes exposing per particle approximately 51 whole antibody molecules conjugated at the distal ends of the PEG-chains were found to be rapidly removed from the circulation (60% after 1 h), whereas the same type of PEG-immunoliposomes, bearing per particle an average of 517 Fab'-fragments derived from the same monoclonal antibody, showed a 6-fold longer circulation time [36]. Moreover, accumulation of Fab'-bearing PEG-liposomes directed against the human carcinoembryonic antigen (CEA) in human gastric cancer tumors in nude mice was 2-fold higher compared to the accumulation of PEG-liposomes bearing whole IgG (approximately 20 and 10% of injected dose/g tissue, respectively) and was comparable with the accumulation of non-targeted PEG-liposomes. These results suggest that either the chemical modification of whole antibody molecules needed for coupling to the liposomes or the Fc-portion of antibody molecules mediate an enhanced clearance rate of antibody-targeted PEG-liposomes. Therefore, the use of Fab'-fragments rather than whole antibodies coupled to the distal ends of PEG-chains seems preferable for directing sterically stabilized liposomes to solid tumors. Nevertheless, many investigators still tend to use whole antibodies as targeting devices for PEG-liposomes.

Immunogenicity

It has become clear that the presence of antibodies on the liposome surface can lead to an immune response. Repeated subcutaneous injections of avidin-liposomes bearing biotinylated murine antibodies elicited isotype-specific antibody responses against the liposome-associated antibodies in mice [37]. Antibodies against phospholipids were not raised. Another study evaluating the immunogenicity of immunoliposomes showed that repeated *i.v.* injections of PEG-immunoliposomes bearing antibodies coupled to the distal ends of PEG-chains via a hydrazide linker elicited antibodies with specificity for the Fc-portion of liposome-conjugated antibodies [38]. This immunogenicity resulted in drastically decreased circulation times of liposomes in case of repeated administration. Remarkably, it was demonstrated that antibodies coupled to the liposomes are more immunogenic than antibodies given in free form [38]. This suggests that either the membrane-associated presentation of antibodies or the chemical modification of the antibodies needed for coupling to the liposomes accounts for the enhanced immunogenicity. Immunogenicity occurring when murine antibodies are used for liposome-targeting may become less of a problem when smaller antibody fragments (like Fab' or scFv) and humanized antibodies are used. Nevertheless, immunogenicity is an important factor to take into account when developing immunoliposomal drug carriers for cancer therapy and deserves more attention in future studies.

Passage of the vascular endothelium

Immunoliposomes need to cross the tumor vasculature in order to reach tumor cells located outside the blood compartment. Normal vascular endothelium consists of a continuous lining of endothelial cells, which are tightly connected with each other by tight junctions. Beneath this cellular layer is the basement membrane and in larger vessels, an additional layer of smooth muscle cells is present. This tight barrier prevents the passage of large molecules and particulates. At certain sites in the body, such as liver and spleen, the vascular anatomy is different. At these sites, the endothelial lining contains fenestrations that are approximately 80 to 120 nm in diameter. In solid tumors the vessels formed by the process of angiogenesis often show an increased permeability due to large fenestrae (up to 400 nm) and an irregularly formed basement membrane [39,40]. At tumor sites with an increased vascular permeability liposomes can extravasate, provided that they are small enough and circulate long enough [41-43]. It has also been suggested that liposomes may extravasate by transcytosis through endothelial cells [44]. However, convincing experimental evidence that this is really the case is still lacking.

Intra-tumoral transport

Unfortunately, tumors often develop in such a way that intratumoral penetration of particles through the interstitium is hindered [45,46]. Rapidly growing tumors often have a poorly developed lymphatic drainage system. This, together with the increased vascular permeability of the neovascular bed results in high interstitial pressure inside tumors opposing the convective transport of extravasated liposomes towards the tumor core. Moreover, though the angiogenesis process may yield vessels that have structural defects, such vessels showing increased permeability are not equally distributed within the tumor. As a consequence, extravasated liposomes are heterogeneously distributed throughout the tumor and are mainly located in perivascular regions [42,43].
There has been controversy whether extravasated PEG-liposomes are taken up by tumor-residing macrophages, thus limiting interaction with tumor cells. A recent study showed that the uptake of extravasated PEG-liposomes by macrophages within the tumor is minor [47]. In contrast to these findings, other reports show perivascular retention of extravasated PEG-liposomes and uptake by tumor-resident macrophages [48,49]. The different findings may be explained by model-related differences in tumor architecture and the number of tumor-residing macrophages. Actively targeted liposomes that expose the constant regions (Fc) of whole antibodies may be even more susceptible to macrophage uptake [50]. The use of antibody fragments lacking the constant part (like Fab' or scFv) may be helpful in reducing the uptake of immunoliposomes by tumor-associated macrophages [48,51].
The binding site barrier hypothesis, proposed by Weinstein et al. [52] for antibodies, suggests that immunoliposomes will not penetrate deeply into the tumor but bind to the first target cells they encounter. In case of solid tumors, the first target cells encountered by *i.v.* administered immunoliposomes will be those located directly behind the endothelial lining (perivascular zone).

Allen et al. showed that non-targeted long-circulating liposomes containing doxorubicin had a better antitumor activity than similar liposomes bearing whole antibodies [53]. According to the binding site barrier hypothesis, it was suggested that the passively targeted long-circulating liposomes may show better penetration into solid tumors compared to the immunoliposomes and therefore show a better antitumor activity [53,54]. However, convincing evidence for the perivascular retention of immunoliposomes limiting tumor penetration has not been provided so far. Recently, Park et al. [48] showed by using colloidal gold-containing PEG-immunoliposomes bearing anti-HER2/neu Fab' fragments at the distal ends of PEG chains that these immunoliposomes were abundantly deposited throughout the tumor mass, indicating that the binding site barrier hypothesis does not hold here. A striking difference with the findings of Allen et al. [53] is that Park et al. did not use whole antibodies but Fab'-fragments. Whole antibodies may have evoked Fc-receptor mediated endocytosis by macrophages located in the perivascular space. Moreover, Fc-receptor expression has been observed on activated endothelial cells [55,56] which may also contribute to the perivascular retention of immunoliposomes targeted with whole antibodies.

BARRIERS ENCOUNTERED IN THE EFFECTOR PHASE

Although much attention has been given in optimizing the transport phase of immunoliposomes in the past years, not much work has been done to improve the next step of effective delivery of immunoliposomal drugs within tumor sites. A dilemma the liposome field is facing is that liposomal drug carrier systems are optimized to retain drugs while in the circulation and not to yield optimal therapeutic availability of encapsulated drugs at tumor sites. Ideally, immunoliposomes should be developed in such a way that they do not release their drug content during the transport phase, but efficiently deliver their encapsulated compounds within the target site. This may require a transformation of the liposomal stability properties once the immunoliposomes have reached the target site to enable the efficient release of entrapped drug at tumor sites. Such a transformation may be induced by an external trigger, like change in pH or temperature, or may involve a slow change of the liposome composition in time, such as loss of the PEG coating resulting in destabilization of liposomes containing non-bilayer forming lipids (*e.g.* DOPE) [57]. Both the target cell binding step and the subsequent therapeutic availability step are discussed in more detail below.

Tumor cell binding

Choice of a target epitope – For the successful delivery of antineoplastic drugs by targeted liposomes, it is required that the drugs are delivered to every malignant cell. Therefore, liposomes

should be targeted to surface molecules that are present on each tumor cell and, most importantly, that are not expressed at similar levels by normal cells. Since tumor cells are notorious for their heterogeneity with respect to phenotype, expression of the target epitope on all malignant cells is very unlikely. Target epitopes on cancer cells may be up- or down-regulated depending on the cell cycle or the differentiation state of cancer cells. Moreover, target epitopes may be shed from the surface of tumor cells. Targeted drug delivery will mainly affect those cells expressing the target epitopes at high densities whereas target epitope-negative cells or those with low-density expression are likely to become less susceptible to the treatment. As a consequence, a target epitope-negative population of cells may be positively selected. Targeting of immunoliposomes to more than one tumor-associated surface antigen may decrease the chance of positively selecting a tumor cell population lacking targeting receptors.

Steric stabilization and specific binding – Targeting of immunoliposomes to solid tumors requires extravasation at the site of the tumor. For efficient extravasation, liposomes with sufficiently long circulation times are necessary. The most popular means to achieve long circulation times is steric stabilization of the liposome surface with PEG. For active targeting of long-circulating liposomes to tumor cells located at extravascular sites, the presence of homing devices on the liposome surface in addition to PEG is required. For maximal prolongation of the circulation time, liposomes should contain 5-10 mol% in case of the use of PEG-2000 [9,58]. Several studies have shown that with increasing PEG content and increasing PEG size, the coupling efficiency of antibodies to the bilayer of liposomes decreases [8,9]. Furthermore, the interaction of antibodies coupled to the bilayer of PEG-grafted liposomes with their target antigens may be hampered due to steric hindrance caused by the long PEG chains [59-62]. Thus, although PEG-liposomes with antibodies coupled directly to the liposome surface show circulation times comparable to non-targeted PEG-liposomes [58,63], the targeting efficiency can be reduced by the presence of long PEG chains on the surface of these liposomes. This problem of steric hindrance has been successfully solved by coupling the antibodies or antibody-fragments to the distal end of functionalized PEG-lipids [9,64]. Such PEG-immunoliposomes combine active targeting capacity with long circulation properties.

Antibody orientation is a major factor influencing the target cell binding of PEG-immunoliposomes. As in most coupling methods the antibodies are randomly derivatized for achieving attachment to functionalized groups on liposomes, part of the antibodies will be attached to the liposomes via their antigen-binding domain resulting in loss of target receptor-binding capacity of at least part of the attached antibodies (Figure 1). Methods that result in specific orientation of either whole antibodies or Fab'-fragments exist [9,65] and are preferred for optimal binding.

Therapeutic availability

After tumor cell binding, the encapsulated drug should become therapeutically available. In principle, the delivery of encapsulated compounds to tumor cells can take place via four different mechanisms: (1) release of encapsulated compounds from cell surface-bound immunoliposomes with subsequent uptake of free drug by the tumor cells, (2) transfer of lipophilic drugs from the immunoliposomal bilayers to the plasma membrane of tumor cells, (3) endocytosis of cell-surface receptor-bound immunoliposomes with subsequent intracellular release of encapsulated compounds, and (4) fusion of the immunoliposomal membrane with the target cell-membrane or endosomal membrane. The first mechanism aims for extracellular release of liposome-encapsulated compounds, whereas in the latter three mechanisms, the drug will be released onto or inside the cell. Intracellular release of antitumor drug has as main advantage that it may overcome multidrug resistence [66-68]. In general, tumor cells circulating in the bloodstream require intracellular delivery as extracellular delivery will result in fast diffusion and redistribution of the drug over the blood compartment [69]. In case of solid tumors, the extracellular release of drug at the tumor site seems preferable as this may lead to diffusion of drug within the tumor mass allowing the drug to reach also those tumor cells that do not express the targeted antigens or that are out of reach for the relatively large immunoliposome carriers. This so-called bystander effect may also occur after intracellular delivery of certain drugs which have physico-chemical characteristics that promote the leakage or active transport of a fraction of these drugs out of the target cells.

Extracellular release of encapsulated drug from surface-bound immunoliposomes – Drug may be released from immunoliposomes which are bound to the surface of tumor cells. The major advantage of this mechanism of delivery is that it does not require that immunoliposomes bind to all tumor cells. The distribution of free drug throughout the tumor will enhance the 'bystander effect' to those tumor cells that do not have any immunoliposomes bound to their surface. In this case, immunoliposomes act as an intratumoral drug depot from which free drug is slowly released and taken up by target cells as well as non-target cells within a solid tumor. For effective delivery, immunoliposomes should retain the encapsulated drug while circulating and efficiently release the drug after tumor cell binding. The recent discovery that PEG-modified lipids can undergo spontaneous transfer between membranes and the development of cleavable PEG-lipids [70] provide the technical tools to develop PEG-immunoliposomes that destabilize in time due to slow shedding of the PEG coat. Based on this PEG-shedding mechanism, liposomes have been developed that, after loss of PEG-modified lipids, become highly fusogenic and unstable [57]. In addition, target-sensitive immunoliposomes have been constructed that were composed of dioleoylphosphatidylethanolamine (DOPE) (80%), dioleoylphosphatidic acid (DOPA) (20%), and a small amount of specific antibody conjugated to *N*- glutaryl-phosphatidylethanolamine. The presence of *N*- glutaryl-phosphatidylethanolamine equally distributed in the liposomal bilayer stabilizes the lipid bilayer containing high amounts of hexagonal phase-forming DOPE. Upon target

cell binding of these target-sensitive immunoliposomes, it is thought that capping of the *N*- glutaryl-phosphatidylethanolamine-conjugated antibodies results in an unequal distribution of *N*-glutaryl-phosphatidylethanolamine within the liposome bilayer inducing rapid destabilization [71]. Whether such liposomes are stable during circulation in the bloodstream remains to be investigated.
An external trigger can potentially enhance the release of immunoliposome-encapsulated drugs in close proximity of tumor cells, provided that the immunoliposomes have an appropriate composition. Such an external trigger may be the local heating of a tumor (thermosensitive immunoliposomes) [72-74],[75] change in pH (pH-sensitive immunoliposomes) [55,76] or laser light (photosensitive immunoliposomes) [77-79]. An obvious requirement for such trigger approaches is that the exact location of the tumor within the body must be known. Besides, the released drug should be able to enter the target cell on its own, as most anticancer agents exert their function inside the cell.

Selective transfer of lipophilic (pro)drugs from immunoliposomes to tumor cells – Immunoliposomes are an attractive carrier system for the delivery of lipophilic anticancer drugs or prodrugs to tumor cells. The liposomal bilayer may be exploited for incorporation of either lipophilic drugs or lipophilic derivatives (prodrugs) of hydrophilic drugs that are not efficiently retained in the aqueous phase of the liposome. Examples of the latter are arabinofuranosylcytosine (Ara-C) [80], 5-fluorouracil (5-FU) [81], 5-fluoro-2'-deoxyuridine (FUdR) [82,83] and methotrexate [84]. To date, only a few studies have been published regarding the incorporation of lipophilic drugs into immunoliposomes for specific targeting [85-87]. One example of lipophilic drug incorporation in immunoliposomes will be further discussed as this shows a unique way by which lipophilic drugs can enter tumor cells. FUdR, which as a free substance is used in the clinic for the treatment of liver metastases of colon cancer, shows rapid leakage after encapsulation in liposomes. Therefore a lipophilic dipalmitoyl prodrug (FUdR-dP) was developed, which remains firmly associated with the liposomes, also under *in vivo* conditions [83,88]. FUdR-dP incorporated in long-circulating immunoliposomes targeted to lung endothelial cells, resulted in increased antitumor activity towards lung metastatic growth of mouse mammary carcinoma [85]. More recently, FUdR-dP incorporated in tumor cell targeted immunoliposomes showed a more than 10-fold increased antiproliferative activity *in vitro* towards rat CC531 colon cancer cells as compared to drug-containing liposomes without antibody [87]. Although these immunoliposomes bind to a high extent to the target cells, they are not internalized. However, the cells rapidly hydrolyze the FUdR-dP yielding the active drug intracellularly. Inhibition of endocytic uptake and intralysosomal degradation resulted in a major inhibition of the hydrolysis of the prodrug, demonstrating that the hydrolysis takes place intracellularly [89]. These results led to the postulation of a selective transfer mechanism for immunoliposomal FUdR-dP (Figure 2). During the high-affinity interaction of the immunoliposomes with the tumor cells the FUdR-dP is selectively transferred from the immunoliposomal bilayer to the plasma membrane (step 1). From there the drug is transferred into the cell by constitutive pinocytic and endocytic processes (step 2) and hydrolyzed intralysosomally

(step 3). Subsequently, the hydrolyzed drug diffuses into the cytoplasm (step 4) from where it is either transferred to the nucleus exerting its cytotoxic effect (step 5) or released into the extracellular compartment (step 6), where it can have cytotoxic effects on other tumor cells ('bystander effect'). An important message to be learnt from these studies is that immunoliposomes can mediate efficient intracellular delivery of a lipophilic drug without being internalized. We believe that this advantage may be more generally applicable for lipophilic (pro)drugs incorporated in immunoliposomes.

Internalization of surface-bound immunoliposomes – Certain proteins that bind to cell-surface receptors are rapidly internalized by the cells before they can dissociate from their receptors. This process, which is called receptor-mediated endocytosis, is recognized as an important and general mechanism by which cells take up nutritional and regulatory proteins from extracellular fluid. This internalization process can also be utilized for the cellular uptake of immuno-liposomes. A number of reports have demonstrated that small immunoliposomes targeted

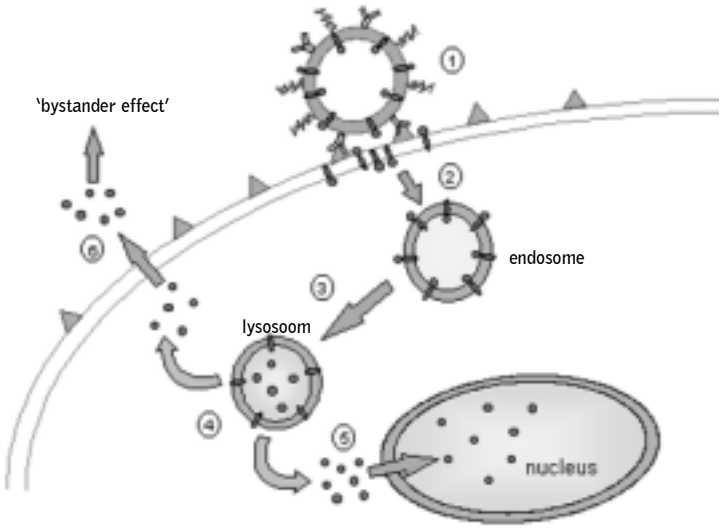

Figure 2 — Schematic presentation of a selective transfer model proposed for a lipophilic dipalmitoyl prodrug of the anticancer agent FUdR from immunoliposomes to the plasma membrane of tumor cells. After target cell binding, the immunoliposome incorporated FUdR-dP is transferred to the plasma membrane of the tumor cell (1). The prodrug is internalized by constitutive endocytic or pinocytic processes (2) and hydrolyzed intralysosomally (3). The active drug FUdR then diffuses into the cytoplasm (4) from where it is either transferred into the site of action, the nucleus (5), or released extracellularly (6) where it can exert a cytotoxic effect on surrounding tumor cells ('bystander effect'). Adapted from Scherphof et al. [51].

to certain cell-surface receptors (*e.g.* epidermal growth factor receptor, transferrin receptor, folate receptor) are internalized in a way that closely resembles receptor-mediated endocytosis [13,35,59,90]. Unfortunately, this route of entry does not guarantee full therapeutic availability of internalized drug molecules. Many therapeutic agents act at an intracellular target site to be reached via the cytosol. Immunoliposomes internalized by the mechanism of receptor-mediated endocytosis will eventually end up in lysosomes where they are degraded together with the encapsulated drug molecules. The physico-chemical nature of the encapsulated drug may be compatible with the need to escape from the endosomal or lysosomal compartment [91]. Molecules like doxorubicin [92] may be resistant to the action of lysosomal enzymes and/or low pH and able to cross the endosomal or lysosomal membranes. Also, lipophilic drugs may transfer from the liposomal to the endosomal membrane during endocytosis via the mechanism described before. Endosomal escape of hydrophilic drugs into the cytosol may be promoted by the incorporation structures with pH-dependent fusogenic properties into the liposomal carrier. These pH-dependent fusion mechanisms will be dealt with in the next section.

Fusion with the plasma membrane of target cells – Fusion of immunoliposomes with cells is an attractive strategy to deliver entrapped drug molecules into the cytosol. In principle, there are two routes by which cytosolic delivery via fusion can be achieved: (1) fusion of immunoliposomes with the plasma membrane of the target cells initiated after the immunoliposomes have bound to the target cells, or (2) pH-dependent fusion of immunoliposomes with the endosomal membrane, after being internalized by the target cells via receptor-mediated endocytosis.
Fusion of surface-bound immunoliposomes with the plasma membrane of the target cells has two major advantages. First, it does not require internalization of the immunoliposomes and consequently there is no need to target to an internalizing epitope, which increases the number of potential target epitopes for immunoliposomes. Second, plasma membrane fusion does not add a restriction to the size of fusogenic immunoliposomes in contrast to immunoliposomes that are designed for internalization. Third, fusion of immunoliposomes with the plasma membrane results in release of encapsulated drug directly into the cytosol. However, it should be realized that immunoliposomes with fusogenic activity at neutral pH may also fuse with non-target cells as a consequence of nonspecific adherence. In addition, unwanted inter-liposomal fusion events may occur.
In contrast to fusion of immunoliposomes directly with the plasma membrane, fusion with the endosomal or lysosomal membranes requires internalization. To this end, immunoliposomes should be targeted to receptors with known internalizing properties. In addition, the size of liposomes should not hamper endocytosis and should therefore be small (preferably < 100 nm) [93]. pH-sensitive liposomes have been used for the cytosolic delivery of encapsulated drug via the pathway of receptor-mediated endocytosis [76]. Liposomes with pH-dependent fusogenic properties can be constructed from dioleoylphosphatidylethanolamine (DOPE) in combination with acidic lipids, such as *N*-palmitoylhomocysteine (PHC), cholesterylhemisuccinate (CHEMS), oleic acid (OA) or

diacylsuccinylglycerols. Under physiological conditions, DOPE preferentially forms inverted hexagonal phase (H_{II}) rather than bilayers. However, admixing DOPE with one of the acidic lipids mentioned above prevents H_{II} phase formation and yields stable bilayers at neutral pH. Upon acidic exposure, however, protonation of the acidic lipids occurs, thereby neutralizing their negative charges and inducing destabilization of the liposomal bilayers and/or induction of pH-dependent lipid mixing [94-97]. Another strategy to achieve pH-dependent membrane destabilization or fusion is to incorporate pH-sensitive polyethylene glycol derivatives having carboxyl groups [98]. It was demonstrated that succinylated poly(glycidol) (SucPG) coupled to egg yolk phosphatidylcholine (PC) liposomes as a fusogenic polymer induced pH-dependent lipid mixing of liposomal lipids and yielded cytoplasmatic delivery of encapsulated calcein [98].

Membrane-active peptides can also be used to promote the cytosolic delivery of liposome-encapsulated drug from the endosomal/lysosomal compartment. Such peptides may act by destabilizing membranes, forming pores or inducing membrane fusion (reviewed by Plank et al. [99]). A number of synthetic, amphipathic peptides have been shown to exert fusogenic activity. For example, the amphipathic 30-residue peptide named GALA mimics the behavior of fusion peptides derived from viral fusion proteins. It was shown that GALA induces fusion of liposomes composed of PC in a pH-dependent fashion [100]. Puyal et al. [101] attached a 14-amino acid residue GALA-type peptide to liposomes and showed that intermixing of lipid between the liposomes occurs in a pH-dependent fashion. Similar results were obtained with a synthetic fusion peptide that induces fusion at neutral pH when coupled to the liposomal membrane [102,103]. The latter two studies clearly show that membrane-active properties can be grafted onto liposomes in order to enhance the cytosolic delivery of encapsulated compounds. Up to now no reports have been published demonstrating the utility of fusogenic peptide-bearing immunoliposomes for cytosolic delivery enhancement.

IMMUNOLIPOSOMES IN VIVO: CURRENT STATUS

In early days, the utility of conventional immunoliposomes *in vivo* was strongly limited by their rapid clearance by the MPS. With the advent of long-circulating liposomes that are able to oppose uptake by the MPS, targeting of liposomes to tissues other than the MPS is now much more realistic. One of the most critical issues to consider when developing immunoliposomal systems is whether the surface-attached antibodies can access the tumor cell surface *in vivo*. Moreover, the route of administration, in relation to the target site, is an important determinant for the degree of *in vivo* targeting that can be achieved. For this reason, the current status of immunoliposomes as drug carriers in cancer therapy will be discussed separately for targeting to cells within the vasculature after *i.v.* administration, to tumor cells residing in the peritoneal cavity after local administration and to solid tumors after *i.v.* administration.

INTRAVENOUS ADMINISTRATION OF IMMUNOLIPOSOMES TO TARGET CELLS WITHIN THE VASCULATURE

Circulating tumor cells

Hematological malignancies (*e.g.* lymphomas, multiple myeloma or leukemias) seem attractive candidates for immunoliposome-based therapy, as circulating tumor cells are likely easily accessible targets for *i.v.* administered immunoliposomes. Lopes de Menezes et al. targeted sterically stabilized immunoliposomes containing doxorubicin (DOX-SSIL) to CD19+ Namalwa cells (human B-cell lymphoma) circulating in the bloodstream of severe combined immune deficiency (SCID) mice. *In vitro* evidence pointed to internalization of tumor cell-bound anti-CD19 SSIL by receptor-mediated endocytosis [69]. Evaluation of the therapeutic effect *in vivo* showed a significant increase in survival (77% increase in lifespan) for the tumor-bearing mice treated with anti-CD19 DOX-SSIL in comparison with non-targeted DOX-SSL or free DOX. The effectiveness of treatment was dependent on the dose of inoculated tumor cells and the time interval between tumor cell inoculation and intervention. Best therapeutic results were obtained with early interventions (*i.e.* 1 h after *i.v.* injection of tumor cells) when inoculated tumor cells are most likely still circulating. Multiple weekly treatments (day 1,8 and 15 after tumor inoculation) only slightly improved the survival times of mice, indicating that target cells may have become relatively inaccessible or have grown to a size that hinders effective penetration of DOX-SSIL formulations into the tumor mass. The better therapeutic effect of DOX-SSIL compared to non-targeted DOX-SSL was explained by efficient internalization of CD19-bound DOX-SSIL by Namalwa cells followed by intracellular release of DOX from the internalized liposomes. As non-targeted DOX-SSL were not internalized by the target cells *in vitro*, the results show the necessity of internalization to increase the efficiency of drug delivery to tumor cells within the vasculature.

Recently, similar results were obtained with DOX-containing sterically stabilized anti-idiotype immunoliposomes in a murine B-cell lymphoma model [104]. Treatment of tumor-bearing mice with DOX-containing anti-idiotype SSIL resulted in a significantly greater median survival time as compared to mice treated with either saline, free DOX or plain DOX-SSL. Also in this study it could be shown *in vitro* that anti-idotype-targeted SSIL were efficiently internalized by the malignant B-cells. Another option to explain the better therapeutic effects of the DOX-anti-idiotype SSIL formulations used in the above-mentioned studies, is that SSIL bound to target cells induced the so-called target cell dragging phenomenon, as proposed by Peeters et al. [105,106]. This phenomenon implies that binding of the immunoliposomes to the circulating target cells induces an opsonic effect leading to rapid uptake of the tumor cells by hepatosplenic macrophages.
A potential clinical hazard related to immunoliposome targeting to circulating cells is the possible occurrence of agglutination. Circulating tumor cells may be agglutinated due to multivalent interaction of immunoliposomes with more than one tumor cell. Moreover, immunoliposomes may be agglutinated in the blood circulation due to the presence of soluble bi- or multivalent target antigens shed from the surface of either circulating tumor cells or tumor cells within a solid tumor. It was demonstrated that immunoliposomes targeted *i.v.* to the idiotype of B-cell receptors on malignant B-cells caused clinical signs of dyspnea, lateral recumbancy, peripheral lymb cyanosis, convulsion and occasional death in tumor-bearing mice [107]. These clinical symptoms were the result of agglutination of circulating immunoliposomes by soluble antibodies produced by the malignant B-cells, which causes occlusion of small capillaries as the *i.v.* injection of purified antibodies from these malignant B-cells after administration of immunoliposomes gave the same symptoms. The above-discussed study on the therapeutic efficacy of CD19 DOX-SSIL in SCID mice bearing CD19+ human B-cell lymphomas did not report any of these symptoms.

Endothelial cells

Endothelial cells lining the vasculature represent an easily accessible target to *i.v.*-administered immunoliposomes. Maruyama et al. have targeted SSIL to lung endothelial cells by conjugating mAb 34A to the distal ends of liposome-attached PEG-molecules [10,64,108]. This mAb is specific for thrombomodulin, which is expressed at high levels on mouse lung endothelial cells. In Balb/c mice up to 60% of *i.v.*-injected 34A-SSIL was found in the lung within 15 min after injection. Liposomes without conjugated antibodies or with irrelevant antibodies conjugated to the PEG-chains did not accumulate in the lung (<0.4% of injected dose), but were mainly found in the liver (50% of injected dose). The excellent targeting efficiency of 34A-SSIL has been utilized to achieve high levels of antineoplastic drugs in lungs of mice bearing metastasized tumors [85]. It was shown that mice treated with FUdR-dP incorporated into 34A-immunoliposomes yielded a significant increase in lifespan compared to mice treated with FUdR-dP dispersed in a lipid emulsion or incorporated in non-targeted liposomes. This study indicates that an indirect targeting approach (*i.e.* active targeting of immunoliposomal antineoplastic drugs to cells other than the tumor cells) may be attractive to obtain high levels of drug in close proximity of tumor cells. The lipophilic

characteristic of the FUdR-dP was proposed to play a role in the transendothelial delivery of the drug.

LOCAL ADMINISTRATION OF IMMUNOLIPOSOMES TO TARGET TUMORS CONFINED TO THE PERITONEAL CAVITY

Tumor cells located in the peritoneal cavity present another accessible target for immunoliposomes if the immunoliposomes are injected intraperitoneally (*i.p.*). This is examplified by studies on the *i.p.* administration of immunoliposomes for the treatment of ovarian carcinoma [86,109,110]. Straubinger et al. attached whole antibodies directed against CA-125 carcinoma antigen to the surface of liposomes [110] and showed that *i.p.* injected OC-125 immunoliposomes were able to bind to ovarian cancer cells (OVCAR-3) growing as an ascitic tumor in the peritoneal cavity of nude mice.

In another study on ovarian carcinoma treatment, the lipophilic prodrug 5'-palmitoyl-5-fluorouridine (PF) was incorporated into immunoliposomes [86]. The investigators utilized whole antibodies directed against the CAR-3 antigen, which is abundantly expressed on human ovarian carcinoma cells and other adenocarcinomas. Intraperitoneal injection of anti-CAR-3 immunoliposomes into the OVCAR-3 xenograft model resulted in a drastic reduction of the tumor mass. The PF-immunoliposomes were 4 times more effective than the corresponding non-targeted PF-liposomes and 18 times more effective than the free drug, illustrating the superiority of PF-immunoliposomes.

Nässander et al. used immunoliposomes bearing covalently coupled Fab' fragments of the monoclonal antibody OV-TL3, which is directed against the antigen OA3 present on more than 90% of all human ovarian carcinoma cells [111]. When these immunoliposomes were administered *i.p.* in the OVCAR-3 xenograft model, a high fraction (about 70%) of the injected dose was bound to the tumor cells within half an hour. Encouraged by these excellent *in vivo* targeting results, doxorubicin (DOX) was loaded into these immunoliposomes (IL) and evaluated in the same *i.p.* xenograft model [109,112]. Although the DOX-IL formulations had a superior antitumor effect compared to non-targeted DOX-liposomes *in vitro,* no difference in antitumor activity between the targeted and non-targeted formulation was observed *in vivo*. As no evidence for cellular internalization of cell-bound immunoliposomes was obtained, a similar degree of premature drug leakage from both the targeted and non-targeted DOX-liposomes within the peritoneal cavity was suggested to underlie the antitumor effects seen. The lack of therapeutic benefit of Fab'-targeted liposomes clearly illustrates the point that even well-targeted immunoliposomes can fail due to lack of cellular internalization and/or uncontrolled drug release patterns.

INTRAVENOUS ADMINISTRATION

TO TARGET SOLID TUMORS AND METASTASES

Liposome investigators have been able to exploit the abnormal blood vessel structures associated with tumor angiogenesis for extravasation of liposomes [46]. It is now well documented that immunoliposomes must both be small and exhibit long circulation times to achieve preferential localization within solid tumors [43,49]. Active targeting of sterically stabilized liposomes (SSL) by conjugating antibodies or antibody fragments to the liposomal surface may further improve the therapeutic efficacy by increasing the specific uptake of liposomal drug formulations into tumor cells within a solid tumor. However, one should realize that the attachment of antibodies or antibody fragments to the surface of SSL and especially to the distal ends of PEG-lipids on SSL may oppose the MPS-avoiding capacities of SSL [8,36].

Maruyama et al. have evaluated the efficiency of tumor targeting of SSIL in a xenograft model of human gastric cancer [10,36]. SSIL were constructed by conjugating either whole antibodies or Fab'-fragments of anti-CEA mAb 21B2 to the distal ends of the PEG-chains. The tumor localization of these SSIL 24 hrs after *i.v.* injection was compared with that of non-targeted SSL and bare liposomes (*i.e.* without any surface-bound molecules). It was shown that the most efficient tumor localization was obtained with non-targeted SSL or Fab'-targeted SSIL (both 20% of injected dose/g tumor). Whole antibody-targeted SSIL localized substantially less at the tumor site (10% of injected dose/g tumor), but tumor accumulation was still higher than for bare liposomes (7% of injected dose/g tumor).

Reports demonstrating an improved efficacy of antitumor agents encapsulated in actively targeted SSIL relative to non-targeted SSL are scarce. Ahmad et al. [113] studied immunoliposomes with avidin covalently coupled to the bilayer of PEG-liposomes. Biotinylated monoclonal antibodies that recognize specific epitopes on the surface of lung squamous carcinoma cells avidly bound to these avidin-bearing liposomes. Such 'two-step-targeted' immunoliposomes were loaded with doxorubicin (DOX) and subsequently injected *i.v.* in mice bearing murine squamous carcinoma cells in the lungs. Treatment with DOX-SSIL resulted in significantly improved antitumor activity compared to treatment with free DOX or non-targeted DOX-liposomes. The success of this approach was ascribed to efficient targeting to tumor cells in combination with the release of DOX from tumor cell-bound immunoliposomes and subsequent uptake of free DOX by tumor cells. Regression of more advanced tumors did not occur, probably as a result of poor tumor penetration or down-regulation/shedding of the target epitope.

Kirpotin at al. [114] treated HER2/neu over-expressing human breast cancer cells *in vitro* with DOX-containing immunoliposomes bearing Fab'-fragments of a humanized mAb directed against the HER2/neu antigen. The Fab'-fragments were either attached to the liposomal surface via a short spacer or to the distal ends of PEG-chains. The *in vitro* tests showed that the immunoliposomes with the Fab'-fragments attached to the distal end of PEG-chains are able to

avidly bind to the HER2/neu over-expressing cells and are rapidly internalized via the coated pit pathway. The DOX-loaded anti-HER2/neu SSIL produced significantly increased antitumor activity as compared to free DOX or DOX-loaded non-targeted liposomes (SSL) in xenograft models of HER2/neu-overexpressing breast cancer. Interestingly, the degree of localization of anti-HER2/neu SSIL in HER2/neu-overexpressing tumor xenografts was not increased over that of non-targeted SSL. However, the use of colloidal gold-containing SSIL revealed that the SSIL were distributed throughout the tumor interstitial spaces as well as localized within tumor cells [115]. In contrast, non-targeted SSL were located predominantly within tumor-associated macrophages. Thus, increased deposition in the intercellular spaces within the tumor tissue outside tumor-associated macrophages, as well as the intracellular delivery of DOX may have contributed to the superior antitumor activity of DOX-loaded anti-HER2/neu SSIL. Therapeutic superiority of targeted SSIL was not observed by Goren et al. [116]. The latter group also studied the *in vivo* antitumor effects of DOX-loaded anti-HER2/neu SSIL and concluded that the antitumor activity of targeted and non-targeted DOX-SSL was similar. Whether this discrepancy in outcome is related to differences in tumor model or to the fact that Goren et al. utilized complete mouse antibody rather than humanized Fab' is not clear.

In another study, mice bearing human ovarian cancer xenografts were treated with either free DOX, DOX-SSL or DOX-SSIL (targeted with mAb B43.13 to human ovarian cancer cells) [53]. Surprisingly, the non-targeted DOX-SSL formulation was more effective than DOX-SSIL in reducing the rate of tumor growth. This finding was ascribed to the binding site barrier phenomenon.

RECENT ADVANCES

A general trend that can be deduced from the previous section is that, despite successful results *in vitro*, the results *in vivo* are somewhat disappointing: only a few examples of successful anticancer applications of immunoliposomes *in vivo* exist. Clearly, the immunoliposomal system needs further improvement in order to obtain an effective drug delivery vehicle for application *in vivo*. Several major problems have been identified. Recent developments to tackle these problems are discussed below.

DEVELOPMENT OF ALTERNATIVE HOMING DEVICES

Antibody-based homing devices

Studies with antibody-targeted PEG-liposomes have shown that a high density of antibodies attached to the distal ends of the PEG chains evoke antibody-mediated recognition and enhanced clearance by the RES [8]. The mechanism of clearance is thought to be - at least in part - Fc-receptor-mediated as the coupling of Fab'-fragments did not induce enhanced clearance at similar high densities. Therefore, the use of Fab'-fragments rather than whole antibodies seems advantageous [10,36]. The presence of antibody-molecules on the liposomal surface can also induce an immune response. Studies from Phillips et al. and Harding et al. show that upon repeated injections of immunoliposomes in mice or rat, isotype-specific antibodies were generated against the antibodies conjugated to the liposomes [37,38]. Likely, immunogenicity problems encountered with antibody therapy in humans, such as human anti-mouse antibody (HAMA) responses [117] will also hold for immunoliposomal drug formulations. Immune responses may severely hinder repeated dosing regimens for immunoliposomes in the clinic. In order to prevent HAMA responses, humanized mAbs have been produced, in which the complementary determining regions (CDRs) of murine mAbs have been grafted onto human antibodies [118,119]. Although such chimeric antibodies largely consist of regions of human origin, still an antibody response may be induced against the grafted variable regions of murine origin. A better option is the use of fully human antibodies, which has become a possibility with the development of transgenic mice that produce antibodies of human origin [120,121]. These mice may provide the optimal source for immuno-tolerant antibodies for targeted drug delivery in humans. However, one should keep in mind that not only the origin of antibodies determines their antigenicity, also coupling chemistry involving the chemical modification of antibodies and the use of anchoring molecules may introduce immunogenicity problems.

Besides whole antibody molecules and Fab'-fragments, smaller antibody fragments have been studied. Lipid-tagged human scFv have been constructed and successfully incorporated into liposomes [122,123] and used to target liposomes to B-lymphocytes *in vitro* [123]. Theoretically,

there are several advantages for using lipid-tagged scFvs over whole antibodies for liposome targeting: (1) The smaller size of scFv antibodies together with their human origin may make immunoliposomes less immunogenic than whole antibodies. (2) The absence of an immunoglobulin constant region will prevent rapid Fc-receptor mediated clearance of scFv-targeted liposomes. (3) There is no need to chemically modify the lipid-tagged scFv-antibodies for coupling to liposomes. They will spontaneously insert into the phospholipid bilayers because of the presence of the lipid tag. Potential loss of binding affinity and/or induction of immunogenicity due to chemical modification procedures are therefore avoided. (4) The availability of large phage display libraries of human scFv-fragments, the possibility of large scale production, and the simple selection procedure of antigen-specific scFv-fragments favor the use of scFv fragments instead of murine antibodies. However, *in vivo* studies utilizing scFv-immunoliposomes are lacking at present; issues like immunogenicity and stable insertion of the lipid tag into the bilayer have not been addressed yet.

Oligonucleotide aptamers

Recently, a novel method for the production of antigen binding molecules has been developed. This method makes use of oligonucleotide aptamers that in their specific three-dimensional conformation can bind to proteins with specificities and affinities comparable to antibody molecules. Aptamers can be selected from large libraries of nucleic acid sequences with the systemic evolution of ligands by the exponential enrichment (SELEX) method [124-126]. From such libraries ($>10^{15}$ different aptamers) aptamers to virtually any structure can be selected and potentially be used as homing devices to target liposomes [16].

Other ligands

Naturally occurring ligands to cell-surface receptors such as vitamins, hormones and growth factors may also be used as immuno-tolerant homing devices. They offer some potential advantages over antibodies, such as lack of immunogenicity and low preparation costs. It was shown that liposomes to which the epidermal growth factor (EGF) was covalently coupled could be targeted to hepatocytes expressing the EGF-receptor [13]. As many carcinoma cells show a relatively high expression of the EGF-receptor, EGF-targeted liposomes may be useful for tumor targeting [12,13].
Folate has also been studied as targeting ligand [11,127]. The folate receptor has been identified as a surface marker in ovarian carcinomas. Receptor overexpression is found in many other types of cancer as well. Folate-targeted liposomes that specifically bind to cells that overexpress the folate receptor have been designed [128]. When folate is directly attached to the phospholipid headgroups or when a short spacer is used to couple folate with the bilayer, the folate-exposing liposomes are not recognized by the folate receptor. A long PEG spacer (MW 3,350) between the folate and the liposome surface is required for receptor recognition. Such folate-targeted liposomes are extensively internalized by receptor-mediated endocytosis after target cell binding. Incorporation of DOX resulted in 86-fold higher cytotoxicity to folate receptor-expressing tumor cells compared to non-targeted liposomes and 2.7-fold higher cytotoxicity relative to free DOX [129].

IMPROVING THE THERAPEUTIC AVAILABILITY OF IMMUNOLIPOSOMAL DRUGS

Thermo-sensitive immunoliposomes

Evidence that the therapeutic efficacy of liposomal drugs can be drastically improved by enhancing the drug release from liposomes extravasated into tumor tissue was elegantly demonstrated with thermo-sensitive liposomes [72-75,130,131]. The heat-induced drug release concept is based on the large increase in permeability of liposomal bilayers around their phase-transition temperature. Local heating of tumor tissue up to this phase transition temperature will enhance drug release out of liposomes present in the heated area [74]. *In vivo* fluorescence videomicroscopy has been used to study the extravasation of thermo-sensitive liposomes and the release of their contents in a rat skin flap window chamber, which contained a well-vascularized mammary adenocarcinoma [72]. Extravasation and contents release were measured at different temperatures (34°C, 42°C, and 45°C). At hyperthermic conditions both the degree of extravasation as well as the rate of drug release from thermosensitive liposomes (composed of DPPC, HSPC, CHOL, PEG-PE) were substantially increased. Improved therapeutic efficacy of thermo-sensitive liposomes in combination with local heat application has been demonstrated in several studies [73,132,133]. The above mentioned studies all used non-targeted thermo-sensitive liposomes. Only one study with thermo-sensitive immunoliposomes *in vitro* has been reported [75]. The *in vivo* applicability of thermo-sensitive immunoliposomes as targeted drug vehicles in cancer therapy remains to be investigated.

pH-sensitive immunoliposomes

pH is another factor that may be utilized to trigger the release of drugs from immunoliposomes. pH-sensitive immunoliposomes have been prepared that became highly unstable and fusogenic at low pH [76,95,96]. pH-sensitive immunoliposomes targeted to internalizing receptors will end up in endosomes where acidification will trigger liposome destabilization and possibly fusion with the endosomal membrane. They have been applied *in vitro* for the delivery of antitumor drugs into the cytoplasm of tumor cells [134,135]. Indeed it was demonstrated that pH-sensitivity strongly increases the potency of immunoliposomal antitumor drugs. pH-sensitive immunoliposomes have never been tested for *in vivo* drug delivery to tumors, probably because of evidence pointing to instability in the circulation [136]. The recent discovery that PEGylation of pH-sensitive liposomes improves their stability *in vivo* may revive the interest in using pH-sensitive immunoliposomes as drug carriers in cancer therapy [94].

Utilization of membrane-active peptides

Membrane-active peptides are short, amphipatic peptides containing hydrophobic amino acid sequences. These sequences have in common that they can fold into specific structures (*e.g.* α-helix, β-sheet) dependent on environmental conditions like pH or the presence of cell membranes. In

nature such peptide stretches are efficiently utilized in many biological events involving membrane interactions [99]. Many of these membrane-active peptides have been synthesized and tested for membrane-destabilizing and/or fusogenic properties [100,103,137-139]. They show membrane destabilizing activities either at low pH [100,101,140,141] or at neutral pH [102,103,138,142]. Certain membrane-active peptides have fusogenic properties as they can induce the mixing of lipids between distinct liposome particles. Several studies have shown the possibility of coupling membrane-active peptides to the bilayer of liposomes [101,102,139]; in some cases an increase in membrane activity of the peptide could be observed after coupling to the bilayer [101,102]. The feasibility of the use of pH-dependent, membrane-active peptides coupled to the surface of immunoliposomes to enhance the cytosolic delivery of anticancer drugs to tumor cells is currently under investigation.

Immuno-enzymosome approach

A totally different approach to obtain high concentrations of therapeutically available drug at tumor sites is the use of immunoliposomes for antibody directed enzyme prodrug therapy (ADEPT) [143,144]. ADEPT was originally described for antibodies [145] but has been adapted for the use of immunoliposomes. Instead of entrapping drug molecules inside immunoliposomes, the immunoliposomes carry anticancer prodrug-activating enzyme molecules on their surface (immunoenzymosomes). In this two-step approach, the first step involves the targeting of the immunoenzymosomes to the tumor site. Subsequently, an anticancer prodrug matched with the enzyme is given, which will then be converted to its cytotoxic parent compound selectively near the tumor cell surface, thereby inducing an antitumor effect without systemic toxicity. Recently, we have coupled the enzyme ?-glucuronidase, capable of activating anthracycline-prodrugs, to the external surface of immunoliposomes directed towards ovarian carcinoma cells [144,146]. It was demonstrated that after tumor cell binding and subsequent addition of prodrug, the immuno-enzymosomes were able to convert almost all prodrug added resulting in killing of ovarian carcinoma cells *in vitro,* whereas enzymosomes (bearing no specific antitumor antibodies) or immunoliposomes (bearing no enzymes) were clearly ineffective. These findings point to the potential usefulness of immuno-enzymosomes in prodrug activation therapy. The immuno-enzymosome approach is a conceptually attractive strategy as it circumvents the problem of insufficient therapeutic availability of the encapsulated drug contents at the target cell surface. The 'bystander effect' described above may promote that also those tumor cells are killed that are not reached by the immuno-enzymosomes. The possible advantages that immuno-enzymosomes offer over antibody-enzyme conjugates usually used in ADEPT are that: (1) one carrier unit of immuno-enzymosomes delivers much more enzyme than one antibody unit, and (2) multivalent interaction of immunoliposomes with target cells may result in higher avidity to the target epitopes as compared to antibodies. Currently, *in vivo* studies are focussed in our group on the *i.p.* administration of immuno-enzymosomes for the therapy of ovarian cancer micrometastases localized in the peritoneal cavity. It is obvious that *i.v.* administration of immuno-enzymosomes to target solid tumors will require steric stabilization of their surfaces to prolong circulation times.

MULTIPLE LAYER LIPOSOMES

As mentioned before, immunoliposomes designed for *in vivo* cancer therapy should be able to retain drug while in the circulation, but upon interaction with the target cells, they should efficiently release the drug. Ironically, those liposomal characteristics needed to retain the drug in circulation, may counteract the efficient release of drug after binding to the target cell. Several paradoxal requirements should be combined into one immunoliposomal carrier system allowing immunoliposomes (1) to retain drug completely following intravenous administration, (2) to avoid rapid clearance by the MPS and allow extravasation, (3) to bind to tumor cells and (4) to efficiently release the entrapped drug after binding to the tumor cells. As these attributes are not needed all at the same time, it may be possible to construct liposomes with different layers in which each layer fulfills one or more of the above mentioned functions (Figure 3). After performing its function a layer should be shed from the liposomes in order to expose the next layer. Such transformable liposomes or 'multiple layer' liposomes have recently been proposed by Bally et al. [147]. The feasibility of these putative 'multiple layer' liposomes has been illustrated in a study in which cleavable PEG (thiolytic cleavage) was used to stabilize liposomes containing the non-bilayer forming fusogenic lipid DOPE [70]. The PEG-lipid in this system has a dual function. First, its presence provides steric stabilization to yield prolonged circulation properties and second, it stabilizes the lipid bilayer, thereby preventing DOPE-mediated destabilization of the bilayer and contents release. The study showed that DOPE-mediated membrane destabilization - and as a result

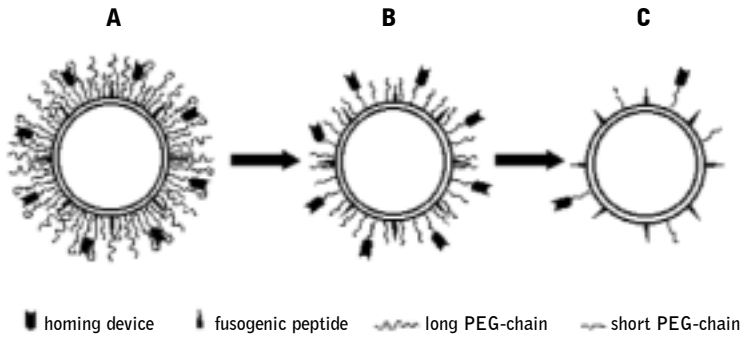

Figure 3 — Schematic representation of 'multiple layer' immunoliposomes at successive stages. Stage A represents a 'multiple layer' immunoliposome while in the circulation. PEG-layers with two different chain lengths preserve the bilayer stability, thereby assuring long circulation properties. After extravasation and penetration into the tumor interstitium, the outermost PEG-coat should be shed in order to expose the homing devices for tumor cell-binding (stage B). Upon transfer of the innermost layer of PEG-lipids or upon PEG-cleavage, the membrane destabilizing properties of liposomes become activated either by exposure of membrane-active peptides (as shown in stage C) or by hexagonal phase formation of DOPE thereby enabling effective delivery of encapsulated drug.

release of contents - only occurs upon cleavage of PEG. The 'multiple layer' liposomes may contain PEG-lipids with different PEG-chain lengths (Figure 3). For instance, the first outermost layer may consist of PEG-5000 for creating the steric stabilization effects**,** the second layer of functionalized PEG-2000 for coupling of homing devices for specific binding and subsequent internalization, and the third layer of 'instable' bilayers, stabilized by the two layers of PEG-lipids. Such 'multiple layer' liposomes should shed their layers one by one, starting with the outermost layer. The shedding of the different layers of PEG may be regulated by using different PEG-lipid conjugates. It has been shown that the rate of transfer of PEG-lipids to a large excess of neutral acceptor liposomes is dependent on the length and degree of saturation of the acyl chains present in the lipid anchor [57]. This difference in PEG-lipid transfer can be utilized to control the shedding of PEG-layers from multiple layer liposomes. In addition, PEG can be coupled to liposomes by using cleavable bonds with different cleavage kinetics (*e.g.* acidic or enzymatic hydrolysis) [70,148].

TARGETING TO THE TUMOR VASCULATURE

An indirect approach to fight tumor cells with immunoliposomes may be to target to the tumor vasculature with the aim to obstruct the blood supply to the tumor (Figure 4). As tumor cells rely on the continuous supply of nutrition and oxygen like normal cells, depriving the blood supply will result in tumor cell death. As compared to tumor cell targeting, vascular targeting has several attractive features: (1) The endothelial cells are directly accessible to circulating immunoliposomes. Therefore, tumor cell accessibility problems related to poor extravasation and tumor tissue penetration are avoided. (2) Even the destruction of a small number of endothelial cells may be enough to induce coagulation, resulting in the formation of an occlusive thrombus interfering with tumor perfusion and leading to necrosis. Therefore, tumor vasculature-directed liposomes can be expected to have a greater capacity to induce antitumor effects than liposomes designed for drug delivery directly to tumor cells. (3) As most solid tumors rely on similar blood vessels for growth, the vascular targeting approach should be applicable to most or possibly all solid tumors. (4) Endothelial cells are genetically stable and therefore expected not to mutate to become resistant to the therapy, as tumor cells often do. A prerequisite for tumor vascular targeting is of course the existence of highly specific tumor vascular markers. In humans, several cell-surface receptors have recently been identified that are specifically expressed on tumor vascular endothelium and are potentially suitable for tumor vascular targeting purposes [149,150].

The feasibility to kill tumors by attacking their blood supply was demonstrated by several investigators, utilizing tumor vasculature-directed antibody or peptide conjugates [151-153]. Successful targeting of immunoliposomes to endothelial cells has been shown *in vitro* [154-157] and *in vivo* [64,158]. The fact that endothelial cells are well accessible for intravenously injected immunoliposomes is illustrated by work of Maruyama et al. showing targeting efficiencies of immunoliposomes targeted to lung endothelial cells of up to 60% of the injected dose [10,108].

However, immunoliposome-based applications of tumor vasculature targeting have not been reported yet.

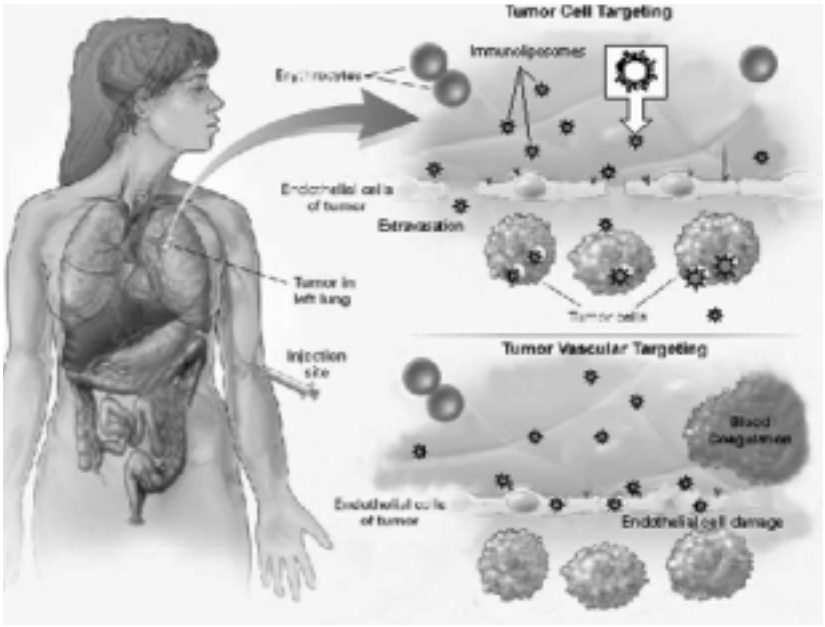

Figure 4 — Concept of tumor vascular targeting versus tumor cell targeting *in vivo*. Immunoliposomes containing cytotoxic drugs are injected intravenously and travel through the circulation where they can reach tumor vessels. If drug-loaded immunoliposomes are targeted to tumor cells (upper panel), they should extravasate in order to reach and kill the tumor cells behind the tumor vascular endothelial lining. Immunoliposomes targeted to specific receptors on tumor endothelium (*e.g.* $\alpha_V\beta$-integrins or VEGF-receptors) can lead to endothelial cell damage and the induction of blood coagulation (lower panel). In this way, the blood supply to the tumor is obstructed, resulting in massive tumor cell death. Adapted from J.E. Schnitzer et al. [150].

FINAL REMARKS

We hope that this review on the current situation regarding the use of immunoliposomes for the use in cancer chemotherapy provides a realistic perspective on where the field is going and where opportunities can be found for rational improvement of the immunoliposome system. Ideally, two objectives should be met: (1) all administered immunoliposomes should bind to their target epitopes *in vivo* and (2) all encapsulated drug molecules should become therapeutically available upon binding of immunoliposomes to the target receptors. To date, all targeted systems, including immunoliposomes, fall short of meeting both criteria. However, recent advances have improved the prospects for immunoliposome-based applications. These advances include development of technological solutions for the preparation of long-circulating immunoliposomes, design of 'immunotolerant' targeting ligands, development of new coupling chemistries for attaching targeting ligands to the liposome and attempts to enhance the therapeutic availability of associated drugs. Certainly, encouraging results have been obtained. Over the last two decades, major pharmaceutical achievements have been realized in liposomology and this maturing process will facilitate the development of immunoliposome formulations that are pharmaceutically acceptable. In conclusion, immunoliposomes still have a long way to go, but the strong need for more effective chemotherapeutics will continue to motivate studies on immunoliposomes.

REFERENCE LIST

1 Kim, S. (1993): **Liposomes as carriers of cancer chemotherapy. Current status and future prospects.** *Drugs,* 46:618-638.

2 Lasic, D.D. (1998): **Novel applications of liposomes**. *Trends.Biotechnol.*, 16:307-321.

3 Storm, G. and Crommelin, D.J.A. (1998): **Liposomes: quo vadis?** *Pharm.Sci.Tech.Today,* 1:19-31.

4 Dass, C.R., Walker, T.L., Burton, M.A., and Decruz, E.E. (1997): **Enhanced anticancer therapy mediated by specialized liposomes**. *J.Pharm.Pharmacol.*, 49:972-975.

5 Harasym, T.O., Bally, M.B., and Tardi, P. (1998): **Clearance properties of liposomes involving conjugated proteins for targeting**. *Adv.Drug.Del.Rev.*, 32:99-118.

6 Forssen, E. and Willis, M. (1998): **Ligand-targeted liposomes**. *Adv.Drug.Del.Rev.*, 29:249-271.

7 Heath, T.D., Bragman, K.S., Matthay, K.K., Lopez-Straubinger, N.G., and Papahadjopoulos, D. (1984): **Antibody-directed liposomes: the development of a cell-specific cytotoxic agent**. *Biochem.Soc.Trans.*, 12:340-342.

8 Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y., and Zalipsky, S. (1995): **A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells.** *Biochim.Biophys.Acta,* 1237:99-108. [published erratum appears in Biochim Biophys Acta 1995 Dec 13;1240(2):285].

9 Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S., and Allen, T.M. (1995): **Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures**. *Biochim.Biophys.Acta,* 1239:133-144.

10 Maruyama, K., Takizawa, T., Takahashi, N., Tagawa, T., Nagaike, K., and Iwatsuru, M. (1997): **Targeting efficiency of PEG-immunoliposome-conjugated antibodies at PEG terminals**. *Adv.Drug.Del.Rev.*, 24:235-242.

11 Lee, R.J. and Low, P.S. (1997): **Folate-targeted liposomes for drug delivery**. *J.Liposome Res.*, 7:455-466.

12 Kikuchi, A., Sugaya, S., Ueda, H., Tanaka, K., Aramaki, Y., Hara, T., Arima, H., Tsuchiya, S., and Fuwa, T. (1996): **Efficient gene transfer to EGF receptor overexpressing cancer cells by means of EGF-labeled cationic liposomes**. *Biochem.Biophys.Res.Commun.*, 227:666-671.

13 Ishii, Y., Aramaki, Y., Hara, T., Tsuchiya, S., and Fuwa, T. (1989): **Preparation of EGF labeled liposomes and their uptake by hepatocytes**. *Biochem.Biophys.Res.Commun.*, 160:732-736.

14 Gyongyossy-Issa, M.I., Muller, W., and Devine, D.V. (1998): **The covalent coupling of Arg-Gly-Asp-containing peptides to liposomes: purification and biochemical function of the lipopeptide**. *Arch.Biochem.Biophys.*, 353:101-108.

15 Oku, N., Tokudome, Y., Koike, C., Nishikawa, N., Mori, H., Saiki, I., and Okada, S. (1996): **Liposomal Arg-Gly-Asp analogs effectively inhibit metastatic B16 melanoma colonization in murine lungs**. *Life Sci.*, 58:2263-2270.

16 Willis, M.C., Collins, B., Zhang, T., Green, L.S., Sebesta, D.P., Bell, C., Kellogg, E., Gill, S.C., Magallanez, A., Knauer, S., Bendele, R.A., Gill, P.S., and Janjic, N. (1998): **Liposome-anchored vascular endothelial growth factor aptamers**. *Bioconjug.Chem.*, 9:573-582.

17 Torchilin, V.P., Khaw, B.A., Smirnov, V.N., and Haber, E. (1979): **Preservation of antimyosin antibody activity after covalent coupling to liposomes**. *Biochem.Biophys.Res.Commun.*, 89:1114-1119.

18 Martin, F.J., Hubbell, W.L., and Papahadjopoulos, D. (1981): **Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds**. *Biochemistry,* 20:4229-4238.

19 Martin, F.J. and Papahadjopoulos, D. (1982): **Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting**. *J.Biol.Chem.*, 257:286-288.

20 Jones, M.N. and Nicholas, A.R. (1991): **The effect of blood serum on the size and stability of phospholipid liposomes**. *Biochim.Biophys.Acta*, 1065:145-152.

21 Schenkman, S., Araujo, P.S., Dijkman, R., Quina, F.H., and Chaimovich, H. (1981): **Effects of temperature and lipid composition on the serum albumin- induced aggregation and fusion of small unilamellar vesicles**. *Biochim.Biophys.Acta*, 649:633-647.

22 Hernandez-Caselles, T., Villalain, J., and Gomez-Fernandez, J.C. (1993): **Influence of liposome charge and composition on their interaction with human blood serum proteins**. *Mol.Cell Biochem.*, 120:119-126.

23 Strejan, G.H., Essani, K., and Surlan, D. (1981): **Naturally occurring antibodies to liposomes. II. Specificity and electrophoretic pattern of rabbit antibodies reacting with sphingomyelin-containing liposomes**. *J.Immunol.*, 127:160-165.

24 Szebeni, J., Wassef, N.M., Rudolph, A.S., and Alving, C.R. (1996): **Complement activation in human serum by liposome-encapsulated hemoglobin: the role of natural anti-phospholipid antibodies**. *Biochim.Biophys.Acta*, 1285:127-130.

25 Derksen, J.T., Morselt, H.W., and Scherphof, G.L. (1988): **Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages**. *Biochim.Biophys.Acta*, 971:127-136.

26 Dijkstra, J., Van Galen, M., and Scherphof, G. (1985): **Influence of liposome charge on the association of liposomes with Kupffer cells *in vitro*. Effects of divalent cations and competition with latex particles**. *Biochim.Biophys.Acta*, 813:287-297.

27 Aragnol, D. and Leserman, L.D. (1986): **Immune clearance of liposomes inhibited by an anti-Fc receptor antibody *in vivo***. *Proc.Natl.Acad.Sci.U.S.A.*, 83:2699-2703.

28 Wallace, P.K., Howell, A.L., and Fanger, M.W. (1994): **Role of Fc gamma receptors in cancer and infectious disease**. *J.Leukoc.Biol.*, 55:816-826.

29 Betageri, G.V., Black, C.D., Szebeni, J., Wahl, L.M., and Weinstein, J.N. (1993): **Fc-receptor-mediated targeting of antibody-bearing liposomes containing dideoxycytidine triphosphate to human monocyte/macrophages**. *J.Pharm.Pharmacol.*, 45:48-53.

30 Torchilin, V.P. (1998): **Polymer-coated long-circulating microparticulate pharmaceuticals**. *J.Microencapsul.*, 15:1-19.

31 Storm, G., Belliot, S.O., Daemen, T., and Lasic, D.D. (1995): **Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system**. *Adv.Drug.Del.Rev.*, 17:31-48.

32 Alving, C.R. and Wassef, N.M. (1992): **Complement-dependent phagocytosis of liposomes: suppression by "stealth" lipids**. *J.Liposome Res.*, 2:383-395.

33 Chonn, A. and Cullis, P.R. (1992): **Ganglioside GM, and hydrophylic polymers increase liposome circulation times by inhibiting the association of blood proteins**. *J.Liposome Res.*, 2:397-410.

34 Blume, G., Cevc, G., Crommelin, D.J.A., Bakker-Woudenberg, I.A., Kluft, C., and Storm, G. (1993): **Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times**. *Biochim.Biophys.Acta*, 1149:180-184.

35 Huwyler, J., Yang, J., and Pardridge, W.M. (1997): **Receptor mediated delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat**. *J.Pharmacol.Exp.Ther.*, 282:1541-1546.

36 Maruyama, K., Takahashi, N., Tagawa, T., Nagaike, K., and Iwatsuru, M. (1997): **Immunoliposomes bearing polyethyleneglycol-coupled Fab' fragment show prolonged circulation time and high extravasation into targeted solid tumors *in vivo***. *FEBS Lett.*, 413:177-180.

37 Phillips, N.C. and Dahman, J. (1995): **Immunogenicity of immunoliposomes: reactivity against species-specific IgG and liposomal phospholipids**. *Immunol.Lett.*, 45:149-152.

38 Harding, J.A., Engbers, C.M., Newman, M.S., Goldstein, N.I., and Zalipsky, S. (1997): **Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)- grafted immunoliposomes**. *Biochim.Biophys.Acta*, 1327:181-192.

39 Dvorak, H.F., Nagy, J.A., Dvorak, J.T., and Dvorak, A.M. (1988): **Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules**. *Am.J.Pathol.*, 133:95-109.

40 Dvorak, H.F. (1990): **Leaky tumor vessels: consequences for tumor stroma generation and for solid tumor therapy**. *Prog.Clin.Biol.Res.*, 354A:317-30:317-330.

41 Wu, N.Z., Braun, R.D., Gaber, M.H., Lin, G.M., Ong, E.T., Shan, S., Papahadjopoulos, D., and Dewhirst, M.W. (1997): **Simultaneous measurement of liposome extravasation and content release in tumors**. *Microcirculation*, 4:83-101.

42 Yuan, F., Leunig, M., Huang, S.K., Berk, D.A., Papahadjopoulos, D., and Jain, R.K. (1994): **Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft**. *Cancer Res.*, 54:3352-3356.

43 Wu, N.Z., Da, D., Rudoll, T.L., Needham, D., Whorton, A.R., and Dewhirst, M.W. (1993): **Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumor tissue**. *Cancer Res.*, 53:3765-3770.

44 Huang, S.K., Martin, F.J., Jay, G., Vogel, J., Papahadjopoulos, D., and Friend, D.S. (1993): **Extravasation and transcytosis of liposomes in Kaposi's sarcoma-like dermal lesions of transgenic mice bearing the HIV tat gene**. *Am.J.Pathol.*, 143:10-14.

45 Brown, J.M. and Giaccia, A.J. (1998): **The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy**. *Cancer Res.*, 58:1408-1416.

46 Jain, R.K. (1998): **Delivery of molecular and cellular medicine to solid tumors**. *J.Contol.Release*, 53:49-67.

47 Mayer, L.D., Dougherty, G., Harasym, T.O., and Bally, M.B. (1997): **The role of tumor-associated macrophages in the delivery of liposomal doxorubicin to solid murine fibrosarcoma tumors**. *J.Pharmacol.Exp.Ther.*, 280:1406-1414.

48 Park, J.W., Hong, K., Kirpotin, D.B., Meyer, O., Papahadjopoulos, D., and Benz, C.C. (1997): **Anti-HER2 immunoliposomes for targeted therapy of human tumors**. *Cancer Lett.*, 118:153-160.

49 Huang, S.K., Lee, K.D., Hong, K., Friend, D.S., and Papahadjopoulos, D. (1992): **Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice**. *Cancer Res.*, 52:5135-5143.

50 Scherphof, G.L., Kamps, J.A.A.M., and Koning, G.A. (1997): **In vivo targeting of surface-modified liposomes to metastatically growing colon carcinoma cells and sinusoidal endothelial cells in the rat liver**. *J.Liposome Res.*, 7:419-432.

51 Kirpotin, D.B., Park, J.W., Hong, K., Shao, Y., Colbern, G., Zheng, W.W., Meyer, O., Benz, C.C., and Papahadjopoulos, D. (1998): Targeting of sterically stabilized liposomes to cancers overexpressing HER2/neu proto-oncogene. In: **Medical Applications of Liposomes**, edited by D.D. Lasic, et al, pp. 325-345. Elsevier, Amsterdam.

52 Weinstein, J.N., Eger, R.R., Covell, D.G., Black, C.D., Mulshine, J., Carrasquillo, J.A., Larson, S.M., and Keenan, A.M. (1987): **The pharmacology of monoclonal antibodies**. *Ann.N.Y.Acad.Sci.*, 507:199-210:199-210.

53 Allen, T.M., Ahmad, I., Lopes de Menezes, D.E., and Moase, E.H. (1995): **Immunoliposome-mediated targeting of anti-cancer drugs *in vivo***. *Biochem.Soc.Trans.*, 23:1073-1079.

54 Allen, T.M. (1997): **Liposome targeting in animal models: problems and opportunities**. *J.Liposome Res.*, 7:315-329.

55 Sedmak, D.D., Davis, D.H., Singh, U., van de Winkel, J.G., and Anderson, C.L. (1991): **Expression of IgG Fc receptor antigens in placenta and on endothelial cells in humans. An immunohistochemical study**. *Am.J.Pathol.*, 138:175-181.

56 Bengualid, V., Hatcher, V.B., Diamond, B., Blumberg, E.A., and Lowy, F.D. (1990): **Staphylococcus aureus infection of human endothelial cells potentiates Fc receptor expression**. *J.Immunol.*, 145:4279-4283.

57 Holland, J.W., Hui, C., Cullis, P.R., and Madden, T.D. (1996): **Poly(ethylene glycol)—lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine**. *Biochemistry*, 35:2618-2624.

58 Suzuki, S., Watanabe, S., Masuko, T., and Hashimoto, Y. (1995): **Preparation of long-circulating immunoliposomes containing adriamycin by a novel method to coat immunoliposomes with poly(ethylene glycol)**. *Biochim.Biophys.Acta*, 1245:9-16.

59 Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. (1997): **Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro***. *Biochemistry*, 36:66-75.

60 Maruyama, K., Mori, A., Bhadra, S., Subbiah, M.T., and Huang, L. (1991): **Proteins and peptides bound to long-circulating liposomes**. *Biochim.Biophys.Acta*, 1070:246-252.

61 Mori, A., Klibanov, A.L., Torchilin, V.P., and Huang, L. (1991): **Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes *in vivo***. *FEBS Lett.*, 284:263-266.

62 Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P., and Huang, L. (1991): **Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target**. *Biochim.Biophys.Acta*, 1062:142-148.

63 Torchilin, V.P., Narula, J., Halpern, E., and Khaw, B.A. (1996): **Poly(ethylene glycol)-coated anti-cardiac myosin immunoliposomes: factors influencing targeted accumulation in the infarcted myocardium**. *Biochim.Biophys.Acta*, 1279:75-83.

64 Maruyama, K., Takizawa, T., Yuda, T., Kennel, S.J., Huang, L., and Iwatsuru, M. (1995): **Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies**. *Biochim.Biophys.Acta*, 1234:74-80.

65 Allen, T.M., Hansen, C.B., and Stuart, D.D. (1998): Targeted sterically stabilized liposomal drug delivery. In: **Medical Applications of Liposomes,** edited by D.D. Lasic, et al, pp. 297-323. Elsevier, Amsterdam.

66 Thierry, A.R., Vige, D., Coughlin, S.S., Belli, J.A., Dritschilo, A., and Rahman, A. (1993): **Modulation of doxorubicin resistance in multidrug-resistant cells by liposomes**. *FASEB J.*, 7:572-579.

67 Sadasivan, R., Morgan, R., Fabian, C., and Stephens, R. (1991): **Reversal of multidrug resistance in HL-60 cells by verapamil and liposome-encapsulated doxorubicin**. *Cancer Lett.*, 57:165-171.

68 Thierry, A.R., Jorgensen, T.J., Forst, D., Belli, J.A., Dritschilo, A., and Rahman, A. (1989): **Modulation of multidrug resistance in Chinese hamster cells by liposome- encapsulated doxorubicin [published erratum appears in Cancer Commun 1989 Dec;1(6):395]**. *Cancer Commun.*, 1:311-316.

69 Lopes de Menezes, D.E., Pilarski, L.M., and Allen, T.M. (1998): **In vitro and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma**. *Cancer Res.*, 58:3320-3330.

70 Kirpotin, D., Hong, K., Mullah, N., Papahadjopoulos, D., and Zalipsky, S. (1996): **Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted ethylene glycol)**. *FEBS Lett.*, 388:115-118.

71 Pinnaduwage, P. and Huang, L. (1992): **Stable target-sensitive immunoliposomes**. *Biochemistry*, 31:2850-2855.

72 Gaber, M.H., Wu, N.Z., Hong, K., Huang, S.K., Dewhirst, M.W., and Papahadjopoulos, D. (1996): **Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks**. *Int.J.Radiat.Oncol.Biol.Phys.*, 36:1177-1187.

73 van Bree, C., Krooshoop, J.J., Rietbroek, R.C., Kipp, J.B., and Bakker, P.J. (1996): **Hyperthermia enhances tumor uptake and antitumor efficacy of thermostable liposomal daunorubicin in a rat solid tumor**. *Cancer Res.*, 56:563-568.

74 Yatvin, M.B., Tegmo-Larsson, I.M., and Dennis, W.H. (1987): **Temperature- and pH-sensitive liposomes for drug targeting**. *Methods Enzymol.*, 149:77-87:77-87.

75 Sullivan, S.M. and Huang, L. (1986): **Enhanced delivery to target cells by heat-sensitive immunoliposomes**. *Proc.Natl.Acad.Sci.U.S.A.*, 83:6117-6121.

76 Huang, L., Connor, J., and Wang, C.Y. (1987): **pH-sensitive immunoliposomes**. *Methods Enzymol.*, 149:88-99:88-99.

77 Bennett, D.E., Lamparski, H., and O'Brien, D.F. (1994): **Photosensitive Liposomes**. *J.Liposome Res.*, 4:331-348.

78 Morgan, J., MacRobert, A.J., Gray, A.G., and Huehns, E.R. (1992): **Use of photosensitive, antibody directed liposomes to destroy target populations of cells in bone marrow: a potential purging method for autologous bone marrow transplantation**. *Br.J.Cancer*, 65:58-64.

79 Khoobehi, B., Char, C.A., Peyman, G.A., and Schuele, K.M. (1990): **Study of the mechanisms of laser-induced release of liposome- encapsulated dye**. *Lasers.Surg.Med.*, 10:303-309.

80 Schwendener, R.A. and Schott, H. (1996): **Lipophilic 1-beta-D-arabinofuranosyl cytosine derivatives in liposomal formulations for oral and parenteral antileukemic therapy in the murine L1210 leukemia model**. *J.Cancer Res.Clin.Oncol.*, 122:723-726.

81 Sasaki, H., Kakutani, T., Hashida, M., and Sezaki, H. (1987): **Characterization of alkylcarbamoyl derivatives of 5-fluorouracil and their application to liposome**. *Int.J.Pharm.*, 36:147-156.

82 Schwendener, R.A., Supersaxo, A., Rubas, W., Weder, H.G., Hartmann, H.R., Schott, H., Ziegler, A., and Hengartner, H. (1985): **5'-O-Palmitoyl- and 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine - novel lipophilic analogues of 5'-fluoro-2'-deoxyuridine: synthesis, incorporation into liposomes and preliminary biological results**. *Biochem.Biophys.Res.Commun.*, 126:660-666.

83 van Borssum Waalkes, M., Van Galen, M., Morselt, H.W.M., Sternberg, B., and Scherphof, G.L. (1993): **In-vitro stability and cytostatic activity of liposomal formulations of 5-fluoro-2'-deoxyuridine and its diacylated derivatives**. *Biochim.Biophys.Acta*, 1148:161-172.

84 Kinsky, S.C. and Loader, J.E. (1987): **Circumvention of the methotrexate transport system by methotrexate-phosphatidylethanolamine derivatives: effect of fatty acid chain length**. *Biochim.Biophys.Acta*, 921:96-103.

85 Mori, A., Kennel, S.J., van Borssum Waalkes, M., Scherphof, G.L., and Huang, L. (1995): **Characterization of organ-specific immunoliposomes for delivery of 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine in a mouse lung- metastasis model**. *Cancer Chemother.Pharmacol.*, 35:447-456.

86 Crosasso, P., Brusa, P., Dosio, F., Arpicco, S., Pacchioni, D., Schuber, F., and Cattel, L. (1997): **Antitumoral activity of liposomes and immunoliposomes containing 5- fluorouridine prodrugs**. *J.Pharm.Sci.*, 86:832-839.

87 Koning, G.A., Gorter, A., Scherphof, G.L., and Kamps, J.A.A.M. (1999): **Antiproliferative effect of immunoliposomes containing 5-fluorodeoxyuridine-dipalmitate on colon cancer cells**. *Br.J.Cancer*, 80:1718-1725.

88 van Borssum Waalkes, M., Fichtner, I., Dontje, B., Lemm, M., Becker, M., Arndt, D., and Scherphof, G.L. (1992): **In vivo distribution and antitumour activity of liposomal 3',5'- O-dipalmitoyl-5-fluoro-2'-deoxyuridine**. *J.Microencapsul.*, 9:335-346.

89 Koning, G.A., Morselt, H.W.M., velinova, M.J., Donga, J., Gorter, A., Allen, T.M., Zalipsky, S., Kamps, J.A.A.M., and Scherphof, G.L. (1999): **Selective transfer of a lipophilic prodrug of 5-fluorodeoxyuridine (FUdR) from immunoliposomes to CC531 colon cancer cells**. *Biochim.Biophys.Acta*, 1420:153-167.

90 Sarti, P., Ginobbi, P., D'Agostino, I., Arancia, G., Lendaro, E., Molinari, A., Ippoliti, R., and Citro, G. (1996): **Liposomal targeting of leukaemia HL60 cells induced by transferrin- receptor endocytosis**. *Biotechnol.Appl.Biochem.*, 24:269-276.

91 Lee, R.J., Wang, S., Turk, M.J., and Low, P.S. (1998): **The effects of pH and intraliposomal buffer strength on the rate of liposome content release and intracellular drug delivery**. *Biosci.Rep.*, 18:69-78.

92 Storm, G., Regts, J., Beynen, F.J., and Roerdink, F.H. (1989): **Processing of doxorubicin-containing liposomes by liver macrophages *in vitro***. *J.Liposome Res.*, 1:195-210.

93 Straubinger, R.M., Hong, K., Friend, D.S., and Papahadjopoulos, D. (1983): **Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles**. *Cell*, 32:1069-1079.

94 Slepushkin, V.A., Simoes, S., Dazin, P., Newman, M.S., Guo, L.S., Pedroso de Lima, M.C., and Duzgunes, N. (1997): **Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation *in vivo***. *J.Biol.Chem.*, 272:2382-2388.

95 Connor, J., Yatvin, M.B., and Huang, L. (1984): **pH-sensitive liposomes: acid-induced liposome fusion**. *Proc.Natl.Acad.Sci.U.S.A.*, 81:1715-1718.

96 Wang, C.Y. and Huang, L. (1987): **pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse**. *Proc.Natl.Acad.Sci.U.S.A.*, 84:7851-7855.

97 Lutwyche, P., Cordeiro, C., Wiseman, D.J., St-Louis, M., Uh, M., Hope, M.J., Webb, M.S., and Finlay, B.B. (1998): **Intracellular Delivery and Antibacterial Activity of Gentamicin Encapsulated in pH-Sensitive Liposomes**. *Antimicrob.Agents Chemother.*, 42:2511-2520.

98 Kono, K., Igawa, T., and Takagishi, T. (1997): **Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative**. *Biochim.Biophys.Acta*, 1325:143-154.

99 Plank, C., Zauner, W., and Wagner, E. **Application of membrane-active peptides for drug and gene delivery across cellular membranes.** *Adv.Drug Del. Reviews* 34(1), 21-35. 1998.

100 Subbarao, N.K., Parente, R.A., Szoka, F.C.J., Nadasdi, L., and Pongracz, K. (1987): **pH-dependent bilayer destabilization by an amphipathic peptide**. *Biochemistry*, 26:2964-2972.

101 Puyal, C., Maurin, L., Miquel, G., Bienvenue, A., and Philippot, J. (1994): **Design of a short membrane-destabilizing peptide covalently bound to liposomes**. *Biochim.Biophys.Acta*, 1195:259-266.

102 Pecheur, E.I., Hoekstra, D., Sainte-Marie, J., Maurin, L., Bienvenue, A., and Philippot, J.R. (1997): **Membrane anchorage brings about fusogenic properties in a short synthetic peptide**. *Biochemistry*, 36:3773-3781.

103 Pecheur, E.I., Martin, I., Ruysschaert, J.M., Bienvenue, A., and Hoekstra, D. (1998): **Membrane fusion induced by 11-mer anionic and cationic peptides: a structure-function study**. *Biochemistry*, 37:2361-2371.

104 Tseng, Y.L., Hong, R.L., Tao, M.H., and Chang, F.H. (1999): **Sterically stabilized anti-idiotype immunoliposomes improve the therapeutic efficacy of doxorubicin in a murine B-cell lymphoma model**. *Int.J.Cancer*, 80:723-730.

105 Peeters, P.A., Oussoren, C., Eling, W.M., and Crommelin, D.J. (1988): **Immunospecific targeting of immunoliposomes, F(ab')2 and IgG to red blood cells *in vivo***. *Biochim.Biophys.Acta*, 943:137-147.

106 Peeters, P.A., Claessens, C.A., Eling, W.M., and Crommelin, D.J. (1988): **Immunospecific targeting of liposomes to erythrocytes**. *Biochem.Pharmacol.*, 37:2215-2222.

107 Bankert, R.B., Yokota, S., Ghosh, S.K., Mayhew, E., and Jou, Y.H. (1989): **Immunospecific targeting of cytosine arabinonucleoside- containing liposomes to the idiotype on the surface of a murine B- cell tumor *in vitro* and *in vivo***. *Cancer Res.*, 49:301-308.

108 Maruyama, K. (1997): **Long-circulating immunoliposome targeting in animal models**. *J.Liposome Res.*, 7:363-389.

109 Vingerhoeds, M.H., Steerenberg, P.A., Hendriks, J.J., Dekker, L.C., Van Hoesel, Q.G., Crommelin, D.J.A., and Storm, G. (1996): **Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma *in vitro* and *in vivo***. *Br.J.Cancer*, 74:1023-1029.

110 Straubinger, R.M., Lopez, N.G., Debs, R.J., Hong, K., and Papahadjopoulos, D. (1988): **Liposome-based therapy of human ovarian cancer: parameters determining potency of negatively charged and antibody-targeted liposomes**. *Cancer Res.*, 48:5237-5245.

111 Nässander, U.K., Steerenberg, P.A., Poppe, H., Storm, G., Poels, L.G., De Jong, W.H., and Crommelin, D.J.A. (1992): **In vivo targeting of OV-TL 3 immunoliposomes to ascitic ovarian carcinoma cells (OVCAR-3) in athymic nude mice**. *Cancer Res.*, 52:646-653.

112 Torchilin, V.P. (1985): **Liposomes as targetable drug carriers**. *Crit.Rev.Ther.Drug Carrier.Syst.*, 2:65-115.

113 Ahmad, I., Longenecker, M., Samuel, J., and Allen, T.M. (1993): **Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice**. *Cancer Res.*, 53:1484-1488.

114 Kirpotin, D.B., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. (1997): **Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro***. *Biochemistry*, 36:66-75.

115 Park, J.W., Hong, K., Carter, P., Asgari, H., Guo, L.Y., Keller, G.A., Wirth, C., Shalaby, R., Kotts, C., and Wood, W.I. (1995): **Development of anti-p185HER2 immunoliposomes for cancer therapy**. *Proc.Natl.Acad.Sci.U.S.A.*, 92:1327-1331.

116 Goren, D., Horowitz, A.T., Zalipsky, S., Woodle, M.C., Yarden, Y., and Gabizon, A. (1996): **Targeting of stealth liposomes to erbB-2 (Her/2) receptor: *in vitro* and *in vivo* studies**. *Br.J.Cancer*, 74:1749-1756.

117 Frodin, J.E., Lefvert, A.K., and Mellstedt, H. (1992): **The clinical significance of HAMA in patients treated with mouse monoclonal antibodies**. *Cell Biophys.*, 21:153-165.

118 Gorman, S.D. and Clark, M.R. (1990): **Humanisation of monoclonal antibodies for therapy**. *Semin.Immunol.*, 2:457-466.

119 Winter, G. and Harris, W.J. (1993): **Humanized antibodies**. *Immunol.Today*, 14:243-246.

120 Lonberg, N. and Huszar, D. (1995): **Human antibodies from transgenic mice**. *Int.Rev.Immunol.*, 13:65-93.

121 Jakobovits, A. (1995): **Production of fully human antibodies by transgenic mice**. *Curr.Opin.Biotechnol.*, 6:561-566.

122 Laukkanen, M.L., Alfthan, K., and Keinanen, K. (1994): **Functional immunoliposomes harboring a biosynthetically lipid-tagged single-chain antibody**. *Biochemistry*, 33:11664-11670.

123 de Kruif, J., Storm, G., van Bloois, L., and Logtenberg, T. (1996): **Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes**. *FEBS Lett.*, 399:232-236.

124 Klug, S.J. and Famulok, M. (1994): **All you wanted to know about SELEX**. *Mol.Biol.Rep.*, 20:97-107.

125 Gold, L., Brown, D., He, Y., Shtatland, T., Singer, B.S., and Wu, Y. (1997): **From oligonucleotide shapes to genomic SELEX: novel biological regulatory loops**. *Proc.Natl.Acad.Sci.U.S.A.*, 94:59-64.

126 Tuerk, C. (1997): **Using the SELEX combinatorial chemistry process to find high affinity nucleic acid ligands to target molecules**. *Methods Mol.Biol.*, 67:219-30:219-230.

127 Wang, S. and Low, P.S. (1998): **Folate-mediated targeting of antineoplastic drags, imaging agents, and nucleic acids to cancer cells**. *J.Contol.Release*, 53:39-48.

128 Lee, R.J. and Low, P.S. (1994): **Delivery of liposomes into cultured KB cells via folate receptor- mediated endocytosis**. *J.Biol.Chem.*, 269:3198-3204.

129 Lee, R.J. and Low, P.S. (1995): **Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro***. *Biochim.Biophys.Acta*, 1233:134-144.

130 Chelvi, T.P. and Ralhan, R. (1995): **Designing of thermosensitive liposomes from natural lipids for multimodality cancer therapy**. *Int.J.Hyperthermia*, 11:685-695.

131 Yatvin, M.B., Cree, T.C., Tegmo-Larsson, I.M., and Gipp, J.J. (1984): **Liposomes as drug carriers in cancer therapy: hyperthermia and pH sensitivity as modalities for targeting**. *Strahlentherapie.*, 160:732-740.

132 Chelvi, T.P., Jain, S.K., and Ralhan, R. (1995): **Hyperthermia-mediated targeted delivery of thermosensitive liposome-encapsulated melphalan in murine tumors**. *Oncol.Res.*, 7:393-398.

133 Sharma, D., Chelvi, T.P., Kaur, J., and Ralhan, R. (1998): **Thermosensitive liposomal taxol formulation: heat-mediated targeted drug delivery in murine melanoma**. *Melanoma Res.*, 8:240-244.

134 Collins, D. and Huang, L. (1987): **Cytotoxicity of diphtheria toxin A fragment to toxin-resistant murine cells delivered by pH-sensitive immunoliposomes**. *Cancer Res.*, 47:735-739.

135 Connor, J. and Huang, L. (1986): **pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs**. *Cancer Res.*, 46:3431-3435.

136 Connor, J., Norley, N., and Huang, L. (1986): **Biodistribution of pH-sensitive immunoliposomes**. *Biochim.Biophys.Acta*, 884:474-481.

137 Nieva, J.L., Nir, S., Muga, A., Goni, F.M., and Wilschut, J. (1994): **Interaction of the HIV-1 fusion peptide with phospholipid vesicles: different structural requirements for fusion and leakage**. *Biochemistry*, 33:3201-3209.

138 Martin, I. and Ruysschaert, J.M. (1997): **Comparison of lipid vesicle fusion induced by the putative fusion peptide of fertilin (a protein active in sperm-egg fusion) and the NH2- terminal domain of the HIV2 gp41**. *FEBS Lett.*, 405:351-355.

139 Bailey, A.L., Monck, M.A., and Cullis, P.R. (1997): **pH-induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin**. *Biochim.Biophys.Acta*, 1324:232-244.

140 Mechtler, K. and Wagner, E. **Gene transfer mediated by influenza virus peptides: The role of peptide sequences.** 1997. Personal Communication

141 Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994): **The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems**. *J.Biol.Chem.*, 269:12918-12924.

142 Singhal, A. and Gupta, C.M. (1986): **Antibody-mediated targeting of liposomes to red cells *in vivo***. *FEBS Lett.*, 201:321-326.

143 Bailey, A.L. (1994): **Antibody-directed enzyme prodrug therapy (Adept)**. *J.Contol.Release*, 28:187-193.

144 Vingerhoeds, M.H., Haisma, H.J., van Muijen, M., van de Rijt, R.B., Crommelin, D.J.A., and Storm, G. (1993): **A new application for liposomes in cancer therapy. Immunoliposomes bearing enzymes (immuno-enzymosomes) for site- specific activation of prodrugs**. *FEBS Lett.*, 336:485-490.

145 Haisma, H.J., Boven, E., van Muijen, M., de Jong, J., van, d., V, and Pinedo, H.M. (1992): **A monoclonal antibody-beta-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer**. *Br.J.Cancer*, 66:474-478.

146 Vingerhoeds, M.H., Haisma, H.J., Belliot, S.O., Smit, R.H., Crommelin, D.J.A., and Storm, G. (1996): **Immunoliposomes as enzyme-carriers (immuno-enzymosomes) for antibody- directed enzyme prodrug therapy (ADEPT): optimization of prodrug activating capacity**. *Pharm.Res.*, 13:604-610.

147 Bally, M.B., Lim, H., Cullis, P.R., and Mayer, L.D. (1998): **Controlling the drug delivery attributes of lipid-based drug formulations**. *J.Liposome Res.*, 8:299-335.

148 Pak, C.C., Ali, S., Janoff, A.S., and Meers, P. (1998): **Triggerable liposomal fusion by enzyme cleavage of a novel peptide-lipid conjugate**. *Biochim.Biophys.Acta*, 1372:13-27.

149 Brekken, R.A., Huang, X., King, S.W., and Thorpe, P.E. (1998): **Vascular endothelial growth factor as a marker of tumor endothelium**. *Cancer Res.*, 58:1952-1959.

150 Molema, G. and Griffioen, A.W. (1998): **Rocking the foundations of solid tumor growth by attacking the tumor's blood supply**. *Immunol.Today*, 19:392-394.

151 Burrows, F.J. and Thorpe, P.E. (1993): **Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature**. *Proc.Natl.Acad.Sci.U.S.A.* , 90:8996-9000.

152 Arap, W., Pasqualini, R., and Ruoslahti, E. (1998): **Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model**. *Science*, 279:377-380.

153 Huang, X., Molema, G., King, S., Watkins, L., Edgington, T.S., and Thorpe, P.E. (1997): **Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature [see comments]**. *Science*, 275:547-550.

154 Bendas, G., Krause, A., Schmidt, R., Vogel, J., and Rothe, U. (1998): **Selectins as new targets for immunoliposome-mediated drug delivery. A potential way of anti-inflammatory therapy**. *Pharm.Acta Helv.*, 73:19-26.

155 Bloemen, P.G.M., Henricks, P.A.J., van Bloois, L., van den Tweel, M.C., Bloem, A.C., Nijkamp, F.P., Crommelin, D.J.A., and Storm, G. (1995): **Adhesion molecules: a new target for immunoliposome-mediated drug delivery**. *FEBS Lett.*, 357:140-144.

156 Mastrobattista, E., Storm, G., van Bloois, L., Reszka, R., Bloemen, P.G.M., Crommelin, D.J.A., and Henricks, P.A.J. (1999): **Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells**. *Biochim.Biophys.Acta*, 1419:353-363.

157 Spragg, D.D., Alford, D.R., Greferath, R., Larsen, C.E., Lee, K.D., Gurtner, G.C., Cybulsky, M.I., Tosi, P.F., Nicolau, C., and Gimbrone, M.A., Jr. (1997): **Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system**. *Proc.Natl.Acad.Sci.U.S.A.*, 94:8795-8800.

158 Kamps, J.A., Morselt, H.W., Swart, P.J., Meijer, D.K., and Scherphof, G.L. (1998): **Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells**. *Proc.Natl.Acad.Sci.U.S.A.*, 94:11681-11685.

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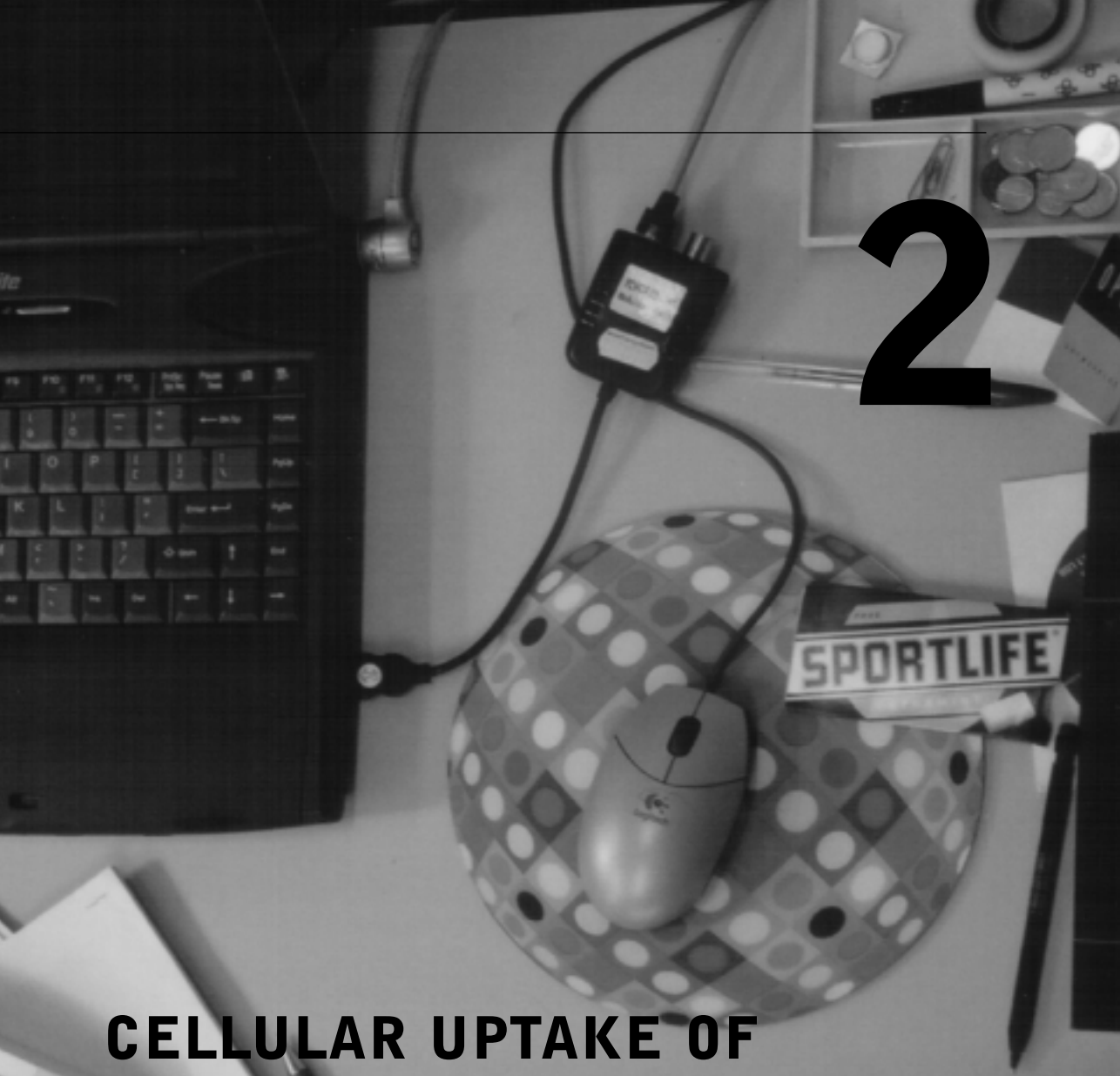

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CELLULAR UPTAKE OF LIPOSOMES TARGETED TO INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) ON BRONCHIAL EPITHELIAL CELLS

CELLULAR UPTAKE OF LIPSOMES TARGETED TO INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) ON BRONCHIAL EPITHELIAL CELLS

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SUMMARY

Previously, it was demonstrated that immunoliposomes, bearing anti-intercellular adhesion molecule-1 (ICAM-1) antibodies (mAb F10.2), can specifically bind to different cell types expressing ICAM-1. In this study, we have quantified the amount of immunoliposomes binding to IFN-γ activated human bronchial epithelial cells (BEAS-2B) *in vitro* and studied the subsequent fate of cell-bound anti-ICAM-1 immunoliposomes. We demonstrate that binding of the immunoliposomes to the epithelial cells is depending on the liposome concentration used. After binding to the cell surface, the anti-ICAM-1 immunoliposomes are rapidly internalized by the epithelial cells. Sixty percent of cell-bound immunoliposomes were internalized by the epithelial cells within one hour of incubation at 37°C. The results indicate that ICAM-1 targeted immunoliposomes may be used as carriers for the intracellular delivery of anti-inflammatory drugs to sites of inflammation characterized by an increased expression of ICAM-1.

INTRODUCTION

Adhesion molecules are cell surface glycoproteins that mediate physical and functional interactions between two cells or between cells and their extracellular matrix. They play an important role in many biological processes as diverse as the complex organization of tissues and organs, the migration of cells during embryogenesis and the regulation of immune responses by mediating the communication between different immune cells and the extravasation of inflammatory cells into inflamed tissues [1-3]. The process of leukocyte extravasation is induced by the local release of pro-inflammatory mediators (vasoactive amines and cytokines) at the site of inflammation. This results in a locally induced or increased expression of a variety of adhesion molecules on endothelial cells, which allows multiple adhesive events with circulating leukocytes to occur. Intercellular adhesion molecule-1 (ICAM-1) is an important adhesion molecule involved in the process of leukocyte adhesion and extravasation. ICAM-1 is an immunoglobulin-like transmembrane glycoprotein that is constitutively expressed at low levels on vascular endothelial cells, epithelial cells and on a subset of leukocytes. Its expression is increased by the pro-inflammatory cytokines interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) or interleukin-1 (IL-1) [4-9]. Numerous studies have demonstrated a locally enhanced ICAM-1 expression in inflammation-related diseases, such as rheumatoid arthritis [10], asthma [11,12], nephritis [13,14], inflammatory dermatosis [15,16], and atherosclerosis [17,18]. The increased expression of ICAM-1 and other cell adhesion molecules at sites of inflammation is under investigation for therapeutic intervention. These molecules may be used as targets for drug carrier systems that can selectively deliver anti-inflammatory compounds to sites of inflammation. In this light, we have previously constructed liposomes bearing antibodies to ICAM-1 [19]. These antibody-containing liposomes (immunoliposomes) were shown to strongly bind to different cell types expressing ICAM-1 on their surface and their binding was positively correlated with the expression level of ICAM-1 on the target cells. After this first report on immunoliposome targeting to cells expressing adhesion molecules, two other reports have appeared confirming the concept of adhesion molecule targeting with immunoliposomes [20,21].

Specific binding of immunoliposomes to their target cells is an important prerequisite to fulfill, but on its own this is not enough for effective drug delivery. As many drugs act at intracellular sites, it is required that immunoliposome-encapsulated drugs are delivered intracellularly. In principle, three routes that may lead to intracellular delivery of encapsulated compounds can be discerned. Firstly, immunoliposomes may, after specific cell binding, release their contents in close proximity of the target cells, with subsequent cellular uptake of released molecules. Secondly, immunoliposomes may fuse with the cell membrane, thereby releasing their contents into the cytosol. Thirdly, cell-bound immunoliposomes may be internalized by the target cells via receptor-mediated endocytosis, followed by the intracellular release of encapsulated agents. Whether immunoliposomes will be internalized or not is dependent on a variety of factors, such as

liposome size [22-25], type of cell, and type of target receptor [26-28]. Targeting of immunoliposomes to receptors with known internalizing capacities (*e.g.* transferrin receptor, low density lipoprotein-receptor, epithelial growth factor receptor) will likely result in internalization of bound immunoliposomes, provided that they are relatively small in size (<0.2 μm) [29-31]. On the other hand, it has been shown that immunoliposomes targeted to non-internalizing cell-surface molecules on tumor cells remain located at the cell surface [32].
Here, we report on the interaction of ICAM-1-directed immunoliposomes with ICAM-1 expressing human bronchial epithelial cells *in vitro*. We have quantified the degree of cell binding of ICAM-1-directed liposomes and we demonstrate that specific binding of these immunoliposomes to ICAM-1 on the bronchial epithelial cell line BEAS-2B triggers rapid internalization of cell bound immunoliposomes. The results are discussed in the context of targeted drug delivery to sites of inflammation, characterized by an increased expression of ICAM-1.

MATERIALS AND METHODS

MATERIALS

Liposome-related materials

Cholesterol (CHOL), *N*-succinimidyl-*S*-acetylthioacetate (SATA), *N*-ethylmaleimide, and calcein were obtained from Sigma Chemical Co. (Rockford, IL, USA). Chloroform and methanol (pro analysi) were obtained from Merck (Darmstadt, Germany), and *N,N*-dimethylformamide (DMF) and hydroxylamine hydrochloride from Janssen Chimica (Geel, Belgium). Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC; Asahi Chemical Industry Co., Ibarakiken, Japan) was prepared as described previously [33]. Egg-phosphatidylglycerol (EPG) was kindly donated by Nattermann GmbH (Cologne, Germany), *N*-[4-(*p*-maleimidophenyl) butyryl]phosphatidyl-ethanolamine (MPB-PE) was synthesized as described before [32,34].

Cell-related materials

Interferon-γ (IFN-γ) was purchased at Boehringer-Mannheim GmbH (Mannheim, Germany), keratinocyte medium (keratynocyte-SFM) and supplements for keratinocyte-SFM, containing recombinant human epithelial growth factor (rhEGF), and bovine pituitary extract came from Gibco. (Grand Island, NY, USA). Bovine serum albumin (fraction V), fluorescein isothiocyanate (FITC)-labeled goat anti-mouse F10.2 IgG (Fab'-specific) and *R*-phycoerythrin (PE)-labeled goat anti-mouse IgG were obtained at Sigma Chemical Company. Anti-ICAM-1 mAb (IgG_1), clone F10.2 was prepared and isolated as described before [35]. Formaldehyde was obtained at Janssen Chimica (Geel, Belgium).

CELL CULTURE

The human bronchial epithelial cell line BEAS-2B obtained from Dr. J.F. Lechner (National Institutes of Health, Bethesda, MD, USA) was cultured in serum-free keratinocyte medium, supplemented with bovine pituary extract (25 µg/ml), recombinant human epidermal growth hormone (rhEGF; 2.5 ng/ml) and gentamicin (50 µg/ml). In order to obtain an enhanced expression of ICAM-1, BEAS-2B cells were stimulated with IFN-γ (200 U/ml) 24 hrs prior to use in immunoliposome binding and internalization experiments [7]. BEAS-2B cells were cultured at 37°C with 5% CO_2 in humidified air.

PREPARATION OF IMMUNOLIPOSOMES

Immunoliposomes, bearing covalently coupled F10.2 mAbs on their surface, were prepared as described previously [19]. In short, liposomes were made from PHEPC, EPG, CHOL, and MPB-PE (38.5:4:16:0.06 molar ratio) by lipid film hydration and subsequent extrusion through 0.2 µm polycarbonate filters [36]. When indicated, calcein was incorporated as an aqueous marker at a concentration of 90 mM and/or rhodamine-PE at an amount of 0.1 mol% of total lipid. Freshly thiolated mAb F10.2 (100 µg/ml final concentration) was added to the liposomes (73.3 µmol phospholipid/8 ml) and incubated for 1 h and 45 min at room temperature under constant rotation. The coupling reaction was terminated by adding 50 µl of freshly prepared 8 mM *N*-ethylmaleimide in HEPES buffer (10 mM HEPES, 1 mM EDTA, 135 mM NaCl, pH 7.4). Liposomes were separated from unconjugated mAbs by ultracentrifugation (4 runs of 30 min at 60,000xg) and stored at 4°C. As a control in binding and internalization experiments, liposomes with the above mentioned composition were used without conjugated F10.2 mAb.

LIPOSOME CHARACTERIZATION

Mean particle size was determined by dynamic light scattering as described before [19]. Mean size of liposomes was 0.2-0.25 µm with a polydispersity index varying from 0.06 to 0.15. Phospholipid concentration was determined by the colorimetric method of Fiske and Subbarow [37]. The amount of conjugated IgG on the liposomal surface was determined with the Biorad *DC* protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with mouse IgG as standard. The protein coupling ratios for the different batches of immunoliposomes were in the range of 2-4 µg IgG/µmol phospholipid, which corresponds with approximately 7-15 IgG molecules per liposome.

QUANTIFICATION OF THE DEGREE OF CELL BINDING

IFN-γ-activated BEAS-2B cells were detached from culture flasks with trypsin/EDTA (Gibco) in phosphate buffered salt solution (PBS, pH 7.4) for 5 min at 37°C, pelleted by centrifugation (5 min at 750xg) and washed once with immunofluorescence (IF)-buffer (1% bovine serum albumin and 0.05% sodium azide in PBS, pH 7.4). A typical washing step involved resuspension of cells into 1 ml of indicated buffer, followed by centrifugation (5 min at 750xg). Cells ($2.5x10^5$ cells for each sample) were resuspended in 300 µl of IF-buffer containing varying concentrations of control liposomes or F10.2 immunoliposomes with entrapped calcein as fluorescent label. Cells were incubated in the presence of liposomes for 1 h on ice, washed three times with IF-buffer to remove unbound liposomes and resuspended in 300 µl IF-buffer before a sample of 50 µl was taken (corresponding with $2.5x10^4$ cells). Samples were diluted with 150 µl of 1% Triton X-100 in PBS and incubated for 15 min at room temperature. Solubilized samples were transferred to a 96 well-plate and the fluorescence intensity of the sample was measured with a LS-50B luminescence spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, England) with excitation wavelength set at 490 nm and emission at 520 nm. A reference curve was constructed from calcein-containing F10.2 immunoliposomes with a known phospholipid concentration. Background value was obtained from solubilized cells without prior incubation with liposomes.

CELLULAR INTERNALIZATION ASSAYS

Determination of the amount of cell-surface bound immunoliposomes as a function of incubation time

Cell surface-bound F10.2 immunoliposomes were determined by indirect immunofluorescence analysis as described by Suzuki et al. [38]. In short, IFN-γ-activated BEAS-2B cells ($2x10^5$) were detached from culture flasks using trypsin/EDTA and $2x10^5$ cells were incubated with F10.2 immunoliposomes (0.5 µmol phospholipid/ml) in a total volume of 200 µl keratinocyte medium for 60 min on ice. Cells were washed twice with ice-cold keratinocyte medium, resuspended into 300 µl medium and cultured for 0-3 hrs at 37°C. Cells were washed once with IF-buffer and incubated with goat anti-mouse IgG-fluorescently labeled with phycoerythrin (100-fold diluted in IF-buffer) for 30 min on ice. After washing twice with IF-buffer, cells were analyzed by flow cytometry using a FACscan flow cytometer (Becton&Dickinson, Mountain View, CA). The procedure above was also performed on formaldehyde-fixed cells (cells were fixed by incubating with 2% formaldehyde in PBS for 10 min at room temperature).

Determination of intracellular calcein

Detached IFN-γ stimulated BEAS-2B cells were incubated with calcein containing F10.2 immunoliposomes. After two washing steps with ice-cold keratinocyte medium to remove the

unbound liposomes, cells were cultured at 37°C for varying time periods (0-2 hrs). Hereafter, cells were incubated for 10 min in citric acid buffer (40 mM citric acid, 120 mM NaCl, pH 3.0), washed twice with IF-buffer and analyzed by flow cytometry using a FACscan flow cytometer for cell-associated calcein fluorescence. The mean fluorescence intensity of 10,000 cells was determined for each sample.

Confocal Laser Microscopy

In these experiments, the F10.2 immunoliposomes contained encapsulated calcein (90 mM) as a fluorescent aqueous content marker and were labeled with rhodamine-PE as a liposomal lipid marker. Detached, IFN-γ stimulated BEAS-2B cells were mixed with F10.2 immunoliposomes in keratinocyte medium on ice as described above. Cells were washed once with IF-buffer and once with keratinocyte SFM medium and resuspended into 300 µl medium. Subsequently, cells were incubated for varying time periods (0-60 min) at 37°C. Thereafter, cells were either washed twice with IF-buffer or 3 times with citric acid buffer (pH 3.0) and once with IF-buffer. Cells were then fixed with 2% formaldehyde in PBS for 1 h on ice and fixed to slides. Fixed cells were analyzed for fluorescence using a laser confocal scanning microscope (Leica TCS NT Laser confocal scanning microscope, Heidelberg GmbH, Germany). By using filters of 525/550 nm and >590 nm, rhodamine and calcein fluorescence could be detected separately.

RESULTS AND DISCUSSION

QUANTIFICATION OF THE DEGREE OF CELL BINDING

In a previous study, we have demonstrated that F10.2 immunoliposomes are able to specifically bind to different cell types expressing ICAM-1. The degree of cell binding appeared to be positively correlated with the degree of ICAM-1 expression [19]. In this study, we have quantified the degree of binding of F10.2 immunoliposomes to IFN-γ-activated human bronchial epithelial cells (BEAS-2B). The activated epithelial cells were used as a model system to study targeting efficiency of F10.2 immunoliposomes to cells with an increased expression of ICAM-1 as found at sites of inflammation. The degree of binding of F10.2 immunoliposomes to IFN-γ-stimulated BEAS-2B cells was fluorometrically determined by analyzing cell-associated calcein fluorescence after cells were incubated with calcein-containing F10.2 immunoliposomes for 1 h on ice. Figure 1 shows that the absolute amount of cell-bound immunoliposomes increased with increasing concentrations of calcein-containing F10.2 immunoliposomes. Control liposomes with the same lipid composition but without conjugated F10.2 mAbs showed a much lower degree of cell binding. From the calcein fluorescence values, the number of bound liposomes per cell can be estimated, assuming an average of 1.5 phospholipid bilayers per liposome and an average size of

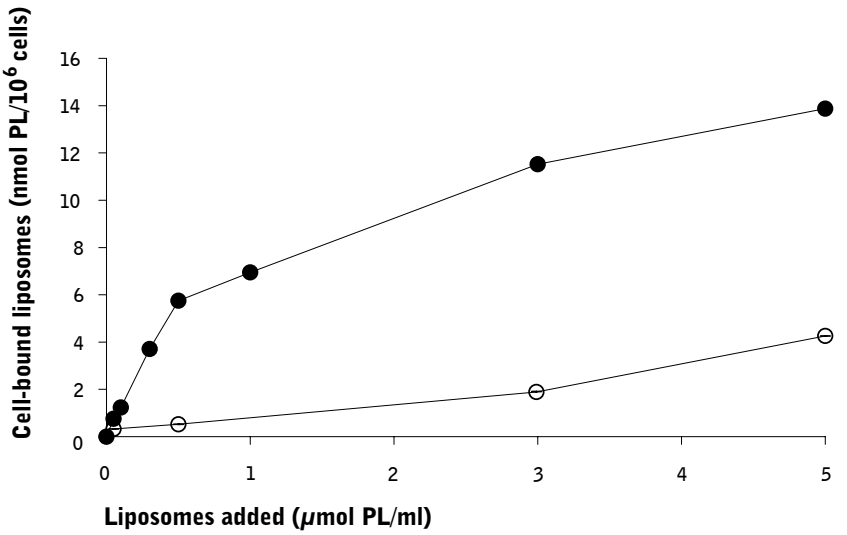

Figure 1 — Effect of liposome concentration on the extent of liposome binding to BEAS-2B cells. INF-γ stimulated BEAS-2B cells (1x10⁶ cell/ml) were incubated with F10.2 immunoliposomes ● or with control liposomes ❍ for 1 h on ice. After removal of unbound liposomes, cell-associated calcein-fluorescence was determined with a fluorometer (n=3).

200 nm (Figure 2). At the highest concentration of F10.2 immunoliposomes used, approximately 15,400 liposome particles are estimated to be bound per cell. However, at such high concentrations, liposomes without conjugated F10.2 mAbs also show considerable cell binding (4,200 liposomes bound per cell), indicating that at high liposome concentrations part of the cell binding is mediated by other interactions than antibody-antigen interaction. Therefore, the experiments described below were carried out with immunoliposome concentrations of 300-500 nmol phospholipid/ml, at which the binding ratio of F10.2 immunoliposome versus control liposomes is maximal. At these concentrations, approximately 4,000-7,000 F10.2 immunoliposomal particles were binding per cell as compared to only 200-300 particles in case of control liposomes.

DETERMINATION OF THE AMOUNT OF CELL SURFACE-BOUND F10.2-IMMUNOLIPOSOMES AS A FUNCTION OF INCUBATION TIME

Cell surface-bound F10.2 immunoliposomes were determined by indirect immunofluorescence analysis as a function of incubation time at 37°C. Cells were incubated with F10.2 immunoliposomes for 1 h on ice and after removal of unbound particles, the cells were cultured

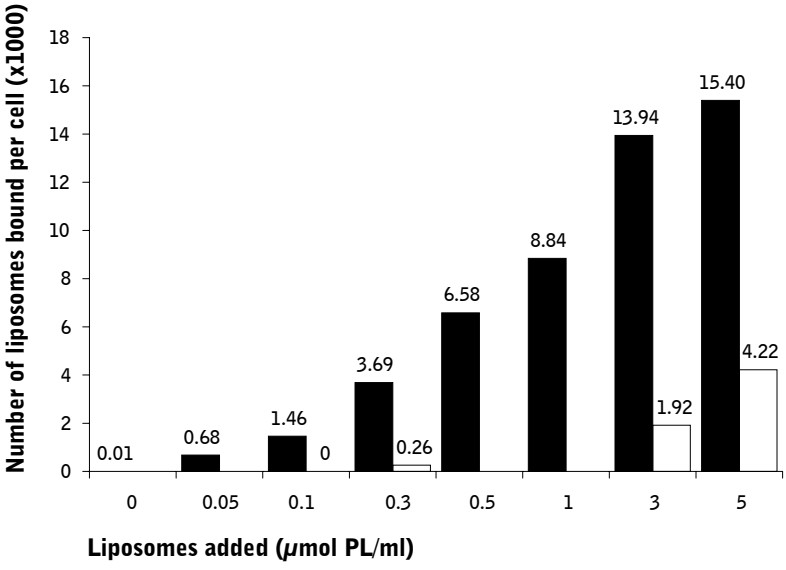

Figure 2 — Effect of liposome concentration on the number of liposomes bound per cell. IFN-γ-stimulated BEAS-2B cells were incubated with F10.2 immunoliposomes (black bars) or with control liposomes (white bars) essentially as described in Figure 1. The exact values of the number of bound liposomes are depicted above each bar. Results of a typical experiment are shown.

at 37°C for varying time periods before analysis by flow cytometry. The amount of immunoliposomes that could be detected on the surface of BEAS-2B cells with FITC-conjugated antibodies directed against the liposome conjugated antibodies decreased during prolonged incubation times at 37°C (Figure 3). After 1 h incubation at 37°C, 60% of the starting amount of cell-bound liposomes had disappeared from the cell surface. Control experiments using cells fixed with formaldehyde prior to liposome binding did not show any reduction in the amount of cell-bound F10.2 immunoliposomes, indicating that the decrease of cell surface-bound immunoliposomes is not caused by dissociation of immunoliposomes from the cell surface, but is related to an active cellular process. This finding strongly suggested that F10.2 immunoliposomes are internalized by BEAS-2B cells.

CALCEIN RELEASE FROM F10.2 IMMUNOLIPOSOMES DURING CELLULAR INTERNALIZATION

The next step was to look at the fate of the encapsulated fluorescent marker calcein upon internalization of F10.2 immunoliposomes by BEAS-2B cells. Cells were incubated with F10.2 immunoliposomes for 1 h on ice and unbound particles were washed away. Hereafter, cell-

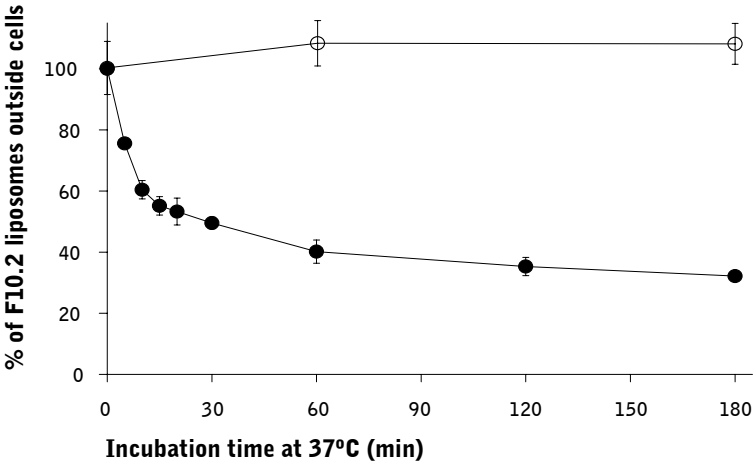

Figure 3 —Determination of cell surface-bound F10.2 immunoliposomes on BEAS-2B cells during prolonged incubation periods at 37°C. Intact (●) or formaldehyde-fixed (❍) cells (1x10⁶/ml) were incubated with F10.2 immunoliposomes (500 nmol phospholipid/ml) for 1 h on ice, washed twice with keratinocyte SFM medium (without serum), and then cultured at 37°C in SFM medium for the indicated time periods. Subsequently, cells were then labeled with phycoerythrin-conjugated goat anti-mouse mAbs and analyzed by flow cytometry (n=4).

associated calcein-fluorescence was determined by flow cytometry, either directly or after 1 h incubation at 37°C (Figure 4). The results show that after 1 h incubation at 37°C, the amount of cell-associated calcein-fluorescence had increased in comparison with the cell-associated calcein-fluorescence before the 1 h incubation at 37°C. As calcein is encapsulated in the liposomes at a quenching concentration (90 mM), an increase of calcein fluorescence intensity can be explained as a dequenching effect due to calcein release from the liposomes.

In order to demonstrate that dequenching of calcein fluorescence is caused by the intracellular release of calcein from internalized F10.2 immunoliposomes and not merely by extracellular leakage of encapsulated calcein from cell-bound F10.2 immunoliposomes, an attempt was made to remove the cell-bound immunoliposomes by short exposure to low pH (10 min at pH 3.0). It is described in literature that such exposure to low pH will liberate immunoliposomes from the cell surface, without disrupting the cell membrane [39,40]. However, confocal laser microscopic analysis showed us that acidic exposure of BEAS-2B cells does not result in the detachment of bound F10.2 immunoliposomes from the cell surface, but rather in release of calcein from the cell-bound liposomes (Figure 6B,D). Nevertheless, acidic treatment of cells can be used to discriminate between immunoliposome-encapsulated calcein located at the cell-surface from internalized immunoliposome-encapsulated calcein as this method completely removes immunoliposome-encapsulated calcein-fluorescence located at the cell surface. The acid

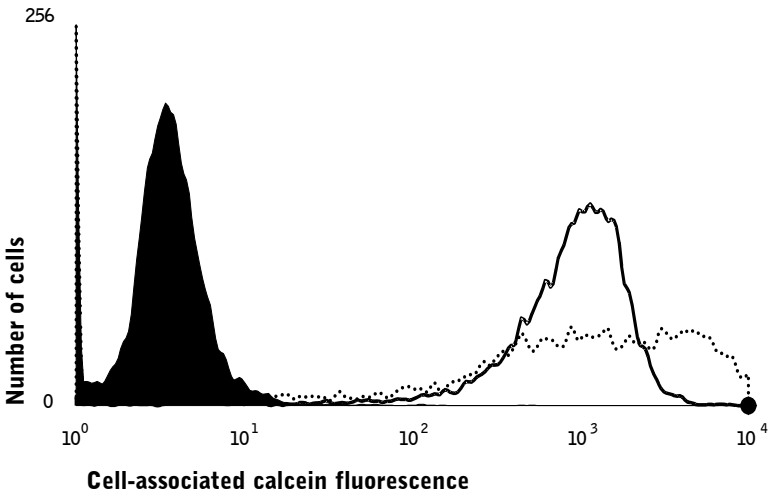

Figure 4 — Flow cytometry analysis of cell-associated calcein fluorescence. BEAS-2B cells were incubated with F10.2 immunoliposomes (500 nmol phospholipid/ml) on ice and unbound liposomes were removed. Thereafter cells were either directly analyzed for associated calcein fluorescence (black line) or after 1 h incubation at 37°C (dotted line). Autofluorescence of BEAS-2B cells are also illustrated (solid filled histogram).

treatment method was used to determine the degree of intracellular calcein-fluorescence as a function of incubation time at 37°C (Figure 5). For this experiment, cells were pre-incubated with F10.2 immunoliposomes for 1 h on ice to load cells with immunoliposomes. Hereafter, cells were cultured at 37°C for varying time periods followed by acid-treatment to remove the calcein from immunoliposomes located at the cell-surface. Figure 5 shows that the amount of intracellular calcein fluorescence increases at increasing incubation times at 37°C. The observation that cell associated calcein fluorescence decreases after 2 hrs incubation at 37°C may be caused by intracellular compounds that are able to quench the calcein fluorescence. For example, Fe^{2+} ions present in the cell will quench calcein fluorescence [41]. However, the possibility that calcein that is released intracellularly will subsequently leak out of the cells cannot be excluded.

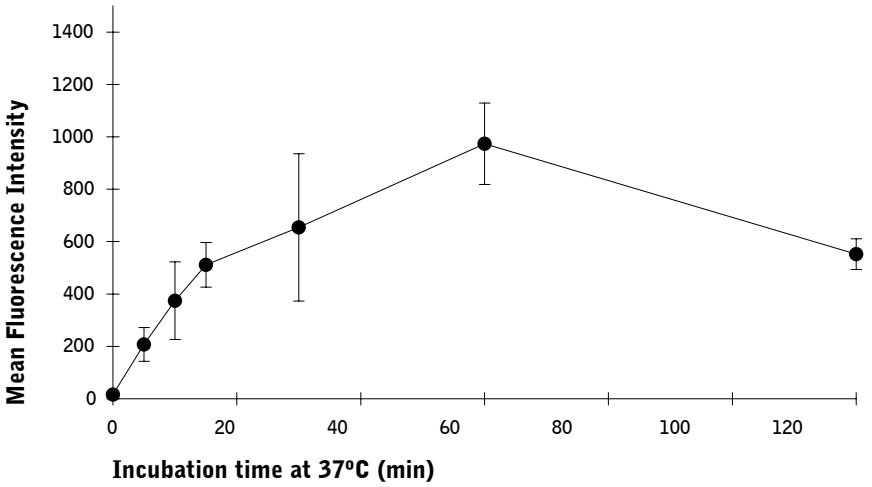

Figure 5 — Flow cytometry analysis of immunoliposome-encapsulated calcein internalization into BEAS-2B cells. Cells ($1x10^6$ cells/ml) were incubated with F10.2 immunoliposomes at a concentration of 500 nmol phospholipid/ml for 1 h on ice, washed twice with keratinocyte SFM medium and incubated at 37°C in SFM medium for the indicated time periods. Thereafter, cells were exposed to acidic condition (citric acid buffer, pH 3.0) for 10 min and washed twice with IF-buffer before flow cytometry analysis (n=2).

CONFOCAL LASER MICROSCOPIC ANALYSIS OF F10.2 IMMUNOLIPOSOME INTERNALIZATION AND SUBSEQUENT INTRACELLULAR RELEASE OF CALCEIN

We have further analyzed the process of liposome internalization by confocal laser microscopy (Figure 6). Cells were incubated with F10.2 immunoliposomes containing two fluorescent markers; rhodamine-PE as a lipid marker and encapsulated calcein as an aqueous marker. After incubation for 1 h at 4°C, both calcein fluorescence and rhodamine fluorescence were observed associated with the cell membrane (Figure 6A). By superimposing two pictures taken with two different filter settings (525-550 nm and >550 nm), it can be seen that the liposomal membrane marker rhodamine-PE and calcein are co-localized as visualized by the yellow fluorescence. Calcein fluorescence, but not rhodamine fluorescence, could be removed from the cell surface by exposing the cells for 10 min to pH 3.0 (Figure 6B). Incubation of cells with surface-bound F10.2 immunoliposomes at 37°C resulted in the appearance of bright fluorescent vacuoles in the cell interior, besides the cell membrane associated fluorescence (Figure 6C). Exposure to low pH showed the disappearance of surface-bound calcein fluorescence, but not of calcein located inside intracellular vacuoles (Figure 6D), confirming the fact that immunoliposomes, together with the encapsulated calcein, are internalized.

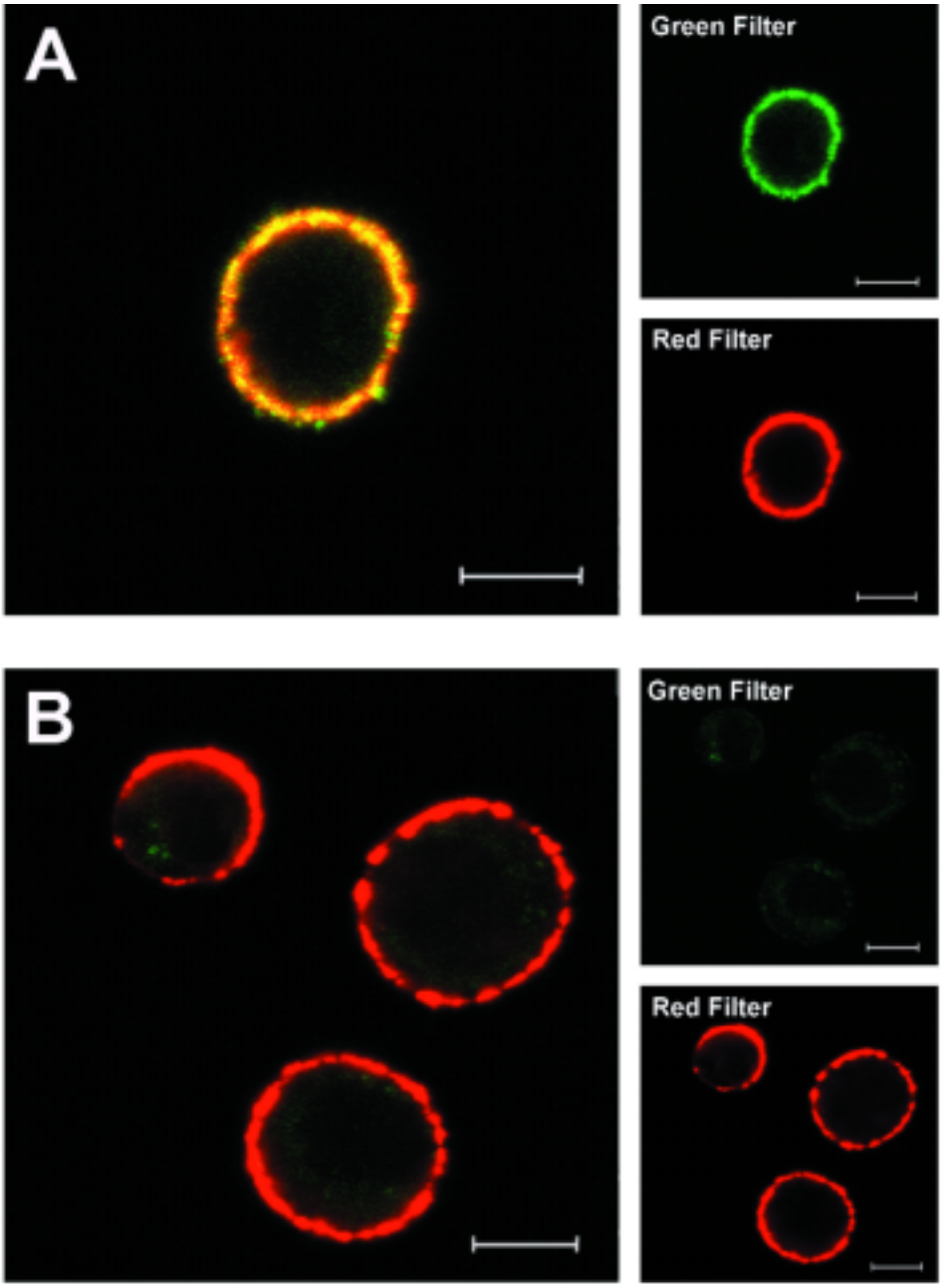

Figure 6 — Confocal laser microscopic analysis of the internalization of F10.2 immunoliposomes containing calcein and rhodamine-PE as fluorescent tracers into IFN-γ-activated BEAS-2B cells. BEAS-2B cells were incubated with F10.2 immunoliposomes either for 1 h on ice (Figures 6A,B) or for 1 h on ice and subsequently for 1 h at 37°C (Figures 6C,D). Thereafter, cells were either directly analyzed by confocal laser microscopy (Figures 6A,C) or exposed to citric acid buffer (pH 3.0) for 10 min (Figures 6B,D) before analysis. Used filter settings were 525-550 nm (green fluorescence) and >590 nm (red fluorescence). Superimposed pictures of both filter settings are also illustrated. Scale bars represent 10 μm.

C
Green Filter
Red Filter
D
Green Filter
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OVERALL CONCLUSIONS

Taking all results together, we can conclude that immunoliposomes targeted to ICAM-1 on IFN-γ activated human bronchial epithelial cells are able to bind to these cells. Depending on the concentration of immunoliposomes added, 1-14 nmol liposomal phospholipid appeared to bind per 10^6 BEAS-2B cells after 1 h incubation on ice, which corresponds with 680-15,400 particles bound per cell.

Furthermore, we studied whether ICAM-1-directed immunoliposomes are internalized by BEAS-2B cells. Little is known about the internalization characteristics of ICAM-1 molecules. ICAM-1 is utilized as a target receptor by the major group of human rhinoviruses. Binding to ICAM-1 results in receptor-mediated endocytosis of the bound virus particles [42]. On the other hand, internalization of monoclonal antibodies directed against ICAM-1 (mAb RR1/1) on human umbilical vein endothelial cells (HUVEC) could not be detected [43].

In this study, it was observed that F10.2 immunoliposomes are rapidly internalized after specific binding to ICAM-1 expressing BEAS-2B cells. After 1 h incubation at 37°C, 60% of the surface-bound immunoliposomes were internalized. These findings have implications for the development of drug targeting strategies to interfere in inflammatory processes. The rapid and extensive internalization of ICAM-1 targeted liposomes make them suitable carriers for the intracellular delivery of anti-inflammatory drugs. Also, ICAM-1-directed immunoliposomes may be used to selectively deliver antisense oligonucleotides to endothelial and epithelial cells to down-regulate adhesion molecule expression at sites of inflammation. As ICAM-1 is involved in the migration of inflammatory cells into inflamed tissues, down-regulation of ICAM-1 expression by antisense oligonucleotides will inhibit the migration of immune cells into inflamed tissues [44], and thereby inhibit the inflammatory response [45,46]. However, it should be realized that the liposomal system needs to be further optimized before application *in vivo*. The immunoliposome composition used in the present *in vitro* study may not be optimal *in vivo*. Also, the presence of whole antibody molecules exposed on the liposome surface may give problems related to immunogenicity when applied repeatedly *in vivo* [47,48]. In addition, circulating time after intravenous injection might be too short to allow efficient interaction with target epitopes.

Although it was observed that calcein is released from the immunoliposomes upon internalization, this does not guarantee that other encapsulated compounds will become available to intracellular targets after internalization of liposomes. The confocal microscopic findings strongly suggest that internalization occurs via receptor-mediated endocytosis. A large amount of endocytosed liposomes will eventually end up in lysosomal, degradative compartments [22,23,49]. For this reason, pH-dependent fusogenic properties have been incorporated into the immunoliposomal carrier, in order to promote delivery over the endosomal membrane and thereby improving the therapeutic availability of the encapsulated compound after cellular internalization. This work is described in chapters 3-5 of this thesis.

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REFERENCE LIST

1 Joseph-Silverstein, J. and Silverstein, R.L. (1998): **Cell adhesion molecules: an overview**. *Cancer Invest.*, 16:176-182.

2 Henricks, P.A.J. and Nijkamp, F.P. (1998): **Pharmacological modulation of cell adhesion molecules**. *Eur.J.Pharmacol.*, 344:1-13.

3 Laffon, A. and Gonzalez-Amaro, R. (1995): **Cell adhesion molecules: an overview**. *Br.J.Rheumatol.*, 34:1101-1102.

4 van de Stolpe, A. and van der Saag, P.T. (1996): **Intercellular adhesion molecule-1**. *J.Mol.Med.*, 74:13-33.

5 Hogg, N., Bates, P.A., and Harvey, J. (1991): **Structure and function of intercellular adhesion molecule-1**. *Chem.Immunol.*, 50:98-115:98-115.

6 Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., and Springer, T.A. (1986): **Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM- 1)**. *J.Immunol.*, 137:245-254.

7 Bloemen, P.G.M., van den Tweel, M.C., Henricks, P.A.J., Engels, F., Wagenaar, S.S., Rutten, A.A.J.J.L., and Nijkamp, F.P. (1993): **Expression and modulation of adhesion molecules on human bronchial epithelial cells**. *Am.J.Respir.Cell Mol.Biol.*, 9:586-593.

8 Atsuta, J., Sterbinsky, S.A., Plitt, J., Schwiebert, L.M., Bochner, B.S., and Schleimer, R.P. (1997): **Phenotyping and cytokine regulation of the BEAS-2B human bronchial epithelial cell: demonstration of inducible expression of the adhesion molecules VCAM-1 and ICAM-1**. *Am.J.Respir.Cell Mol.Biol.*, 17:571-582.

9 Paolieri, F., Battifora, M., Riccio, A.M., Pesce, G., Canonica, G.W., and Bagnasco, M. (1997): **Intercellular adhesion molecule-1 on cultured human epithelial cell lines: influence of proinflammatory cytokines**. *Allergy*, 52:521-531.

10 Seidel, M.F., Keck, R., and Vetter, H. (1997): **ICAM-1/LFA-1 expression in acute osteodestructive joint lesions in collagen-induced arthritis in rats**. *J.Histochem.Cytochem.*, 45:1247-1253.

11 Wegner, C.D., Gundel, R.H., Reilly, P., Haynes, N., Letts, L.G., and Rothlein, R. (1990): **Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma**. *Science*, 247:456-459.

12 Montefort, S., Roche, W.R., Howarth, P.H., Djukanovic, R., Gratziou, C., Carroll, M., Smith, L., Britten, K.M., Haskard, D., and Lee, T.H. (1992): **Intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in the bronchial mucosa of normal and asthmatic subjects**. *Eur.Respir.J.*, 5:815-823.

13 Wuthrich, R.P., Jevnikar, A.M., Takei, F., Glimcher, L.H., and Kelley, V.E. (1990): **Intercellular adhesion molecule-1 (ICAM-1) expression is upregulated in autoimmune murine lupus nephritis**. *Am.J.Pathol.*, 136:441-450.

14 Muller, G.A., Markovic-Lipkovski, J., and Muller, C.A. (1991): **Intercellular adhesion molecule-1 expression in human kidneys with glomerulonephritis**. *Clin.Nephrol.*, 36:203-208.

15 Karashima, T., Hachisuka, H., Okubo, K., and Sasai, Y. (1992): **Epidermal keratinocytes of bullous pemphigoid express intercellular adhesion molecule-1 (ICAM-1)**. *J.Dermatol.*, 19:82-86.

16 Little, M.C., Gawkrodger, D.J., and Mac Neil, S. (1996): **Differentiation of human keratinocytes is associated with a progressive loss of interferon gamma-induced intercellular adhesion molecule-1 expression**. *Br.J.Dermatol.*, 135:24-31.

17 Duplaa, C., Couffinhal, T., Labat, L., Moreau, C., Petit-Jean, M.E., Doutre, M.S., Lamaziere, J.M., and Bonnet, J. (1996): **Monocyte/macrophage recruitment and expression of endothelial adhesion proteins in human atherosclerotic lesions**. *Atherosclerosis*, 121:253-266.

18 O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D., and Alpers, C.E. (1996): **Neovascular expression of E-selectin, intercellular adhesion molecule- 1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content**. *Circulation*, 93:672-682.

19 Bloemen, P.G.M., Henricks, P.A.J., van Bloois, L., van den Tweel, M.C., Bloem, A.C., Nijkamp, F.P., Crommelin, D.J.A., and Storm, G. (1995): **Adhesion molecules: a new target for immunoliposome-mediated drug delivery**. *FEBS Lett.*, 357:140-144.

20 Spragg, D.D., Alford, D.R., Greferath, R., Larsen, C.E., Lee, K.D., Gurtner, G.C., Cybulsky, M.I., Tosi, P.F., Nicolau, C., and Gimbrone, M.A., Jr. (1997): **Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system**. *Proc.Natl.Acad.Sci.U.S.A.*, 94:8795-8800.

21 Bendas, G., Krause, A., Schmidt, R., Vogel, J., and Rothe, U. (1998): **Selectins as new targets for immunoliposome-mediated drug delivery. A potential way of anti-inflammatory therapy**. *Pharm.Acta Helv.*, 73:19-26.

22 Daleke, D.L., Hong, K., and Papahadjopoulos, D. (1990): **Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay**. *Biochim.Biophys.Acta*, 1024:352-366.

23 Straubinger, R.M., Hong, K., Friend, D.S., and Papahadjopoulos, D. (1983): **Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles**. *Cell*, 32:1069-1079.

24 Lee, K.D., Nir, S., and Papahadjopoulos, D. (1993): **Quantitative analysis of liposome-cell interactions *in vitro*: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes**. *Biochemistry*, 32:889-899.

25 Machy, P. and Leserman, L.D. (1983): **Small liposomes are better than large liposomes for specific drug delivery *in vitro***. *Biochim.Biophys.Acta*, 730:313-320.

26 Vingerhoeds, M.H., Steerenberg, P.A., Hendriks, J.J., Dekker, L.C., Van Hoesel, Q.G., Crommelin, D.J.A., and Storm, G. (1996): **Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma *in vitro* and *in vivo***. *Br.J.Cancer*, 74:1023-1029.

27 Storm, G. and Crommelin, D.J.A. (1998): **Liposomes: quo vadis?** *Pharm.Sci.Tech.Today*, 1:19-31.

28 Matthay, K.K., Abai, A.M., Cobb, S., Hong, K., Papahadjopoulos, D., and Straubinger, R.M. (1989): **Role of ligand in antibody-directed endocytosis of liposomes by human T- leukemia cells**. *Cancer Res.*, 49:4879-4886.

29 Lundberg, B., Hong, K., and Papahadjopoulos, D. (1993): **Conjugation of apolipoprotein B with liposomes and targeting to cells in culture**. *Biochim.Biophys.Acta*, 1149:305-312.

30 Ishii, Y., Aramaki, Y., Hara, T., Tsuchiya, S., and Fuwa, T. (1989): **Preparation of EGF labeled liposomes and their uptake by hepatocytes**. *Biochem.Biophys.Res.Commun.*, 160:732-736.

31 Suzuki, S., Inoue, K., Hongoh, A., Hashimoto, Y., and Yamazoe, Y. (1997): **Modulation of doxorubicin resistance in a doxorubicin-resistant human leukaemia cell by an immunoliposome targeting transferring receptor**. *Br.J.Cancer*, 76:83-89.

32 Nässander, U.K., Steerenberg, P.A., De Jong, W.H., Van Overveld, W.O., Te Boekhorst, C.M., Poels, L.G., Jap, P.H., and Storm, G. (1995): **Design of immunoliposomes directed against human ovarian carcinoma**. *Biochim.Biophys.Acta*, 1235:126-139.

33 Lang, J., Vigo-Pelfrey, C., and Martin, F. (1990): **Liposomes composed of partially hydrogenated egg phosphatidylcholines: fatty acid composition, thermal phase behavior and oxidative stability**. *Chem.Phys.Lipids*, 53:91-101.

34 Martin, F.J. and Papahadjopoulos, D. (1982): **Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting**. *J.Biol.Chem.*, 257:286-288.

35 Bloemen, P.G.M., Moldenhauer, G., van Dijk, M., Schuurman, H.J., and Bloem, A.C. (1992): **Multiple ICAM-1 (CD54) epitopes are involved in homotypic B-cell adhesion**. *Scand.J.Immunol.*, 35:517-523.

36 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., and Papahadjopoulos, D. (1979): **Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes**. *Biochim.Biophys.Acta*, 557:9-23.

37 Fiske, C.H. and Subbarow, Y. (1925): **The colorimetric determination of phosphorus**. *J.Biol.Chem.*, 66:375-400.

38 Suzuki, S., Watanabe, S., Uno, S., Tanaka, M., Masuko, T., and Hashimoto, Y. (1994): **Endocytosis does not necessarily augment the cytotoxicity of adriamycin encapsulated in immunoliposomes**. *Biochim.Biophys.Acta*, 1224:445-453.

39 Matthay, K.K., Heath, T.D., Badger, C.C., Bernstein, I.D., and Papahadjopoulos, D. (1986): **Antibody-directed liposomes: comparison of various ligands for association, endocytosis, and drug delivery**. *Cancer Res.*, 46:4904-4910.

40 Suzuki, H., Zelphati, O., Hildebrand, G., and Leserman, L. (1991): **CD4 and CD7 molecules as targets for drug delivery from antibody bearing liposomes**. *Exp.Cell Res.*, 193:112-119.

41 Breuer, W., Epsztejn, S., and Cabantchik, Z.I. (1995): **Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II)**. *J.Biol.Chem.*, 270:24209-24215.

42 Grunert, H.P., Wolf, K.U., Langner, K.D., Sawitzky, D., Habermehl, K.O., and Zeichhardt, H. (1997): **Internalization of human rhinovirus 14 into HeLa and ICAM-1- transfected BHK cells**. *Med.Microbiol.Immunol.(Berl)*, 186:1-9.

43 von Asmuth, E.J., Smeets, E.F., Ginsel, L.A., Onderwater, J.J., Leeuwenberg, J.F., and Buurman, W.A. (1992): **Evidence for endocytosis of E-selectin in human endothelial cells**. *Eur.J.Immunol.*, 22:2519-2526.

44 Glover, J.M., Leeds, J.M., Mant, T.G., Amin, D., Kisner, D.L., Zuckerman, J.E., Geary, R.S., Levin, A.A., and Shanahan, W.R., Jr. (1997): **Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302)**. *J.Pharmacol.Exp.Ther.*, 282:1173-1180.

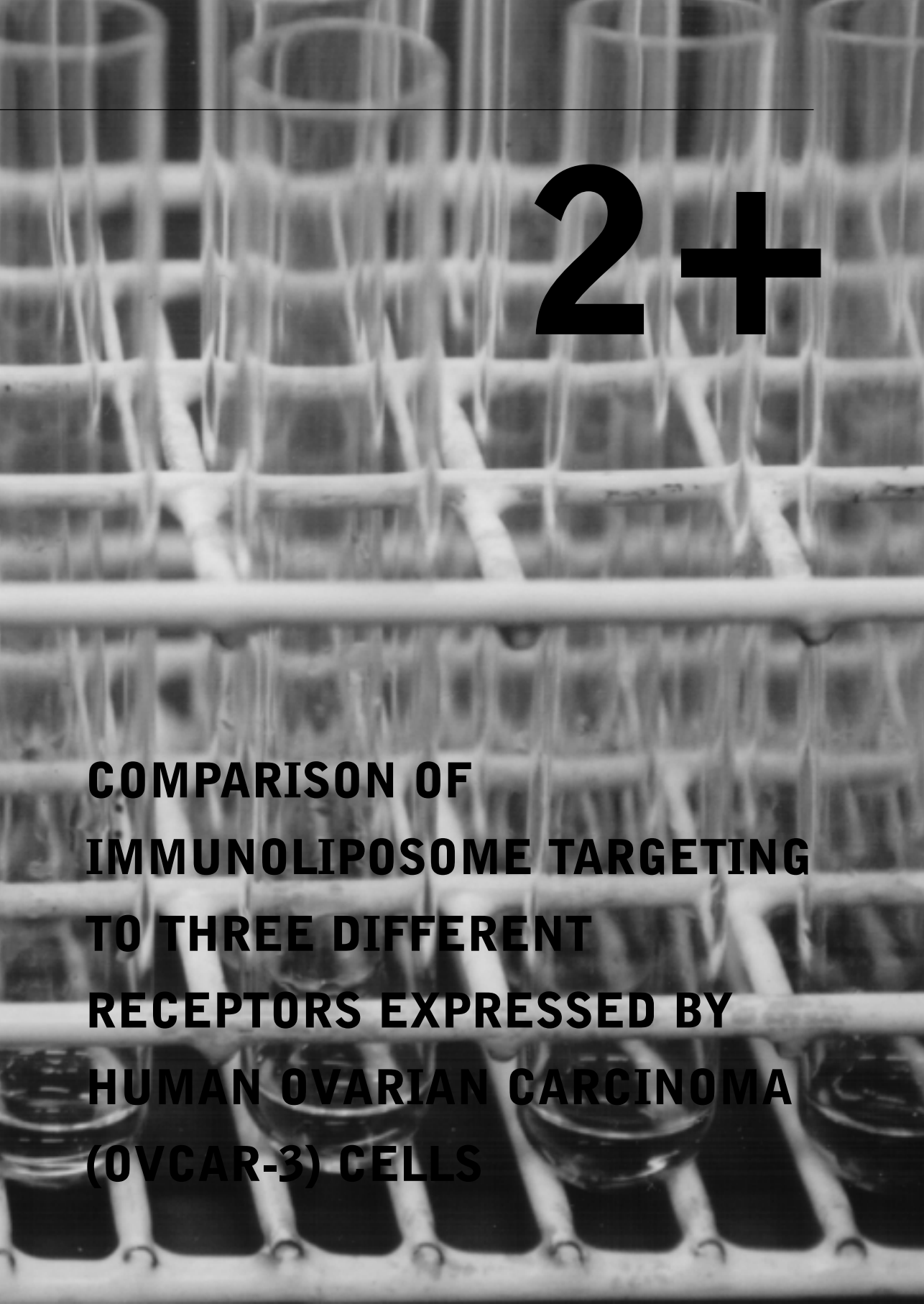
45 Kumasaka, T., Quinlan, W.M., Doyle, N.A., Condon, T.P., Sligh, J., Takei, F., Beaudet Al, Bennett, C.F., and Doerschuk, C.M. (1996): **Role of the intercellular adhesion molecule-1(ICAM-1) in endotoxin- induced pneumonia evaluated using ICAM-1 antisense oligonucleotides, anti-ICAM-1 monoclonal antibodies, and ICAM-1 mutant mice**. *J.Clin.Invest.*, 97:2362-2369.

46 Chiang, M.Y., Chan, H., Zounes, M.A., Freier, S.M., Lima, W.F., and Bennett, C.F. (1991): **Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms**. *J.Biol.Chem.*, 266:18162-18171.

47 Harding, J.A., Engbers, C.M., Newman, M.S., Goldstein, N.I., and Zalipsky, S. (1997): **Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)- grafted immunoliposomes**. *Biochim.Biophys.Acta*, 1327:181-192.

48 Phillips, N.C. and Dahman, J. (1995): **Immunogenicity of immunoliposomes: reactivity against species-specific IgG and liposomal phospholipids**. *Immunol.Lett.*, 45:149-152.

49 Yoshimura, T., Shono, M., Imai, K., and Hong, K. (1995): **Kinetic analysis of endocytosis and intracellular fate of liposomes in single macrophages**. *J.Biochem.(Tokyo).*, 117:34-41.



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COMPARISON OF IMMUNOLIPOSOME TARGETING TO THREE DIFFERENT RECEPTORS EXPRESSED BY HUMAN OVARIAN CARCINOMA (OVCAR-3) CELLS

COMPARISON OF IMMUNOLIPOSOME TARGETING TO THREE DIFFERENT RECEPTORS EXPRESSED BY HUMAN OVARIAN CARCINOMA (OVCAR-3) CELLS

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To be submitted

SUMMARY

Immunoliposomes were targeted to three different cell-surface receptors: intercellular adhesion molecule-1 (ICAM-1), epithelial glycoprotein-2 (EGP-2), and epidermal growth factor receptor (EGFR) expressed on OVCAR-3 ovarian carcinoma cells. Binding and internalization of these immunoliposomes was analyzed qualitatively by flow cytometry and confocal laser scanning microscopy to determine which cell-surface receptor is most suitable in intracellular delivery of immunoliposomal drugs to ovarian carcinoma cells. Under the tested conditions, the EGFR turned out to be the best candidate to obtain receptor-mediated endocytosis of a large amount of immunoliposomes.

INTRODUCTION

The research described in this thesis aims at delivering therapeutic compounds encapsulated in antibody-directed liposomes (immunoliposomes; IL) into the cytoplasm of tumor cells, in particular of ovarian carcinoma cells (OVCAR-3) cells. This is attempted by exploiting the route of receptor-mediated endocytosis followed by endosomal escape of immunoliposome-entrapped drug molecules, induced by pH-dependent fusogenic peptides or proteins incorporated in the liposomal carrier. This 'Trojan horse' approach of delivery requires binding and internalization of immunoliposomes as a first step in the cytosolic delivery of entrapped drugs.

Both the occurrence of internalization and lack of internalization of immunoliposomes specifically bound to target cell surface receptors have been reported. The occurrence of receptor-mediated endocytosis of receptor-bound immunoliposomes is determined by the type of receptor, provided that immunoliposomes are small enough (<150 nm). Therefore, the choice of the target receptor for intracellular delivery of immunoliposomal drugs is of major importance. However, not all monoclonal antibodies recognizing the same receptor mediate internalization of the receptor/mAb complex. It was demonstrated that three different mAbs recognizing different epitopes on the same receptor (folate-binding protein) on ovarian carcinoma cells were processed by the cells in different ways. Two out of three mAbs were internalized, whereas the other remained located on the cell surface. Thus, not all mAbs directed against receptors with internalizing capacity will be able to trigger internalization as these mAb recognize different epitopes on the same receptor. In addition, the internalizing capacity of cell-surface receptors is also dependent on the type of cell that expresses the cell-surface receptor. For instance, immunoliposomes targeted to the HER-2 antigen were reported to be efficiently internalized by human breast cancer cells, less efficiently by MKN-7 human gastric or SKOV-3 ovarian carcinoma cells, and not at all by N-87 human gastric carcinoma cells. Consequently, internalization of antibody-directed liposomes cannot be predicted *a priori*.

For targeting immunoliposomes to ovarian carcinoma cells, mAbs directed against several receptors have been used. In our group Fab' fragments of mAb OV-TL3 directed against the tumor-associated antigen OA3, which is present on more than 90% of all human ovarian carcinomas and mAb 323/A3 directed against the epithelial glycoprotein-2 (EGP-2) were conjugated to the surface of doxorubicin-laden liposomes. For both OV-TL3 immunoliposomes and 323/A3 immunoliposomes specific binding to OVCAR-3 was demonstrated *in vitro* and *in vivo*. Binding of OV-TL3 immunoliposomes to OVCAR-3 cells residing in the peritoneal cavity of nude mice after *i.p.* injection was high: after 5 hrs incubation >80% of immunoliposomes recovered from the peritoneal cavity was found to be associated with tumor cells. In addition, electron microscopy analysis demonstrated that OV-TL3 immunoliposomes were bound to the surface of OVCAR-3 cells that were isolated from the peritoneal cavity of athymic mice. Internalization of cell-bound immunoliposomes was not detected after this 5 hrs incubation.

Internalization of 323/A3-IL by OVCAR-3 cells has not been investigated so far. It has been shown in the immunotoxin literature that targeting to the EGP-2 receptor can lead to internalization: 25% of cell bound immunotoxins consisting of doxorubicin conjugated to mAb MOC31 (α-EGP-2), were internalized by small lung cancer cells after 5 hrs.
Besides the above mentioned target receptors, other receptors may be used to target immunoliposomes to OVCAR-3 cells. In a study from our group immunoliposomes were targeted to intercellular adhesion molecule-1 (ICAM-1) expressed on human bronchial epithelial cells. It was demonstrated that the immunoliposomes were capable to specifically bind to these cells; in addition, they were efficiently internalized (see chapter 2 and). As ICAM-1 expression is not only up-regulated at sites of inflammation but is also involved in the metastatic spread of many types of tumor cells , this receptor may also be used for targeting immunoliposomes to OVCAR-3 cells. The epidermal growth factor receptor (EGFR), has been found to be over-expressed on many carcinoma cells and is often used as target for drug delivery to carcinoma cells.

In this study we have targeted immunoliposomes to the cell-surface receptors ICAM-1, EGP-2 and EGFR expressed on OVCAR-3 cells. Binding and internalization by OVCAR-3 cells was monitored qualitatively by flow cytometry and confocal laser scanning microscopy to determine which antibody is most suitable to achieve specific internalization of immunoliposomes by OVCAR-3 cells. The results show that the EGFR is the most suitable candidate to deliver immunoliposomes into the endocytic pathway of OVCAR-3 cells.

MATERIALS & METHODS

MATERIALS

Cholesterol (CHOL), *N*-succinimidyl-*S*-acetylthioacetate (SATA), *N*-ethylmaleimide, bovine serum albumin (fraction V), fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Fab'-specific) and *R*-phycoerythrin (PE)-labeled goat anti-mouse IgG were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Chloroform and methanol were obtained from Merck (Darmstadt, Germany), and *N,N*-dimethylformamide (DMF) and hydroxylamine hydrochloride from Janssen Chimica (Geel, Belgium). Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC; Asahi Chemical Industry Co., Ibarakiken, Japan) was prepared as described previously. *N*-[4-(*p*-maleimidophenyl) butyryl]phosphatidylethanolamine (MPB-PE) and lissamine rhodamine B-labeled glycerophosphoethanolamine (Rho-PE) were purchased at Avanti Polar Lipids Inc. (Alabaster, AL, USA). Anti-ICAM-1 mAb (IgG_1), clone F10.2 was a gift from Dr. Henricks (department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands). Murine mAb 425 of isotype IgG2b (EMD55900) (Merck KGaA, Bernstadt, Germany) directed against the human epidermal growth factor receptor (EGFR) was kindly donated by Dr. G.A. van Dongen (Department of Otolaryngology/Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). Monoclonal antibody 323/A3, directed against the epithelial glycoprotein-2 (EGP-2) was kindly donated by Centocor Europe BV (Leiden, The Netherlands). Formaldehyde was obtained from Janssen Chimica (Geel, Belgium). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) was purchased from Molecular Probes (Leiden, The Netherlands).

CELL CULTURE

The human ovarian carcinoma cell line NIH:OVCAR-3 was originally obtained from Dr. Hamilton (National Cancer Institute, Bethesda, MD). OVCAR-3 cells were cultured in DMEM containing 3.7 g/l sodium bicarbonate, 4.5 g/l L-glucose (Gibco, Grand Island, NY, USA) and supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% (v/v) heat inactivated fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml; all purchased at Gibco). Cells were maintained at 37°C in humidified air with 5% CO_2.

PREPARATION AND CHARACTERIZATION OF IMMUNOLIPOSOMES

Immunoliposomes were prepared by conjugating mAb 425 (IgG2b), 323/A3 (IgG1), and F10.2 (IgG1) to the membrane of liposomes according to the method described previously with some minor modifications (see chapter 2). In short, liposomes were made by hydrating a lipid film composed of PHEPC, CHOL and MPB-PE at a molar ratio of 1.925:1:0.075, respectively in 5 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.4 (HBS/EDTA). When indicated either NBD-PE or Rho-PE were included in the liposomes as a fluorescent marker at a molar amount of 0.1% relative to the total amount of lipid. Subsequently, liposomes were sized by extrusion through 0.1 µm pore filters. Freshly thiolated mAbs (for details see chapter 2) at a final concentration of 450 µg/ml were added to the liposomes (40 mM) and incubated for 2 hrs at room temperature under constant rotation. The coupling reaction was terminated by adding 50 µl of freshly prepared 8 mM *N*-ethylmaleimide in HEPES buffer (10 mM HEPES; 1mM EDTA; 135 mM NaCl pH 7.4). Liposomes were separated from unconjugated mAbs by ultracentrifugation (4 runs of 30 min at 60,000xg) and stored at 4°C. Mean particle size of (immuno)liposomes was determined by dynamic light scattering as described before. Phospholipid concentration was determined by the colorimetric method of Fiske and Subbarow and the amount of conjugated IgG on the liposomal surface was determined with the Bio-Rad *DC* protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with mouse IgG as standard.

FLOW CYTOMETRY ANALYSIS OF IMMUNOLIPOSOME BINDING TO OVCAR-3 CELLS

Immunoliposomes and control liposomes (without conjugated mAb) were added to $2.5x10^5$ OVCAR-3 cells and incubated for 1 h at the indicated temperature. Cells were washed 2 times with 1 ml of immunofluorescence (IF) buffer (1% (w/v) BSA in PBS pH 7.4). Hereafter, cells that had been incubated with fluorescently labeled immunoliposomes (*i.e.* immunoliposomes containing Rho-PE as a lipidic fluorescent marker) were resuspended in 500 µl of IF-buffer and analyzed by flow cytometry. To cells that were incubated with unlabeled (immuno)liposomes, 100 µl of appropriately diluted FITC-labeled goat anti-mouse IgG were added and incubated for 1 h on ice. After two additional wash steps with IF-buffer, these cells were resuspended into 500 µl IF-buffer and analysed for cell-associated fluorescence by flow cytometry. Quantification of the degree of cellular internalization of cell-bound liposomes was performed as previously described by us and another group (see also chapter 2).

CONFOCAL LASER SCANNING MICROSCOPY (CLSM) ANALYSIS OF CELLULAR UPTAKE OF IMMUNOLIPOSOMES

For CLSM analysis, OVCAR-3 cells, either in suspension or adhered to chamber slides (Nalge Nunc International Corp., Naperville, IL, USA), were incubated with Rho-PE-labeled (immuno)liposomes for 1 h after which cells were washed twice with 1 ml of PBS and subsequently fixed with 4% (v/v) of formaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed twice with PBS. Suspension cells were mounted on object slides and overlaid with cover slides that were sealed with nail polish. Cells were analyzed on a Leica TCS-SP confocal laser-scanning microscope equipped with a 488 nm Argon, 568 nm Krypton and 647 nm HeNe laser.

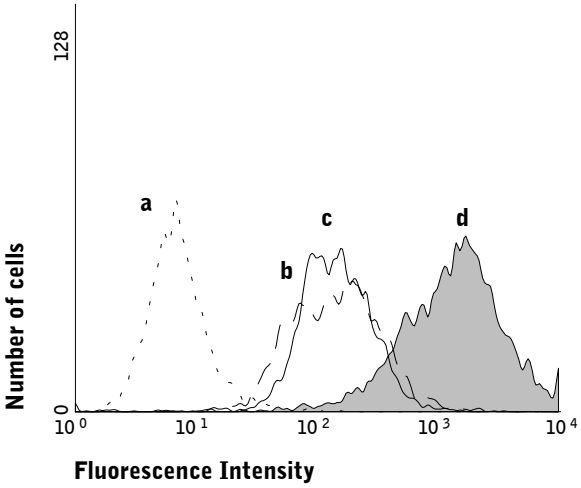

Figure 1 – Expression pattern of different cell-surface receptors on the surface of OVCAR-3 cells determined by indirect immunofluorescence staining and flow cytometry. Shown are OVCAR-3 cells incubated with FITC-labeled goat anti-mouse secondary antibody (a), with mAb 425 (αEGFR) and FITC-labeled secondary antibody (b); with mAb F10.2 (αICAM-1) and FITC-labeled secondary antibody (c), and with mAb 323/A3 and FITC-labeled secondary antibody (d). Histograms show the mean fluorescent intensity of 4000 cells.

RESULTS AND DISCUSSION

In this study, immunoliposomes were targeted to three different cell surface receptors on OVCAR-3 cells to explore which receptor is most efficient in delivering immunoliposomes into the endosomal pathway of the cell as the first step in a strategy for cytosolic drug delivery. Based on previous experience with immunoliposome targeting, three different surface receptors were chosen: the intercellular adhesion-molecule-1 (ICAM-1), the epithelial glycoprotein-2 (EGP-2) and the epidermal growth factor receptor (EGFR). The expression pattern of these cell-surface receptors on OVCAR-3 cells was determined by flow cytometry with indirect immunofluorescence using monoclonal antibodies directed against EGP-2 (mAb 323/A3), EGFR (mAb 425) and ICAM-1 (mAb F10.2). The expression levels of the different cell surface receptors are shown in Figure 1. The expression level of EGP-2 was 10 times higher than the expression levels of ICAM-1 and EGFR, which showed comparable expression levels. For liposome targeting, the 3 mAbs were chemically modified with SATA to allow covalent conjugation to the surface of liposomes. The average size of liposomes and the amount of conjugated IgG are given in Table 1.

IMMUNOLIPOSOME BINDING TO OVCAR-3 CELLS

Binding of fluorescently labeled immunoliposomes to OVCAR-3 cells at 4°C is shown in Figure 2. Both 323/A3-IL and 425-IL showed concentration-dependent binding to OVCAR-3 cells. Binding of 323/A3-IL was higher than binding of 425-IL at concentrations >1 µmol

Table 1 – Characterization of control liposomes and immunoliposomes. Mean particle size and amount of conjugated protein are given for each liposome preparation.

Type of Liposomes	Average particle size (nm)/ Polydispersity Index	Amount of conjugated mAb (µg/µmol PL)
Control Liposomes	133/0.08	—
425-Immunoliposomes (α-EGFR)	128/0.09	10
323/A3-Immunoliposomes (α-EGP-2)	155/0.13	18
F10.2-Immunoliposomes (α-ICAM1)	131/0.09	14

PL/ml. Binding of control liposomes (*i.e.* liposomes without conjugated antibody) was negligible at the concentration range tested. Surprisingly, immunoliposomes targeted to ICAM-1 did not show any binding to OVCAR-3 cells despite the fact that ICAM-1 cell-surface expression on these cells was detected. It is not clear which factors underlie this lack of cell binding. One potential reason may be that modification of mAb F10.2 with SATA necessary to conjugate the antibody to maleimide-derivatized phospholipids in the liposome bilayer has resulted in loss of binding activity of this mAb. In chapter 2, however, it was demonstrated that modification of F10.2 with SATA was possible with preservation of binding activity. Yet, F10.2 used in chapter 2 was modified at a lower SATA to antibody ratio (8:1) compared to SATA modification of mAb F10.2 used in this study (30:1). Due to the inability of F10.2 IL to bind to OVCAR-3 cells, these immunoliposomes were excluded from further experiments.

Analysis of immunoliposome binding by confocal laser scanning microscopy (CLSM) confirmed that both 323/A3-IL and 425-IL are able to bind to a monolayer of OVCAR-3 cells adhered to polystyrene chamber slides as indicated by the presence of rhodamine-fluorescence on the surface of the cells (Figure 3). Fluorescent staining of OVCAR-3 cells incubated with 425-IL is relatively weak compared to cells incubated with 323/A3-IL, indicating a lower degree of cell binding of

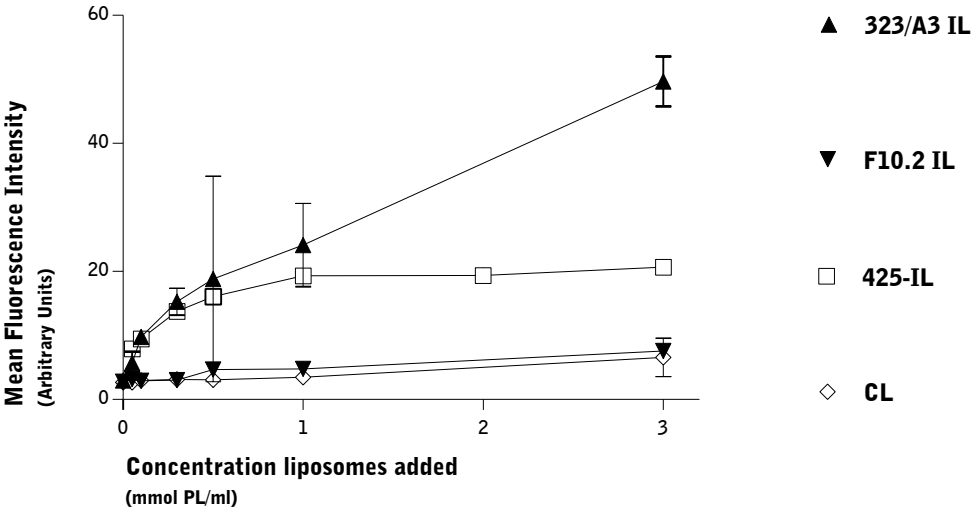

Figure 2 – Binding of immunoliposomes to OVCAR-3 cells at 4°C. OVCAR-3 cells were incubated with liposomes labeled with the lipophilic fluorescent probe Rho-PE for 1 h at 4°C. After removal of unbound liposomes cells were analyzed for cell-associated rhodamine fluorescence by flow cytometry. Each point represents the mean fluorescence intensity of 4000 cells analyzed (n=3). 323/A3 IL: mAb 323/A3-directed immunoliposomes; F10.2 IL: mAb F10.2-directed immunoliposomes; 425-IL: mAb 425-directed immunoliposomes; CL: control liposomes without conjugated antibodies, but with the same lipid composition as the immunoliposomes. Error bars indicate SEM (n=3).

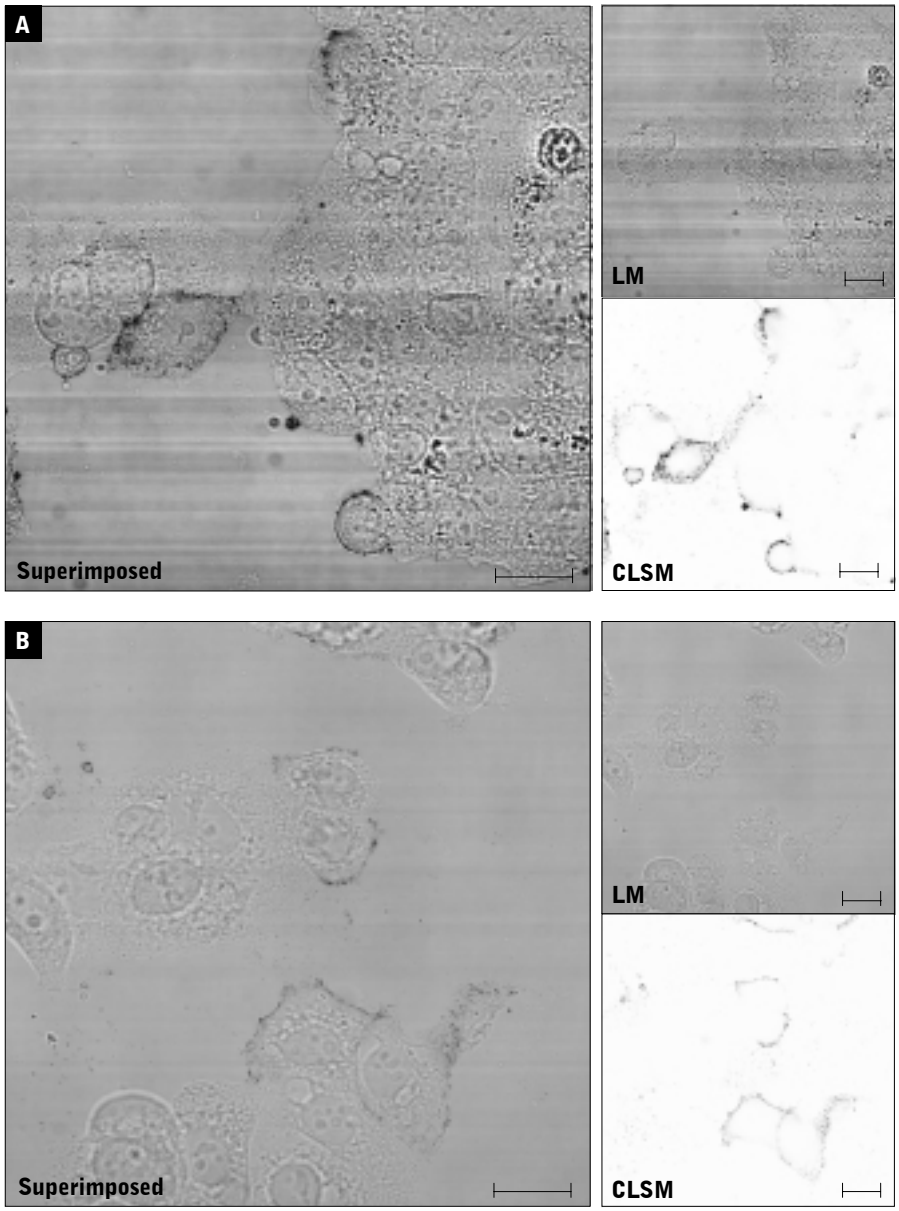

Figure 3 – Confocal laser scanning microscopy (CLSM) analysis of immunoliposome binding to OVCAR-3 cells at 4°C. A monolayer of OVCAR-3 cells adhered to chamber slides was incubated with Rho-PE-labeled 323/A3-IL (Panel A) or 425-IL (Panel B) for 60 min at 4°C (500 nmol PL/ml). After removal of unbound liposomes, cells were fixed in 4% formaldehyde solution in PBS and analyzed by CLSM and light microscopy (LM). For clarity, cell-associated rhodamine fluorescence is indicated in greyscale (white indicates no fluorescence, black indicates highest fluorescence). Pictures obtained with CLSM and LM have been superimposed to show the localization of fluorescence inside cells. Scale bar represents 25 μm.

425-IL at 4°C. The observed cell-associated fluorescence is primarily located at the plasma membrane of OVCAR-3 cells with no detectable spots of fluorescence located intracellularly. Binding of immunoliposomes to both OVCAR-3 cells and A-2780 cells (ovarian carcinoma cells lacking expression of EGP-2) was also evaluated at 37°C both with flow cytometry and CLSM. For flow cytometry analysis, 323/A3 immunoliposomes and 425-immunoliposomes were labeled with the fluorescent probe DiD. Figure 4 shows that the degree of binding of 323/A3-IL to OVCAR-3 cells is again higher compared to that of 425-IL. In addition, 323/A3-IL binding was restricted to OVCAR-3 cells, whereas 425-IL also bound to A2780 cells, although less than binding to OVCAR-3 cells. CLSM analysis with Rho-PE-labeled immunoliposomes demonstrates that cell-associated fluorescence is more intense at 37°C compared to binding at 4°C (Figure 5), indicating that the degree of binding is higher at 37°C. Interestingly, the distribution of cell-bound 323/A3-IL is primarily confined to cells located at the edges of the monolayer, whereas 425-IL binding is more equally distributed over the cells present in the monolayer. Basolateral expression of EGP-2 epithelial cells has been demonstrated in a study comparing the expression patterns of EGP-2 in 84 different human colorectal adenomas and adeno-carcinomas as well as healthy tissue. Although the complex organization of epithelial cells *in vivo* cannot be compared with the *in vitro* situation it might well be that EGP-2 expression on adherent OVCAR-3 cells growing as a monolayer is restricted to the basolateral side of the cells, which may explain the

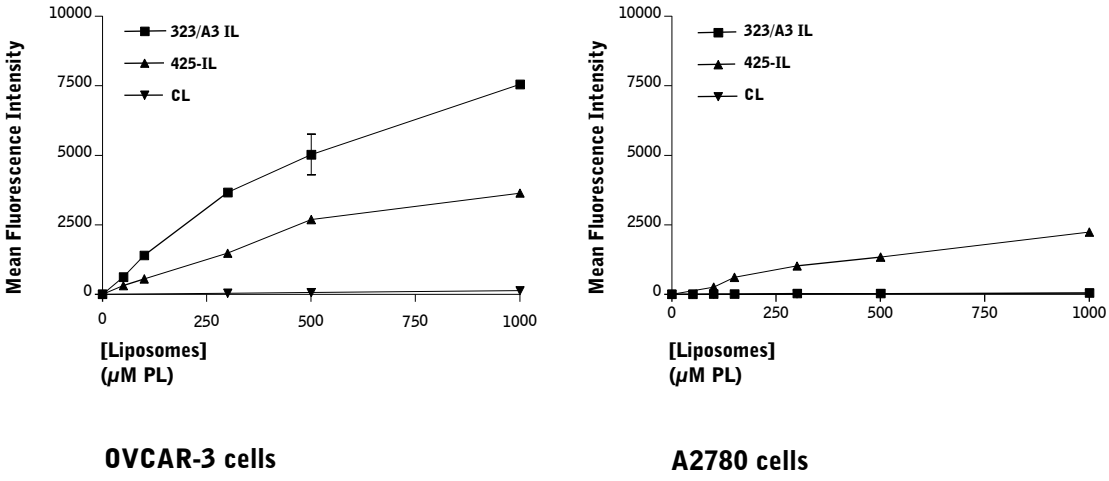

Figure 4 – Flow cytometry analysis of immunoliposome binding to OVCAR-3 and A2780 cells as a function of liposome concentration. Cells were incubated with increasing concentrations of DiD-labeled immunoliposomes or control liposomes for 60 min at 37°C. Cells were analyzed by flow cytometry after removal of unbound liposomes. Each point represents the average of the mean fluorescence intensity of 4000 cells. Error bars indicate SEM (n=3).

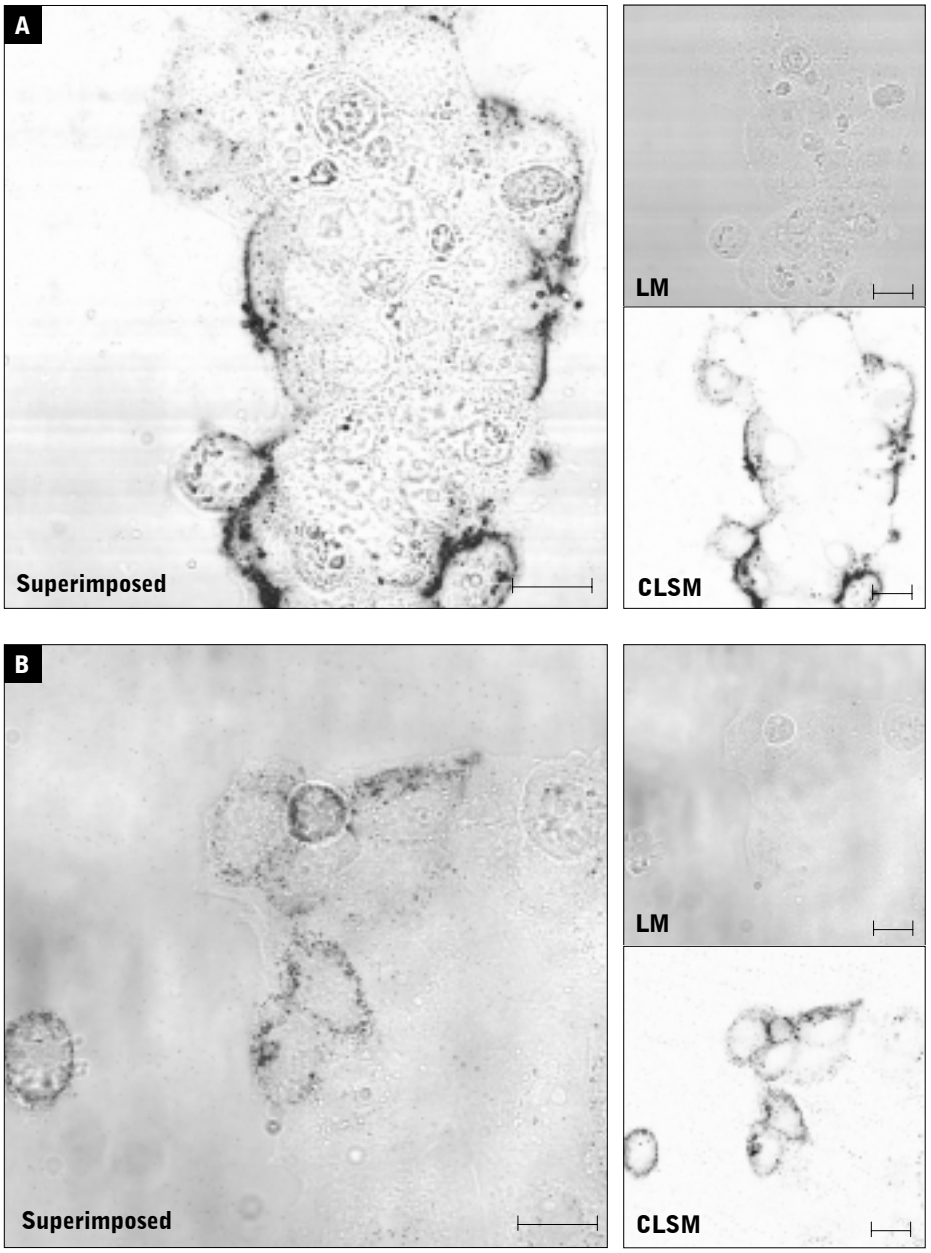

Figure 5 – CLSM analysis of immunoliposome binding to OVCAR-3 cells at 37°C. A monolayer of OVCAR-3 cells adhered to chamber slides were incubated with rhodamine-labeled 323/A3-IL (Panel A) or 425-IL (Panel B) for 60 min at 37°C (500 nmol PL/ml). After removal of unbound liposomes, cells were fixed in 4% formaldehyde solution in PBS and analyzed by CLSM and light microscopy (LM). For clarity, cell-associated rhodamine fluorescence is indicated in greyscale (white indicates no fluorescence, black indicates highest fluorescence). Pictures obtained with CLSM and LM have been superimposed to show the localization of fluorescence inside cells. Scale bar represents 25 μm.

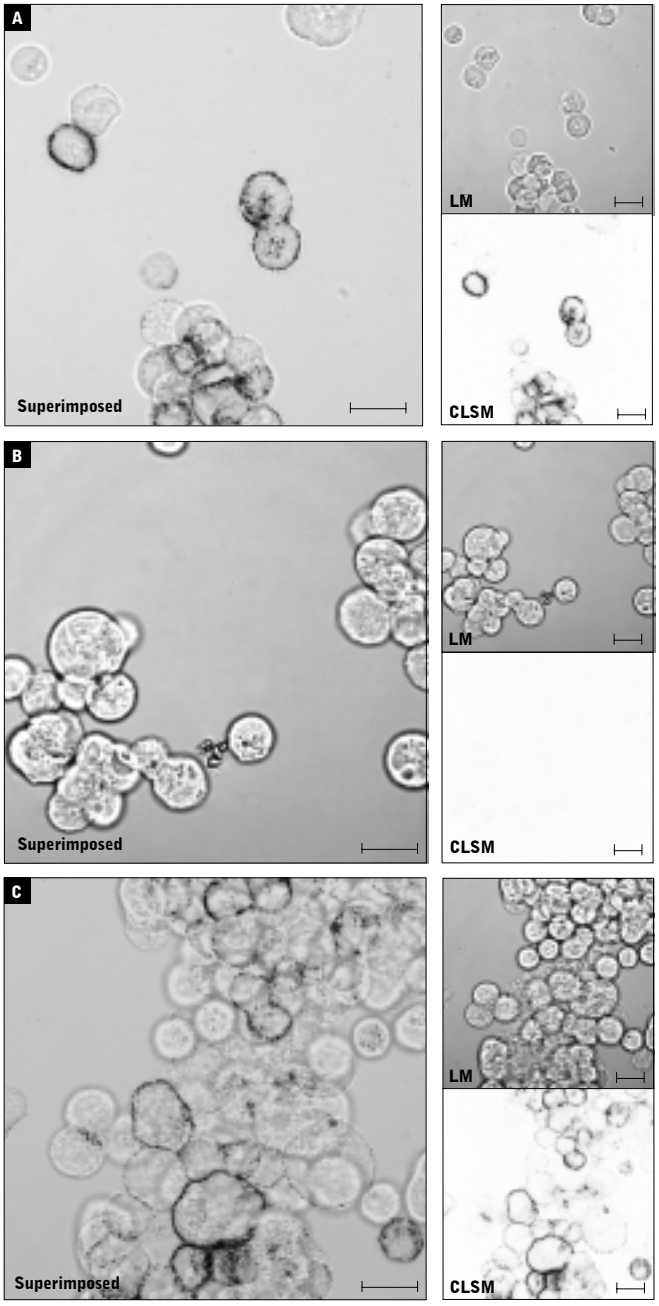

Figure 6 – CLSM analysis of immunoliposome binding to OVCAR-3 cells in suspension. Panel A: OVCAR-3 cells incubated with 500 μmol/ml PL of 425-IL for 1 h at 37°C. Panel B: same as panel A but in the presence of 1 mg/ml free mAb 425; Panel C: same as panel A but in the presence of 1 mg/ml of free mAb 323/A3. Scale bars represent 25 μm.

lower degree of cell binding. Besides cell membrane-associated fluorescence also spots of fluorescence located inside cells can be discerned when the cells were incubated with 425-IL. The frequency and intensity of intracellular fluorescent spots is much less when cells were incubated with 323/A3-IL, indicating that 425-IL are more efficiently internalized by OVCAR-3 cells than 323/A3-IL.

Binding of 425-IL to OVCAR-3 cells could be blocked by adding high concentrations (1 mg/ml) of free mAb 425 to the cells prior to the addition of the immunoliposomes. Pre-incubation with the same concentration of mAb 323/A3 did not have an effect on the degree of 425-IL binding to OVCAR-3 cells (Figure 6). These results strongly indicate that 425-IL binding to OVCAR-3 cells is exclusively mediated by the conjugated monoclonal antibodies present on the surface of 425-IL.

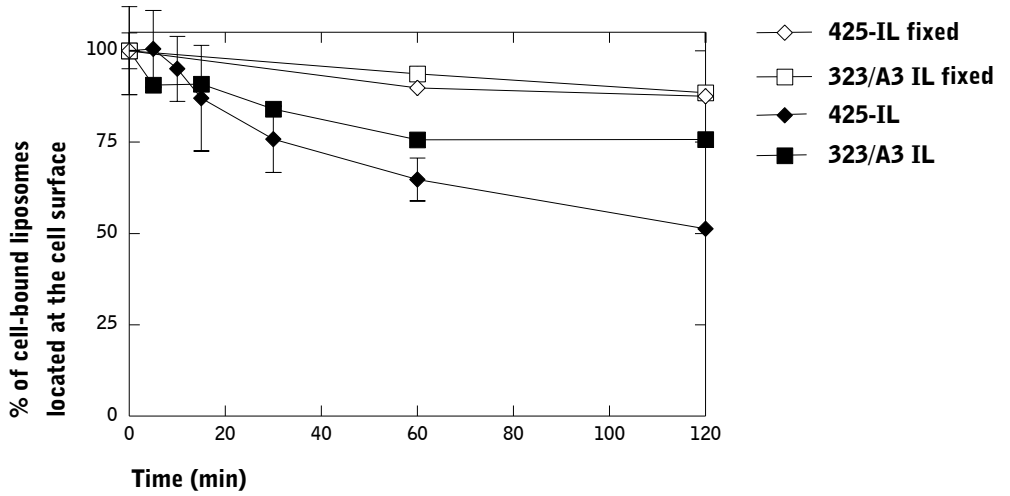

Figure 7 – Determination of the amount of immunoliposomes bound to the surface of OVCAR-3 cells. OVCAR-3 cells, both vital and fixed with 4% formaldehyde, were incubated with immunoliposomes (500 μmol PL/ml) for 1 h at 4°C. After removal of unbound liposomes, cells were further incubated in culture medium at 37°C for indicated time periods. Hereafter, immunoliposomes still present on the cell surface were stained with a FITC-labeled goat anti-mouse antibody and analyzed by flow cytometry. The amount of immunoliposomes present on the cell surface is expressed relative to the initial amount of cell-bound immunoliposomes (*i.e.* at t=0 min, directly after incubation at 4°C). Error bars indicate SEM (n=3).

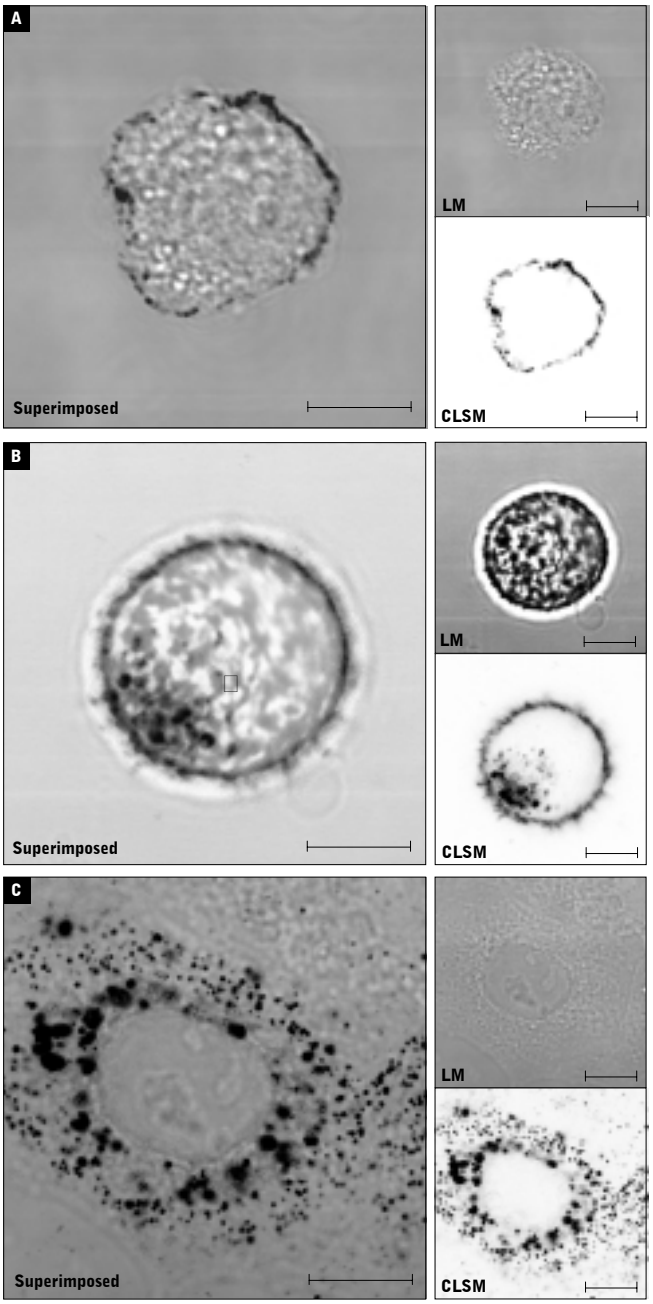

Figure 8 – CLSM close-up analysis of immunoliposome internalization into OVCAR-3 cells. OVCAR-3 cells were incubated with 500 μmol PL/ml of immunoliposomes for 2 hrs at 37°C. Unbound immunoliposomes were removed prior to fixation of cells with 4% formaldehyde. Panel A: OVCAR-3 cells grown in suspension and incubated with 323/A3 IL. Panel B: OVCAR-3 cells grown in suspension and incubated with 425-IL. Panel C: OVCAR-3 cells grown as a monolayer and incubated with 425-IL. Scale bars represent 10 μm.

INTERNALIZATION OF IMMUNOLIPOSOMES BY OVCAR-3 CELLS

Internalization of cell-bound immunoliposomes was also confirmed by indirect immunofluorescence analysis as described previously. Immunoliposomes were incubated with OVCAR-3 cells at 4°C for 1 h. After removal of unbound immunoliposomes, cells were incubated at 37°C for the indicated time period. Then the immunoliposomes still attached to the cell-surface of OVCAR-3 cells were labeled with FITC-conjugated antibodies directed against the liposome-conjugated mAbs. The results of this experiment are shown in Figure 7. It can be seen that the amount of immunoliposomes located on the surface of OVCAR-3 cells decreased within time. After 2 hrs, the amount of 323/A3-IL initially bound to OVCAR-3 cells was decreased with approx. 25% and the amount of 425-IL with approx. 50%. Control experiments using cells fixed with formaldehyde prior to immunoliposome binding show a marginal reduction in immunoliposome binding (approx. 10%). As fixed cells do not internalize particles, differences in reduction of immunoliposomes bound to the surface of OVCAR-3 cells between fixed and non-fixed cells can be ascribed to an active process of the cell, which strongly suggests that both 323/A3-IL and 425-IL are internalized by OVCAR-3 cells.

To ascertain the occurrence of internalization, close-ups of OVCAR-3 cells incubated with either 323/A3-IL or 425-IL were made by confocal laser scanning microscopy to check for intracellularly localized immunoliposomes (Figure 8). 323/A3-IL incubated with OVCAR-3 cells at 37°C are primarily located at or near the cell membrane of OVCAR-3 cells as illustrated by a rim of fluorescence around OVCAR-3 cells. Only minor amounts of internalized spots of fluorescence can be observed (Figure 8A). In contrast, 425-IL incubation yield many fluorescent spots inside OVCAR-3 cells besides fluorescence at the cell surface (Figure 8B). Internalization of 425-IL by OVCAR-3 cells is more pronounced when adherent OVCAR-3 cells are used as compared to OVCAR-3 cells in suspension (Figure 8C).

Taking these results together it can be concluded that both EGP-2 and EGFR can be used as receptors to target immunoliposomes into the endosomal pathway of OVCAR-3 cells. Although the expression level of EGP-2 is much higher than the expression level of EGFR, the capacity of this receptor to internalize immunoliposomes is much lower (~15% of cell-bound IL after 2 hrs) as compared to the EGFR (~40% after 2 hrs). This finding together with the poor accessibility of EGP-2 on OVCAR-3 cells observed *in vitro* suggest that the EGF-receptor is the most suitable candidate of the 3 tested for achieving receptor-mediated endocytosis of immunoliposomes and their entrapped drugs by OVCAR-3 cells.

REFERENCE LIST

1 Huwyler, J., Wu, D., and Pardridge, W.M. (1996): **Brain drug delivery of small molecules using immunoliposomes**. *Proc.Natl.Acad.Sci.U.S.A.*, 93:14164-14169.

2 Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. (1997): **Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro***. *Biochemistry*, 36:66-75.

3 Lopes de Menezes, D.E., Pilarski, L.M., and Allen, T.M. (1998): **In vitro and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma**. *Cancer Res.*, 58:3320-3330.

4 Mastrobattista, E., Storm, G., van Bloois, L., Reszka, R., Bloemen, P.G., Crommelin, D.J., and Henricks, P.A. (1999): **Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells**. *Biochim.Biophys.Acta*, 1419:353-363.

5 Park, J.W., Hong, K., Carter, P., Asgari, H., Guo, L.Y., Keller, G.A., Wirth, C., Shalaby, R., Kotts, C., and Wood, W.I. (1995): **Development of anti-p185HER2 immunoliposomes for cancer therapy**. *Proc.Natl.Acad.Sci.U.S.A.*, 92:1327-1331.

6 Suzuki, S., Uno, S., Fukuda, Y., Aoki, Y., Masuko, T., and Hashimoto, Y. (1995): **Cytotoxicity of anti-c-erbB-2 immunoliposomes containing doxorubicin on human cancer cells**. *Br.J.Cancer*, 72:663-668.

7 Koning, G.A., Morselt, H.W., Velinova, M.J., Donga, J., Gorter, A., Allen, T.M., Zalipsky, S., Kamps, J.A., and Scherphof, G.L. (1999): **Selective transfer of a lipophilic prodrug of 5-fluorodeoxyuridine from immunoliposomes to colon cancer cells**. *Biochim.Biophys.Acta*, 1420:153-167.

8 Nässander, U.K., Steerenberg, P.A., De Jong, W.H., Van Overveld, W.O., Te Boekhorst, C.M., Poels, L.G., Jap, P.H., and Storm, G. (1995): **Design of immunoliposomes directed against human ovarian carcinoma**. *Biochim.Biophys.Acta*, 1235:126-139.

9 Casalini, P., Mezzanzanica, D., Canevari, S., Della, T.G., Miotti, S., Colnaghi, M.I., and Matzku, S. (1991): **Use of combination of monoclonal antibodies directed against three distinct epitopes of a tumor-associated antigen: analysis of cell binding and internalization**. *Int.J.Cancer*, 48:284-290.

10 Goren, D., Horowitz, A.T., Zalipsky, S., Woodle, M.C., Yarden, Y., and Gabizon, A. (1996): **Targeting of stealth liposomes to erbB-2 (Her/2) receptor: *in vitro* and *in vivo* studies**. *Br.J.Cancer.*, 74:1749-1756.

11 Straubinger, R.M., Lopez, N.G., Debs, R.J., Hong, K., and Papahadjopoulos, D. (1988): **Liposome-based therapy of human ovarian cancer: parameters determining potency of negatively charged and antibody-targeted liposomes**. *Cancer Res.*, 48:5237-5245.

12 Vingerhoeds, M.H., Steerenberg, P.A., Hendriks, J.J., Dekker, L.C., Van Hoesel, Q.G., Crommelin, D.J.A., and Storm, G. (1996): **Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma *in vitro* and *in vivo***. *Br.J.Cancer*, 74:1023-1029.

13 Nässander, U.K., Steerenberg, P.A., Poppe, H., Storm, G., Poels, L.G., De Jong, W.H., and Crommelin, D.J.A. (1992): **In vivo targeting of OV-TL 3 immunoliposomes to ascitic ovarian carcinoma cells (OVCAR-3) in athymic nude mice**. *Cancer Res.*, 52:646-653.

14 Vingerhoeds, M. H. Immunoliposomes against ovarian carcinoma: from targeting to antitumor activity. Thesis. 1995. Utrecht University.

15 Froesch, B.A., Stahel, R.A., and Zangemeister-Wittke, U. (1996): **Preparation and functional evaluation of new doxorubicin immunoconjugates containing an acid-sensitive linker on small-cell lung cancer cells**. *Cancer Immunol.Immunother.*, 42:55-63.

16 Okamoto, Y., Tsurunaga, T., and Ueki, M. (1999): **Serum soluble ICAM-1 levels in the patients with cervical cancer**. *Acta Obstet.Gynecol.Scand.*, 78:60-65.

17 Regidor, P.A., Callies, R., Regidor, M., and Schindler, A.E. (1998): **Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera**. *Eur.J.Gynaecol.Oncol.*, 19:377-383.

18 Maurer, C.A., Friess, H., Kretschmann, B., Wildi, S., Muller, C., Graber, H., Schilling, M., and Buchler, M.W. (1998): **Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer**. *Int.J.Cancer*, 79:76-81.

19 Davies, D.E. and Chamberlin, S.G. (1996): **Targeting the epidermal growth factor receptor for therapy of carcinomas**. *Biochem.Pharmacol.*, 51:1101-1110.

20 Engebraaten, O., Sivam, G., Juell, S., and Fodstad, O. (2000): **Systemic immunotoxin treatment inhibits formation of human breast cancer metastasis and tumor growth in nude rats**. *Int.J.Cancer*, 88:970-976.

21 Kikuchi, A., Sugaya, S., Ueda, H., Tanaka, K., Aramaki, Y., Hara, T., Arima, H., Tsuchiya, S., and Fuwa, T. (1996): **Efficient gene transfer to EGF receptor overexpressing cancer cells by means of EGF-labeled cationic liposomes**. *Biochem.Biophys.Res.Commun.*, 227:666-671.

22 Mendelsohn, J. (1997): **Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy**. *Clin.Cancer Res.*, 3:2703-2707.

23 Ishii, Y., Aramaki, Y., Hara, T., Tsuchiya, S., and Fuwa, T. (1989): **Preparation of EGF labeled liposomes and their uptake by hepatocytes**. *Biochem.Biophys.Res.Commun.*, 160:732-736.

24 Lang, J., Vigo-Pelfrey, C., and Martin, F. (1990): **Liposomes composed of partially hydrogenated egg phosphatidylcholines: fatty acid composition, thermal phase behavior and oxidative stability**. *Chem.Phys.Lipids*, 53:91-101.

25 Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R., Green, J.A., Chu, E.W., Whang-Peng, J., Rogan, A.M., Green, W.R., and Ozols, R.F. (1983): **Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors**. *Cancer Res.*, 43:5379-5389.

26 Bloemen, P.G.M., Henricks, P.A.J., van Bloois, L., van den Tweel, M.C., Bloem, A.C., Nijkamp, F.P., Crommelin, D.J.A., and Storm, G. (1995): **Adhesion molecules: a new target for immunoliposome-mediated drug delivery**. *FEBS Lett.*, 357:140-144.

27 Suzuki, S., Watanabe, S., Uno, S., Tanaka, M., Masuko, T., and Hashimoto, Y. (1994): **Endocytosis does not necessarily augment the cytotoxicity of adriamycin encapsulated in immunoliposomes**. *Biochim.Biophys.Acta*, 1224:445-453.

28 Schwendener, R.A., Trub, T., Schott, H., Langhals, H., Barth, R.F., Groscurth, P., and Hengartner, H. (1990): **Comparative studies of the preparation of immunoliposomes with the use of two bifunctional coupling agents and investigation of *in vitro* immunoliposome-target cell binding by cytofluorometry and electron microscopy**. *Biochim.Biophys.Acta.*, 1026:69-79.

29 Duncan, R.J., Weston, P.D., and Wrigglesworth, R. (1983): **A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay**. *Anal.Biochem.*, 132:68-73.

30 Ogura, E., Senzaki, H., Yoshizawa, K., Hioki, K., and Tsubura, A. (1998): **Immunohistochemical localization of epithelial glycoprotein EGP-2 and carcinoembryonic antigen in normal colonic mucosa and colorectal tumors**. *Anticancer Res.*, 18:3669-3675.

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TARGETING OF INFLUENZA VIROSOMES TO OVARIAN CARCINOMA CELLS

TARGETING OF INFLUENZA VIROSOMES TO OVARIAN CARCINOMA CELLS

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SUMMARY

Reconstituted influenza virus envelopes (virosomes) containing the viral hemagglutinin (HA) have attracted attention as delivery carriers for cytosolic drug delivery as they possess membrane fusion activity. Here, we show that influenza virosomes can be targeted towards ovarian carcinoma cells (OVCAR-3) with preservation of fusion activity by incorporating PEG-derivatized lipids into the virosome membrane. This PEG-layer shields the interaction of HA with ubiquitous sialic acid residues and also serves as spatial anchor for antibody attachment. Coupling of Fab' fragments of mAb 323/A3 (anti-epithelial glycoprotein-2) to the distal ends of the PEG-lipids resulted in specific antibody-mediated binding of virosomes to OVCAR-3 cells. These antibody-redirected virosomes fused with membranes of OVCAR-3 cells in a pH-dependent fashion.

INTRODUCTION

The cytosol of cells is an important, but relatively inaccessible compartment for many therapeutic macromolecules. As these molecules often show restricted ability to pass through cell membranes due to their unfavorable physicochemical characteristics, in particular large size and/or excessive charge, there is a strong need for a carrier that can deliver these membrane-impermeable macromolecules into the cytosol of target cells. Although liposomes have been widely used for the delivery of therapeutic compounds to target cells, thus far liposome-mediated delivery of membrane-impermeable macromolecular agents into the cytosol has been inefficient. This is mainly due to the poor endosomal/lysosomal escape of these agents after cellular uptake in liposome-encapsulated form. In fact, often the fate of liposome-encapsulated macromolecules is intralysosomal degradation [1-3]. One approach to enhance cytosolic delivery through escape from the endosomes is the use of reconstituted envelopes (virosomes) of the human influenza virus A [4-7].

The human influenza virus is an enveloped virus, which enters its host cell by receptor-mediated endocytosis [8,9]. The acidic environment within the endosomes triggers fusion of the viral envelope with the endosomal membrane resulting in release of viral nucleic acids into the cytosol of the host cell. Both receptor binding and low pH-induced membrane fusion are mediated by the viral integral membrane protein hemagglutinin (HA), which forms trimers in the viral envelope [10,11]. Each HA monomer consists of two subunits: HA1, which contains the sialic acid binding pocket and HA2, which mediates membrane fusion. At low pH a conformational change occurs within HA resulting in the exposure of the hydrophobic N-termini of the HA2 subunits at the distal ends of the HA trimers. These hydrophobic sequences interact with target membranes thereby inducing membrane fusion. Reconstituted influenza virus envelopes (virosomes) containing HA have been developed that retain fusogenic activity and can be utilized as carriers for the delivery of normally membrane-impermeable substances into the cytosol of cells [5,12-14]. However, as influenza virosomes have a tropism for sialic acid-bearing cells and sialic acid residues are ubiquitous on the cell surface of many different cell types, targeting of influenza virosomes to specific cell types for the purpose of drug delivery is a challenge. In order to obtain cell specificity, influenza virosomes have to be redirected. Redirection involves two steps. First, the binding to the natural receptor of influenza virosomes (*i.e.* sialic acid residues) has to be prevented and second, specific homing devices (*e.g.* antibodies) have to be introduced. Despite both modifications fusogenic activity must be preserved. Although binding and fusion functionalities are integrated in one protein, studies have shown that HA-mediated fusion can occur without HA-mediated binding to sialic acid cell surface receptors as long as the HA protein is present in its native form [15,16].

This study shows for the first time that influenza virosomes can be redirected to specific cell types without loss of fusogenic activity. For redirection we used the steric shielding capacity of

poly(ethylene glycol) (PEG) that, when exposed on the virosome membranes, may effectively prevent HA from binding to sialic acid residues on target cell membranes. Dependent on the PEG-chain length and the PEG density on the surface, HA-mediated binding to sialic acid residues was fully inhibited. By conjugating antibody Fab' fragments of mAb 323/A3 (directed against epithelial glycoprotein-2) to the distal ends of surface-exposed PEG molecules antigen-specific binding of influenza virosomes to ovarian carcinoma cells with full retention of fusogenic activity was obtained.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Octa(ethylene glycol)-mono(*n*-dodecyl) ether ($C_{12}E_8$) was from Calbiochem (Darmstadt, Germany). The hydrophobic resin Bio-Beads SM-2 were from Bio-Rad (Hercules, CA, USA). Before use, Bio-Beads kept in methanol were extensively washed with HEPES-buffered salt solution (HBS; 5 mM HEPES, 150 mM NaCl, pH 7.4). PyrPC (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine) was from Molecular Probes (Eugene, OR, USA). Neuraminidase from Clostridium perfringens was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). PEG-5000-DSPE (1,2-distearoyl-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)5000], PEG-2000-DSPE (1,2-distearoyl-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] and L-α-phosphatidylethanolamine–*N*-(lissamine rhodamine B sulfonyl) (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL, USA). Maleimide-PEG-2000-DSPE was obtained from Shearwater polymers (Huntsville, AL, USA). Iso-Octylphenoxypolyethoxyethanol (Triton X-100) was from BDH (Poole, England). All other chemicals were from the highest grade available.

CELLS AND VIRUSES

The X47 recombinant strain of influenza virus, carrying the HA of influenza A/Victoria/3/75, was grown and purified as described elsewhere [17]. Human erythrocytes were isolated from whole blood of a healthy volunteer as described elsewhere [18]. The human ovarian cancer cell lines NIH:OVCAR-3 [19] and A2780 [20] were maintained in DMEM (Gibco) containing 3.7 g/l sodium bicarbonate, 4.5 g/l L-glucose and supplemented with 10% heat inactivated fetal calf serum, L-glutamine (4.5 g/l), penicillin (100 IU/ml) and streptomycin (100 µg/ml) and amphothericin B (0.25 µg/ml).

VIROSOME PREPARATION

Reconstitution of influenza virus envelopes was carried out according to [4], with some minor modifications. Briefly, an amount of virus corresponding to 1.5 mmol of phospholipid was pelleted by ultracentrifugation for 1 h at 100,000xg using a fixed angle rotor. Pelleted virus was resuspended in 0.5 ml of 30 mM $C_{12}E_8$ in HBS and incubated for 30 min at room temperature. To incorporate pyrPC and PEG-DSPE conjugates into the virosome membrane, solubilized viral components were added to either pyrPC or a mixture of pyrPC and PEG-DSPE after removal of

the nucleoprotein complex by centrifugation at 100,000xg for 30 min. The amount of pyrPC relative to the total amount of phospholipid was 10%, the amount of PEG-DSPE varied from 0-20%. To be able to conjugate Fab'-molecules to the virosomes, the sulfhydryl-reactive maleimide-PEG2000-DSPE was incorporated at a 2% lipid density. The mixture was added to 45 mg (dry weight) of Bio-Beads prewashed in HBS and incubated for 2 hrs on a shaking-device (1,400 rpm) at room temperature while protected from light. Subsequently, 2 batches of 25 mg of fresh Bio-Beads were applied to the mixture within a time interval of 30 min while shaking was continued. For targeting purposes, Fab'-fragments of mAb 323/A3 were covalently conjugated to the distal ends of maleimide-derivatized PEG-DSPE incorporated in the virosome membrane as previously described for liposomes [21,22]. The formed virosomes were layered atop a discontinuous sucrose gradient (10-40-50-60 % w/v) and centrifuged at 100,000xg for 90 min at 4°C. Hereafter, the virosomes sedimented atop the 40% sucrose band were collected and dialyzed overnight against HBS at 4°C. In all cases, formed virosome particles had an average size of approx. 100-200 nm with a polydispersity index varying between 0.1-0.4 as determinded by dynamic light scattering.

PREPARATION OF LIPOSOMES FROM RECONSTITUTED MEMBRANES OF OVARIAN CARCINOMA CELLS

Liposomes composed of the reconstituted membranes of OVCAR-3 cells were prepared essentially as described before [23]. A total amount of $1x10^7$ OVCAR-3 cells were collected from culture flasks and washed twice with 10 ml of PBS by subsequent centrifugation (200xg, 5 min) and resuspension steps. Pelleted cells were resuspended in 5 ml of hypotonic buffer (10 mM Tris-HCl; 1mM $MgCl_2$; 1 mM KCl; 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.3) and subjected to 4 freeze/thaw cycles with liquid nitrogen and lukewarm water, respectively. Hereafter, cells were vigorously shaken for 5 min and centrifuged at 1,000xg for 5 min to remove cell debris. Supernatant was collected and remaining pellets were washed twice with isotonic buffer (10 mM Tris-HCl; 140 mM NaCl; 0.5 mM PMSF, pH 7.3) and supernatants were pooled. Pooled supernatants were centrifuged at 100,000xg and pellet was solubilized into 3 ml 150 mM OG in HBS by incubating at 37°C for 30 min. After removal of insoluble cell debris by centrifugation (700xg; 5 min), the clear solution was applied to a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA) with a molecular weight cut off of 10,000 Da and dialyzed against 1 l of HBS overnight at 4°C. The formed liposomes were collected and analyzed for size distribution, phospholipid and protein contents. OVCAR-3 liposomes had an average size of 140 nm with a polydispersity index of 0.2.

MEMBRANE FUSION ASSAY

Membrane fusion experiments were carried out essentially as described before [15]. In short, virosomes at a final concentration of 2.5 μM phospholipid and donor membranes (either erythrocyte ghosts or OVCAR-3 liposomes at a final concentration of 50 μM or 200 μM phospholipid, respectively) were added to a thermostatted and stirred cuvette that contained HBS. After 2 min, the pH of the medium in the cuvette was lowered to 5.1 by adding 1/20 volume of fusion buffer (0.1 M 2-[*N*-morpholino]ethanesulfonic acid] (Mes); 0.1 M Acetic Acid pH 4.1). Fusion was continuously monitored at 37°C by measuring the decrease in pyrPC excimer fluorescence with an LS50B fluorescence spectrophotometer (Perkin Elmer) set at an excitation wavelength of 345 nm and an emission wavelength of 480 nm. The decrease of fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at infinite dilution, which was obtained by adding 70 μl of 10% (v/v) Triton X-100 in HBS.

HEMAGGLUTINATION (HA) ASSAY

The hemagglutination assay was carried out essentially as described [24]. In short, 100 μl of virosomes at a starting concentration of 68 μM phospholipid were applied to the first well of each row in a V-shaped 96-wells plate and a 2-fold serial dilution was made to obtain a concentration of virosomes corresponding to 8.3 nM of phospholipid in the last well of each row. Human erythrocytes were isolated from whole blood as described by Yoneda et al. [18]. A 0.5% suspension of freshly isolated erythrocytes in HBS was prepared and 100 μl of this suspension was applied to each well. Erythrocytes were allowed to sediment after which the occurrence of agglutination was screened by visual inspection.

CELL BINDING OF VIROSOMES, PEG-VIROSOMES AND PEG-IMMUNOVIROSOMES

Cell binding of virosomes, PEG-virosomes and antibody-targeted PEG-virosomes (all fluorescently labeled with NBD-PE) was assessed by flow cytometry. Virosomes (10 μM of phospholipid) were added to $2x10^5$ OVCAR-3 or A2780 cells and incubated for 120 min at 4°C in the dark. Hereafter, cells were washed twice with wash-buffer (1% BSA in HBS) and cells were resuspended in 500 μl wash-buffer before analysis with a FACscan flow cytometer (Becton and Dickinson, Mountain View, CA, USA).

RESULTS

EFFECT OF SURFACE-EXPOSED PEG ON SIALIC ACID BINDING

The influence of the presence of PEG molecules present on the surface of virosomes on the capacity to agglutinate human red blood cells was determined with a hemagglutination assay [24]. This test is based on HA-mediated binding to sialic-acid-containing receptors present on red blood cells. Influenza virosomes bearing HA bind to these receptors and cause clumping or agglutination of red blood cells in suspension. It is demonstrated that the presence of PEG molecules on the surface of virosomes completely inhibits HA-mediated sialic-acid binding (Table 1). Binding of influenza virosomes to red blood cells negatively correlated with the molecular weight and density of surface-exposed PEG. For PEG-2000-DSPE, 20 mol% was needed to fully block agglutination of red blood cells; the same result could be achieved with 8 mol% PEG-5000-DSPE.

Table 1 — Effect of PEG exposed on the surface of virosomes on the capacity of virosomes to agglutinate human red blood cells (hemagglutination) as a measure for the degree of sialic acid residue binding. A serial dilution of the different virosome preparations was made and added to a 0.5% suspension of human red blood cells. After incubation for 3-4 hrs at room temperature to allow sedimentation of non-agglutinated red blood cells, the occurrence of agglutination was scored visually. + indicate wells positive for agglutination; – indicate wells negative for agglutination and +/- indicate wells where agglutination is observed together with sedimentation of red blood cells.

	Dilution	2	4	8	16	32	64	128	256	512	1024	2048	4096
Virosome preparation	**25 μM**	1	2	3	4	5	6	7	8	9	10	11	12
0% PEG	**A**	+	+	+	+	+	+	+	+	+	+	+	+
2% PEG-5000	**B**	+	+	+	+	+	+	+	±	–	–	–	–
5% PEG-5000	**C**	±	±	–	–	–	–	–	–	–	–	–	–
8% PEG-5000	**D**	–	–	–	–	–	–	–	–	–	–	–	–
10% PEG-5000	**E**	+	+	+	+	+	±	–	–	–	–	–	–
13,5% PEG-5000	**F**	±	±	–	–	–	–	–	–	–	–	–	–
20% PEG-5000	**G**	–	–	–	–	–	–	–	–	–	–	–	–
No virosomes	**H**	–	–	–	–	–	–	–	–	–	–	–	–

EFFECT OF SURFACE-EXPOSED PEG ON MEMBRANE FUSION

Knowing that PEG molecules present at the surface of virosomes can inhibit HA-mediated sialic acid binding, we investigated the effect of surface-exposed PEG on the rate and extent of fusion with erythrocyte membranes induced by low pH exposure. Figure 1 shows that PEG inclusion reduced the degree of lipid mixing between erythrocyte ghosts and PEG-virosomes. At PEG densities that fully block hemagglutination (8 mol% PEG-5000-DSPE and 20 mol% PEG-2000-DSPE) the rate of lipid mixing was drastically reduced compared to virosomes without membrane-coupled PEG.

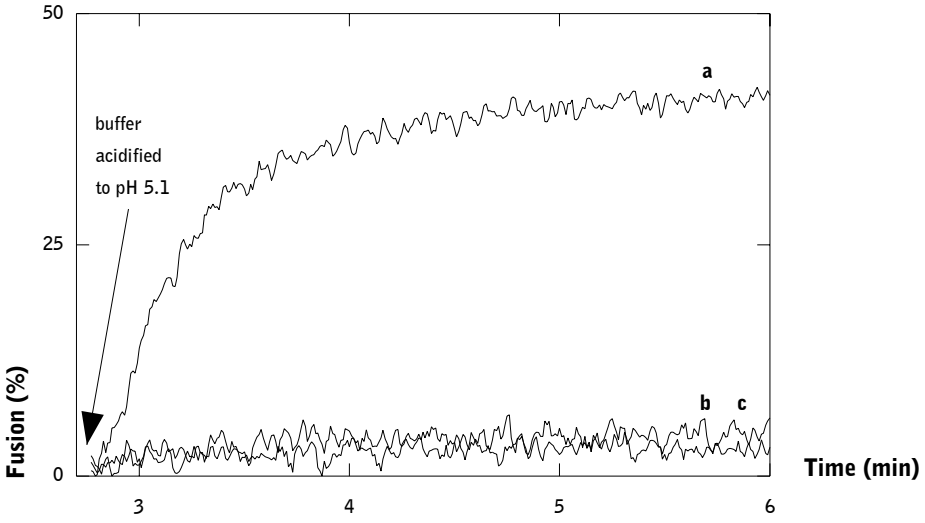


Figure 1 — Effect of surface-exposed PEG on fusion of influenza virosomes with erythrocyte ghosts. Erythrocyte ghosts (50 μM phospholipid) were added to pyrPC-labeled virosomes (2.5 μM phospholipid). At the time-point indicated with the arrow the medium was acidified to pH 5.1. Pyrene excimer fluorescence was continuously monitored at 37°C, whilst stirring the cuvette contents. The decrease of fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at infinite dilution (*i.e.* after vesicle solubilization with detergent). Curve a: unmodified virosomes; curve b: virosomes prepared with 8 mol% of PEG-5000-DSPE; curve c: virosomes prepared with 20 mol% PEG-2000-DSPE.

COUPLING OF ANTIBODY FRAGMENTS TO PEGYLATED VIROSOMES

Fab' fragments of mAb 323/A3 directed against the epithelial glycoprotein-2 (EGP-2) were conjugated to the distal ends of lipid-anchored PEG-chains present in the virosome membrane in order to achieve antibody-mediated binding to OVCAR-3 cells. Figure 2 shows that influenza virosomes without PEG bound to both A-2780 (EGP-2 negative) and OVCAR-3 (EGP-2 positive) cells most likely by HA-mediated binding to sialic acid residues present on the tumor cells. Incorporation of 20 mol% PEG-2000-DSPE (hemagglutination-inhibitory concentration) almost completely inhibited the binding to both types of tumor cells. 323/A3-PEG-virosomes only bound to EGP-2-expressing OVCAR-3 cells and not to A-2780 cells lacking this cell surface receptor. Thus, by conjugating 323/A3 Fab'-fragments to the distal ends of PEG-chains present on the virosome surface, influenza virosomes could be specifically redirected to OVCAR-3 cells.

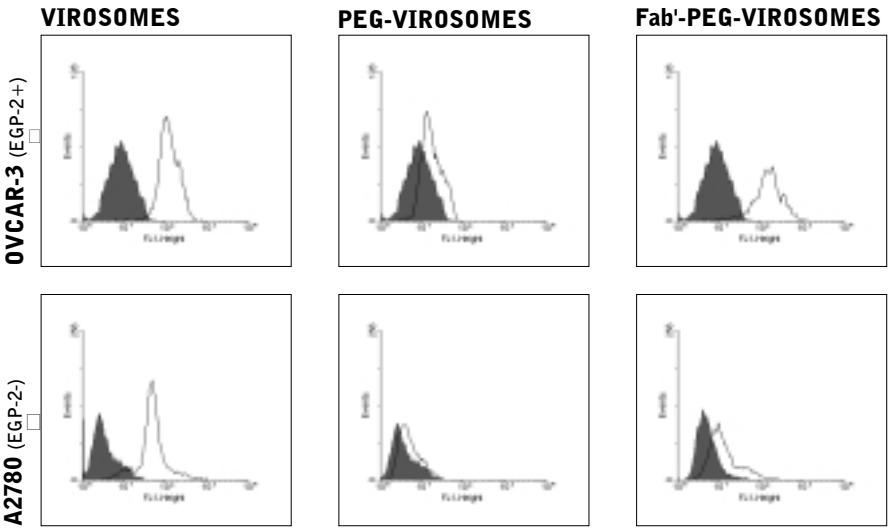
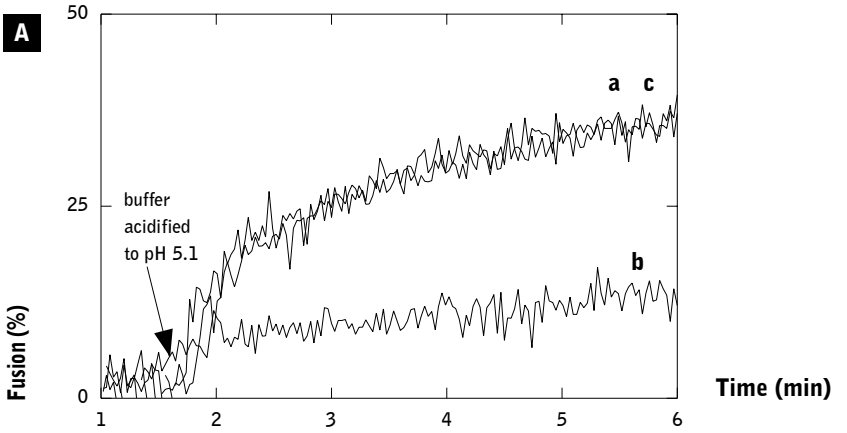


Figure 2 — Virosome binding to OVCAR-3 cells (expressing high levels of the target receptor EGP-2) and A2780 cells (no EGP-2 expression). Virosomes, PEG-2000-virosomes (prepared with 20 mol% PEG-2000-DSPE) and 323/A3 Fab'-PEG-2000-virosomes (prepared with 20% PEG-2000-DSPE), all labeled with 0.1% NBD-PE were incubated with either OVCAR-3 cells or A2780 cells ($1x10^6$ cells/ml) for 1 h at 4°C at a concentration of 10 μM phospholipid. Unbound virosomes were removed by 2 centrifugal wash steps before cells were analyzed by flow cytometry. Histograms in black show the fluorescence intensity of 3,000 cells incubated in buffer. Transparent overlays show the fluorescence intensity of 3,000 cells after incubation with the indicated virosome preparation.

FUSOGENIC ACTIVITY OF REDIRECTED INFLUENZA VIROSOMES

To determine whether binding of 323/A3-PEG-virosomes to OVCAR-3 cells via antibody/antigen interaction results in comparable levels of fusion as virosomes that bind to target membranes via the natural HA/sialic-acid interaction, the degree of lipid mixing induced by virosomes, PEG-virosomes or 323/A3-PEG-virosomes was measured. As we were unable to measure lipid mixing between virosomes and intact cells with the pyrPC assay, due to a very high scattering signal when intact cells were used, liposomes prepared from crude membrane extracts of OVCAR-3 cells according to the method of Bergers et al. [23] were used. These reconstituted cell membranes (OVCAR-3 liposomes) bear all the membrane proteins and lipids of the native cell membranes among which the EGP-2 cell surface antigen and sialylated proteins and lipids. Because of their relatively small size (100-200 nm) their scattering signal is much lower than that of whole cells. The results show that PEG virosomes containing 20 mol% of PEG-2000-DSPE were not able to induce lipid mixing with OVCAR-3 liposomes upon lowering the pH of the incubation medium to 5.1. As expected, plain virosomes induced lipid mixing with OVCAR-3 liposomes. The extent of lipid mixing was lower compared to lipid mixing with erythrocyte ghosts as acceptor membranes. This may be related to differences in the density of sialylated receptors expressed on the surface of the membrane vesicles. It is well known that erythrocyte membranes are a rich source of sialic acid bearing glycolipids and glycoproteins. When 323/A3-PEG-virosomes were added to OVCAR-3 liposomes and the pH of the medium was lowered to 5.1, a very low degree of lipid mixing was observed (results not shown). After pre-binding of the 323/A3-PEG-virosomes to the surface of OVCAR-3 liposomes for 1 h at room temperature, the extent of lipid mixing was as high as with unmodified virosomes (Figure 3A). Treatment of OVCAR-3 liposomes with neuraminidase to remove the sialic acid residues resulted in a drastically decreased level of lipid mixing induced by virosomes but only slightly altered the degree of lipid mixing observed for 323/A3-PEG-virosomes, indicating that in the latter case target cell binding is mainly mediated by antibody/antigen interaction and not by binding of HA to sialic acid residues (Figure 3B).



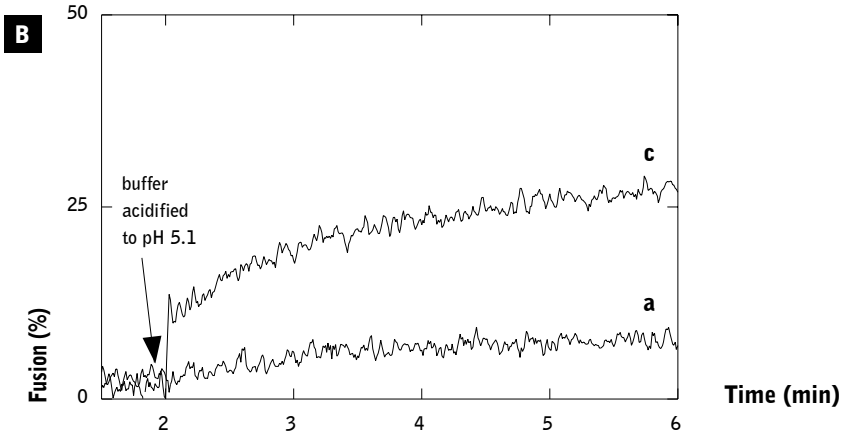


Figure 3 — Fusion of influenza virosomes with OVCAR-3 liposomes (Panel A) or OVCAR-3 liposomes treated with neuraminidase to remove sialic acid residues (Panel B). OVCAR-3 liposomes (200 μM phospholipid) were added to pyrPC-labeled virosomes (2.5 μM phospholipid). At the time point indicated with an arrow, the medium was acidified to pH 5.1 and fusion was monitored as previously described (see legend Figure.1). Results were obtained with virosomes (a); virosomes prepared with 20% PEG-2000-DSPE (b) and antibody-targeted virosomes also prepared with 20% PEG-2000-DSPE (c).

DISCUSSION

The results presented here show that influenza virosomes can be redirected to ovarian carcinoma cells with full retention of fusogenic activity. In the past, unsuccessful attempts have been made to target virosomes prepared from the reconstituted envelopes of Sendai virus and influenza virus to specific cell surface receptors [25-28]. The lack of success of targeting can be ascribed to the dominance of the natural binding tropism of the virosomes. Studies with influenza virus have demonstrated that removal or modification of the HA1 domain responsible for binding to the ubiquitous sialic acid residues, results in an impaired fusogenic activity mediated by the HA2 domain [29,30]. In addition, introduction of new, specific targeting elements by chemical conjugation of proteins to HA (personal observation) or by creating chimeric HA-scFv proteins at a genetic level also result in loss of fusogenic activity of the HA protein [25].

In this study we have chosen a different approach to redirect influenza virosomes without the need to modify HA. Inspired by the well-described use of PEG to shield surface-exposed proteins on colloidal systems as recently demonstrated for influenza virosomes by Chams et al. [31], we have anchored PEG to the surface of influenza virosomes by incorporating PEG-lipids into the viral envelope for two reasons: (1) to sterically shield HA thereby preventing it to interact with its natural ligand sialic acid and (2) to use the surface-grafted PEG layer as a spacer to conjugate specific homing devices to the distal end of the PEG-chains in order to obtain specific binding to target cells. The present results confirm that high concentrations of surface-grafted PEG (20 mol% PEG-2000-DSPE or 8 mol% PEG-5000-DSPE) on the virosomes fully block the binding of virosomes to sialic acid bearing receptors on human erythrocytes. Whether this inhibition of binding is caused by steric shielding of the sialic acid binding pocket of HA by incorporated PEG-lipids is not completely certain. X-ray crystallography has demonstrated that HA molecules protrude 13.5 nm from the viral membrane surface [32]. The effective thickness of the PEG coat has been reported to be approximately 6.5-7 nm at a surface coverage of 9 mol% PEG-1900 and 15 nm for PEG- 5000 at 7 mol% surface coverage [33]. Considering the dimensions of the PEG-layer and the HA protein one would expect that only PEG-5000 would be able to sterically shield the sialic acid binding pocket of HA. However, we observed that PEG-2000-DSPE incorporated in the virosome membrane at a density of 20 mol% also blocks the binding of influenza virosomes to erythrocytes. As high densities of surface-grafted PEG cause the PEG-chains to stretch out (*i.e.* brush formation) [34], it may well be possible that — at a density of 20 mol% PEG-2000 — the PEG-chains are reaching the fully stretched conformation which has a theoretical dimension of 15.8 nm (45 units of ethylene glycol of 3.5 Å each). However, it cannot be excluded that other mechanisms than steric shielding also play a role in the mechanism of sialic acid binding hindrance by the PEG-layer [35].

It should be noted that the amount of PEG-lipids that can be incorporated in bilayers is limited due to the tendency of PEG-lipids to form mixed micelles above a certain concentration [36].

Indeed, we observed that at the highest PEG-lipid concentration used (20 mol% PEG-2000), the recovery of viral phospholipids present in the virosome particles after removal of micelles and protein aggregates by density gradient centrifugation was low (recovery between 30-40%) compared to virosome preparations without PEG-lipids (recovery between 70-80%). This indicates that — at the highest concentration of PEG lipids used — solubilization of components from the viral envelope (lipids, spike proteins) into mixed micelles has occurred. Nevertheless, functional virosome particles containing PEG-lipids could be isolated.

Previous studies have shown that influenza virus HA-mediated fusion is independent of binding to sialic acid [15]. The binding step can be replaced by any other molecular interaction such as biotin/streptavidin interactions, which indicates that fusion is independent of any specific behavioral property of the underlying (cell surface) protein or lipid to which the sialic acid is linked. Presumably, the only function of the HA binding to sialic acid residues is to initiate receptor-mediated endocytosis of the virosome particles and then to hold the virosome in close proximity of the endosomal membrane while the membrane fusion takes place. This study demonstrates that binding of influenza virosomes via antibody-antigen interaction results in specific binding and subsequent membrane fusion. The antibody Fab'-fragments are displayed on the outer edge of the PEG layer whereas the sialic acid binding pocket of HA is shielded by the same PEG-layer. Therefore, the initial binding event of the virosomes to the target cell surface will be accomplished by the exposed antibody fragments. In view of the dimensions of the HA molecule (see above) it cannot be excluded that the initial binding of antibody to cell surface antigen, which brings the virosome particles in close proximity of the glycocalyx of the cell membrane favors the chance of HA to interact with sialic acid residues as a second binding mode.

The choice of targeting ligand for redirecting influenza virosomes is critically important. It should not only specifically bind to target cell surface receptors but binding should also lead to receptor-mediated endocytosis, as the virosome will only become fusogenic at the low pH environment within endosomes. Indeed, it has been reported that the antibody used here as targeting ligand is internalized after binding to EGP-2 [37,38].

Besides the advantage of grafted PEG-lipids to shield the binding function of HA, it may also prevent or inhibit unwanted immune recognition of the antigenic viral HA proteins after systemic administration of PEG-virosomes. Studies with PEGylated adenoviruses have demonstrated that surface PEG molecules can inhibit the antibody response against viral proteins [39,40]. *In vivo* pharmacokinetic studies on repeated injections of PEGylated influenza virosomes should be performed to address this issue.

In conclusion, this study demonstrates that influenza virosomes can be redirected towards ovarian carcinoma cells by incorporating PEG-lipids into the virosome membrane with conjugated antibodies at the distal ends of PEG-chains. Studies to address whether these antibody-redirected influenza virosomes can be utilized for enhancement of the cytosolic delivery of otherwise cell-impermeable substances are currently underway in our laboratory.

REFERENCE LIST

1 Yoshimura, T., Shono, M., Imai, K., and Hong, K. (1995): **Kinetic analysis of endocytosis and intracellular fate of liposomes in single macrophages**. *J.Biochem.(Tokyo).*, 117:34-41.

2 Straubinger, R.M., Hong, K., Friend, D.S., and Papahadjopoulos, D. (1983): **Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles**. *Cell*, 32:1069-1079.

3 Szoka, F.C., Jr. and Jones, C.S. (1987): **Uptake of liposome-encapsulated agents**. *Methods Enzymol.*, 149:143-7:143-147.

4 Stegmann, T., Morselt, H.W., Booy, F.P., van Breemen, J.F., Scherphof, G., and Wilschut, J. (1987): **Functional reconstitution of influenza virus envelopes**. *EMBO J.*, 6:2651-2659.

5 Bron, R., Ortiz, A., and Wilschut, J. (1994): **Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza virus envelopes (virosomes)**. *Biochemistry*, 33:9110-9117.

6 Schoen, P., Chonn, A., Cullis, P.R., Wilschut, J., and Scherrer, P. (1999): **Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles**. *Gene Ther.*, 6:823-832.

7 Daemen, T., de Haan, A., Arkema, A., and Wilschut, J. (1998): **Liposomes and virosomes as immunoadjuvant and antigen-carrier systems in vaccine formulations.** In: *Medical Applications of Liposomes*, edited by D.D. Lasic, et al, pp. 117-143. Elsevier, Amsterdam.

8 Matlin, K.S., Reggio, H., Helenius, A., and Simons, K. (1981): **Infectious entry pathway of influenza virus in a canine kidney cell line**. *J.Cell Biol.*, 91:601-613.

9 Yoshimura, A. and Ohnishi, S. (1984): **Uncoating of influenza virus in endosomes**. *J.Virol.*, 51:497-504.

10 White, J., Helenius, A., and Gething, M.J. (1982): **Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion**. *Nature*, 300:658-659.

11 Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A., and Wiley, D.C. (1982): **Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion**. *Proc.Natl.Acad.Sci.U.S.A.*, 79:968-972.

12 Arkema, A., Huckriede, A., Schoen, P., Wilschut, J., and Daemen, T. (2000): **Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes**. *Vaccine*, 18:1327-1333.

13 Waelti, E.R. and Gluck, R. (1998): **Delivery to cancer cells of antisense L-myc oligonucleotides incorporated in fusogenic, cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes)**. *Int.J.Cancer*, 77:728-733.

14 Tikchonenko, T.I., Glushakova, S.E., Kislina, O.S., Grodnitskaya, N.A., Manykin, A.A., and Naroditsky, B.S. (1988): **Transfer of condensed viral DNA into eukaryotic cells using proteoliposomes**. *Gene*, 63:321-330.

15 Schoen, P., Leserman, L., and Wilschut, J. (1996): **Fusion of reconstituted influenza virus envelopes with liposomes mediated by streptavidin/biotin interactions**. *FEBS Lett.*, 390:315-318.

16 Wharton, S.A., Skehel, J.J., and Wiley, D.C. (1986): **Studies of influenza haemagglutinin-mediated membrane fusion**. *Virology*, 149:27-35.

17 Stegmann, T., Schoen, P., Bron, R., Wey, J., Bartoldus, I., Ortiz, A., Nieva, J.L., and Wilschut, J. (1993): **Evaluation of viral membrane fusion assays. Comparison of the octadecylrhodamine dequenching assay with the pyrene excimer assay**. *Biochemistry* , 32:11330-11337.

18 Yoneda, Y. (1993): **Microinjection of macromolecules into cultured cells by erythrocyte ghost-cell fusion**. *Methods Enzymol.*, 221:306-317:306-317.

19 Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R., Green, J.A., Chu, E.W., Whang-Peng, J., Rogan, A.M., Green, W.R., and Ozols, R.F. (1983): **Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors**. *Cancer Res.*, 43:5379-5389.

20 Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C., and Aaronson, S.A. (1982): **Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells**. *Nature*, 295:116-119.

21 Nässander, U.K., Steerenberg, P.A., De Jong, W.H., Van Overveld, W.O., Te Boekhorst, C.M., Poels, L.G., Jap, P.H., and Storm, G. (1995): **Design of immunoliposomes directed against human ovarian carcinoma**. *Biochim.Biophys.Acta*, 1235:126-139.

22 Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. (1997): **Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro***. *Biochemistry*, 36:66-75.

23 Bergers, J.J., Den Otter, W., De Groot, J.W., De Blois, A.W., Dullens, H.F., Steerenberg, P.A., and Crommelin, D.J. (1992): **Reconstituted membranes of tumour cells (proteoliposomes) induce specific protection to murine lymphoma cells**. *Cancer Immunol.Immunother.*, 34:233-240.

24 Compans, R.W. and Roberts, P.C. (1994): **Viruses as model systems in cell biology.** In: *Methods in cell biology*, edited by M.G. Roth, pp. 3-42. Academic Press Inc.,

25 Patterson, S.M., Swainsbury, R., and Routledge, E.G. (1999): **Antigen-specific membrane fusion mediated by the haemagglutinin protein of influenza A virus: separation of attachment and fusion functions on different molecules**. *Gene Ther.*, 6:694-702.

26 Gitman, A.G., Graessmann, A., and Loyter, A. (1985): **Targeting of loaded Sendai virus envelopes by covalently attached insulin molecules to virus receptor-depleted cells: fusion-mediated microinjection of ricin A and simian virus 40 DNA**. *Proc.Natl.Acad.Sci.U.S.A.*, 82:7309-7313.

27 Gitman, A.G., Kahane, I., and Loyter, A. (1985): **Use of virus-attached antibodies or insulin molecules to mediate fusion between Sendai virus envelopes and neuraminidase-treated cells**. *Biochemistry*, 24:2762-2768.

28 Gitman, A.G. and Loyter, A. (1984): **Construction of fusogenic vesicles bearing specific antibodies. Targeting of reconstituted Sendai virus envelopes towards neuraminidase- treated human erythrocytes**. *J.Biol.Chem.*, 259:9813-9820.

29 Gray, C. and Tamm, L.K. (1997): **Structural studies on membrane-embedded influenza hemagglutinin and its fragments**. *Protein Sci.*, 6:1993-2006.

30 Godley, L., Pfeifer, J., Steinhauer, D., Ely, B., Shaw, G., Kaufmann, R., Suchanek, E., Pabo, C., Skehel, J.J., and Wiley, D.C. (1992): **Introduction of intersubunit disulfide bonds in the membrane-distal region of the influenza hemagglutinin abolishes membrane fusion activity**. *Cell*, 68:635-645.

31 Chams, V., Bonnafous, P., and Stegmann, T. (1999): **Influenza hemagglutinin mediated fusion of membranes containing poly(ethylene-glycol) grafted lipids: new insights into the fusion mechanism**. *FEBS Lett.*, 448:28-32.

32 Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J., and Wiley, D.C. (1988): **Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid**. *Nature*, 333:426-431.

33 Woodle, M.C., Collins, L.R., Sponsler, E., Kossovsky, N., Papahadjopoulos, D., and Martin, F.J. (1992): **Sterically stabilized liposomes. Reduction in electrophoretic mobility but not electrostatic surface potential**. *Biophys.J.*, 61:902-910.

34 Kenworthy, A.K., Hristova, K., Needham, D., and McIntosh, T.J. (1995): **Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol)**. *Biophys.J.*, 68:1921-1936.

35 Papisov, M.I. (1998): **Theoretical considerations of RES-avoiding liposomes: Molecular mechanics and chemistry of liposome interactions**. *Adv.Drug.Del.Rev.*, 32:119-138.

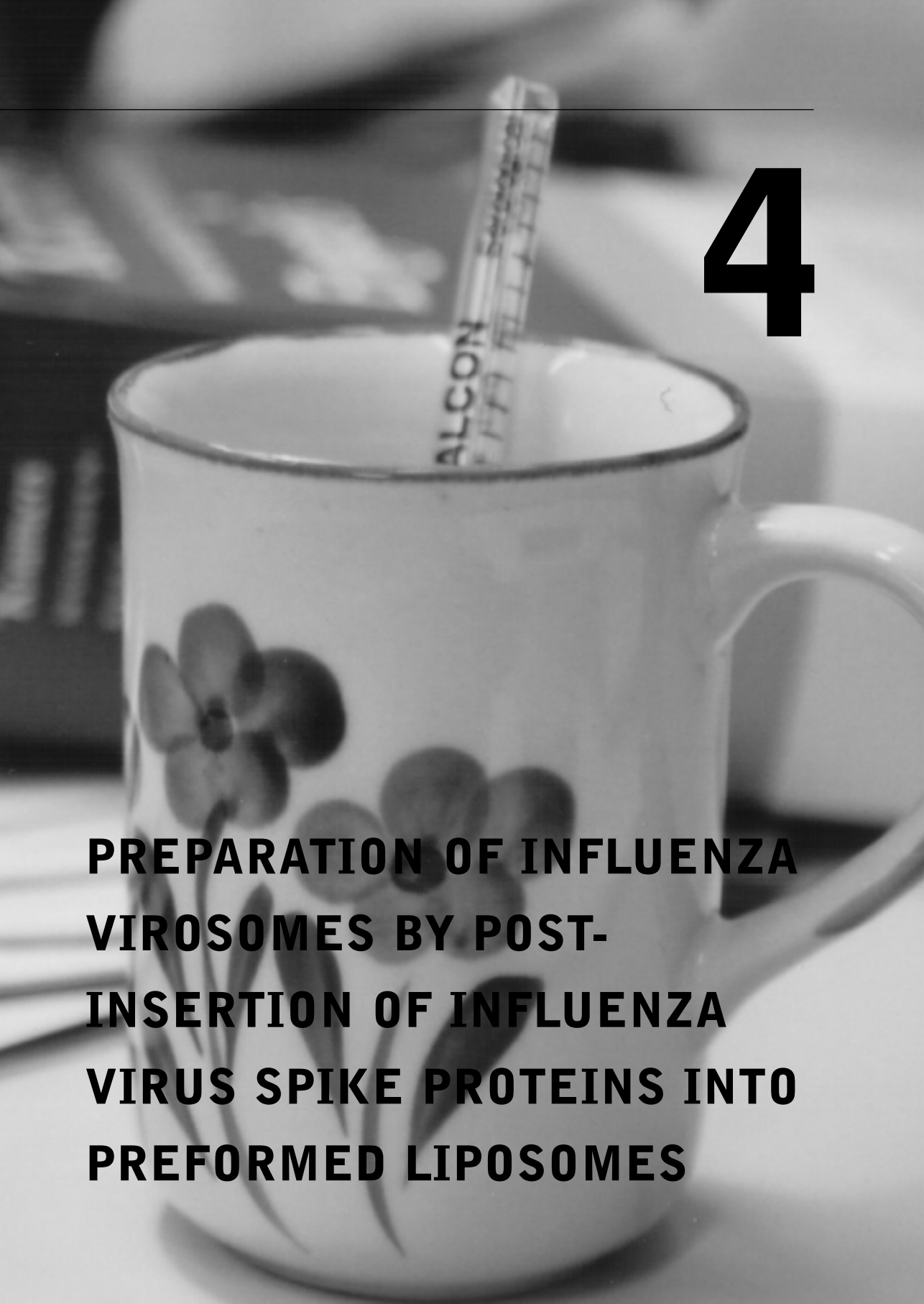
36 Edwards, K., Johnsson, M., Karlsson, G., and Silvander, M. (1997): **Effect of polyethyleneglycol-phospholipids on aggregate structure in preparations of small unilamellar liposomes**. *Biophys.J.*, 73:258-266.

37 Zimmermann, S., Wels, W., Froesch, B.A., Gerstmayer, B., Stahel, R.A., and Zangemeister-Wittke, U. (1997): **A novel immunotoxin recognising the epithelial glycoprotein-2 has potent antitumoural activity on chemotherapy-resistant lung cancer**. *Cancer Immunol.Immunother.*, 44:1-9.

38 Haisma, H.J., Pinedo, H.M., Rijswijk, A., der, M., Sosnowski, B.A., Ying, W., Beusechem, V.W., Tillman, B.W., Gerritsen, W.R., and Curiel, D.T. (1999): **Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM**. *Gene Ther.*, 6:1469-1474.

39 O'Riordan, C.R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S.C., Smith, A.E., and Francis, G.E. (1999): **PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo***. *Hum.Gene Ther.*, 10:1349-1358.

40 Romanczuk, H., Galer, C.E., Zabner, J., Barsomian, G., Wadsworth, S.C., and O'Riordan, C.R. (1999): **Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice**. *Hum.Gene Ther.*, 10:2615-2626.



4

PREPARATION OF INFLUENZA VIROSOMES BY POST-INSERTION OF INFLUENZA VIRUS SPIKE PROTEINS INTO PREFORMED LIPOSOMES

PREPARATION OF INFLUENZA VIROSOMES BY POST-INSERTION OF INFLUENZA VIRUS SPIKE PROTEINS INTO PREFORMED LIPOSOMES

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SUMMARY

In this study, a novel method of influenza virosome preparation is presented. This method is based on the post-insertion of influenza virus envelope components (consisting predominantly of hemagglutinin (HA) spike proteins) that are solubilized with short-chain phosphatidylcholines into preformed liposomes. It was hypothesized that with the 'post-insertion' method of virosome preparation, the problem of low encapsulation efficiency of water-soluble compounds related to the traditional method of virosome preparation (*i.e.* reconstitution of influenza virus envelopes solubilized with $C_{12}E_8$) could be circumvented. It is demonstrated that influenza virus spike proteins can be post-inserted into preformed liposomes with full retention of fusion activity of HA. Unfortunately, under the conditions needed for effective post-insertion of viral spike proteins into liposomes, the post-insertion procedure induced extensive leakage of liposome-entrapped molecules, both small (calcein) and large (DNA). Nevertheless, the use of dicaproyl phosphatidylcholine (DCPC) as detergent in the post-insertion method is advantageous as bilayer-associated DCPC molecules can be readily removed from the virosomes by dialysis, in contrast to the low CMC detergent used in the traditional method of virosome preparation. It is anticipated that post-insertion virosomes are applicable for cytosolic delivery of molecules which can be entrapped in virosomes via remote loading procedures.

INTRODUCTION

Reconstituted membranes of enveloped viruses (virosomes) have attracted attention as drug carriers because they possess fusogenic activity towards target membranes enabling the cytosolic deposition of entrapped drug molecules. Virosomes have been prepared from a variety of enveloped viruses, among which vesicular stomatitis virus (VSV) [1,2], Sendai virus [3-5], Semliki Forest virus (SFV) [6] and influenza virus [7-13]. Influenza virosomes have been prepared in different ways, but only a few methods resulted in reconstitution of viral envelopes with preservation of fusogenic activity [7,9,10]. One successful reconstitution procedure that is commonly used is based on solubilization of the viral envelope with $C_{12}E_8$ detergent with a low critical micelle concentration (CMC), removal of the viral nucleocapsid by ultracentrifugation, and subsequent controlled removal of the detergent from the supernatant by adsorption to hydrophobic, polystyrene beads [10]. SDS-PAGE analysis of influenza virosomes prepared via the traditional method utilizing the $C_{12}E_8$ detergent has demonstrated that the proteins reconstituted in virosomes primarily consist of the spike proteins hemagglutinin (HA) and neuraminidase (NA) in a ratio of 5:1. Cryo-EM analysis showed that virosomes are unilamellar vesicles of approximately 150-200 nm in size and densely covered with spikes, which appear to be present on both sides of the membrane. The presence of fusion-active HA molecules in the bilayer of influenza virosomes allows fusion of the virosome membrane with the endosomal membrane after internalization by the target cells. In this way virosome-entrapped compounds can be delivered into the cytosol of cells. Indeed, several groups have reported that influenza virosomes can be used as carriers for the cytosolic delivery of bacterial toxins [14,15], peptides [16,17], and DNA [18]. Yet, virosome-mediated cytosolic delivery of molecules is limited by a poor encapsulation efficiency of water-soluble compounds into the virosome carrier system [14]. Moreover, as fusogenic influenza virosomes are prepared with detergents having a low CMC, the presence of residual detergent molecules in the virosome membrane is to be expected. Residual detergent in influenza virosomes prepared with the standard method has been determined to be 7.5-9 mol% relative to total virosomal lipid [10]. Favored by the presence of residual detergent monomers, the virosomal bilayers are rather leaky for entrapped molecules. An attempt to prepare influenza virosomes using the detergent octyl glucoside, which can be readily removed by dialysis yielding low residual amounts in the virosomal bilayers, resulted in the formation of vesicles lacking pH-dependent membrane fusion activity [10].

Different methods of liposome preparation are available that provide efficient entrapment of water-soluble (macro)molecules as well as flexibility in choice of bilayer constituents [19]. Ideally, one would like to combine the flexibility in choice of lipid composition and preparation method with the pH-dependent membrane fusion capacity of virosomes. One way to achieve this may be the incorporation of influenza virus spike proteins into preformed liposomes with the desired bilayer composition. Viral spike proteins of VSV and Sindbis virus have been successfully

incorporated into preformed liposomes [20,21]. In addition, several other membrane proteins have been incorporated into liposomes by spontaneous insertion of the purified protein [22,23] or by detergent-aided insertion [24-27]. Several detergents, among which short-chain lecithins, have been used for facilitating insertion of membrane proteins [28,29]. The latter have the advantage that they can be readily removed from the phospholipid bilayers by dialysis and do not seem to impair the biological activity of membrane proteins during solubilization [28,29].
In this study, an attempt is made to circumvent the problems of low encapsulation efficiency and poor bilayer stability related to the traditional influenza virosome preparation methods by preparing influenza virosomes in two separate steps. In the first step, small-sized liposomes were prepared by lipid-film hydration and subsequent extrusion [30], which allows for more efficient entrapment of water-soluble compounds. The second step involved post-insertion of influenza virus spike proteins that were solubilized with short-chain phospholipids (dicaproyl phosphatidylcholines; DCPC) into the preformed liposomes with subsequent removal of the short-chain PCs by dialysis. This study shows for the first time that membrane fusion-active influenza virosomes can be prepared by post-insertion of viral spike proteins utilizing a high CMC detergent.

MATERIALS AND METHODS

MATERIALS

The X47 recombinant strain of influenza virus, carrying the HA of influenza A/Victoria/3/75, was grown and purified as described elsewhere [31]. Octa(ethylene glycol)-mono(*n*-dodecyl) ether ($C_{12}E_8$; CMC: 0.11 mM) was purchased at Calbiochem (Darmstadt, Germany). PyrPC (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine) and the green fluorescent cell-impermeant SYTOX green nucleic acid stain were from Molecular Probes (Eugene, OR, USA). Bovine brain gangliosides purified from bovine brain, cholesterol, 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DCPC; CMC: 14.4 mM) and calcein were from Sigma-Aldrich Co. (St. Louis, MO, USA). Egg phosphatidylcholine (EPC), egg phosphatidylethanolamine (EPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) were all from Avanti Polar Lipids Inc. (Alabaster, AL, USA). DNase I from bovine pancreas was obtained from Boehringer Mannheim (Mannheim, Germany). Sepharose CL-4B came from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

PREPARATION OF LIPOSOMES

Lipids used for all liposome preparations were dissolved in $CHCl_3$:MeOH (2:1 v/v ratio) and stored at –20°C. Lipids from stock solutions were mixed at the appropriate molar ratio in a round bottom flask and dried under vacuum by rotary evaporation. The obtained lipid films were further dried for at least 1 h under a stream of N_2 to remove all traces of organic solvent. Lipid films were hydrated with 1 ml of HEPES-buffered salt solution (HBS; 5 mM HEPES, 150 mM NaCl, pH 7.4), containing the substances to be encapsulated (DNA or calcein), by shaking in the presence of glass beads. The formed multilamellar liposomes were extruded through 0.2 µm filters using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). If needed, extruded liposomes were separated from non-encapsulated materials by column chromatography using a Sepharose CL-4B column. Lipid concentration of liposomes was determined by measuring the amount of lipid phosphate according to Fiske et al. [32]. Liposomes used for post-inserting influenza virus spike proteins were composed of EPC, EPE and cholesterol at a molar ratio of 4:2:3, respectively. For lipid mixing experiments 10 mol% (relative to phospholipid) of pyrPC was incorporated in these liposomes. Liposomes used as 'target' membranes in lipid mixing studies had the same lipid composition as liposomes used for post-insertion, supplemented with 10 mol% or 30 mol% gangliosides (relative to the total amount of phospholipid).

VIRUS SOLUBILIZATION

An amount of virus corresponding to 200 nmol of phospholipid was pelleted by ultracentrifugation (1 h; 100,000xg; 4°C) using a fixed angle rotor. Pelleted virus was resuspended in 400 µl of high salt HNE buffer (5 mM HEPES; 1 M NaCl; 0.1 mM EDTA; pH 7.4) using a 26G needle. Resuspended virus was transferred to a tube containing a dry film of 16 µmol DCPC and incubated for 30 min at ambient temperature. Insoluble nucleocapsids were removed by ultracentrifugation at 100,000xg for 20 min at 4°C and supernatant containing the solubilized viral envelope components was collected. After lipid phosphate determination (corrected for the presence of DCPC), solubilized viral envelopes were directly used for post-insertion.

PREPARATION OF POST-INSERTION (PI)-VIROSOMES

Preformed liposomes (200 µM of phospholipid) were incubated in HBS containing 8 mM of DCPC (below CMC of detergent) for 30 min at room temperature. Detergent-containing liposomes were admixed with viral envelopes solubilized in 40 mM DCPC at such volume ratios that the final detergent concentration did not exceed 11 mM. Mixtures were incubated for 30 min at room temperature and gently shaken every 5 min. Samples were dialyzed overnight using a Slide-A-Lyzer dialysis cassette with a molecular weight cut-off (MWCO) of 10,000 (Pierce, Rockford, IL, USA) at 4°C against two changes of HBS in 1000-fold volume excess.

LIPID MIXING ASSAY OF MEMBRANE FUSION

The extent of membrane fusion was determined as described in chapter 3 [33]. Briefly, PI-virosomes labeled with the fluorescent probe pyrPC (10 mol% relative to the total amount of lipid) at a concentration of 10 or 20 µM of PL were admixed with an excess of donor membranes (*i.e.* erythrocyte ghosts prepared as described in [34] (100 µM of PL) or ganglioside liposomes in a total volume of 1.4 ml of HBS). The virosome mixture was placed into a cuvette and stirred at a temperature of 37°C. After 2 min, the pH of the medium in the cuvette was lowered to 5.1 by adding 1/20 volume of fusion buffer (0.1 M 2-[*N*-morpholino]ethanesulfonic acid] (MES), 0.1 M acetic acid, pH 4.1). Fusion was continuously monitored at 37°C by measuring the decrease in pyrPC excimer fluorescence with a LS50B fluorescence spectrophotometer (Perkin Elmer, Beaconsfield, UK) set at an excitation wavelength of 345 nm and an emission wavelength of 480 nm. The decrease in fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at infinite dilution, which was determined by adding 70 µl of 10% (v/v) Triton X-100 in HBS to the PI-virosome dispersion.

LEAKAGE ASSAYS

Leakage of calcein

Liposomes (500 nmol PL/ml) containing 100 mM of the fluorescent probe calcein (at this concentration calcein is >95% self-quenched) were incubated for indicated time periods at 37°C with increasing DCPC concentrations (0-20 mM) to determine detergent-induced leakage. In addition, leakage of calcein from liposomes was also investigated in the presence of DCPC-solubilized influenza virus spike proteins (0.5 mM PL) at increasing final detergent concentrations (0-20 mM) to determine the effect of post-insertion on the extent of calcein leakage. Calcein leakage was expressed relative to the difference in fluorescence of calcein-containing liposomes incubated at 37°C in the presence of 0.5% (v/v) of Triton X-100 in HBS (defined as 100% leakage) and incubated in the absence of DCPC at 37°C (defined as 0% leakage). Fluorescence was measured with an LS50B fluorescence spectrophotometer set at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Leakage of DNA

Plasmid DNA (5,800 basepairs in size) was entrapped in liposomes composed of EPC, EPE and cholesterol (molar ratio 4:2:3, respectively) as described by Schoen et al. [35]. After two centrifuge steps (100,000xg for 1 h at 4°C) to remove non-encapsulated DNA, these DNA liposomes were used for post-insertion of viral spike proteins. DNA-containing liposomes and, after post-insertion and dialysis, DNA-containing PI-virosomes were analyzed for the presence of DNA not entrapped within the lipid vesicles (*i.e.* either free in solution or associated with the liposome surface) with the membrane-impermeant SYTOX Green nucleic acid stain. Liposomes or PI-virosomes (50 µM of PL) were added to a cuvette containing 1 mM $MgCl_2$ and SYTOX Green nucleic acid stain (1 µM in HBS, pH 7.4) and fluorescence was measured with an LS50B fluorometer set at an excitation wavelength of 504 $\pm$ 2.5 nm and an emission wavelength of 523 $\pm$ 4 nm. The amount of DNA that was not entrapped within liposomes before and after post-insertion was expressed relative to the difference in SYTOX Green fluorescence obtained after disruption of the DNA-liposomes or PI-virosomes with 0.5% (v/v) of Triton X-100 and the SYTOX Green fluorescence obtained with DNA-liposomes or PI-virosomes from which the externally located DNA had been degraded by DNase I (10 U/ml).

Electron microscopy

Influenza virus particle, influenza virosomes and liposomes were adsorbed to glow-discharged carbon-stabilized formvar-coated grids, negatively stained using 2.0% potassium phosphotungstate (pH 5,2) and analyzed using a Philips TEM 400 electron microscope at an operating voltage of 80 kV. Images were digitally stored and analyzed using analySIS (Soft-imaging software, Germany).

RESULTS

EFFECT OF DCPC ON LEAKAGE OF CALCEIN FROM LIPOSOMES

Several membrane proteins have been successfully inserted into preformed liposomes aided by bilayer-associated amphipathic molecules such as cholesterol, short-chain phosphatidylcholines, detergents, and lyso-derivatives of fatty acids into the liposomal bilayer [36]. Similarly, in this study we focused on the post-insertion of influenza virus spike proteins (approximately 80% HA and 20% NA) solubilized with dicaproyl phosphatidylcholine (DCPC) into preformed liposomes, whose bilayers contained sub-solubilizing amounts of DCPC molecules to facilitate spike protein insertion. As leakage of liposome-entrapped compounds during the post-insertion procedure is undesirable, we first determined the maximum concentration of DCPC that can be utilized without causing extensive leakage of liposome-entrapped compounds. Figure 1 shows that the degree of leakage of the fluorescent probe calcein from liposomes composed of EPC, EPE and cholesterol (molar ratio 4:2:3, respectively) is dependent on the concentration of DCPC added to the dispersion and the incubation time at 37°C. Extensive leakage of calcein is observed at DCPC concentrations >12 mM. Dynamic light scattering measurements were performed to monitor integrity of liposome particles (results not shown). At 20 mM of DCPC a decrease in average particle size was observed, indicating that at this detergent concentration liposome solubilization had occurred. For all experiments described hereafter, a final concentration of 8 mM DCPC was used to facilitate viral spike protein insertion, as at this concentration leakage of calcein is marginal, even after 6 hrs incubation at 37°C (Figure. 1).

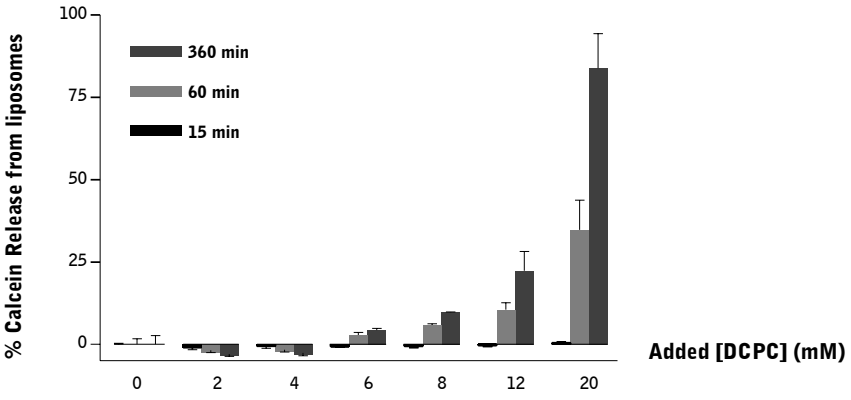

Figure 1 — Calcein release from liposomes at increasing concentrations of DCPC. Liposomes (EPC:EPE:CHOL 4:2:3; 100 nmol PL) were incubated for 15, 60 or 360 min at 37°C at increasing concentrations of DCPC. Calcein release was determined relative to the difference in release between Triton X-100-solubilized liposomes (100% release) and liposomes incubated for 360 min in the absence of DCPC (0% release).

ELECTRON MICROSCOPY ANALYSIS

The morphology of influenza virosomes prepared by post-inserting influenza virus spike proteins into preformed liposomes (PI-virosomes) was analyzed by electron microscopy and compared with the morphology of native influenza virus particles, liposomes without post-inserted spike proteins and influenza virosomes prepared with the $C_{12}E_8$ method [10]. The EM-pictures (Figure 2) show that the size of both PI-virosomes (panel D) and $C_{12}E_8$ virosomes (panel C) is approximately 150-200 nm, which is comparable to the size of intact influenza virus particles (panel A). The viral spike proteins in the bilayer of virosomes prepared with the $C_{12}E_8$ method are both facing inwards and outwards. In contrast, the spike proteins post-inserted into preformed liposomes are primarily pointing outwards, similar to the orientation of spike proteins in native influenza virus particles.

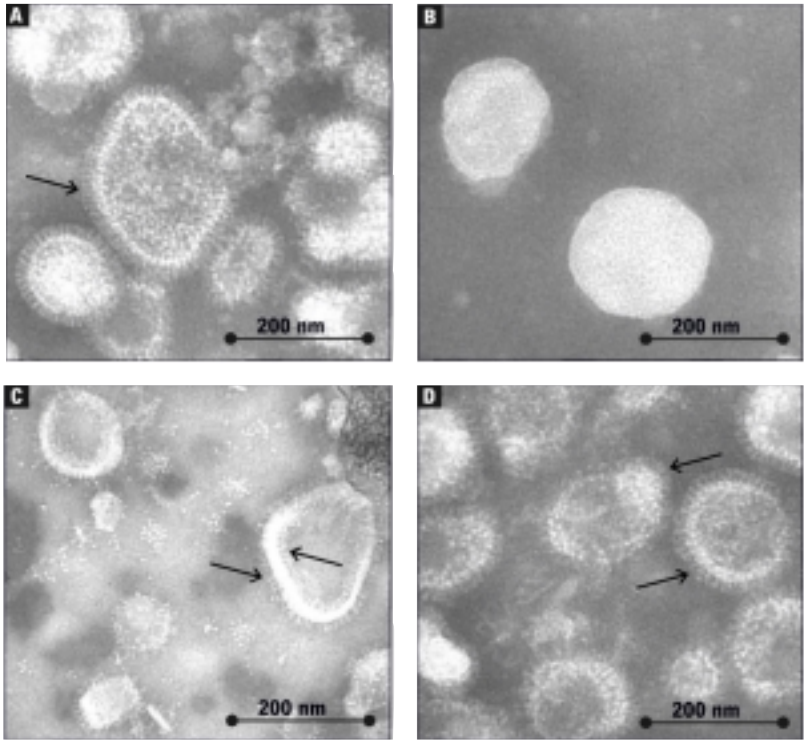

Figure 2 — Electron micrographs of influenza virus particles (A), liposomes (B), $C_{12}E_8$ virosomes (C), and PI-virosomes (D) negatively stained with 1% sodium phosphotungstate, pH 7.0. The bar in each micrograph represents 200 nm. Arrows indicate viral spike proteins inserted in the virosome bilayer. Note that spike proteins located in the bilayer of virosomes prepared with the $C_{12}E_8$ method are facing inwards as well as outwards.

PI-VIROSOME-INDUCED LIPID MIXING

Effect of viral spike protein density

A lipid mixing assay was performed to determine whether PI-virosomes are fusogenic. PI-virosomes were prepared by post-inserting increasing amounts of solubilized influenza virus spike proteins into a fixed amount of pyrPC-labeled liposomes. Bilayers of liposomes were composed of EPC, EPE and cholesterol at a molar ratio of 4:2:3, respectively. Lipid mixing between PI-virosomes and an excess of erythrocyte ghosts was monitored continuously for 5 min after lowering the pH of the buffer to 5.1. It can be seen that the degree of lipid mixing between PI-virosomes and erythrocyte ghosts is proportional to the amount of spike proteins added for post-insertion (Figure 3). PI-virosomes prepared at a lipid to protein (w/w) ratio of 1:2 show the same extent of lipid mixing as observed for influenza virosomes prepared with the traditional $C_{12}E_8$ method [10]. Control liposomes with the same composition as used for the PI-virosomes but without spike proteins do not show lipid mixing, indicating that lipid mixing is exclusively mediated by the post-inserted viral components. In addition, lipid mixing is pH-dependent as it only occurs after lowering the pH of the buffer.

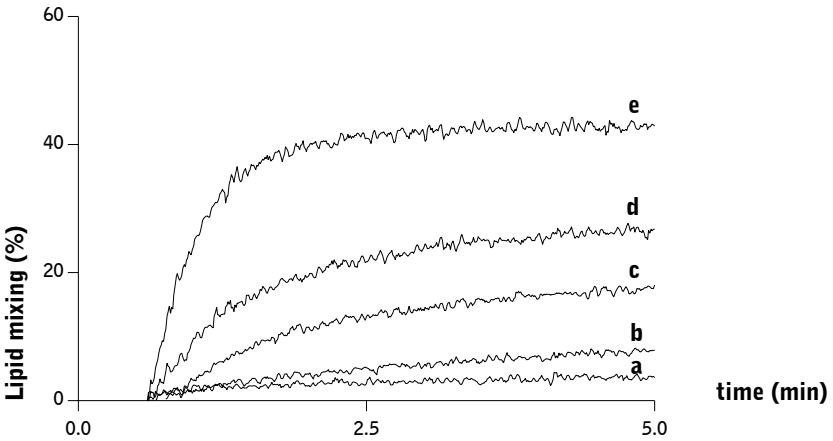

Figure 3 — Lipid mixing between PI-virosomes and erythrocyte ghosts. PyrPC-labeled PI-virosomes (20 μM of PL) were mixed with erythrocyte ghosts (100 μM of PL) in a cuvette kept at a temperature of 37°C while continuously stirring the mixture. The occurrence of lipid mixing expressed as the decrease in pyrene excimer fluorescence was continuously monitored (after acidification of the buffer to pH 5.1) with an LS50B fluorescence spectrophotometer set at an excitation wavelength of 345 nm and an emission wavelength of 480.nm The decrease of fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at infinite dilution (*i.e.* after vesicle solubilization with detergent). Curves indicate lipid mixing obtained with PI-virosomes prepared at phospholipid to spike protein (w/w) ratios of 1:0 **(a)**, 1:0.25 **(b)**, 1:0.5 **(c)**, 1:1 **(d)**, and 1:2 **(e)**, respectively.

Effect of bilayer rigidity

As the efficiency of insertion of influenza virus spike proteins into preformed liposomes may be dependent on the bilayer rigidity, PI-virosomes were prepared by post-inserting spike proteins (lipid-to-protein ratio: 1:2) into liposomes with different bilayer rigidity (EPC:EPE:CHOL, DPPC:DPPE:CHOL, and DSPC:DSPE:CHOL, molar ratio of 4:2:3 for all compositions). These PI-virosomes were tested for their ability to induce lipid mixing with erythrocyte membranes (Figure 4). The relative extent of lipid mixing of PI-virosomes composed of DSPC:DSPE:CHOL was 55% compared to 40% for PI-virosomes composed of DPPC:DPPE:CHOL and 40% for PI-virosomes composed of EPC:EPE:CHOL. Thus, the extent of lipid mixing between PI-virosomes and erythrocyte ghosts was highest in case of the most rigid bilayer composition.

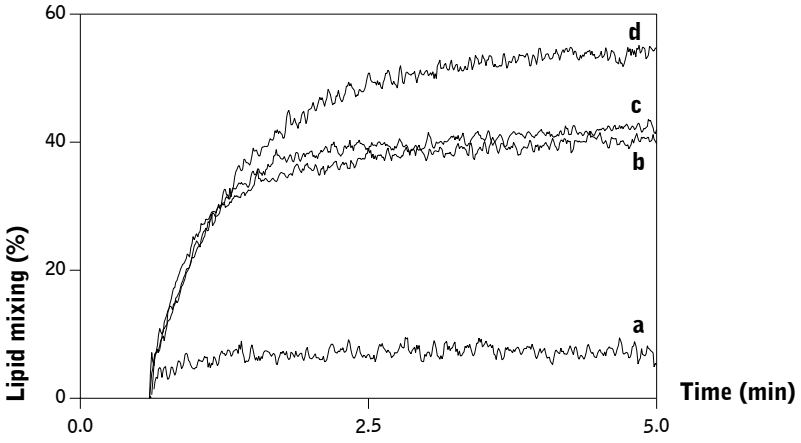

Figure 4 — Influence of lipid bilayer rigidity of PI-virosomes on the extent of lipid mixing with erythrocyte ghosts. Either liposomes or PI-virosomes (20 μM of PL) were mixed with erythrocyte ghosts (100 μM of PL) in a cuvette kept at a temperature of 37°C. The mixture was continuously stirred. Lipid mixing was monitored after lowering the pH of the medium to 5.1. Lipid mixing between erythrocyte ghosts and preformed DSPC:DSPE:CHOL liposomes **(a)**, EPC:EPE:CHOL PI-virosomes **(b)**, DPPC:DPPE:CHOL PI-virosomes **(c)** and DSPC:DSPE:CHOL PI-virosomes **(d)** are shown.

Effect of target membranes: erythrocyte ghosts versus ganglioside liposomes

Erythrocyte ghosts are frequently used as target membranes to study lipid mixing with influenza virus particles or virosomes, as they are a rich source of sialylated lipids (gangliosides) and glycoproteins that form the natural receptors for influenza virus HA. To determine the effect of target membrane composition on the extent of lipid mixing induced by PI-virosomes, liposomes (EPC:EPE:CHOL molar ratio 4:2:3) containing 10 or 30 mol% gangliosides (purified from bovine brain) as a source of sialic acid residues were used as target membranes. Lipid mixing of PI-virosomes with ganglioside-liposomes was compared with that of erythrocyte ghosts. Figure 5 shows that lipid mixing of PI-virosomes with ganglioside liposomes is less efficient than with erythrocyte membranes. Increasing the amount of gangliosides present in the liposomal bilayers did not result in an increase in lipid mixing, indicating that the amount of natural receptors for HA present on the liposomes is not the limiting factor. Probably, differences in bilayer composition of erythrocyte ghost membranes and ganglioside-liposomes underlie the differences in lipid mixing with PI-virosomes. It is noteworthy that lipid mixing still occurs despite the presence of high amounts of cholesterol (33 mol%) in the bilayer of target membranes.

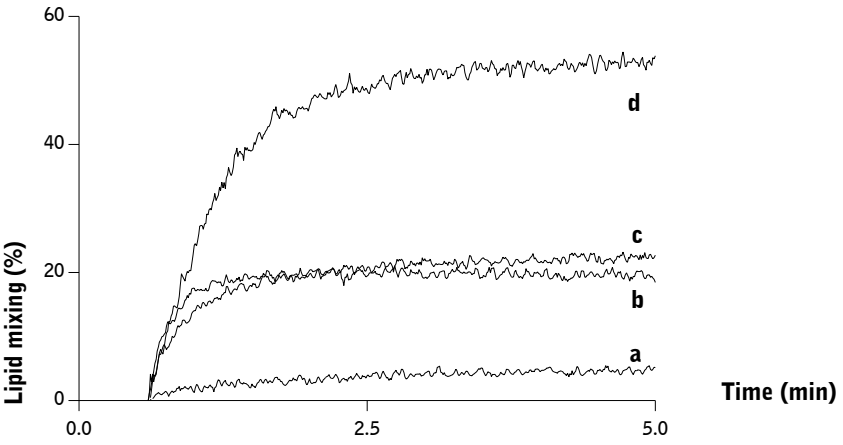

Figure 5 — Lipid mixing of PI-virosomes with different target membranes. PI-virosomes were mixed with an excess of target membrane vesicles (100 μM of erythrocyte ghosts or 500 μM of (ganglioside) liposomes) in a cuvette kept at 37°C. Lipid mixing was continuously monitored upon acidification of the buffer to pH 5.1. During measurements cuvette contents were continuously stirred. Target membrane vesicles used for lipid mixing were EPC:EPE:CHOL (4:2:3 molar ratio) liposomes **(a)**, EPC:EPE:CHOL liposomes containing 10 mol% gangliosides (relative to PL) **(b)**, EPC:EPE:CHOL liposomes containing 30 mol% gangliosides **(c)** and erythrocyte ghosts **(d)**.

LEAKAGE DURING POST-INSERTION

Leakage of calcein

The previous experiments show that, in the presence of 8 mM of DCPC, influenza virus spike proteins can be effectively inserted into the bilayers of preformed liposomes with retention of fusogenic activity as demonstrated by the occurrence of lipid mixing. Destabilization of liposomal bilayers, in the presence of low amounts of DCPC (8 mM) to facilitate spike protein insertion, resulted in only low levels of calcein leakage from the liposomes (Figure 1). As the insertion of membrane proteins into the liposome bilayer may also influence the retention of liposome-entrapped solutes, the extent of calcein leakage from liposomes during the viral spike protein insertion process was determined. Influenza virus spike proteins solubilized with 40 mM of DCPC were added to calcein-containing liposomes (2:1 lipid to protein ratio) at increasing volume ratios to obtain final detergent concentrations varying from 2.5 mM DCPC to 20 mM DCPC. Figure 6 shows that calcein leakage during post-insertion increased at increasing final concentrations of DCPC. Leakage of calcein from liposomes at comparable DCPC concentration (10 mM final concentration) as used for effective spike protein insertion (8 mM final concentration) was high: 80% of calcein was released during the 1 h post-insertion process. No leakage of calcein was observed at 2.5 mM DCPC and 25% of calcein was released at 5 mM DCPC. However, PI-virosomes prepared at final DCPC concentrations (2.5 mM and 5 mM DCPC) that allowed retention of most of the liposome-entrapped calcein during the post-insertion process, did not

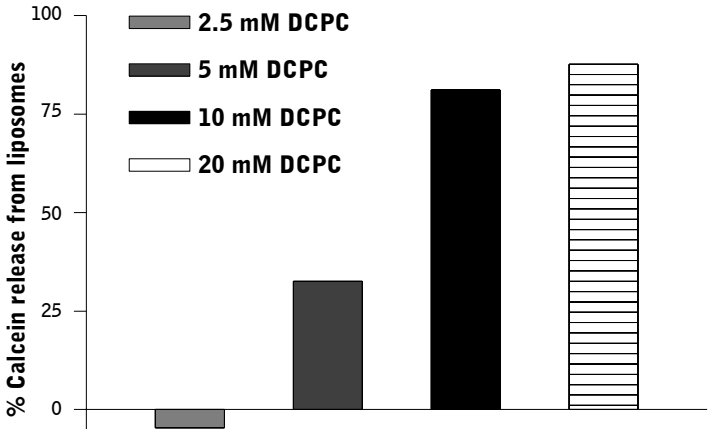

Figure 6 — Calcein leakage from liposomes during post-insertion of influenza virus spike proteins in the presence of varying DCPC concentrations for a period of 60 min.

show much fusogenic activity (Figure 7). Clearly, effective post-insertion of influenza virus spike proteins into preformed liposomes is accompanied by extensive leakage of encapsulated calcein.

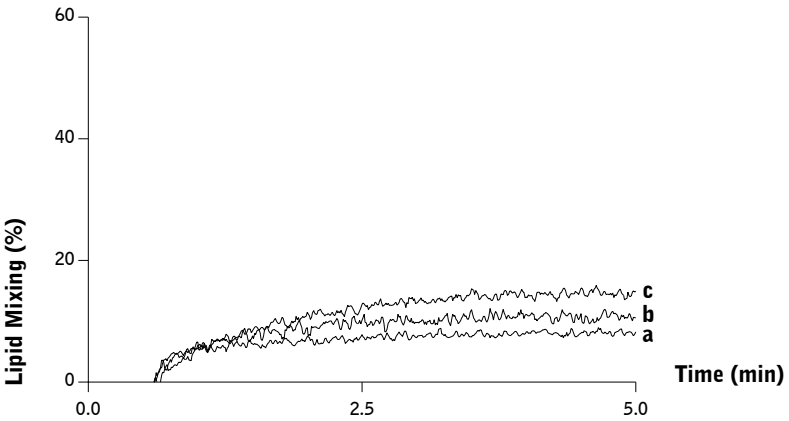

Figure 7 — Lipid mixing between PI-virosomes prepared at final DCPC concentrations of 2.5 and 5 mM and erythrocyte ghosts. PI-virosomes were prepared by solubilizing influenza virus spike proteins with 40 mM DCPC and mixing solubilized spike proteins with preformed liposomes at different volume ratios to obtain varying final DCPC concentration in the PI-virosome mixture. Lipid mixing between an excess of erythrocyte ghosts (100 μM of PL) and PI-virosomes (20 μM of PL) prepared at final DCPC concentrations of 2.5 mM **(curve b)** and 5 mM **(curve c)** obtained after detergent dialysis are shown as well as lipid mixing between erythrocyte ghosts and preformed liposomes without post-inserted spike proteins **(curve a).**

DNA leakage from liposomes during post-insertion

To determine whether the calcein leakage phenomenon during post-insertion of influenza virus spike proteins also applies to membrane-impermeant macromolecules, influenza virus spike proteins were post-inserted into liposomes in which DNA had been encapsulated according to the method of Schoen et al. [35]. The percentage of externally located DNA (*i.e.* DNA associated with the surface of liposomes and DNA free in solution) before and after post-insertion of spike proteins into DNA-containing liposomes was determined with the membrane-impermeant SYTOX Green nucleic acid stain (Figure 8). The results show that before post-insertion, approx. 40% of liposome-associated DNA was entrapped within liposomes, whereas after post-insertion of viral spike proteins all DNA could be stained with SYTOX Green, indicating that all DNA had been released from the liposomes during the post-insertion process.

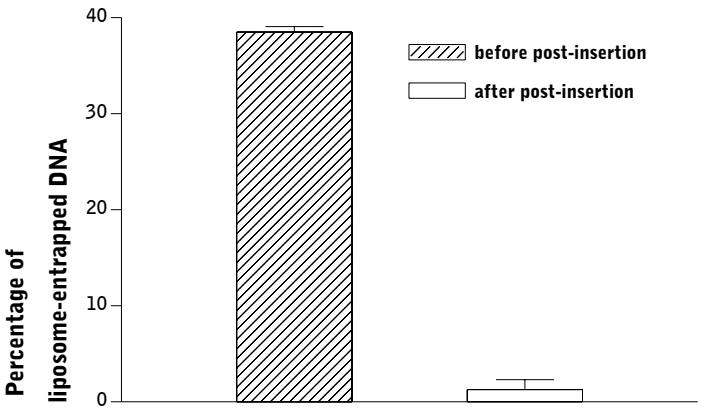

Figure 8 — Leakage of DNA from liposomes during post-insertion of influenza virus spike proteins. The amount of DNA entrapped within liposomes (EPC:EPE:CHOL, molar ratio 4:2:3) was determined before and after post-insertion of influenza virus spike proteins at a lipid-to-spike protein ratio of 1:2. The amount of entrapped DNA is expressed relative to the total amount of liposome- or PI-virosome-associated DNA.

DISCUSSION

In an attempt to obtain fusogenic influenza virosomes with a higher encapsulation efficiency of water-soluble (macro)molecules, we have post-inserted influenza virus spike proteins solubilized with DCPC into preformed liposomes. This method has several advantages over the traditional method of influenza virosome preparation that involves viral envelope solubilization with $C_{12}E_8$ and subsequent controlled removal of detergent by adsorption to hydrophobic, polystyrene beads [10]. First, the use of preformed liposomes as basis to construct fusogenic virosomes allows substantially higher encapsulation efficiencies for water-soluble compounds by using standard liposome preparation techniques. Second, the post-insertion method of virosome preparation provides flexibility in the choice of lipid composition of the virosomal bilayer. Third, post-insertion of viral spike proteins into preformed liposomes favors the correct orientation of these integral membrane proteins in the bilayer of virosomes with no spike proteins facing inwards as is the case for the traditional method of virosome preparation. Fourth, the use of DCPC as a mild detergent to solubilize influenza virus spike proteins allows more efficient removal of detergent molecules from the virosomal membranes by dialysis, which favors membrane stability and provide the possibility to entrap drug molecules inside these PI-virosomes with remote loading procedures [28,29,37].

Post-inserting membrane proteins into preformed liposomes either directly or aided by detergents have been described for integral membrane proteins, among which viral spike proteins [22-27]. The underlying principle of the method appears to be that membrane proteins insert spontaneously into small unilamellar phospholipid vesicles in which there are defects in the packing of the acyl chains, induced either spontaneously or by amphipathic molecules such as detergents, short-chain lecithins or lyso-derivatives.

In this study, it was demonstrated that detergent-aided post-insertion of influenza virus spike proteins into preformed liposomes results in the formation of fusion-active virosomes with a morphology similar to the native virus particles. Post-insertion resulted in correct orientation of the viral spike protein in the bilayer with most (if not all) spikes facing outwards (Figure 2). This is in contrast to the orientation of spike proteins in virosomes prepared with the traditional $C_{12}E_8$ method. In the latter case, approximately half of the spike proteins are facing inwards as seen on EM micrographs (Figure 2). The correct orientation of influenza virus spike proteins is obviously important when influenza virosomes are used as model systems to study the HA-mediated membrane fusion mechanism [38,39].

PI-virosome-induced lipid mixing was dependent on the amount of post-inserted spike proteins and was exclusively pH-dependent with no lipid mixing observed at neutral pH. Lipid mixing was only observed with target membrane vesicles displaying sialic acid residues on their surface. These findings strongly suggest that fusion is mediated by the post-inserted viral HA. Several studies have reported on the effect of HA density on the kinetics of fusion of influenza virus [40-

42]. Although it is not clear whether one trimer of HA is enough to induce fusion [40] or the concerted action of several HA trimers is needed to induce membrane fusion [41,42], they all show positive correlation between HA density and rate and extent of fusion. Furthermore, it was demonstrated that membrane fusion is dependent on the composition of target membranes as has been reported for native virus [43,44].

Effective post-insertion of spike proteins required destabilization of the liposome bilayers with short-chain phospholipids at a concentration of 8 mM prior to protein insertion. Unfortunately, the post-insertion procedure was accompanied by extensive leakage of both small (calcein) as well as large (DNA) molecules entrapped inside the liposomes. Due to this leakage we were unable to determine the occurrence of aqueous contents mixing between PI-virosomes and ganglioside liposomes (results not shown). It was demonstrated that the retention of liposome-encapsulated substances was not compromised by the presence of DCPC detergent (Figure 1), but leakage was caused by the detergent-aided insertion of viral spike proteins (Figure 6 and 8). It is believed that the wedge-shaped DCPC molecule destabilizes membranes by inserting into the outer leaflet of phospholipid bilayers thereby causing considerable tension between the two membrane leaflets [29]. This tension facilitates the insertion of membrane proteins. As both influenza virus HA and NA are integral membrane proteins that span the bilayer, it is well possible that insertion of these proteins causes extensive leakage by temporarily forming bilayer perturbations that allow the passage of entrapped molecules. It was not expected, however, that also large macromolecules such as DNA would be released during the post-insertion process. Lowering the final concentration of DCPC present during post-insertion from 8 mM to 5 or 2.5 mM resulted in a decrease in calcein leakage. However, it also reduced the extent of lipid mixing observed with PI-virosomes, most likely caused by less efficient insertion of the viral spike proteins into liposomes (Figure 7).

As demonstrated in this study, the post-insertion procedure to prepare PI-virosomes is not compatible with retention of entrapped (macro)molecules during the post-insertion procedure. Nevertheless, after PI-virosomes have been formed and the detergent has been removed by dialysis, these PI-virosomes may be subjected to remote loading of drugs [45-47]. As the remote loading procedure is only effective when a transbilayer gradient (either pH or potential) can be maintained, virosomes with stable, non-leaky bilayers are required. Due to the flexibility in choice of bilayer composition and the ease of detergent removal with the post-insertion procedure of virosome preparation, both favorable for bilayer stability, it is expected that PI-virosomes will be more suitable for remote loading of drugs than virosomes prepared with the $C_{12}E_8$ method. The latter method yields virosomes with high amounts (7.5-9 mol%) of residual detergent present in the virosome bilayer [10]. The possibility to incorporate water-soluble agents in PI-virosomes via remote loading procedures requires further investigation.

REFERENCE LIST

1 Metsikko, K., van Meer, G., and Simons, K. (1986): **Reconstitution of the fusogenic activity of vesicular stomatitis virus**. *EMBO J.*, 5:3429-3435.

2 Paternostre, M., Viard, M., Meyer, O., Ghanam, M., Ollivon, M., and Blumenthal, R. (1997): **Solubilization and reconstitution of vesicular stomatitis virus envelope using octylglucoside**. *Biophys.J.*, 72:1683-1694.

3 Vainstein, A., Razin, A., Graessmann, A., and Loyter, A. (1983): **Fusogenic reconstituted Sendai virus envelopes as a vehicle for introducing DNA into viable mammalian cells**. *Methods Enzymol.*, 101:492-512.

4 Ozawa, M. and Asano, A. (1981): **The preparation of cell fusion-inducing proteoliposomes from purified glycoproteins of HVJ (Sendai virus) and chemically defined lipids**. *J.Biol.Chem.*, 256:5954-5956.

5 Hosaka, Y. and Shimizu, Y.K. (1972): **Artificial assembly of envelope particles of HVJ (Sendai virus). I. Assembly of hemolytic and fusion factors from envelopes solubilized by Nonidet P40**. *Virology*, 49:627-639.

6 Helenius, A., Sarvas, M., and Simons, K. (1981): **Asymmetric and symmetric membrane reconstitution by detergent elimination. Studies with Semliki-Forest-virus spike glycoprotein and penicillinase from the membrane of Bacillus licheniformis**. *Eur.J.Biochem.*, 116:27-35.

7 Nüssbaum, O., Lapidot, M., and Loyter, A. (1987): **Reconstitution of functional influenza virus envelopes and fusion with membranes and liposomes lacking virus receptors**. *J.Virol.*, 61:2245-2252.

8 Kawasaki, K., Sato, S.B., and Ohnishi, S. (1983): **Membrane fusion activity of reconstituted vesicles of influenza virus hemagglutinin glycoproteins**. *Biochim.Biophys.Acta*, 733:286-290.

9 Huang, R.T., Wahn, K., Klenk, H.D., and Rott, R. (1980): **Fusion between cell membrane and liposomes containing the glycoproteins of influenza virus**. *Virology*, 104:294-302.

10 Stegmann, T., Morselt, H.W., Booy, F.P., van Breemen, J.F., Scherphof, G., and Wilschut, J. (1987): **Functional reconstitution of influenza virus envelopes**. *EMBO J.*, 6:2651-2659.

11 Trudel, M. and Nadon, F. (1981): **Virosome preparation: differences between influenza and rubella hemagglutinin adsorption**. *Can. J. Microbiol.*, 27:958-962.

12 Almeida, J.D., Edwards, D.C., Brand, C.M., and Heath, T.D. (1975): **Formation of virosomes from influenza subunits and liposomes**. *Lancet*, 2:899-901.

13 Hosaka, Y., Yasuda, Y., and Fukai, K. (1983): **Hemolysis by liposomes containing influenza virus hemagglutinins**. *J. Virol.*, 46:1014-1017.

14 Bron, R., Ortiz, A., and Wilschut, J. (1994): **Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza virus envelopes (virosomes)**. *Biochemistry*, 33:9110-9117.

15 Schoen, P., Bron, R., and Wilschut, J. (1993): **Delivery of foreign substances to cells mediated by fusion- active reconstituted influenza virus envelopes (virosomes)**. *J.Liposome Res.*, 3:767-792.

16 Poltl-Frank, F., Zurbriggen, R., Helg, A., Stuart, F., Robinson, J., Glück, R., and Pluschke, G. (1999): **Use of reconstituted influenza virus virosomes as an immunopotentiating delivery system for a peptide-based vaccine**. *Clin.Exp.Immunol.*, 117:496-503.

17 Arkema, A., Huckriede, A., Schoen, P., Wilschut, J., and Daemen, T. (2000): **Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes**. *Vaccine*, 18:1327-1333.

18 Schoen, P., Chonn, A., Cullis, P.R., Wilschut, J., and Scherrer, P. (1999): **Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles**. *Gene Ther.*, 6:823-832.

19 New, R.R.C. (1990): ***Liposomes: a practical approach***. Oxford University Press, New York.

20 Scheule, R.K. (1986): **Novel preparation of functional Sindbis virosomes**. *Biochemistry*, 25:4223-4232.

21 Petri, W.A. and Wagner, R.R. (1980): **Glycoprotein micelles isolated from vesicular stomatitis virus spontaneously partition into sonicated phosphatidylcholine vesicles**. *Virology*, 107:543-547.

22 Simon-Plas, F., Venema, K., Grouzis, J.P., Gibrat, R., Rigaud, J., and Grignon, C. (1991): **Spontaneous insertion of plant plasma membrane (H+)ATPase into a preformed bilayer**. *J.Membr.Biol.*, 120:51-58.

23 Zakim, D. and Scotto, A.W. (1989): **Spontaneous insertion of integral membrane proteins into preformed unilamellar vesicles**. *Methods Enzymol.*, 171:253-264.

24 Knol, J., Veenhoff, L., Liang, W.J., Henderson, P.J., Leblanc, G., and Poolman, B. (1996): **Unidirectional reconstitution into detergent-destabilized liposomes of the purified lactose transport system of Streptococcus thermophilus**. *J.Biol.Chem.*, 271:15358-15366.

25 Jung, H., Tebbe, S., Schmid, R., and Jung, K. (1998): **Unidirectional reconstitution and characterization of purified Na+/proline transporter of Escherichia coli**. *Biochemistry*, 37:11083-11088.

26 Levy, D., Gulik, A., Bluzat, A., and Rigaud, J.L. (1992): **Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents**. *Biochim.Biophys.Acta*, 1107:283-298.

27 Angrand, M., Briolay, A., Ronzon, F., and Roux, B. (1997): **Detergent-mediated reconstitution of a glycosyl-phosphatidylinositol- protein into liposomes**. *Eur.J.Biochem.*, 250:168-176.

28 Hauser, H. (2000): **Short-chain phospholipids as detergents**. *Biochim.Biophys.Acta*, 1508:164-181.

29 Kessi, J., Poiree, J.C., Wehrli, E., Bachofen, R., Semenza, G., and Hauser, H. (1994): **Short-chain phosphatidylcholines as superior detergents in solubilizing membrane proteins and preserving biological activity**. *Biochemistry*, 33:10825-10836.

30 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., and Papahadjopoulos, D. (1979): **Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes**. *Biochim.Biophys.Acta*, 557:9-23.

31 Stegmann, T., Schoen, P., Bron, R., Wey, J., Bartoldus, I., Ortiz, A., Nieva, J.L., and Wilschut, J. (1993): **Evaluation of viral membrane fusion assays. Comparison of the octadecylrhodamine dequenching assay with the pyrene excimer assay**. *Biochemistry*, 32:11330-11337.

32 Fiske, C.H. and Subbarow, Y. (1925): **The colorimetric determination of phosphorus**. *J.Biol.Chem.*, 66:375-400.

33 Mastrobattista, E., Wilschut, J., Schoen, P., Crommelin, D.J.A., and Storm, G. (2001): **Targeting influenza virosomes to ovarian carcinoma cells**. *FEBS Lett.*, Submitted.

34 Steck, T.L. and Kant, J.A. (1974): **Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes**. *Methods Enzymol.*, 31:172-180.

35 Schoen, P., Bijl, L., and Wilschut, J. (1998): **Efficient encapsulation of plasmid DNA in anionic liposomes by a freeze/thaw-extrusion procedure**. *J.Liposome Res.*, 8:485-497.

36 Rigaud, J.L., Pitard, B., and Levy, D. (1995): **Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins**. *Biochim.Biophys.Acta*, 1231:223-246.

37 Dencher, N.A. (1986): **Spontaneous transmembrane insertion of membrane proteins into lipid vesicles facilitated by short-chain lecithins**. *Biochemistry*, 25:1195-1200.

38 Chams, V., Bonnafous, P., and Stegmann, T. (1999): **Influenza hemagglutinin mediated fusion of membranes containing poly(ethylene-glycol) grafted lipids: new insights into the fusion mechanism**. *FEBS Lett.*, 448:28-32.

39 Klotz, K.H., Bartoldus, I., and Stegmann, T. (1996): **Membrane asymmetry is maintained during influenza-induced fusion**. *J.Biol.Chem.*, 271:2383-2386.

40 Gunther-Ausborn, S., Schoen, P., Bartoldus, I., Wilschut, J., and Stegmann, T. (2000): **Role of hemagglutinin surface density in the initial stages of influenza virus fusion: lack of evidence for cooperativity**. *J.Virol.*, 74:2714-2720.

41 Danieli, T., Pelletier, S.L., Henis, Y.I., and White, J.M. (1996): **Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers**. *J.Cell Biol.*, 133:559-569.

42 Ellens, H., Bentz, J., Mason, D., Zhang, F., and White, J.M. (1990): **Fusion of influenza hemagglutinin-expressing fibroblasts with glycophorin-bearing liposomes: role of hemagglutinin surface density**. *Biochemistry*, 29:9697-9707.

43 Stegmann, T., Nir, S., and Wilschut, J. (1989): **Membrane fusion activity of influenza virus. Effects of gangliosides and negatively charged phospholipids in target liposomes**. *Biochemistry*, 28:1698-1704.

44 Stegmann, T., Hoekstra, D., Scherphof, G., and Wilschut, J. (1986): **Fusion activity of influenza virus. A comparison between biological and artificial target membrane vesicles**. *J.Biol.Chem.*, 261:10966-10969.

45 Haran, G., Cohen, R., Bar, L.K., and Barenholz, Y. (1993): **Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases**. *Biochim.Biophys.Acta*, 1151:201-215.

46 Hwang, S.H., Maitani, Y., Qi, X.R., Takayama, K., and Nagai, T. (1999): **Remote loading of diclofenac, insulin and fluorescein isothiocyanate labeled insulin into liposomes by pH and acetate gradient methods**. *Int.J.Pharm.*, 179:85-95.

47 Hwang, S.H., Maitani, Y., Takayama, K., and Nagai, T. (2000): **High entrapment of insulin and bovine serum albumin into neutral and positively-charged liposomes by the remote loading method**. *Chem.Pharm.Bull.(Tokyo)*, 48:325-329.


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300
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FFERDE
UMCHLORID
8,2 g
1,9 g
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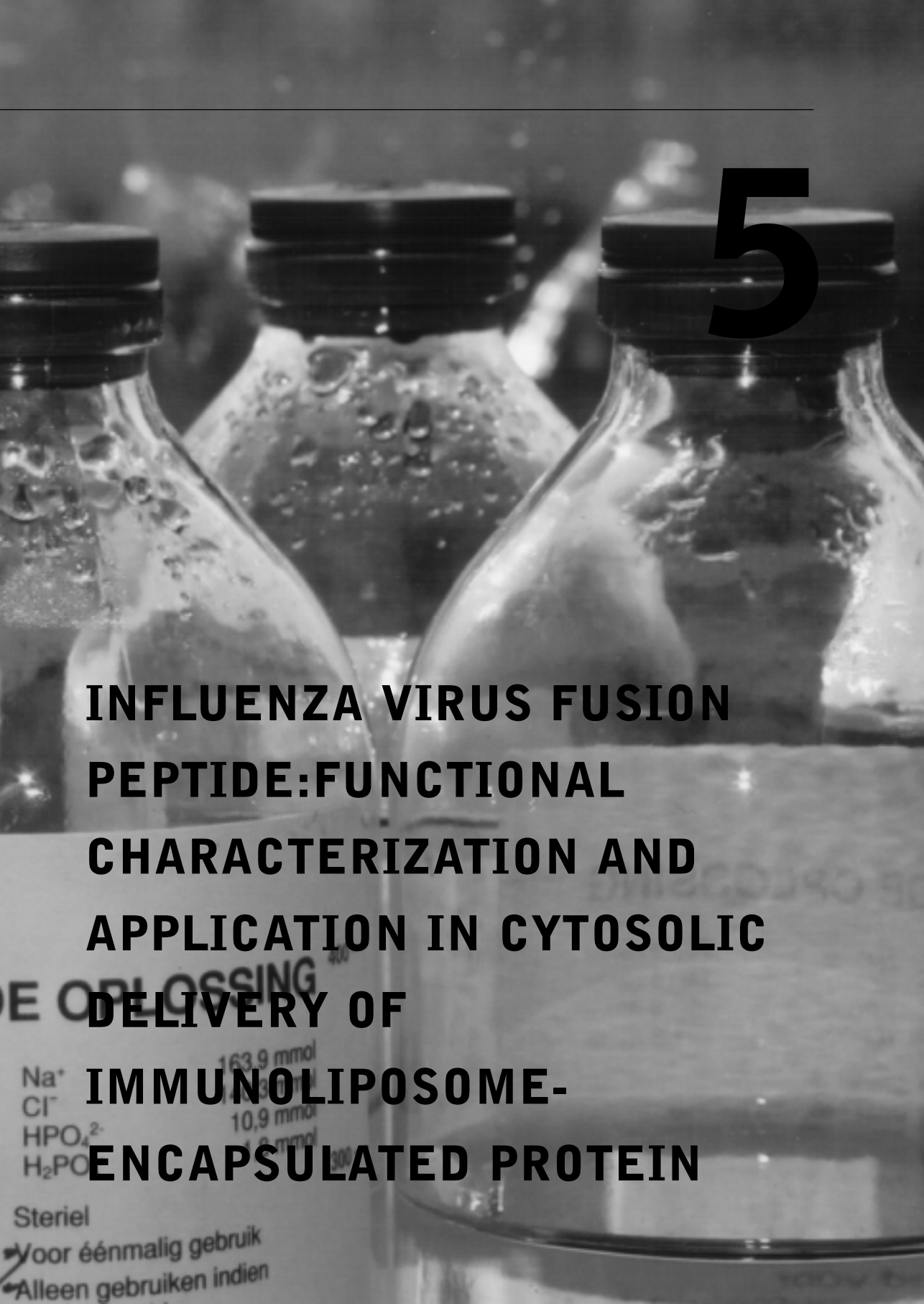

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INFLUENZA VIRUS FUSION PEPTIDE:FUNCTIONAL CHARACTERIZATION AND APPLICATION IN CYTOSOLIC DELIVERY OF IMMUNOLIPOSOME-ENCAPSULATED PROTEIN

INFLUENZA VIRUS FUSION PEPTIDE:

Functional characterization and application in cytosolic delivery of immunoliposome-encapsulated protein

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SUMMARY

With advances in biotechnology, a growing number of biotherapeutic molecules are becoming available. As these biotherapeutic macromolecules (proteins and nucleic acids) often act on intracellular targets that are out of reach for these large, membrane-impermeable molecules, a carrier is needed that can specifically deliver these kind of drugs into the cytosol of target cells. Antibody-targeted liposomes (immunoliposomes) are frequently used for cell-specific drug delivery. However, delivery of large biotherapeutic molecules with immunoliposomes is often hampered by an inefficient release of entrapped macromolecules after target cell binding and subsequent immunoliposome internalization. To enhance cytosolic drug delivery from immunoliposomes present inside endosomes a pH-dependent fusogenic peptide (diINF-7) resembling the N-terminal domain of influenza virus hemagglutinin HA2 subunit was used. Functional characterization of this dimeric peptide showed its ability to induce fusion between model liposome membranes and leakage of liposome entrapped compounds. More importantly, liposome-encapsulated fusogenic peptides were able to induce leakage of compounds from liposomes other than those in which the fusion peptides were entrapped. In a second series of experiments diINF-7 peptides were encapsulated in immunoliposomes to enhance the endosomal escape of diphtheria toxin A chain (DTA), which can inhibit protein synthesis when delivered into the cytosol of target cells. Immunoliposomes with encapsulated DTA targeted to the internalizing epidermal growth factor receptor (EGFR) on the surface of ovarian carcinoma cells (OVCAR-3) did not show any cytotoxicity towards OVCAR-3 cells. Only when diINF-7 peptides were co-encapsulated together with DTA in liposomes cytotoxicity was observed. Thus, synthetic fusion peptides when entrapped inside liposomes can greatly enhance cytosolic delivery of liposomal macromolecules by pH-dependent destabilization of endosomal membranes after cellular uptake of liposomes.

INTRODUCTION

To exert an optimal therapeutic effect an administered drug must safely reach not only its target cell but also the appropriate location within that cell. Many biotherapeutic agents (*e.g.* peptides, proteins and nucleic acids) act at sites in the cytosol or nucleus that are difficult to reach due to poor membrane permeation characteristics resulting from unfavorable physicochemical characteristics, such as large size and/or excessive charge. Therefore, these biotherapeutic agents rely on drug carriers that allow cytosolic delivery of these agents into diseased cells.

One attractive strategy to obtain cytosolic delivery is the development of fusion-competent antibody-directed liposomes (immunoliposomes) that, after receptor binding, can fuse with the plasma-membrane of target cells or, after endocytosis of liposomes, with the endosomal membranes. This membrane fusion process should result in cytosolic access of liposome-entrapped drug molecules. Such fusion-competent liposomes can be obtained by disassembling enveloped viruses, isolating their fusion-promoting components and re-assembling these into vesicles to obtain so-called virosomes (see chapter 3 and 4 of this thesis) [1-4]. Another, more refined approach involves the use of synthetic peptides designed to resemble the putative fusion peptide of viruses to promote fusion of the liposome membrane with the host plasma membrane or – after receptor-mediated endocytosis of immunoliposomes – fusion with the endosomal membrane [5-8]. It is hypothesized that destabilization of endosomal membranes is less damaging than plasma membrane destabilization. The latter may result in cytotoxicity through eradication of the electrochemical potential generated by the asymmetric distribution of ions across the plasma membrane. Therefore, drug delivery into the cytosol of target cells via endosomal escape is the preferred route. It is expected that fusion and/or membrane destabilizing mechanisms that allow endosomal escape of liposome-entrapped therapeutic macromolecules will increase the therapeutic availability at the target site and consequently therapeutic efficacy of liposomal drug formulations.

Many viral fusion peptides have been identified. Their mechanistic and structural role in the fusion process occurring between the envelopes of viruses and membranes of host-cells has been extensively studied, both with intact viral fusion proteins and with synthetic analogs of the fusion protein domains. One of the best-characterized viral fusion peptides is the N-terminal domain of influenza virus hemagglutinin subunit HA2 [9,10]. It is believed that this short stretch of hydrophobic amino acids interacts with target membranes after a low pH-induced conformational change in the HA protein. At low pH, the fusion peptide domain adopts an α-helical structure that is thought to favor the oblique insertion of the fusion peptide in the endosomal membrane of host cells that have endocytosed the influenza virus particles.

Studies with synthetic peptides resembling the native sequence of the influenza virus N-terminal domain of the HA2 subunit have clearly demonstrated that such peptides are able to destabilize both model membranes (such as liposomes) and natural membranes in a pH-dependent manner.

Fusion peptide-induced lipid mixing and aqueous contents mixing between liposomes have been demonstrated, indicating that the peptide analogs have fusogenic capacities [11,12]. Influenza virus-derived fusion peptides have been used to enhance the endosomal escape of oligonucleotides [13,14] and polycation-condensed DNA complexes [5,15,16] after cellular uptake. Surprisingly, despite the wealth of reports on the use of liposomes to functionally characterize fusogenic peptides, only one study reported on the utility of these peptides in enhancing cytosolic delivery of liposome-entrapped drugs [17].

In this article the possibility to utilize co-encapsulation of pH-dependent fusion peptides into liposomal drug formulations as a means to obtain endosomal escape of liposome-entrapped drug molecules and release of these molecules into the cytosol of tumor cells was studied. For this purpose a dimeric peptide (diINF-7) resembling the N-terminal domain of influenza virus hemagglutinin was synthesized. Firstly, the membrane-destabilizing and fusogenic capacity of diINF-7 either in free form or encapsulated in liposomes was demonstrated by using liposomes as model membranes. Next, the capacity of these peptides to induce endosomal escape of immunoliposome-entrapped bioactive molecules was demonstrated by measuring cytosolic delivery of diphtheria toxin A chain (DTA) as model compound. Co-encapsulation of diINF-7 into DTA-containing immunoliposomes resulted in drastically increased cytotoxicity towards ovarian carcinoma cells, indicating that this peptide can be used to obtain cytosolic delivery of liposome-entrapped drugs with poor membrane permeation capacities.

MATERIALS AND METHODS

MATERIALS

Cholesterol (CHOL), 2',7'-[bis(carboxymethyl)amino]methyl-fluorescein (calcein), *N*-succinimidyl-*S*-acetylthioacetate (SATA), and sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Egg phosphatidylcholine and 1,2-distearoyl-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-DSPE) were from Avanti Polar Lipids (Birmingham, AL, USA). Maleimide-PEG2000-DSPE (MAL-PEG2000-DSPE) was obtained from Shearwater Polymers (Huntsville, AL, USA). 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (PyrPC) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Iso-octylphenoxypolyethoxyethanol (Triton-X100) was obtained from BDH Laboratory Supplies (Poole, England). Dithiothreitol (DTT) was from Pierce (Rockford, IL, USA). Formaldehyde solution was obtained from Janssen Chimica (Geel, Belgium). Murine mAb 425 of isotype IgG2b (EMD55900) (Merck KGaA, Bernstadt, Germany) directed against the human epidermal growth factor receptor (EGFR) was kindly donated by Dr. G.A. van Dongen (Department of Otolaryngology/Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). Irrelevant isotype-matched murine mAb directed against the influenza virus HA (clone 12CA5) was kindly donated by Ms E. Boot (Immunology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands).

CELL CULTURE

The human ovarian carcinoma cell line NIH:OVCAR-3 originated from the laboratory of Dr. Hamilton (National Cancer Institute, Bethesda, MD) [18]. OVCAR-3 cells were cultured in Dulbecco's modified Eagle's medium containing 3.7 g/l sodium bicarbonate, 4.5 g/l L-glucose and supplemented with L-glutamine (2 mM), HEPES (10 mM), 10% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) at 37°C with 5% CO2 in humidified air. All cell-culture related material was obtained from Gibco (Grand Island, NY, USA).

PEPTIDE SYNTHESIS

The 24 amino acid peptide INF-7 was synthesized by standard Fmoc solid phase synthesis essentially as described in [19]. Crude peptide was precipitated by dropwise addition of ether and collected by centrifugation. The peptide was washed three times with ether and subsequently dried under a stream of argon followed by high vacuum. Subsequently, the peptide was dissolved in 1 ml of 20 mM ammonium bicarbonate, pH 8.5 and freeze-dried. Lyophilized peptide was stored at –20°C. Purity and identity of the peptide were confirmed by HPLC (Waters 486, Millipore), amino acid analysis, and fast atom bombardment mass spectrometry (FAB-MS). The presence of a cysteine residue at the carboxy-terminus resulted in dimerization of the INF-7 peptide by disulfide bond formation.

DTA PURIFICATION

The A chain of diphtheria toxin (DTA) was produced essentially as described by Oeltmann et al. [20] and Carroll et al. [21]. In short, supernatant of cultures of *Corynebacterium diphtheriae* containing high amounts of diphtheria toxin (DT), which was kindly donated by Dr. Kersten (The National Institute of Public Health and The Environment, Bilthoven, The Netherlands) was dialyzed against two 1 liter changes of HEPES-buffered salt solution (HBS; 5 mM HEPES, 150 mM NaCl, pH 7.4) overnight at 4°C using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL, USA) with a molecular weight cut off (MWCO) of 10,000. Four milligram of dialyzed DT was nicked with trypsin (1 µg/ml in HBS) for 30 min at room temperature after which the trypsin was inactivated with soybean trypsin inhibitor (5 µg/ml final concentration). DT was reduced and denatured in HBS containing 8 M urea and 0.1 M dithiothreitol (DTT) and applied to a 1.5x50 cm column of Sephacryl S-200 equilibrated in elution buffer (50 mM Tris-HCl, 2 M urea, 10 mM DTT and 1 mM EDTA; pH 7.5). Fractions of 5 ml were collected and analyzed for the presence of DTA by SDS-PAGE under reducing conditions. Fractions containing DTA were pooled and dialyzed overnight at 4°C against two 1-liter changes of HBS containing 0.1 M 2,2'-dihydroxyethyl disulfide to reversibly block the free sulfhydryl groups. The concentration of DTA was determined spectrometrically, versus appropriate blanks, using the extinction coefficient at 280 nm of 1.5 $lxg^{-1}xcm^{-1}$. The biological activity of DTA to inhibit protein synthesis was determined with a rabbit reticulocyte lysate *in vitro* translation system (Promega, Madison, WI, USA) [22].

LIPOSOME PREPARATION AND CHARACTERIZATION

Lipids used for all liposome preparations were dissolved in $CHCl_3$:MeOH (2:1 v/v ratio) and stored at –20°C under a nitrogen atmosphere. Egg PC and CHOL (30 µmol of lipid) dissolved in the organic solvent were mixed at a molar ratio of 2:1 in a round bottom flask. When indicated, 0.1 mol% (relative to PL) of the lipidic fluorescence probe DiD, 10 mol% pyrPC (relative to PL), or varying amounts of PEG2000-DSPE were added to the lipid mixture prior to solvent evaporation using a rotary evaporator device. The formed lipid films were further dried under a stream of nitrogen for at least 1 h to remove traces of organic solvent. Lipid films were hydrated with 1 ml solutions of HBS, calcein (90 mM), diINF-7 (500 µg/ml in HBS), DTA (250 µg/ml in HBS) or combinations of the latter two substances. Hydration of lipids was facilitated by shaking the flasks in the presence of glass beads. The formed liposomes were subsequently extruded through polycarbonate filters with pore sizes varying from 0.05 µm – 0.6 µm using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). Non-entrapped material was removed from liposomes by centrifugation steps (2x45 min at 100,000xg; 4°C). Pelleted liposomes were resuspended in 1 ml of HBS. The phospholipid concentration of liposome formulations was determined by the colorimetric method of Fiske and Subbarow [23]. The amount of encapsulated protein (DTA and diINF-7) was determined with the BCA protein assay reagent kit (Pierce) after disruption of the liposomes with 0.5% (v/v) TX-100.

IMMUNOLIPOSOME PREPARATION

For targeting purposes conjugates of mAb425-PEG2000-DSPE were transferred to liposomes essentially as described by Ishida et al. [24]. In short, 2.5 mg of mAb 425 was modified with a 8-fold molar excess of SATA to randomly introduce thiol groups. SATA-modified mAb 425 was allowed to react with micelles containing PEG2000-DSPE and MAL-PEG2000-DSPE at a 4:1 molar ratio after deacetylation. One micromol of liposomes was incubated with 425-PEG-DSPE micelles corresponding to an amount of 30 µg of conjugated protein for 90 min at 40°C to allow transfer of 425-PEG2000-DSPE to the liposomes. Purification of liposomes was performed on a Sepharose CL-4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). With this technique, approximately 70% of targeting ligands could be reproducibly transferred into preformed liposomes.

FLOW CYTOMETRY AND CONFOCAL LASER SCANNING MICROSCOPY ANALYSIS

OVCAR-3 cells grown to a confluent monolayer were detached from culture flasks by incubating the cells for 5 min at 37°C with trypsin/EDTA solutions from Gibco. Detached OVCAR-3 cells ($2x10^5$ cells) were incubated with DiD-labeled liposomes in a final volume of 300 µl culture medium for 1 h at 37°C. Hereafter, cells were washed with immunofluorescence (IF) buffer (1% BSA in PBS, pH 7.4) by 2 centrifugation steps (5 min at 750xg) and resuspended into 500 µl IF-buffer before being analyzed by flow cytometry with a FACSCalibur flow cytometer (Becton&Dickinson, Mountain View, CA, USA). For confocal laser-scanning microscopy analysis, cells that were incubated with liposomes, were washed 3 times with 1 ml of PBS before fixation with 2% (v/v) of formaldehyde in PBS for 20 min at room temperature. After fixation, cells that were adhered to chamberslides were washed twice with PBS and overlaid with cover slides that were sealed with nail polish. Cells were analyzed on a Leica TCS-SP confocal laser-scanning microscope equipped with a 488 nm Argon, 568 nm Krypton and 647 nm HeNe laser.

CD MEASUREMENTS

Circular dichroism (CD) measurements were performed with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. Cuvettes with a path length of 1 mm were used and the peptide concentration was 100 µg/ml. CD spectra were recorded from 250-200 nm at 25°C. Each measurement was the average of six repeated scans (step resolution 1 nm, 1s each step) from which the corresponding background spectrum was subtracted. Quantitative prediction of the helical content was accomplished by fitting CD data with Hennessey-Johnson (H.-J.) algorithm [25], by the program CDNN in "simple spectra"-configuration [26], using a protein database with known secondary structure as the basis set.

LIPOSOME TURBIDITY ASSAY

Liposomes composed of EPC and CHOL (molar ratio of 2:1) and with increasing amounts of PEG2000-DSPE (0, 2.5, 5 and 10 mol%) were added to a cuvette at a concentration of 300 µM or 1000 µM in a final volume of 1.5 ml of HBS. Immediately after the addition of diINF-7 (lipid to peptide molar ratio of 50), peptide-induced aggregation of liposomes at the indicated pH

values was determined by measuring the increase in optical density of the liposome dispersion at 500 nm with a spectrophotometer over a period of 8 min at room temperature.

LIPOSOME LEAKAGE ASSAY

A serial dilution (1:5) of free or liposome-encapsulated diINF-7 peptide was prepared in a 96-well microtiter plate by transferring 20 µl of the initial peptide solution (50 µg/ml for free peptide and 0.3 µg/ml for liposome-entrapped peptide) from one well to the next well containing 80 µl of HBS. Calcein-containing liposomes were added to each well at a final concentration of 40 µM. After 60 min incubation at room temperature samples were analyzed for fluorescence with an LS50B fluorimeter (Perkin Elmer) set at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Calcein leakage was expressed relative to the difference in the fluorescence of calcein liposomes incubated in the absence of diINF-7 peptide (0% leakage) and the fluorescence of calcein liposomes that had been disrupted with 0.5% (v/v) Triton X-100 (100% leakage). The effect of Triton X-100 on calcein fluorescence intensity was negligible at the tested concentration [27,28].

LIPID MIXING ASSAY

diINF-7-induced lipid mixing was monitored with the pyrene excimer assay developed by Galla et al. [29]. Liposomes (5 µM) labeled with 10 mol% PyrPC (Molecular Probes) were mixed in a total volume of 1.4 ml of HBS with a 20-fold molar excess of non-labeled donor liposomes in a stirred cuvette kept at 37°C. diINF-7 peptide was added at the amounts indicated and after a 2 min equilibration period, the pH of the medium in the cuvette was lowered to 5.2 by adding 1/20 volume of fusion buffer (0.1 M 2-[*N*-morpholino]ethanesulfonic acid] (MES), 0.1 M acetic acid, pH 4.1). Fusion was continuously monitored at 37°C by measuring the decrease in pyrPC excimer fluorescence with a LS50B fluorimeter set at an excitation wavelength of 345 nm and an emission wavelength of 480 nm. The decrease of fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at infinite dilution, which was obtained by disrupting the liposomes with 0.5% (v/v) Triton X-100 (final concentration).

AQUEOUS CONTENTS MIXING ASSAY

The occurrence of diINF-7-induced intermixing of liposome-entrapped solutes was determined with a fluorescent assay based on quenching of calcein with bivalent metal ions [30]. Unilamellar liposomes of 150 nm in size containing calcein (0.8 mM) and $CuSO_4$ (1 mM) in HBS were admixed at a concentration of 50 µM with a five-fold molar excess of liposomes containing 20 mM EDTA in a stirred cuvette kept at a temperature of 37°C. diINF-7 was added at a concentration of 5 µg/ml. The pH of the buffer was lowered to 5.2 by adding 1/20 volume of fusion buffer. Aqueous contents mixing and/or leakage were continuously monitored at 37°C by measuring the increase in calcein fluorescence with a fluorimeter set at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. To be able to determine the contribution of both leakage and aqueous content mixing to the total increase in calcein fluorescence, 1/20 volume of 1 mM $CuSO_4$ was added to the external medium during the fusion process.

CYTOTOXICITY ASSAY

OVCAR-3 cells were plated in 96-well cell culture plates at $5x10^3$ cells/well and cultured for 24 hrs. Culture medium was removed by decanting the plates and cells were overlaid with 100 µl of fresh culture medium. To the first well of each column 100 µl of liposome formulation were added at a final lipid concentration of 1 mM (corresponding to 0.5 µg/ml of DTA). Two-fold serial dilutions of liposome formulations were prepared by transferring 100 µl of the first well in a column to the next and so forth. After 1 h incubation at 37°C liposomes were removed from the cells by decanting the plates, cells were washed with 100 µl/well of fresh culture medium and overlaid with 100 µl of fresh culture medium before incubation was continued. Forty eight hrs after addition of liposome formulations cell viability was assessed with the colorimetric XTT assay as previously described [31].

RESULTS

PRIMARY AND SECONDARY STRUCTURE OF diINF-7

The primary amino acid sequence of diINF-7 is presented in Table 1. Compared to the native sequence of the N-terminal domain of the influenza virus HA2 subunit, diINF-7 differs in only two amino acids: glycine on position 4 and alanine on position 7 have been substituted by glutamic acid residues. These substitutions favor the pH dependency of the fusogenic activity of the peptide. As fusogenic activity is thought to be directly correlated with α-helix formation of the peptide [6,32-34], the secondary structure of diINF-7 was determined by circular dichroism spectropolarimetry at both pH 7.4 and pH 5.2 in the presence or absence of detergent-solubilized phospholipids. Since Subbarao et al. [35] showed that interpretation of CD-spectra below 222 nm in the presence of phospholipid vesicles is ambiguous due to liposome scattering, we used mixed micelles of phospholipids and detergent to determine the effect of a hydrophobic environment on the helical content of the peptide. The CD-spectrum (Figure 1) of the free peptide at pH 5.2 shows minima at 208 and 222 nm, typical of a highly α-helical structure; in contrast, at neutral pH the diINF-7 peptide is primarily randomly coiled (Figure 1A and Table 2). Interestingly, the presence of detergent-solubilized phospholipids can also induce α-helix formation of diINF-7 (Figure 1B and Table 2).

Table 1 — Amino acid sequences of fusion peptides. INF HA2: amino-terminal sequence of influenza virus X-31 (H3N2) hemagglutinin subunit HA2; diINF-7: peptide analogue resembling Inf HA2 dimerized at the C-terminus by disulfide bond formation between C-terminal cysteine residues. Differences in amino acid sequence of diINF-7 compared to the native INF HA2 sequence are highlighted in bold.

INF HA2	GLF	GAI	AGFI	ENGW	EGMI	DGWYG -------
diINF-7	GLF	**E**AI	**E**GFI	ENGW	EGMI	DGWYGC]
	GLF	**E**AI	**E**GFI	ENGW	EGMI	DGWYGC]

Table 2 — Molar Absorbance at 222 nm ($\Delta\varepsilon_{222}$) and helical content of diINF-7 in absence or presence of mixed micelles and at different pH values.

Mixed Micelles	pH	$\Delta\varepsilon_{222}$ ($M^{-1}cm^{-1}$)	Helical content (%)
—	7.4	– 0.675	15
—	5.2	– 2.751	31
EPC/OG (1 mM)/(40 mM)	7.4	– 2.538	29
EPC/OG (1 mM)/(40 mM)	5.2	– 4.686	53

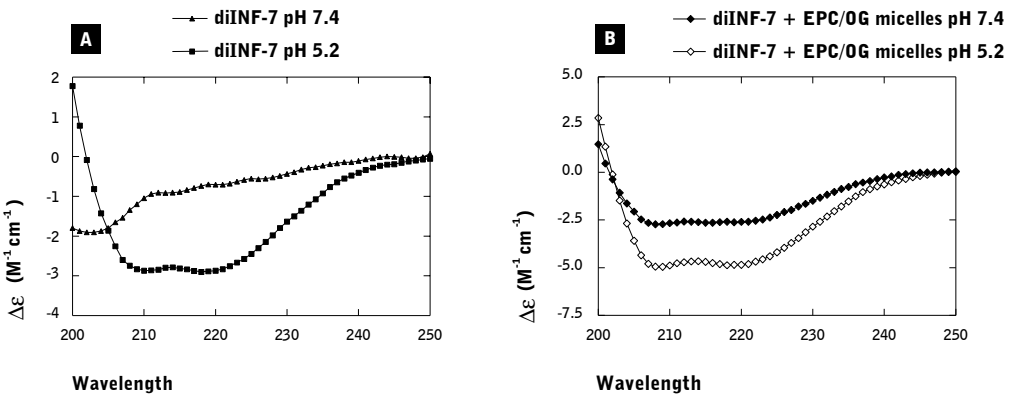

Figure 1 — Secondary structure of diINF-7 peptides as a function of pH and presence of mixed micelles of phospholipids and detergent. CD spectra of diINF-7 at pH 5.2 and 7.4 in the absence (A) and presence (B) of phospholipid micelles against appropriate blanks at 25°C. In all cases, the concentration of diINF-7 peptide was 18.6 μM. Mixed micelles consisted of 1 mM of indicated phospholipids and 40 mM of OG. As judged from the molar absorbance at 222 nm, alpha helicity of diINF-7 peptides increased with decreasing pH and in the presence of detergent or mixed micelles. EPC: egg phosphatidyl choline; OG: octyl glucoside.

LIPOSOME CHARACTERIZATION

In this study neutral liposomes (EPC:CHOL molar ratio 2:1) with and without incorporated PEG2000-DSPE were used as model membranes to test the membrane-destabilizing capacity of diINF-7 and targeted drug carriers to deliver diphtheria toxin A chain (DTA) into the cytosol of tumor cells. Liposomes were prepared by hydrating lipid films of 30 μmol total lipid (TL) with 1 ml of the appropriate buffer after which the formed liposomes were sized by extrusion [36]. The lipid composition, phospholipid concentration, entrapped contents and liposome size of the batches of liposomes used throughout this study are given in Table 3.

Table 3 — Characterization of liposome preparations used throughout this study.

Liposome Composition (molar ratio)	Lipid Concentration (mM of PL)	Entrapped Contents	Average Size in nm (polydispersity index)
EPC:CHOL (2:1)	8.2	—	90 (0.110)
EPC:CHOL (2:1)	8.8	—	150 (0.101)
EPC:CHOL (2:1)	7.6	—	270 (0.326)
EPC:CHOL (2:1)	7.1	—	748 (0.555)
EPC:CHOL (2:1)	6.2	diINF-7 (12 μg/μmol PL)	194 (0.397)
EPC:CHOL (2:1)	10.3	DTA (0,5 μg/μmol PL)	152 (0.123)
EPC:CHOL (2:1)	7.7	DTA diINF-7 (12 μgtotal protein/μmol PL)	193 (0.405)
EPC:CHOL (2:1)	8.0	Calcein (90 mM)	130 (0.096)
EPC:CHOL (2:1)	6.8	Calcein (90 mM) diINF-7 (12 μg/μmol PL)	188 (0.368)
EPC:CHOL:PEG2000-DSPE (1.9:1:0.5)	6.2	—	127 (0.091)
EPC:CHOL:PEG2000-DSPE (1.9:1:0.5)	7.1	—	128 (0.084)
EPC:CHOL:PEG2000-DSPE (1.9:1:0.5)	7.4	—	127 (0.021)

diINF-7 PEPTIDE-INDUCED AGGREGATION OF LIPOSOMES

diINF-7 peptide-induced aggregation of unilamellar liposomes as a function of pH, and presence of PEG molecules on the surface of liposomes was monitored by measuring the changes in turbidity of liposome dispersions at 500 nm (Figure 2). The results show that liposome aggregation increased with decreasing pH. No aggregation of liposomes was observed at pH 8.4. The presence of PEG molecules (Mw 2000) exposed on the surface of liposomes (2.5, 5 and 10 mol% relative to total lipid) fully inhibited diINF-7-induced liposome aggregation at low pH.

LIPID MIXING ASSAY

The capacity of diINF-7 to induce membrane fusion between liposomes was determined with the pyrene excimer lipid-mixing assay [29]. 'Donor' liposomes, containing 10 mol% of pyrene-labeled phosphatidylcholine (pyrPC) were admixed with non-labeled 'acceptor' liposomes. Upon intermixing of membrane lipids between donor and acceptor liposomes, the pyrPC label will be diluted over a larger membrane area, resulting in a decrease in excimer formation and thus a decrease in pyrPC fluorescence. Figure 3 shows that the extent of lipid mixing increased with increasing concentrations of diINF-7. Lipid mixing was pH-dependent. No lipid mixing was observed at pH 7.4. At acidic environment, the rate and extent of lipid mixing increased with increasing diINF-7 concentration (panel A) and decreasing pH (panel B). Furthermore, the

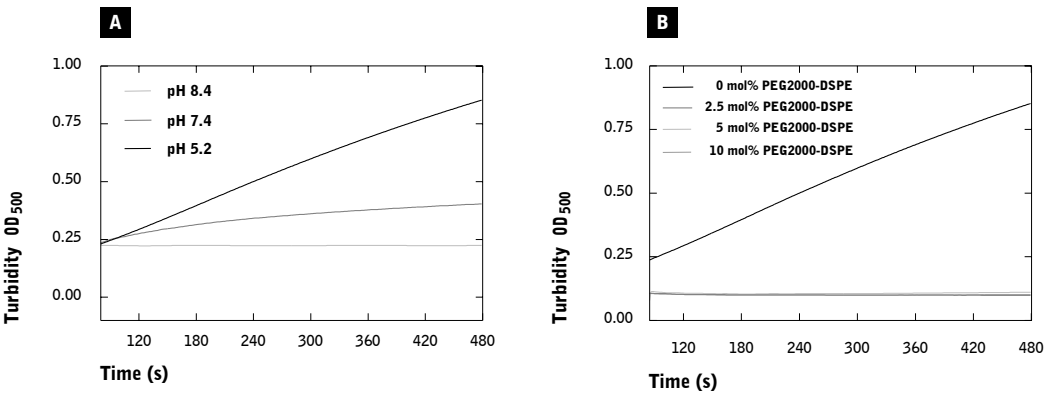

Figure 2 — diINF-7 peptide-induced aggregation of liposomes as a function of pH, and liposome composition. *Panel A*: Aggregation of liposomes (300 μM) when exposed to diINF-7 peptides dissolved in the aqueous dispersion (molar ratio lipid-to-peptide: 50) at pH 5.2, 7.4 and 8.4. ***Panel B***: Effect of PEG2000 exposed to the surface of liposome on diINF-7 induced aggregation of liposomes. Concentrations of liposomes and peptide were the same as in Panel A.

degree of lipid mixing depended on the size of 'acceptor' and 'donor' liposomes (panel C). Small liposomes with an average size of 70 nm showed the highest degree of lipid mixing (75%), whereas the extent of lipid mixing between liposomes with an average size of approximately 700 nm was only 22%. Furthermore, the extent of lipid mixing was negatively correlated with the amount of PEG2000 exposed on the surface of liposomes (panel D).

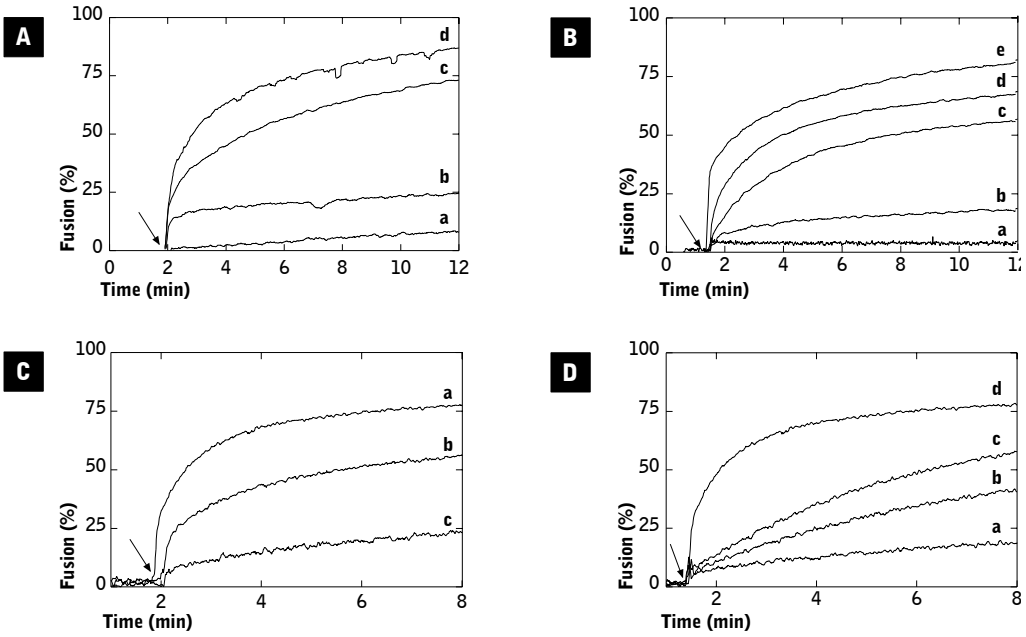

Figure 3 — Effect of diINF-7 peptide concentration, buffer pH, liposome size and surface-exposed PEG molecules on the extent of diINF-7-induced lipid mixing. 'Donor' liposomes (EPC:CHOL:pyrPC, molar ratio 1.8:1:0.2) were admixed with a 20-fold molar excess of 'acceptor' liposomes (EPC:CHOL, molar ratio 2:1) at a final lipid concentration of 105 μM. diINF-7 peptides were added to the liposome dispersion at the indicated concentration. At the time point indicated with a black arrow, the external medium was acidified to pH 5.2 (unless mentioned otherwise) and lipid mixing was continuously monitored as measured by a decrease of pyrPC excimer fluorescence. The decrease of pyrPC excimer fluorescence was expressed relative to the initial fluorescence and the excimer fluorescence at infinite dilution (*i.e.* after vesicle solubilization with detergent). ***Panel A***: Extent of lipid mixing as a function of diINF-7 peptide concentration: 0 (a), 1, (b), 10 (c), 20 (d) μg/ml diINF-7. ***Panel B***: Influence of pH on degree of lipid mixing induced by diINF-7 (10 μg/ml): pH 7.4 (a), 6.5 (b), 5.2 (c), 5.0 (d), and 4.6 (e). ***Panel C***: Influence of liposome size on the degree of lipid mixing induced by diINF-7 (10 μg/ml). Curves show lipid mixing between liposomes of 90 nm (a), 270 nm (b) and 750 nm (c) in size. ***Panel D***: Effect of surface-exposed PEG molecules on the degree of diINF-7-induced lipid mixing between 100 nm liposomes. Peptide concentration was 10 μg/ml. Relative amount of PEG2000 molecules grafted on the surface of liposomes was 10 mol% (a), 5 mol% (b), 2.5 mol% (c), and 0 mol% (d). Each curve respresents the average of three individual measurements.

diINF-7-MEDIATED MEMBRANE DESTABILIZATION RESULTS IN LEAKY MEMBRANE FUSION

A fluorescent assay based on the method of Kendall et al. [30] to monitor vesicle fusion and lysis was performed to determine whether diINF-7-induced lipid mixing is accompanied by intermixing of aqueous contents between liposomes and/or leakage of liposome-entrapped materials. Calcein/Cu^{2+} complexes, which are non-fluorescent, were entrapped in one set of liposomes and admixed with a 5-fold excess of a second set of liposomes containing entrapped EDTA. When EDTA comes in contact with Cu^{2+}/calcein complexes, either due to aqueous contents mixing between liposomes or due to leakage of both compounds to the external medium, EDTA will chelate the Cu^{2+} ions, thereby liberating the highly fluorescent calcein. The results of diINF-7-induced aqueous contents mixing between EDTA-containing liposomes and Cu^{2+}/calcein-containing liposomes are presented in Figure 4. An increase in calcein fluorescence was observed directly after lowering the pH of the external medium to 5.2. To be able to discriminate which part of the observed increase in calcein fluorescence was caused by leakage of entrapped calcein and EDTA and which part was caused by intermixing of aqueous contents, an excess of Cu^{2+} ions was added to the external medium. This resulted in an instantaneous decrease in calcein fluorescence, followed by a slow decline in calcein fluorescence. This biphasic decrease of calcein upon addition of Cu^{2+} ions to the external medium may be explained by instantaneous quenching of calcein located outside liposomes, whereas quenching of

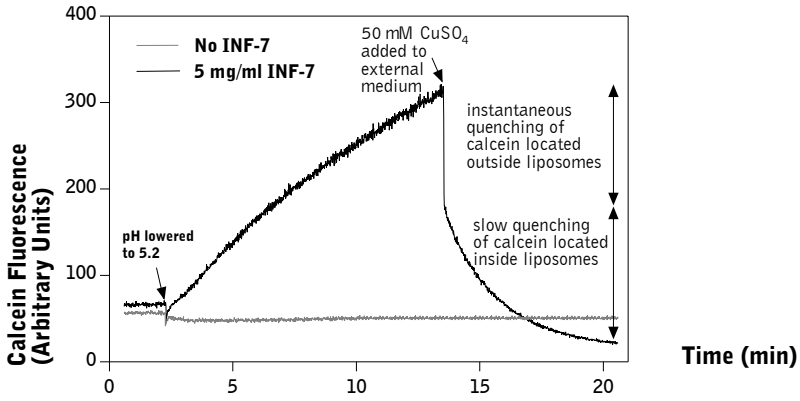

Figure 4 — diINF-7-induced aqueous contents mixing and leakage of liposomes. Liposomes (50 μM of TL) containing 1 mM $CuSO_4$ and 0.8 mM calcein were admixed with a five-fold excess of liposomes containing 20 mM EDTA. Fluorescence of calcein was continuously monitored with an LS50B fluorescent spectrophotometer set at excitation wavelength of 488 nm and an emission wavelength of 520 nm. At indicated time points, pH of the external medium was lowered from 7.4 to 5.2 and 1/20 vol of 1 mM $CuSO_4$ was added to the external medium. Black curve: calcein fluorescence pattern obtained in the presence of 5 μg/mL diINF-7. Gray curve: calcein fluorescence pattern obtained in the absence of diINF-7 peptide. Results of a typical experiment are shown.

free calcein located inside lipidic vesicles occurred at a slower rate, as this requires passage of Cu^{2+} ions over the diINF-7 peptide-destabilized liposome membranes. No increase in calcein fluorescence could be observed in the absence of diINF-7 peptides.

EFFECT OF LIPOSOME ENCAPSULATION ON THE MEMBRANE DESTABILIZING CAPACITY OF diINF-7

Previous studies with synthetic analogs of influenza virus fusion peptides have demonstrated that peptide-induced membrane destabilization is accompanied by leakage of liposome-entrapped materials when peptides were added in free form to the liposomes [16,19,37,38]. Here, we tested the capacity of diINF-7 to induce leakage of calcein from liposomes under two different conditions: (1) by adding diINF-7 peptide free in solution to a dispersion of calcein-containing liposomes or (2) by adding liposome-entrapped diINF-7 to calcein-containing liposomes. Figure 5A presents the results of calcein leakage from liposomes composed of EPC and CHOL (molar ratio 2:1) after 1 h incubation at room temperature in the presence of increasing diINF-7 peptide concentrations. Calcein leakage occurred both at pH 5.2 and pH 7.4. However, at pH 7.4 30-fold higher concentrations of diINF-7 were needed to obtain the same level of calcein leakage. diINF-7 also induced leakage of calcein from liposomes at pH 5.2 when encapsulated in liposomes other than the calcein-liposomes, though less efficient than free diINF-7 (Figure 5B).

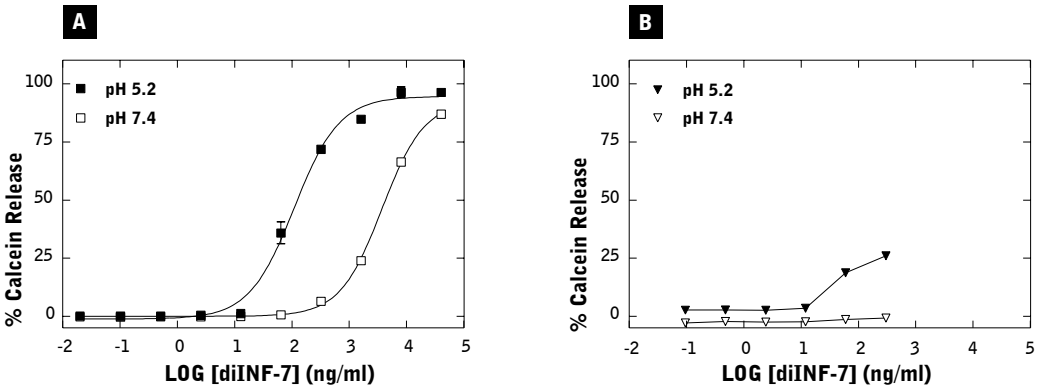

Figure 5 — Effect of liposome-encapsulated diINF-7 peptide compared to free diINF-7 peptide on the extent of calcein leakage from liposomes at pH 5.2 and pH 7.4. Leakage of calcein from liposomes composed of EPC and CHOL (molar ratio 2:1) and approx 130 nm in size induced by A: diINF-7 peptides free in solution and B: diINF-7 peptides encapsulated in liposomes other than the liposomes containing the calcein. Leakage of calcein from liposomes was measured both at pH 7.4 and pH 5.2 after 1-h incubation at room temperature. Leakage of calcein is expressed relative to the difference between leakage of calcein from liposomes after disruption with detergent (100% value) and leakage of calcein from liposomes in the absence of peptides (0% value). Each data point represents the average of three individual measurements.

EFFECT OF LIPOSOME-ENCAPSULATED diINF-7 ON CYTOSOLIC DTA DELIVERY

An important finding in this study is that diINF-7 induced leakage of calcein from liposomes exclusively at low pH when these peptides were encapsulated in liposomes other than the calcein-containing liposomes. This finding implies that diINF-7 peptides may be able to destabilize not only the carrier liposomes in which these peptides are entrapped, but also the endosomal membranes when these fusion-peptide bearing liposomes are endocytosed by target cells.

To evaluate the endosomolytic activity of diINF-7, we co-encapsulated DTA in diINF-7-containing liposomes. DTA/diINF-7 liposomes were targeted to the internalizing human epidermal growth factor receptor (hEGFR) by conjugating mAb 425 to the liposome surface. These immunoliposomes were incubated with ovarian carcinoma cells (OVCAR-3) expressing the EGFR on their cell surface. Binding of DTA/diINF-7 liposomes labeled with the fluorescent probe DiD is illustrated in Figure 6A. The figure shows that the immunoliposomes are able to bind to OVCAR-3 cells. Cell binding could be partially blocked by adding free mAb 425 at a concentration of 500 µg/ml. Liposomes lacking surface-grafted antibodies showed only low levels of cell binding and liposomes bearing an isotype-matched irrelevant antibody (mAb 12CA5 directed against influenza virus HA) grafted on their surface showed moderate binding. Confocal laser scanning microscopy experiments show that liposomes targeted to the EGFR on OVCAR-3 cells are efficiently internalized (Figure 6B). Cytotoxicity of native DT, free DTA and liposomal DTA formulations towards OVCAR-3 cells is shown in Figure 7. DTA in free form was not toxic to OVCAR-3 cells in the concentration range tested, whereas the nicked DT showed concentration-dependent cytotoxicity towards OVCAR-3 cells (Figure 7A). Importantly, the cytotoxicity of diINF-7/DTA-containing immunoliposomes (DTA-425-FIL) was even higher compared to native DT, indicating that the amount of toxin molecules delivered by immunoliposomes via EGFR-mediated endocytosis and subsequent endosomal escape is higher than the amount of toxin delivered via the native HB-EGF receptor (Figure 7B and C). DTA-immunoliposome formulations devoid of diINF-7 (DTA-425-IL) did not show cytotoxicity. Cytotoxicity was not due to diINF-7-induced endosomal membrane disruption, as immunoliposomes bearing diINF-7 but no DTA (425-FIL) did not show toxicity towards OVCAR-3 cells (Figure 7C). Non-targeted diINF-7/DTA-liposomes or targeted with an irrelevant monoclonal antibody did not show cytotoxicity (Figure 7B). Inhibition of acidification of endosomes by adding 20 mM NH_4Cl to the culture medium 30 min prior to adding the diINF-7/DTA immunoliposomes resulted in loss of cytotoxicity of this DTA formulation (Figure 7B). The addition of 20 mM NH_4Cl to the culture medium had no effect on cell viability (results not shown). In conclusion, the results show that cytosolic delivery of DTA into OVCAR-3 cells requires both receptor-mediated endocytosis of DTA/diINF-7 immunoliposomes and subsequent low pH-induced activation of the endosomolytic activity of diINF-7.

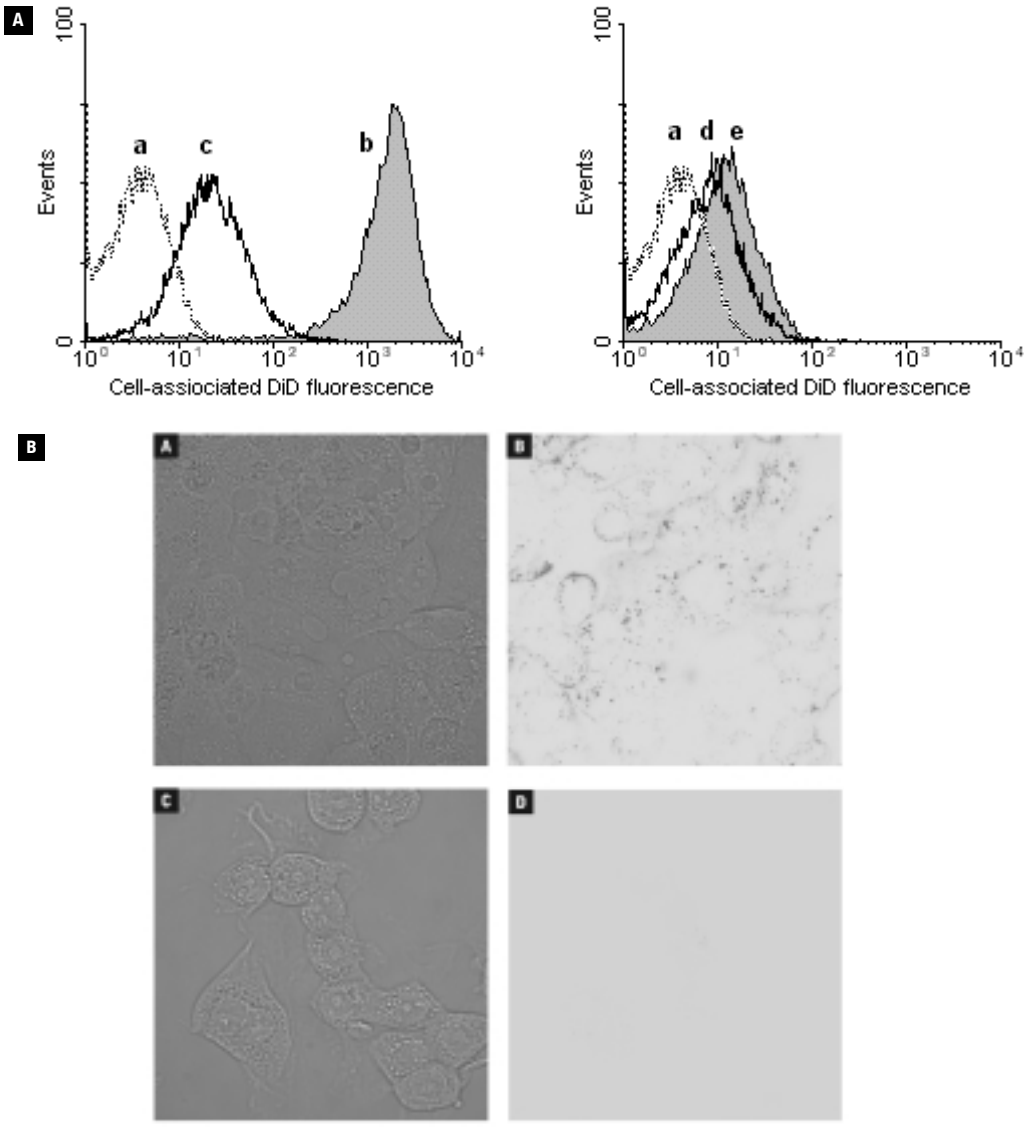

Figure 6 — Flow cytometry (A) and confocal laser scanning microscopy (CLSM;B) analysis of cellular uptake of diINF-7/DTA-containing immunoliposomes by OVCAR-3 cells. Cells ($2x10^5$) were incubated with DiD-labeled liposomes (EPC:CHOL:DiD 2:1:0.002) or immunoliposomes (500 μM) for 1 h at 37°C. Liposomes contained 0.5 μg/μmol PL of DTA and 12 μg/μmol PL of diINF-7. After unbound liposomes were washed away, cells were analyzed for associated DiD fluorescence by flow cytometry. ***Panel A***: Cell-associated DiD fluorescence of OVCAR-3 cells incubated without liposomes (a), with 425-IL (b) with 425-IL in the presence of free mAb 425 (500 μg/ml) (c), liposomes without surface-conjugated antibodies (d) and immunoliposomes bearing isotype matched irrelevant mAb 12CA5 (e). ***Panel B***: Light contrast (A and C) and CLSM (B and D) pictures taken from OVCAR-3 cells incubated for 60 min with 425-IL with encapsulated DTA and diINF-7 (A and B) or incubated with non-targeted liposomes containing encapsulated DTA and diINF-7 (C and D).

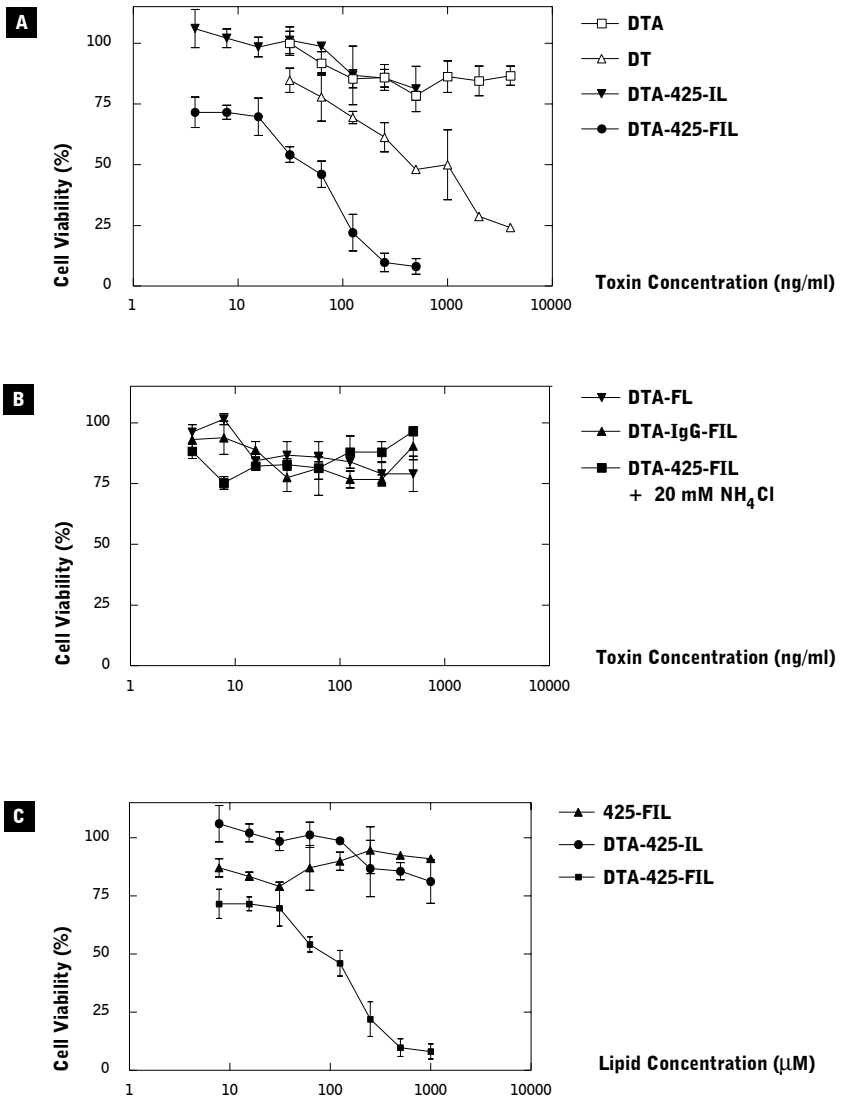

Figure 7 — Cytotoxicity of free or liposome-encapsulated DTA towards OVCAR-3 cells. OVCAR-3 cells, plated at a density of $5x10^4$ cells/mL were exposed to several DT or DTA formulations for 1 h at 37°C. Hereafter, toxin formulations were removed and cells were incubated for 47 hrs before cell viability was assessed with the XTT assay. Cell viability is expressed relative to the difference in viability of untreated cells (100% value) and empty wells (0% viability). ***Panel A***: DTA: diphtheria toxin A chain; DT: crude diphtheria toxin activated with trypsin; DTA-425-IL: immunoliposomes targeted with an anti-EGFR antibody (mAb 425) and containing DTA (0.5 μg/μmol PL); DTA-425-FIL: 425-IL containing DTA (0.5 μg/μmol PL) and diINF-7 (12 μg/μmol PL). ***Panel B***: DTA-FL: non-targeted liposomes containing DTA (0.5 μg/μmol PL) and diINF-7 (12 μg/μmol PL); DTA-IgG-FIL: immunoliposomes targeted with an isotype-matched irrelevant antibody and containing DTA (0.5 μg/μmol PL) and diINF-7 (12 μg/μmol PL); DTA-425-FIL + 20 mM NH_4Cl: DTA-425-FIL incubated with OVCAR-3 cells in the presence of 20 mM NH_4Cl to inhibit endosome acidification. ***Panel C***: 425-FIL: 425-IL containing diINF-7 (12 μg/μmol PL).

DISCUSSION

In the work presented here, a dimerized synthetic fusion peptide (diINF-7) resembling the N-terminal domain of influenza virus hemagglutinin subunit HA2 was used for the purpose to enhance cytosolic delivery of immunoliposome-encapsulated macromolecules after cellular uptake of immunoliposomes by receptor-mediated endocytosis. A previous study demonstrated that dimerization of synthetic influenza virus fusion peptides resulted in 20-fold enhanced liposome leakage activity and high, pH specific lysis of erythrocytes [10]. For this reason, we have chosen to use the dimerized form of the fusion peptide analogue INF-7. Structural characterization of diINF-7 showed that the dimeric peptide adopts an α-helix when exposed to low pH and when exposed to mixed micelles of detergent and phospholipids. The pH-dependent membrane destabilizing capacity of diINF-7 was demonstrated by the induction of leakage of calcein from liposomes. An important finding for the application of diINF-7 as endosomolytic agent in liposomal drug delivery is that — though less effective than free diINF-7 peptide — liposome-encapsulated diINF-7 peptides are able to induce release of calcein from another set of liposomes (Figure 5). This indicates that the liposome-encapsulated peptides are not only able to destabilize the liposomes in which they are encapsulated, but subsequently also other lipid membranes.

Besides leakage, the diINF-7 peptide also induces liposome aggregation (Figure 2) and subsequent intermixing of lipids between liposome vesicles, indicative for membrane fusion (Figure 3). The degree of lipid mixing induced by diINF-7 seemed to be dependent on the size of liposomes. An explanation for this may be that small liposomes have a high curvature and membrane strain, which favors merging with other vesicles to release the strain. Conversely, multilamellarity of large liposomes may account for the decrease in observed lipid mixing. An interesting observation is that incorporation of only small amounts of PEG-lipids in the liposome membranes (2.5 mol%) completely inhibited liposome aggregation and caused a large reduction in lipid mixing (Figure 2B and 3D). Presumably, the hydrophilic PEG polymers prevented the close juxtaposition of membranes required for peptide-induced aggregation of liposomes and subsequent membrane exchange between aggregated liposomes. Surprisingly, the presence of PEG on the surface of liposomes has a less pronounced inhibitory effect on peptide-induced calcein leakage, suggesting that leakage is not merely due to liposome aggregation. diINF-7 peptide-induced fusion is a rather leaky process resulting in excessive leakage of liposome-entrapped compounds to the external medium in addition to mixing of aqueous contents between fused liposomes (Figure 4). Similar findings have been reported for fusion induced by intact influenza virus particles with liposomes [39].

Indeed, when monomeric INF-7 was covalently conjugated to maleimide-derivatized PE that was incorporated into the liposomal bilayer, the fusion peptide showed fusogenic activity already at neutral pH thereby causing undesired aggregation and lipid mixing of liposomes (results not shown).

In a second set of experiments diINF-7 was tested on its capacity to enhance endosomal escape of immunoliposome-entrapped compounds. diINF-7 together with DTA were entrapped inside immunoliposomes targeted to the EGFR expressed on the surface of OVCAR-3 cells. We have utilized encapsulation of diINF-7 as a means to guarantee delivery of sufficient quantities of peptides into the endosomal compartment, which is required for effective destabilization of liposomal as well as endosomal membranes with subsequent cytosolic drug release. The possibility to attach the fusogenic peptide to the liposome membrane has been explored by us (unpublished results) and several other groups [37,40-42]. However, the general conclusion is that lipid-modification of fusogenic peptides leads to an altered conformation of the peptides thereby influencing their fusogenic behavior [37,42]. The results presented in this study demonstrate that co-encapsulation of diINF-7 into DTA-containing immunoliposomes results in cytosolic delivery of DTA into OVCAR-3 cells. As inhibition of endosome acidification results in loss of cytotoxicity of diINF-7/DTA-containing immunoliposomes, it is most likely that delivery of DTA into the cytosol of OVCAR-3 cells is mediated by diINF-7-induced endosomal membrane destabilization after cellular uptake of the diINF-7/DTA immunoliposomes.

In conclusion, encapsulation of the fusogenic peptide diINF-7 into immunoliposomes is an attractive strategy to obtain endosomal escape of liposome-entrapped therapeutic compounds after receptor-mediated endocytosis of these immunoliposomes by target cells. Future research will focus on the *in vivo* applicability of this Trojan horse concept of liposomal drug delivery.

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REFERENCE LIST

1 Scheule, R.K. (1986): **Novel preparation of functional Sindbis virosomes**. *Biochemistry*, 25:4223-4232.

2 Stegmann, T., Morselt, H.W., Booy, F.P., van Breemen, J.F., Scherphof, G., and Wilschut, J. (1987): **Functional reconstitution of influenza virus envelopes**. *EMBO J.*, 6:2651-2659.

3 Schoen, P., Chonn, A., Cullis, P.R., Wilschut, J., and Scherrer, P. (1999): **Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles**. *Gene Ther.*, 6:823-832.

4 Kaneda, Y. (2000): **Virosomes: evolution of the liposome as a targeted drug delivery system**. *Adv.Drug.Del.Rev.*, 43:197-205.

5 Plank, C., Zauner, W., and Wagner, E. (1998): **Application of membrane-active peptides for drug and gene delivery across cellular membranes**. *Adv.Drug.Del.Rev.*, 34:21-35.

6 Pecheur, E.I., Sainte-Marie, J., Bienven, and Hoekstra, D. (1999): **Peptides and membrane fusion: towards an understanding of the molecular mechanism of protein-induced fusion**. *J.Membr.Biol.*, 167:1-17.

7 Fujii, G. (1999): **To fuse or not to fuse: the effects of electrostatic interactions, hydrophobic forces, and structural amphiphilicity on protein-mediated membrane destabilization**. *Adv.Drug.Del.Rev.*, 38:257-277.

8 Wagner, E. (1999): **Application of membrane-active peptides for nonviral gene delivery**. *Adv.Drug.Del.Rev.*, 38:279-289.

9 Mechtler, K. and Wagner, E. (1997): **Gene transfer mediated by influenza virus peptides: The role of peptide sequences**. *New J.Chem.*, 21:105-111.

10 Oberhauser, B., Plank, C., and Wagner, E. (1995): **Enhancing endosomal exit of nucleic acids using pH-sensitive viral fusion peptides.** In: *Delivery strategies for antisense oligonucleotide therapeutics*, edited by S. Akhtar, pp. 247-266. CRC Press, Boca Raton.

11 Matsumoto, T. (1999): **Membrane destabilizing activity of influenza virus hemagglutinin-based synthetic peptide: implications of critical glycine residue in fusion peptide**. *Biophys.Chem.*, 79:153-162.

12 Düzgünes, N. and Shavnin, S.A. (1992): **Membrane destabilization by N-terminal peptides of viral envelope proteins**. *J.Membr.Biol.*, 128:71-80.

13 Pichon, C., Freulon, I., Midoux, P., Mayer, R., Monsigny, M., and Roche, A.C. (1997): **Cytosolic and nuclear delivery of oligonucleotides mediated by an amphiphilic anionic peptide**. *Antisense Nucleic Acid Drug Dev.*, 7:335-343.

14 Freulon, I., Roche, A.C., Monsigny, M., and Mayer, R. (2001): **Delivery of oligonucleotides into mammalian cells by anionic peptides: comparison between monomeric and dimeric peptides**. *Biochem.J.*, 354:671-679.

15 Kichler, A., Mechtler, K., Behr, J.P., and Wagner, E. (1997): **Influence of membrane-active peptides on lipospermine/DNA complex mediated gene transfer**. *Bioconjug.Chem.*, 8:213-221.

16 Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M.L. (1992): **Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle**. *Proc.Natl.Acad.Sci.U.S.A.*, 89:7934-7938.

17 Baru, M., Nahum, O., Jaaro, H., Sha'anani, J., and Nur, I. (1998): **Lysosome-disrupting peptide increases the efficiency of in-vivo gene transfer by liposome-encapsulated DNA**. *J.Drug Target.*, 6:191-199.

18 Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R., Green, J.A., Chu, E.W., Whang-Peng, J., Rogan, A.M., Green, W.R., and Ozols, R.F. (1983): **Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors**. *Cancer Res.*, 43:5379-5389.

19 Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994): **The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems**. *J.Biol.Chem.*, 269:12918-12924.

20 Oeltmann, T.N. and Wiley, R.G. (1988): **Preparation of diphtheria toxin fragment A coupled to hormone**. *Methods Enzymol.*, 165:204-10:204-210.

21 Carroll, S.F., Barbieri, J.T., and Collier, R.J. (1988): **Diphtheria toxin: purification and properties**. *Methods Enzymol.*, 165:68-76:68-76.

22 Carroll, S.F. and Collier, R.J. (1988): **Diphtheria toxin: quantification and assay**. *Methods Enzymol.*, 165:218-25:218-225.

23 Fiske, C.H. and Subbarow, Y. (1925): **The colorimetric determination of phosphorus**. *J.Biol.Chem.*, 66:375-400.

24 Ishida, T., Iden, D.L., and Allen, T.M. (1999): **A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs**. *FEBS Lett.*, 460:129-133.

25 Hennessey, J.P.J. and Johnson, W.C.J. (1981): **Information content in the circular dichroism of proteins**. *Biochemistry*, 20:1085-1094.

26 Böhm, G. **CDNN CD Spectra** Deconvolution Software. (2.1). 1997. Computer Program

27 Memoli, A., Palermiti, L.G., Travagli, V., and Alhaique, F. (1999): **Effects of surfactants on the spectral behaviour of calcein (II): a method of evaluation**. *J.Pharm.Biomed.Anal.*, 19:627-632.

28 Memoli, A., Palermiti, L.G., Travagli, V., and Alhaique, F. (1994): **Effects of surfactants on the spectral behaviour of calcein**. *J.Pharm.Biomed.Anal.*, 12:307-312.

29 Galla, H.J. and Hartmann, W. (1980): **Excimer-forming lipids in membrane research**. *Chem.Phys.Lipids*, 27:199-219.

30 Kendall, D.A. and MacDonald, R.C. (1982): **A fluorescence assay to monitor vesicle fusion and lysis**. *J.Biol.Chem.*, 257:13892-13895.

31 Arigita, C., Zuidam, N.J., Crommelin, D.J., and Hennink, W.E. (1999): **Association and dissociation characteristics of polymer/DNA complexes used for gene delivery**. *Pharm.Res.*, 16:1534-1541.

32 Düzgünes, N. and Gambale, F. (1988): **Membrane action of synthetic N-terminal peptides of influenza virus hemagglutinin and its mutants**. *FEBS Lett.*, 227:110-114.

33 Wharton, S.A., Martin, S.R., Ruigrok, R.W., Skehel, J.J., and Wiley, D.C. (1988): **Membrane fusion by peptide analogues of influenza virus haemagglutinin**. *J.Gen.Virol.* ,69(Pt 8):1847-1857.

34 Brasseur, R. (2000): **Tilted peptides: a motif for membrane destabilization (hypothesis)**. *Mol.Membr.Biol.*, 17:31-40.

35 Subbarao, N.K., Parente, R.A., Szoka, F.C.J., Nadasdi, L., and Pongracz, K. (1987): **pH-dependent bilayer destabilization by an amphipathic peptide**. *Biochemistry*, 26:2964-2972.

36 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., and Papahadjopoulos, D. (1979): **Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes**. *Biochim.Biophys.Acta*, 557:9-23.

37 Bailey, A.L., Monck, M.A., and Cullis, P.R. (1997): **pH-induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin**. *Biochim.Biophys.Acta*, 1324:232-244.

38 Steinhauer, D.A., Wharton, S.A., Skehel, J.J., and Wiley, D.C. (1995): **Studies of the membrane fusion activities of fusion peptide mutants of influenza virus hemagglutinin**. *J.Virol.*, 69:6643-6651.

39 Shangguan, T., Alford, D., and Bentz, J. (1996): **Influenza-virus-liposome lipid mixing is leaky and largely insensitive to the material properties of the target membrane**. *Biochemistry*, 35:4956-4965.

40 Puyal, C., Maurin, L., Miquel, G., Bienvenue, A., and Philippot, J. (1994): **Design of a short membrane-destabilizing peptide covalently bound to liposomes**. *Biochim.Biophys.Acta*, 1195:259-266.

41 Pecheur, E.I., Hoekstra, D., Sainte-Marie, J., Maurin, L., Bienvenue, A., and Philippot, J.R. (1997): **Membrane anchorage brings about fusogenic properties in a short synthetic peptide**. *Biochemistry*, 36:3773-3781.

42 Laczko, I., Hollosi, M., Vass, E., and Toth, G.K. (1998): **Liposome-induced conformational changes of an epitopic peptide and its palmitoylated derivative of influenza virus hemagglutinin**. *Biochem.Biophys.Res.Commun.*, 249:213-217.


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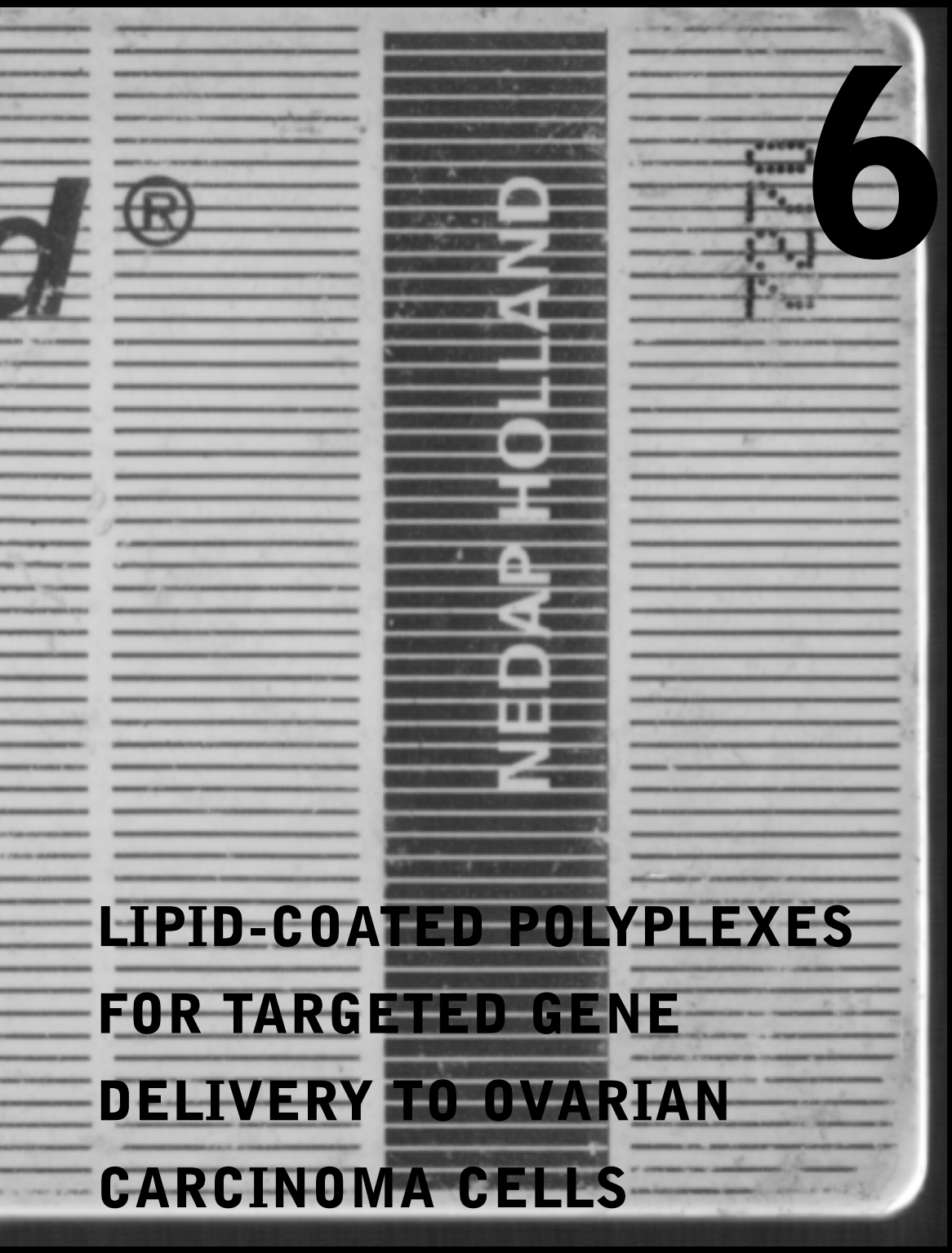

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LIPID-COATED POLYPLEXES FOR TARGETED GENE DELIVERY TO OVARIAN CARCINOMA CELLS

LIPID-COATED POLYPLEXES FOR TARGETED GENE DELIVERY TO OVARIAN CARCINOMA CELLS

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Cancer Gene Therapy, In press

SUMMARY

A non-viral gene delivery vector has been developed in our laboratory based on the cationic polymer poly(2-(dimethylethylamino)ethylmethacrylate) (pDMAEMA). p(DMAEMA)-based polyplexes have been successfully used for the transfection of OVCAR-3 cells *in vitro*. However, these polyplexes were unable to transfect OVCAR-3 cells growing in the peritoneal cavity of nude mice after *i.p.* administration, which could be ascribed to inactivation by components (including hyaluronic acid) present in the tumor ascitic fluid.

The present work aimed at (1) protecting p(DMAEMA)-based polyplexes against destabilization or inactivation by polyanions such as hyaluronic acid present in tumor ascitic fluid and (2) to enhance cellular uptake of the protected p(DMAEMA)-based polyplexes by targeting with antibody Fab' fragments. To fulfill these requirements we have developed a detergent removal method to coat polyplexes with anionic lipids. With this method spherical particles of ~125 nm were obtained that were protected from destabilization by polyanions. More importantly, the transfection efficiency of LPP was unaffected in the presence of hyaluronic acid (HyAc) indicating that lipid coating of polyplexes protects against destabilization by HyAc. By conjugating antibody Fab'-fragments directed against the epithelial glycoprotein-2 to the lipidic surface of these LPP, target cell-specific transfection of OVCAR-3 cells could be obtained *in vitro*.

INTRODUCTION

Ovarian cancer is one of the most common and fatal human gynecological malignancies. As ovarian cancer remains confined to the peritoneal cavity throughout most of its clinical course, this type of cancer is an attractive candidate for intraperitoneal cancer gene therapy [1,2]. Naked DNA does not easily pass cell membranes. Therefore, a carrier is needed that can deliver genes into cells. Viral vectors have been successfully utilized for the delivery of genetic material as they contain all features necessary for efficient gene transfer (*i.e.* cell adhesion, membrane translocation, efficient transcription and translation) [3,4]. However, despite efficient gene transfer, viral vectors have some limitations, in particular their relatively small packaging capacity of therapeutic DNA, safety concerns and immunogenicity [5,6]. In addition, the broad tropism of viruses used for gene delivery prevents cell-specific gene delivery. These limitations of viral vectors have led to the development and evaluation of alternative gene carriers based on synthetic, non-viral components [5,7]. Most non-viral vectors to date are based on the complexation of plasmid DNA with polycations by electrostatic interaction. The advantage of this method is that relatively small and positively charged particles can be obtained that can be taken up by cells. Moreover, complexation of DNA with polycations such as cationic peptides, polymers (polyplexes) or lipids (lipoplexes) protects to a large extent the DNA from degradation by nucleases.

In our laboratory a non-viral gene delivery vector has been developed based on the cationic polymer poly(2-(dimethylethylamino)ethylmethacrylate) (pDMAEMA) that forms stable and small (~100 nm) particles upon complexation with plasmid DNA [8-10]. These cationic polyplexes have been used successfully to introduce reporter genes into ovarian carcinoma (OVCAR-3) cells cultured *in vitro*. The p(DMAEMA)-based polyplexes were subsequently used *in vivo* for the transfection of OVCAR-3 cells growing in the peritoneal cavity of nude mice [11]. The results of the *in vivo* transfection experiments were disappointing: p(DMAEMA)-based polyplexes showed negligible transfection of intraperitoneally localized OVCAR-3 cells after *i.p.* administration. It has been reported that interaction of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans has an inhibitory effect on gene transfer [12]. Indeed, with our polyplexes evidence was obtained that one or more components present in the peritoneal ascitic fluid (among which hyaluronic acid) induced a detrimental effect on the *in vivo* transfection capability of p(DMAEMA)-based polyplexes. Clearly, in order to obtain effective gene transfer of ovarian carcinoma cells under *in vivo* conditions, polyplexes need to be protected from the inactivating effects of tumor ascitic components.

In this study we aimed at protecting p(DMAEMA)-based polyplexes against inactivation induced by components such as hyaluronic acid that are present in tumor ascitic fluid. In addition to protection, we also aimed at enhancing the transfection efficiency of protected polyplexes by conjugating targeting ligands to the transfection system to facilitate the uptake of protected

polyplexes by ovarian carcinoma cells. For the protection of p(DMAEMA)-based polyplexes against inactivation we have developed a detergent removal method to coat the cationic polyplexes with anionic lipids. This method is based on the preferential formation of a lipid coat around the positively charged polyplexes upon transition of phospholipids from mixed micellar to lamellar state induced by slow removal of the detergent. Lipid coating of polyplexes should fulfill several functions. First, the coating should shield the positive surface charge of polyplexes that may cause aggregation of polyplexes upon exposure to the biological environment. Second, it should protect the polyplexes from interaction with inactivating substances such as hyaluronic acid. Third, as it is expected that shielding of positive surface charges prevents the aspecific binding to cells [13], tumor-specific antibodies or antibody fragments can be conjugated to the lipid coating to obtain specific cellular uptake. Here, we show that the intended protection and targeting effects can be realized for p(DMAEMA)-based polyplexes using the proposed lipid coating and antibody coupling procedures.

MATERIALS AND METHODS

CHEMICALS

Egg phosphatidylglycerol (EPG) and egg phosphatidylcholine (EPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (CHOL), *n*-Octyl β-D-glucopyranoside (OG), o-nitrophenyl-β-D-galactopyranoside (ONPG), poly(aspartic acid) (p(Asp)), hyaluronic acid, DNase (grade I) and sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) were obtained from Sigma (St. Louis, MA, USA). *N*-[4-(p-maleimidophenyl)butyryl]phosphatidylethanol-amine (MPB-PE) was synthesized as described before [14]. BioBeads SM-2 non-polar polystyrene adsorbents came from Bio-Rad Laboratories (Hercules, CA, USA). The PicoGreen DNA detection kit was obtained from Molecular Probes (Eugene, Oregon, USA). All other reagents were of analytical grade.

PLASMID DNA

The plasmid pCMV*LacZ* containing a bacterial *LacZ* gene preceded by a nuclear localization signal under control of a CMV promoter was kindly donated by Dr. A. Bout (IntroGene, Rijswijk, The Netherlands) [15]. The plasmid was amplified in *E. Coli* (strain DH5α) and purified by column chromatography using a purification method that fully removes endotoxins (Qiagen Endofree Kit, Qiagen, Leusden, The Netherlands). The plasmid DNA was shown to be absent of RNA or bacterial DNA as analyzed by gel electrophoresis and absent of endotoxins as determined by the limulus amebocyte lysate (LAL) assay. Purity of the plasmid was determined by UV spectroscopy giving a A_{260}/A_{280} ratio of 1.84. Furthermore, gel electrophoresis and anion exchange chromatography showed that the plasmid DNA was in the supercoiled and open circular form in a ratio of about 1:1 [16].

CELL CULTURE

The human ovarian carcinoma cell line NIH:OVCAR-3 originated from the laboratory of Dr. Hamilton (National Cancer Institute, Bethesda, MD, USA) [17]. OVCAR-3 cells were cultured in DMEM containing 3.7 g/l sodium bicarbonate, 4.5 g/l L-glucose and supplemented with L-glutamine (2 mM), HEPES (10 mM), 10% (v/v) FCS, penicillin (100 IU /ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml).

POLYPLEX FORMATION AND CHARACTERIZATION

For condensation of the pCMV*LacZ* plasmid DNA the polymer poly(2-dimethylamino)ethylmethacrylate (p(DMAEMA)) synthesized at our laboratory was used [8]. The weight average molecular weight (Mw) and number average molecular weight (Mn) of p(DMAEMA) were determined by gel permeation chromatography (GPC). The used batch of p(DMAEMA) had a M_w of $2.1x10^6$ and a M_n of $4.1x10^4$.
Stock solutions of p(DMAEMA) at a concentration of 37.5 µg/ml were prepared in HEPES-buffer (5 mM HEPES, pH 7.0). Two and a half µg of pCMV*LacZ* plasmid DNA at a final concentration of 10 µg/ml in HEPES-buffer (5 mM HEPES, pH 7.4) was condensed by adding the p(DMAEMA) at a 3:1 (w/w) ratio to the DNA, followed by vigorously shaking for 5 seconds on a vortex. This ratio was chosen as at this ratio small-sized particles were obtained without the presence of free polymer [18]. After 30 minutes of maturation at room temperature, the formed polyplexes were analyzed for size distribution by dynamic light scattering and ζ-potential as previously described [8]. The polyplexes had a mean diameter of approximately 80 nm with a ζ-potential of +25-30 mV (in 5 mM HEPES; pH 7.0).

LIPID SOLUBILIZATION WITH OG: TURBIDITY ASSAY

Lipid films (3 µmol of total lipid) of different compositions were prepared from lipid stocks in a mixture of chloroform/methanol (2:1) by solvent evaporation under low pressure using a rotavapor device. A series of lipid films were hydrated in 1 ml of HEPES-buffer (5 mM HEPES, pH 7.0) at increasing concentrations of OG (0-30 mM) for 30 min at room temperature under gentle agitation. Hereafter, the turbidity of all solutions was measured (OD_{400}) spectrophotometrically. As liposomes have a much higher turbidity than mixed micelles [19], turbidity determination can be used to assess the minimum OG concentration required to achieve complete lipid solubilization into mixed micelles. The stability of polyplexes in the presence of OG detergent at a concentration just high enough to completely solubilize the lipids was evaluated by monitoring the change in particle size in time with dynamic light scattering. The size of polyplexes was measured every 15 min for 2 hrs. Polyplexes were considered to be stable if particle size did not increase with more than 10% after 2 hrs incubation in the presence of detergent.

PREPARATION OF LIPOPOLYPLEXES (LPP) AND IMMUNO-LIPOPOLYPLEXES (ILPP)

Lipid films containing 3 μmol of total lipid (EPC:EPG:CHOL molar ratio 2:2:1, unless mentioned otherwise) were formed from lipid stocks in a mixture of chloroform/methanol (2:1) by solvent evaporation under low pressure using a rotavapor device. The lipid films were further dried under a stream of nitrogen for one h. Lipid films were solubilized by adding 1 ml of 18 mM OG in HEPES-buffer (5 mM HEPES, pH 7.0). Subsequently, p(DMAEMA)-based polyplexes prepared as described above were added to the solubilized lipid films in an amount corresponding with 2.5 μg of plasmid DNA. Twenty five mg of BioBeads were added and the polyplex/lipid micelle mixtures were shaken for 1 h at 1400 rpm using a IKA MTS2 shaker. Hereafter, 200 mg of BioBeads were added and again the samples were shaken for 1 h, then samples were separated from the BioBeads and final traces of detergent were removed by overnight dialyisis at 4°C against 1 l of HEPES-buffered salt solution (HBS; 5 mM HEPES, 150 mM NaCl, pH 7.4).
For coupling antibody Fab'-fragments to the lipid surface of LPP, 2.5 mol% (of total lipid) of *N*-[4-(p-maleimidophenyl)butyryl] phosphatidylethanolamine (MPB-PE) was added to the lipids before solvent evaporation. MPB-PE is an anchor molecule commonly used to couple antibodies or enzymes to liposomes and is thiol reactive [14,20]. Three mg of $(Fab)_2$ fragments of mAb 323/A3 were reduced with 20 mM DTT in a total volume of 1 ml of 100 mM NaCl, 35 mM sodium phosphate, 20 mM citric acid, and 1 mM EDTA, pH 5.5 for 90 min at room temperature under nitrogen atmosphere. Reduced Fab' fragments containing free thiol groups were separated from DTT by column chromatography using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with deoxigenated PIPES-buffer (20 mM PIPES, 140 mM NaCl, pH 6.5) and added to freshly prepared LPP containing the MPB-PE functionalized phospholipid in a Slide-A-Lyzer dialysis slide (MWCO 10,000; Pierce, Rockford, IL, USA). The coupling reaction was proceeded overnight at 4°C while dialysis against 0.8 liter of Pipes-buffer (20 mM PIPES, 140 mM NaCl, pH 6.5) was continued. The formed immunolipopolyplex particles (ILPP particles) were separated from unconjugated Fab'-fragments by ultracentrifugation (40 min at 60,000 rpm).
LPP and ILPP were characterized for phospholipid and protein amount, size distribution and surface charge. Phospholipid amount was determined by the colorimetric method of Fiske and Subbarow [21]. The contribution of DNA to the total phosphate amount of LPP particles was negligible as determined with phosphate determination after phospholipid extraction according to Bligh and Dyer [22]. The amount of conjugated Fab' on the surface of the ILPP was determined with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with mouse IgG as standard (obtained from Sigma). Size distribution and surface charge properties of LPP and ILPP were determined by DLS and ζ-potential measurements, respectively.

ELECTRON MICROSCOPY

Polyplexes and LPP were adsorbed to glow-discharged carbon-stabilized formvar-coated grids, negatively stained using 2.0% potassium phosphotungstate (pH 5,2) and analyzed in a Philips TEM 400 electron microscope at an operating voltage of 80 kV. Images were digitally stored and analyzed using analySIS (Soft-imaging software, Germany). Images were printed after grey value modification.

NUCLEASE RESISTANCE ASSAY

Freshly prepared LPP and a mixture of polyplexes and empty anionic liposomes were exposed to a large excess of p(Asp) (1 mg/ml final concentration in HEPES buffer pH 7.0) to liberate DNA from polyplexes [9,23]. Subsequently, 1/10 volume of 10x DNase I reaction buffer (1 M sodium acetate, 50 mM $MgCl_2$) and 4U of DNase I were added and samples were incubated for 3 hrs at 37°C. Hereafter, DNase I was inactivated by adding EDTA (40 mM final concentration) and lipid vesicles (LPP or empty liposomes) were disrupted by the addition of OG (50 mM final concentration). An additional amount of p(Asp) was added before analyzing the samples by gel electrophoresis on a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide, in TAE buffer (pH 7.4).

QUANTITATIVE DETERMINATION OF PLASMID DNA IN LPP

Poly(aspartic acid) (p(Asp)) is able to dissociate polyplexes but not LPP as they possess a protective lipid coat. This feature of p(Asp) is utilized to determine indirectly the amount of DNA present in LPP. The LPP dispersion was incubated for 3.5 hrs at 40°C either with p(Asp) (1 mg/ml) to liberate the DNA from polyplexes present in the LPP dispersion or with p(Asp) (1 mg/ml) and 1% (v/v) Triton X-100 to liberate DNA from LPP. Triton X-100 disrupts the lipid coat of LPP allowing p(Asp) to liberate the DNA from polyplexes inside LPP. The amount of liberated DNA was quantified with the PicoGreen dsDNA detection kit (Molecular Probes, Eugene, USA) according to the manufacturer's protocol. PicoGreen fluorescence was measured with a LS50B fluorometer (Perkin Elmer), set at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. By subtracting the amount of non-LPP associated DNA determined by incubation with p(Asp) from the total amount of DNA determined by incubation with p(Asp) and Triton X-100 the amount of DNA inside LPP could be derived.

TRANSFECTION STUDIES

Cells were plated in 96-wells plates at $1.1x10^4$ cells/well and cultured for 24 h prior to transfection, by which time the adherent cells were ~60-80% confluent. Cells were overlaid with 100 µl of prewarmed culture medium containing 10% FCS before the addition of polyplexes, LPP or ILPP particles (corresponding to 1 µg of DNA) in triplicates. From here, three different methods were followed: The 'short-term exposure' method cells were exposed to the DNA-particles for 1 h at 37°C after which the transfection medium containing the DNA-particles was replaced by fresh culture medium containing 10% FCS and incubation was continued for another 47 hrs. In the 'prolonged exposure' method DNA-particles were not removed from the cells but cells remained exposed for the total 48 h incubation time. The third method is the same as the 'short-term exposure' method with the difference that hyaluronic acid (2.5 mg/ml) is additionally present in the wells during DNA-particle exposure. This concentration is sufficient to block the transfection capability of p(DMAEMA)-based polyplexes [11]. Cells were evaluated for β-galactosidase expression and viability using a slightly modified ONPG-assay and the XTT-assay, essentially as described before [9].

RESULTS

SIZE STABILITY OF P(DMAEMA)-BASED POLYPLEXES IN THE PRESENCE OF DETERGENT

In this study a procedure was developed to coat small-sized, cationic polyplexes with lipids using a detergent removal method (Figure 1). Initially, lipid-coated polyplexes (further referred to as lipopolyplexes (LPP)) were formed by adding p(DMAEMA)-based polyplexes (3:1 w/w ratio of polymer:DNA) to 3 µmol of lipids solubilized in 150 mM octyl glucoside (OG) and subsequent slow removal of the detergent by adsorption to hydrophobic polystyrene BioBeads. The used OG concentration is far above the critical micelle concentration (14.5 mM according to manufacturer) and it turned out that this high concentration of detergent had a negative influence on the stability of the formed polyplexes as a substantial increase in size of polyplexes occurred in time (results not shown). Therefore, in an attempt to avoid this problem, the minimal concentration of OG necessary to completely solubilize the lipids was determined and size stability of polyplexes at this concentration was monitored.
Lipid films (3 µmol of total lipid) of different compositions were prepared: EPG:EPC:CHOL at

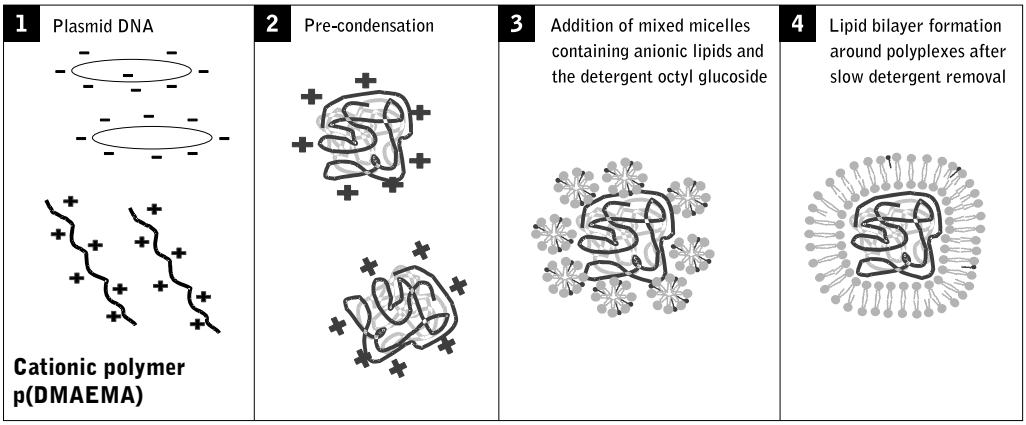

Figure 1 — Schematic representation of lipopolyplex formation. (1) Plasmid DNA is condensed by adding the cationic polymer p(DMAEMA) to the DNA at a weight/weight ratio of 3:1, respectively. The formed polyplexes (2) are added to mixed micelles containing the detergent OG and a total amount of 3 μmol detergent-solubilized lipids (3). Upon slow removal of detergent by adsorption to hydrophobic BioBeads, lipid coats are preferentially formed around positively charged polyplexes due to electrostatic interactions (4).

the molar ratios of 2:2:1 (40% EPG), 1:7:2 (10% EPG) and 0:4:1 (0% EPG). For each composition lipids were hydrated at increasing concentrations of OG. Figure 2 shows that the amount of PG included in the lipid film influences the solubilization capacity of OG for the lipids. At decreasing amounts of PG in the lipid film, increasing concentrations of OG are needed in order to totally solubilize the lipids into mixed micelles. Table 1 shows that, at the different amounts of PG tested, polyplexes do not change in size within the OG concentration range of 18-32 mM (stable for at least 2 hrs). Thus, low OG concentrations can be used to solubilize anionic lipid compositions into mixed micelles with preservation of the size stability of the polyplexes.

Table 1 — Size stability of polyplexes at OG concentrations necessary for lipid solubilization.

Amount of lipid (μmol)	**% of PG in lipid coat**	**[OG]** (for total solubilization into mixed micelles (mM))	Size of polyplexes in nm (after 2 hrs at required OG concentration)
0	—	—	91±0.4
3	0	32	101±1.2
3	10	22.5	104±0.8
3	40	18	113±1.6

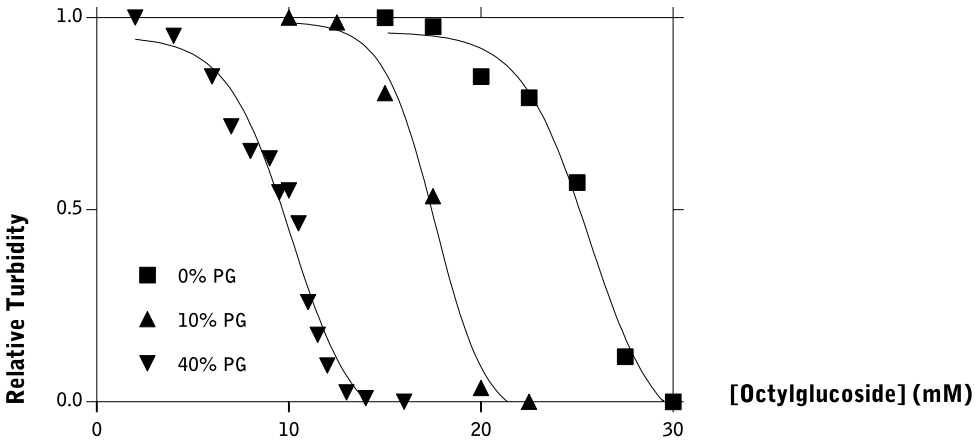

Figure 2 — Assessment of the minimal amount of OG necessary for complete lipid solubilization. Lipid films containing 3 μmol TL were hydrated with OG in the concentration range of 0-30 mM in HEPES, pH 7.0. After 30 min incubation at room temperature the turbidity of samples was spectrophotometrically determined at 400 nm. ■, EPC:CHOL (2:1) lipids ; ▲, EPC:EPG:CHOL (6:1:3) lipids; ▼, EPC:EPG:CHOL (2:2:1) lipids.

EFFECT OF LIPID/POLYPLEX RATIO ON THE SIZE OF LPP

The influence of the total amount of lipid on the size of formed LPP was determined. LPP were prepared at a fixed polyplex concentration (corresponding to 2 µg/ml of DNA and 6 µg/ml p(DMAEMA)) and increasing concentrations of OG-solubilized lipids (0-4 µmol/ml of TL; PC:PG:CHOL molar ratio 2:2:1). After slow removal of the detergent by adsorption to BioBeads and exhaustive dialysis against HBS, the mean size of the LPP was determined (Figure 3). It can be seen that the apparent mean size of LPP varied with the lipid/polyplex ratio. At lipid amounts > 1 µmol TL, small LPP were obtained with an average size of ~100-200 nm. LPP prepared at lipid amounts < 1 µmol TL showed larger particle sizes. In the experiments described below, LPP prepared at 3 µmol of lipids were used.

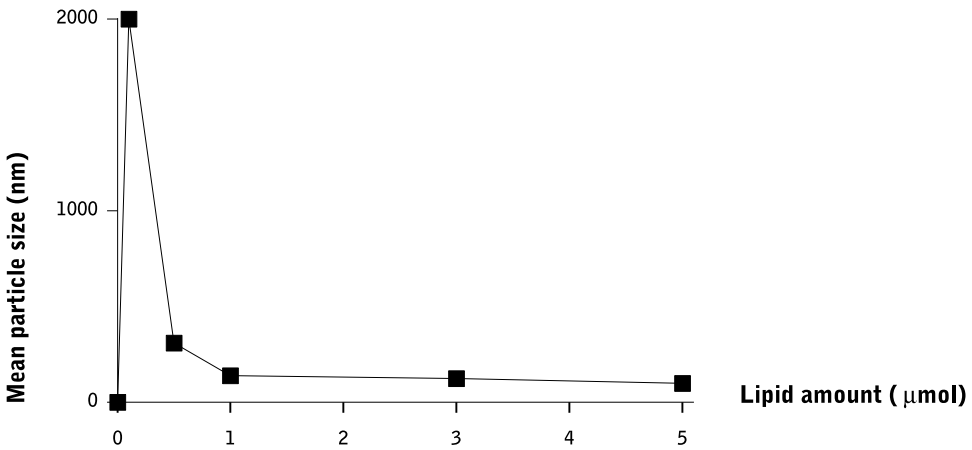

Figure 3 — Effect of lipid/polyplex ratio on the size of LPP. LPP were prepared at a fixed amount of polyplexes (corresponding to 2.5 µg DNA) with increasing amounts of lipids. Mean size was determined by dynamic light scattering.

CHARACTERIZATION OF LPP

LPP prepared with the detergent removal method were characterized for mean particle size, ζ-potential, and morphology. At 3 μmol TL, the formed LPP had an average size of 125 nm and a ζ-potential between –50 and –60 mV. Both parameters did not change during storage at 4°C for at least 2 weeks. The morphology of LPP was compared with that of polyplexes by negative-stain electron microscopy. As shown in Figure 4A, polyplexes are small, spherical particles with an average diameter of 80 ± 9 nm. Most of these particles are roundish of shape. Due to the negative staining technique, they show a mark halo of stain and appear as moderately electron dense as they attract much of the negative stain onto their positive charged surface. In contrast, LPP appear as electron light particles in a background of stain. Due to their negative surface charge there is a general failure of PTA to penetrate into these particles resulting in the presence of a white halo (Figure 4B). Besides small particles with a size variable from 50 to 70 nm (most likely empty liposomes) also larger (100-150 nm) polymorphic structures can be observed, which consist of condensed structures surrounded by a lipid coat (LPP; marked with arrows in Figure 4B). The presence of these polymorph structures probably accounts for the large size distribution range of LPP as indicated by the polydispersity index of 0.3-0.4.

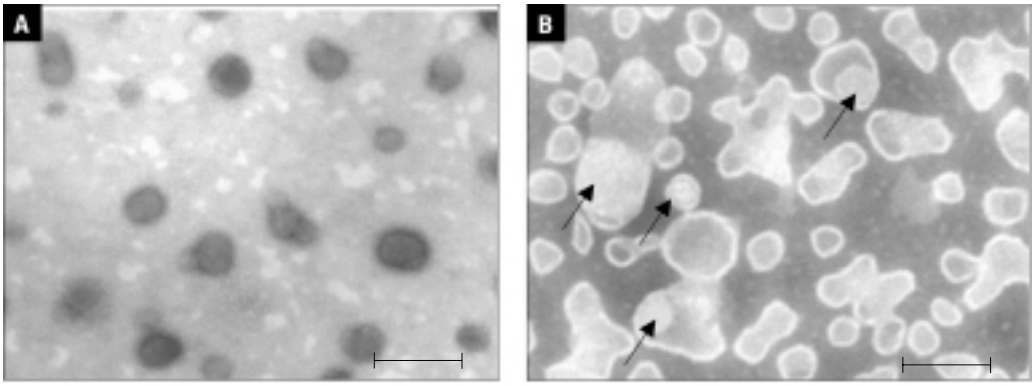

Figure 4 — Electron microscopic images of negatively stained polyplexes and LPP. Polyplexes (A) are seen as roundish spherical structures with a mark halo of stain and LPP (B) are seen as electron light particles within a stained background. These differences in staining characteristics reflect the differences in surface electrostatic forces of these particles. The arrows point at polyplexes surrounded by a phospholipid bilayer. Indicated bar represents 100 nm.

LPP ARE PROTECTED AGAINST POLYANION DESTABILIZATION

Previous work from our group has demonstrated that p(DMAEMA)-based polyplexes are destabilized when exposed to p(Asp), which liberates the DNA from the polyplexes and making it susceptible to DNase I induced degradation [9]. Here, we investigated whether polyplexes present in LPP are protected against destabilization with p(Asp). LPP and polyplexes admixed with empty anionic liposomes were incubated with DNase I in the presence of a large excess of p(Asp). Before and after the addition of DNase I samples were analyzed by gel electrophoresis for the presence of non-degraded plasmid DNA (Figure 5). It is demonstrated that there is no difference in DNA staining intensity between LPP before (lane 2) and after (lane 1) treatment with DNase I, indicating that a large amount (if not all) of the polyplexes within LPP is protected from destabilization with p(Asp). In contrast, polyplexes that were admixed with empty preformed liposomes with the same lipid composition as in the LPP coat appeared very sensitive to destabilization with p(Asp), as DNase I completely degraded complexed DNA (lanes 3 and 4). These results indicate that coating of polyplexes with lipids protects the polyplexes from destabilization by p(Asp).

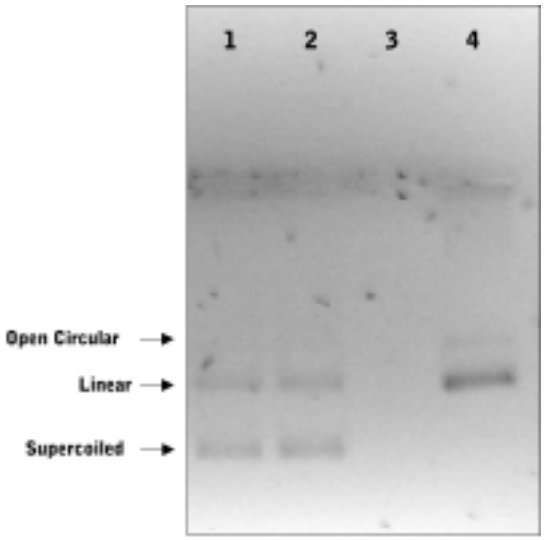

Figure 5 — Nuclease resistance assay of DNA in polyplexes and in LPP in the presence of p(Asp). LPP (lanes 1 and 2) and polyplexes admixed with empty liposomes (lanes 3 and 4) were incubated with DNase I in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of p(Asp) (1 mg/ml) for 30 min at 37°C and subsequently analysed by gel electrophoresis to visualize the presence of intact plasmid DNA. Image colors have been inverted for clarity.

QUANTITATIVE DETERMINATION OF DNA IN LPP

To quantify the efficiency of the lipid coating procedure, the amount of polyplexes effectively coated with lipids (LPP) was determined with an assay which is based on the observation that polyplexes in LPP are protected from dissociation in the presence of a large excess of p(Asp). Figure 6 shows that the degree of protection conferred by the lipid coating is positively correlated with the amount of negatively charged PG used for the coating. Complete protection of polyplexes was obtained when the lipid coat of LPP contained 40 mol% PG, 70% protection was obtained when the coat contained 10 mol% PG and 50% protection when the coat lacked negatively charged PG. These results suggest that electrostatic interactions between the cationic polyplexes and negatively charged phospholipids promote the coating of polyplexes with lipids occurring during the slow removal of detergent from the detergent-solubilized lipids.

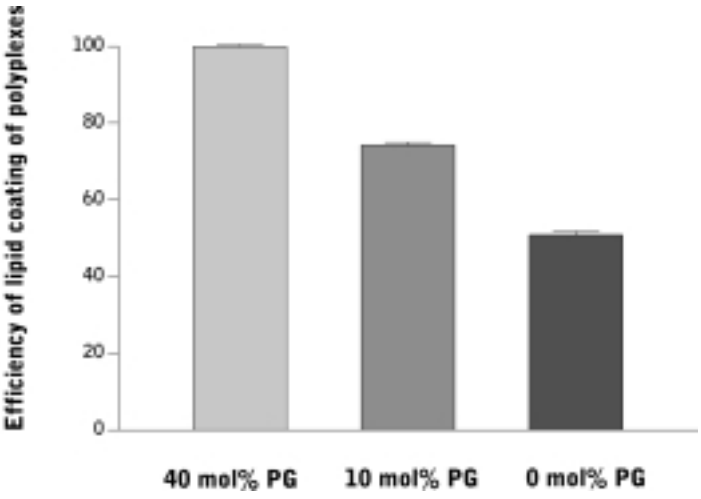

Figure 6 — Effect of anionic lipids on the efficiency of lipid coating in protecting cationic polyplexes. The coating efficiency of polyplexes with lipid compositions containing different amounts of PG (0,10 and 40%) was determined by comparing the total amount of DNA present in the sample and the amount of DNA inside polyplexes not protected against destabilization with p(Asp) (=outside LPP). The amount of DNA was determined with the Picogreen dsDNA detection assay (see materials & methods section).

CONJUGATION OF FAB'-FRAGMENTS TO LPP (ILPP)

As our aim is to target genes to ovarian carcinoma cells, we have conjugated Fab'-fragments of mAb 323/A3 to the surface of LPP. This mAb binds specifically to the epithelial glycoprotein-2 (EGP-2, EPCAM), which is expressed at high levels on the surface of OVCAR-3 cells [24,25]. After conjugation, the amount of Fab'-molecules coupled to the surface of LPP was determined. These antibody-targeted LPP (further referred to as immunolipopolyplexes, ILPP) contained an average of 20-30 conjugated Fab'-molecules per vesicle.

COMPARATIVE TRANSFECTION CAPABILITY OF POLYPLEXES, LPP AND ILPP

The capability of polyplexes, LPP and ILPP (both LPP and ILPP both contained 40mol % PG) to transfect cultured OVCAR-3 cells was investigated under three different transfection conditions. Cells were exposed to the DNA formulations for (1) 1h, (2) 48 h and (3) 1 h in the presence of hyaluronic acid (2.5 mg/ml). Hyaluronic acid was used as this component has been shown to have an adverse effect on the transfection capability of plain polyplexes [11]. However, it is

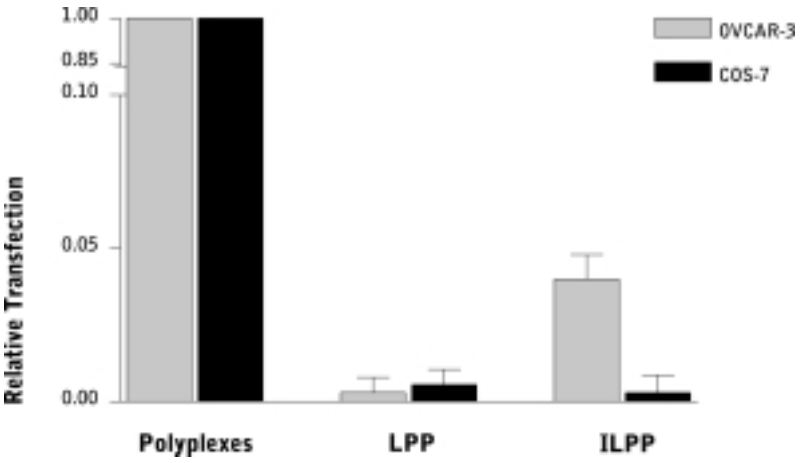

Figure 7 — Transfection of OVCAR-3 cells (EGP-2 +) and COS-7 cells (EGP-2 -) after short term (1h) exposure to polyplexes, LPP or ILPP. Cells ($1.1x10_4$ cells/well) were exposed for 1 h at 37°C to either polyplexes, LPP or ILPP (at 1 μg of plasmid DNA). Gene carriers were removed by washing and cell culture was continued for another 47 h prior to evaluation of β-galactosidase expression.

hypothesized that hyaluronic acid will not adversely affect the transfection capability of LPP and ILPP due to the protective lipid layer.

Figure 7 shows the transfection results obtained when cells were exposed for 1 h to the different DNA formulations. It is clear that both negatively charged LPP and ILPP are much less efficient in transfecting OVCAR-3 cells compared to positively charged polyplexes under the tested conditions. Nevertheless, the antibody-targeted ILPP are 10-fold more efficient than LPP in transfecting OVCAR-3 cells. In addition, the efficiency of ILPP to transfect COS-7 cells (lacking EGP-2 expression) is not enhanced compared to non-targeted LPP indicating that antibody-mediated targeting to OVCAR-3 cells can be achieved. No cytotoxicity was observed with all DNA formulations used (polyplexes, LPP and ILPP) in the short-term exposure setting (results not shown).

Figure 8 shows the results of transfection of OVCAR-3 cells after long-term exposure (=48 hrs)

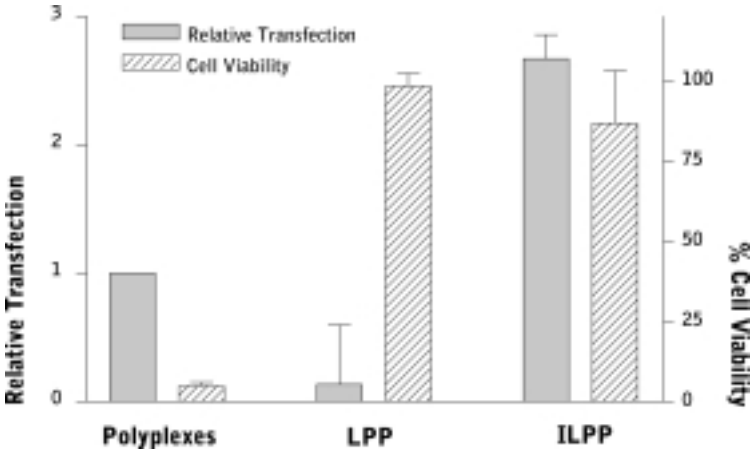

Figure 8 — Transfection and cell viability of OVCAR-3 cells after long term exposure (48h) to polyplexes, LPP or ILPP. OVCAR-3 cells (1.1×10^4 cells/well) were exposed for 48 hrs at 37°C to either polyplexes, LPP or ILPP (at 1 μg of plasmid DNA), and subsequently evaluated for β-galactosidase expression and cell viability using the ONPG and XTT assay, respectively.

to polyplexes, LPP or ILPP. It can be seen that long-term exposure of cells to the polyplexes was detrimental for the transfection efficiency of this transfection system (compare with effect in Figure. 7). The reason for this negative effect lies in increased cytotoxicity: the cell viability was only about 5%. Cytotoxicity was not observed when cells are exposed for 48 hrs to LPP and ILPP. In this long-term exposure setting, the transfection efficiency of ILPP was about 2.5 times higher than that of plain polyplexes. LPP hardly gave any transfection in contrast to the ILPP, indicating that antibody-mediated binding of the LPP to OVCAR-3 cells is critical for achieving transfection of OVCAR-3 cells.

As we have shown that coating of polyplexes with lipids protects the polyplexes from polyanion-induced destabilization, we have compared transfection results obtained with polyplexes and ILPP in the absence and presence of the polyanion hyaluronic acid, which is abundantly present in tumor ascitic fluid (Figure 9). Whereas the transfection efficiency of polyplexes is drastically reduced in the presence of hyaluronic acid (Figure 9A), this reduction is not observed with ILPP (Figure 9B), indicating that hyaluronic acid does not negatively affect the transfection efficiency of ILPP.

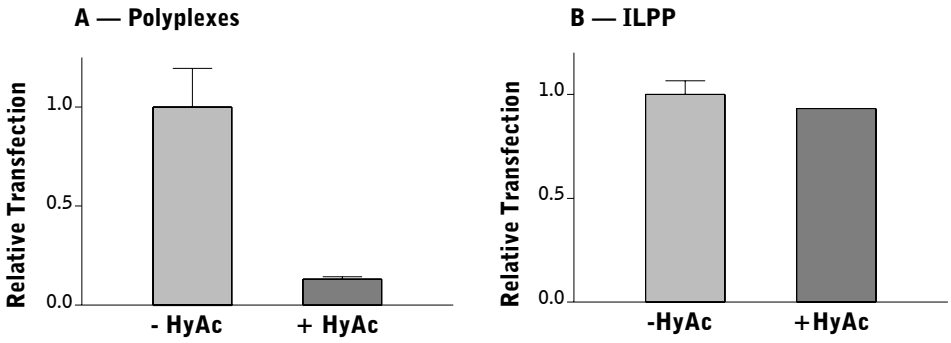

Figure 9 — Influence of hyaluronic acid on the transfection efficiency of polyplexes vs ILPP. OVCAR-3 cells (1.1×10^4 cells/well) were exposed for 1 h at 37°C to (A) polyplexes or (B) ILPP in the absence (-HyAc) or presence (+HyAc) of 2.5 mg/ml hyaluronic acid. Gene carriers were removed by washing and cell culture was continued for another 47 hrs prior to evaluation for β-galactosidase expression.

DISCUSSION

Local delivery of therapeutic genes into ovarian carcinoma cells is an attractive strategy for the treatment of ovarian carcinoma [1]. As naked DNA is very unstable in the biological environment, is poorly taken up by target cells and lacks cell specificity, a gene delivery carrier is needed to fulfill the requirements of stability and cellular uptake. For this purpose, the cationic polymer p(DMAEMA) has been developed in our laboratory. p(DMAEMA) can condense large-sized DNA into small and stable complexes of approximately 100 nm in size and which are protected from DNA degradation by DNase *in vitro* [8,9]. Despite encouraging transfection results obtained with p(DMAEMA)-based polyplexes *in vitro*, *in vivo* transfection of ovarian carcinoma cells growing in the peritoneal cavity of nude mice is negligible [11]. Evidence exists that one of the components of the peritoneal ascitic fluid, hyaluronic acid, induced a detrimental effect on the *in vivo* transfection capability of p(DMAEMA)-based polyplexes.

To address the problem of low *in vivo* transfection activity of p(DMAEMA)-based polyplexes we present here a method of coating the p(DMAEMA)-based cationic polyplexes with anionic lipids and show that the anionic lipid coating confers protection against destabilization of polyplexes by polyanionic molecules such as p(Asp) and hyaluronic acid. However, the LPP lack significant transfection capability likely due to the loss of cationic charge-mediated electrostatic interaction with the cells. This problem can be overcome by coupling specific antibody fragments to the LPP surface thus restoring cellular uptake and transfection. Both aspects, *i.e.* protection and targeting, will be discussed below.

The first requirement to meet was to create protection against polyanion-induced destabilization or inactivation. Previous transfection studies with plain p(DMAEMA)-based polyplexes have shown that hyaluronic acid, which is present at high levels in tumor ascitic fluid, has a dose-dependent negative effect on the transfection efficiency of plain polyplexes (see Figure 9A and van de Wetering et al.[11]). With the detergent removal method used in this study it is possible to coat polyplexes with lipids with efficiencies of up to 100% as reflected by complete protection against polyanion-induced destabilization. Moreover, this method yields stable particles with a small mean size (~120 nm) and a net negative surface charge. The degree of protection conferred by the lipid coating is positively correlated with the amount of negatively charged lipids present in the coat, which indicates that electrostatic interactions promote the lipid coating process. Similarly, Lee et al. [26,27] took advantage of electrostatic interactions for coating polylysine-condensed DNA complexes with pre-formed anionic liposomes. Although analysis by negative stain electron microscopy revealed anionic lipid-coated particles, it was not shown whether the lipid coat really caused protection of the condensed DNA against degradation or destabilization. Here, we show that LPP prepared with the detergent removal method are protected from destabilization by polyanions such as p(Asp) and hyaluronic acid. In addition, the transfection efficiency of ILPP is not negatively influenced by the presence of high concentrations of

hyaluronic acid, in contrast to the transfection efficiency of plain, unprotected polyplexes (Figure 9).

Unfortunately, besides the positive effect of conferring protection, the presence of a negatively charged lipid coat around LPP has a negative impact on cellular uptake of LPP. Cationic polyplexes are thought to electrostatically interact with cell membranes thereby triggering cellular uptake mechanisms [13]. The observed lack of transfection capability and cytotoxicity of LPP is most likely due to absence of cellular association. Indeed, this is confirmed by our observation that targeting of LPP, which promotes specific cellular uptake of LPP is a prerequisite to obtain transfection of ovarian carcinoma cells. Targeting of LPP to ovarian carcinoma cells (OVCAR-3) was realized by coupling Fab'-fragments of mAb 323/A3 (anti-EGP-2) to the surface of LPP (ILPP). However, despite the targeting effect the transfection efficiency of ILPP is 20-fold lower than obtained with plain polyplexes in case of 'short term' exposure (1 h) of cells to the gene carriers. This may well be explained by the quantitative difference in degree of cellular uptake between polyplexes and ILPP. The mechanism of nonspecific cellular uptake of plain polyplexes may be much more efficient than the mechanism of specific cellular uptake of ILPP (antibody-mediated binding and internalization), the latter being dependent on antigen-density and kinetics of internalization of the target surface antigen EGP-2. The EGP-2 surface receptor is slowly internalized (50% after 48 hrs) [28].

However, it should be realized that the 1 h exposure period does by far not reflect the *in vivo* conditions in which peritoneal tumor cells are expected to be exposed to *i.p.*-injected ILPP for much longer periods of time. For this reason we have compared the transfection levels of ovarian carcinoma cells after 48 h exposure to either plain polyplexes, LPP or ILPP. Under these 'long-term exposure' conditions the transfection efficiency of the antibody-targeted ILPP, which were 20 times less efficient in transfection ovarian cancer cells under 'short term exposure' are now 2.5-fold more efficient in transfecting ovarian carcinoma cells compared to plain polyplexes. Moreover, even under 'long-term exposure' conditions non-targeted LPP does not show any transfection, which illustrates the necessity of achieving target receptor-mediated internalization. Apparently, the presence of the targeting ligand mediates cellular binding and uptake of the coated particles and compensates for the loss of electrostatic interaction with the cell membrane caused by the introduction of the lipid coating on the polyplex surface. It is noteworthy that — in sharp contrast with the plain polyplexes — ILPP did not induce any cytotoxicity to the cells (Figure 8). These findings indicate that antibody-targeted ILPP are better suited for intraperitoneal transfection of ovarian carcinoma cells than plain polyplexes.

In conclusion, the ILPP system introduced in this study features colloidal stability and transfection capability under conditions mimicking the *in vivo* situation and therefore shows promise for use in plasmid-based approaches to gene therapy of ovarian cancer. At present, the ILPP system is being investigated in our laboratory for its efficiency to deliver genes to ovarian carcinoma cells for the purpose of gene-dependent enzyme prodrug therapy [29].

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REFERENCE LIST

1 Robertson, M.W., Barnes, M.N., Rancourt, C., Wang, M., Grim, J., Alvarez, R.D., Siegal, G.P., and Curiel, D.T. (1998): **Gene therapy for ovarian carcinoma.** *Semin.Oncol.* , 25:397-406.

2 Gomez-Navarro, J., Siegal, G.P., Alvarez, R.D., and Curiel, D.T. (1998): **Gene therapy: ovarian carcinoma as the paradigm.** *Am.J.Clin.Pathol.*, 109:444-467.

3 Cristiano, R.J. (1998): **Viral and non-viral vectors for cancer gene therapy.** *Anticancer Res.*, 18:3241-3245.

4 Robbins, P.D. and Ghivizzani, S.C. (1998): **Viral vectors for gene therapy.** *Pharmacol.Ther.*, 80:35-47.

5 Luo, D. and Saltzman, W.M. (2000): **Synthetic DNA delivery systems.** *Nat.Biotechnol.*, 18:33-37.

6 Crystal, R.G. (1995): **Transfer of genes to humans: early lessons and obstacles to success.** *Science*, 270:404-410.

7 Cristiano, R.J. (1998): **Targeted, non-viral gene delivery for cancer gene therapy.** *Front.Biosci.*, 3:D1161-D1170

8 van de Wetering, P., Cherng, J.-Y., Talsma, H., and Hennink, W.E. (1997): **Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid complexes.** *J.Control.Release*, 49:59-69.

9 Arigita, C., Zuidam, N.J., Crommelin, D.J., and Hennink, W.E. (1999): **Association and dissociation characteristics of polymer/DNA complexes used for gene delivery.** *Pharm.Res.*, 16:1534-1541.

10 De Smedt, S.C., Demeester, J., and Hennink, W.E. (2000): **Cationic polymer based gene delivery systems.** *Pharm.Res.*, 17:113-126.

11 van de Wetering, P., Schuurmans-Nieuwenbroek, N.M.E., Hennink, W.E., and Storm, G. (1999): **Comparative transfection studies of human ovarian carcinoma cells in vitro, ex vivo and *in vivo* with poly(2-(dimethylamino)ethylmethacrylate)-based polyplexes.** *J.Gen.Med.*, 1:156-165.

12 Ruponen, M., Yla-Herttuala, S., and Urtti, A. (1999): **Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies.** *Biochim.Biophys.Acta*, 1415:331-341.

13 Zuidam, N.J., Posthuma, G., de Vries, E.T., Crommelin, D.J., Hennink, W.E., and Storm, G. (2000): **Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization.** *J.Drug Target.*, 8:51-66.

14 Martin, F.J. and Papahadjopoulos, D. (1982): **Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting.** *J.Biol.Chem.*, 257:286-288.

15 Bout, A., Valerio, D., and Scholte, B.J. (1993): **In vivo transfer and expression of the lacZ gene in the mouse lung**. *Exp.Lung Res.*, 19:193-202.

16 Hinrichs, W.L., Schuurmans-Nieuwenbroek, N.M., van de Wetering, P., and Hennink, W.E. (1999): **Thermosensitive polymers as carriers for DNA delivery.** *J.Control.Release*, 60:249-259.

17 Hamilton, T.C., Young, R.C., McKoy, W.M., Grotzinger, K.R., Green, J.A., Chu, E.W., Whang-Peng, J., Rogan, A.M., Green, W.R., and Ozols, R.F. (1983): **Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors.** *Cancer Res.*, 43:5379-5389.

18 Cherng, J.Y., van de Wetering, P., Talsma, H., Crommelin, D.J., and Hennink, W.E. (1996): **Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles.** *Pharm.Res.*, 13:1038-1042.

19 Levy, D., Gulik, A., Seigneuret, M., and Rigaud, J.L. (1990): **Phospholipid vesicle solubilization and reconstitution by detergents. Symmetrical analysis of the two processes using octaethylene glycol mono-n-dodecyl ether.** *Biochemistry*, 29:9480-9488.

20 Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S., and Allen, T.M. (1995): **Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures.** *Biochim.Biophys.Acta*, 1239:133-144.

21 Fiske, C.H. and Subbarow, Y. (1925): **The colorimetric determination of phosphorus.** *J.Biol.Chem.*, 66:375-400.

22 Bligh, E.G. and Dyer, E.J. (1959): **A rapid method of total lipid extraction and purification.** *Can.J.Biochem.Physiol.*, 37:911-917.

23 Katayose, S. and Kataoka, K. (1997): **Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer.** *Bioconjug.Chem.*, 8:702-707.

24 Edwards, D.P., Grzyb, K.T., Dressler, L.G., Mansel, R.E., Zava, D.T., Sledge, G.W.J., and McGuire, W.L. (1986): **Monoclonal antibody identification and characterization of a Mr 43,000 membrane glycoprotein associated with human breast cancer.** *Cancer Res.*, 46:1306-1317.

25 Langmuir, V.K., Mendonca, H.L., and Woo, D.V. (1992): **Comparisons between two monoclonal antibodies that bind to the same antigen but have differing affinities: uptake kinetics and 125^{I}- antibody therapy efficacy in multicell spheroids.** *Cancer Res.*, 52:4728-4734.

26 Lee, R.J. and Huang, L. (1996): **Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer.** *J.Biol.Chem.*, 271:8481-8487.

27 Lee, R.J. and Huang, L. (1997): **Lipidic vector systems for gene transfer.** *Crit.Rev.Ther.Drug Carrier.Syst.*, 14:173-206.

28 Haisma, H.J., Pinedo, H.M., Rijswijk, A., der, M., Sosnowski, B.A., Ying, W., Beusechem, V.W., Tillman, B.W., Gerritsen, W.R., and Curiel, D.T. (1999): **Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM.** *Gene Ther.*, 6:1469-1474.

29 Fonseca, M.J., Storm, G., Hennink, W.E., Gerritsen, W.R., and Haisma, H.J. (1999): **Cationic polymeric gene delivery of β-glucuronidase for doxorubicin prodrug therapy**. *J.Gen.Med.*, 1:407-414.

prevent repeated injections of these fusogenic drug carriers *in vivo*. One way to circumvent the problem of immunogenicity is to avoid immune recognition by using different strains of influenza virus for preparing the virosomes. In this way, the potential presence of antibodies raised against the first virosome batch will not be able recognize the second batch of virosomes prepared from another influenza virus strain, as the antigenic determinants of this strain are different. Alternatively, the presence of high amounts of PEG-molecules on the surface of virosomes may effectively prevent immune recognition of the influenza virus HA molecules by sterically shielding the HA molecules as was demonstrated for PEGylated adenoviruses [21,22].

Antibody-targeted influenza virosomes may be of great use for vaccination purposes. For this application the immunogenicity of HA is highly desired in order to raise an immune response against the antigens delivered by the virosomal carriers. Due to their fusogenic capacity, influenza virosomes are able to introduce antigen into the MHC class I presentation pathway of antigen presenting cells [23]. Active targeting of influenza virosomes may enhance the delivery of antigens to specific subsets of antigen presenting cells *in vivo*.

In an attempt to specifically deliver water-soluble agents into the cytosol of OVCAR-3 cells, we have encapsulated the bacterial toxin DTA inside antibody-targeted virosomes. No increased cytotoxicity of virosomal DTA over free DTA could be observed (results not shown). This can be ascribed to poor encapsulation efficiency of DTA into virosomes, which is inherent to the method of virosome preparation (detergent removal at low lipid concentrations) in combination with loss of biological activity when exposed to hydrophobic beads that are used to remove detergent during the preparation of virosomes [24]. It was estimated that approximately 0.1% of the bacterial toxin was associated with targeted influenza virosomes. Due to the low amounts of virosome-associated DTA it was impossible to determine the amount of DTA, which was present inside virosomes. Furthermore, as the influenza virosomes are prepared with a detergent having a low critical micelle concentration (CMC), high amounts of residual detergent will be present in the virosome membranes, which makes the membranes rather leaky [25].

To circumvent the problems of low encapsulation efficiency and membrane instability a novel method of virosome preparation was explored in which HA proteins solubilized with short-chain PC molecules (DCPC) were post-inserted into preformed

SUMMARIZING DISCUSSION

Advances in molecular and cellular biology have led to the development of a new class of biotherapeutic macromolecules (*i.e.* proteins, (poly)peptides and nucleic acids) that can be designed de novo to modulate functions of target cells. As many of these new biotherapeutics have intrinsically unfavorable properties for accessing targets within the cytoplasm or nucleus, achieving their full potential ultimately requires drug carriers that can efficiently deliver these agents across cellular membrane barriers into the cytosol of target cells.
This thesis describes the optimization of immunoliposomes (IL) for cytosolic delivery of biotherapeutic macromolecules to tumor cells, in particular ovarian carcinoma cells, via the route of receptor-mediated endocytosis. Two important requirements to be met for efficient cytosolic delivery via this route that are investigated in this thesis are (1) specific interaction with target cells leading to receptor-mediated uptake of the immunoliposomes and (2) release of IL-entrapped drug into the cytosol of target cells by endosomal membrane destabilization. Although there are several critical steps involved in the transport of immunoliposomes from the site of injection to the target site (see chapter 1), this thesis primarily focuses on the delivery of the macromolecular drug into the cytosol of target cells after immunoliposomes have reached their target cells.

RECEPTOR-MEDIATED ENDOCYTOSIS OF IMMUNOLIPOSOMES

As reviewed in chapter 1, a variety of receptor-specific monoclonal antibodies have been conjugated to the surface of liposomes to achieve site-specific delivery. Dependent on the type of target receptor and size of the IL particles, receptor binding can result in internalization of the receptor/IL complexes by receptor-mediated endocytosis. Although numerous receptors with internalizing capacity have been identified, the liposome-internalizing capacity of most of the tumor-associated receptors used for tumor cell-directed IL targeting is unknown. Therefore, in pursuing cytosolic drug delivery with IL via the route of receptor-mediated endocytosis, target receptors need to be identified that enable not only selective binding to tumor cells but also sufficient internalization of the cell surface-bound IL. In **Chapter 2** and the **Appendix to Chapter 2** we have explored three target receptors expressed on OVCAR-3 cells that may be used to deliver IL into the endocytic pathway. These receptors are the intercellular adhesion molecule-1 (ICAM-1), the epithelial glycoprotein-2 (EGP-2) and the epidermal growth factor receptor (EGFR).
It is well established that the expression of ICAM-1 is up-regulated on endothelial and epithelial cells at sites of inflammation. Thus, targeting liposomes to ICAM-1 may have potential for

specific drug delivery to sites of inflammation. In previous work from our group it was demonstrated that liposomes could be specifically targeted to cells expressing ICAM-1 by conjugating the monoclonal antibody F10.2 to their surface. These F10.2-IL were shown to strongly bind to different cell types expressing ICAM-1 on their surface and binding was positively correlated with the expression level of ICAM-1 [1]. In **Chapter 2** of this thesis it was demonstrated that F10.2-IL were efficiently internalized by bronchial epithelial cells. Sixty percent of cell-bound IL were internalized within 1 h of incubation at 37°C. As ICAM-1 expression is up-regulated at sites of inflammation, selective drug delivery with ICAM-1-directed IL to sites of inflammation has been proposed. ICAM-1 not only plays a role in localization of immune cells into inflamed tissue but is also involved in the pathogenesis and metastatic spread of many types of tumor cells [2-6]. For this reason we have also investigated the possibility to target F10.2-IL to ovarian carcinoma cells (OVCAR-3) (**Appendix to Chapter 2**). Flow cytometry analysis confirmed the presence of ICAM-1 on the surface of OVCAR-3 cells. Unfortunately, binding of F10.2-IL to OVCAR-3 cells was not observed. As the flow cytometry analysis of ICAM-1 expression on OVCAR-3 cells was performed with the same mAb as used for IL targeting, lack of IL binding is likely related to loss of binding capacity of the F10.2 mAb during the chemical modification step necessary to conjugate the mAb to the surface of liposomes.
An important aspect in selecting a suitable target receptor for tumor targeting is not only tumor-selective expression but also accessibility for the targeted drug carriers. EGP-2, also referred to as epithelial cell adhesion molecule (Ep-CAM), mediates Ca^{2+}-independent homotypic cell-cell adhesions and has been found to be expressed on the basolateral cell membrane of normal epithelial cells and a variety of adenomas and adenocarcinomas [7-10]. Similarly, we observed *in vitro* that 323/A3-IL targeted to EGP-2 on OVCAR-3 cells efficiently bound to these cells when cells were in suspension but when grown as a monolayer adhered to plastic culture flasks binding of the IL was restricted to the edges of the monolayer **(Appendix to Chapter 2)**. Clearly, under these *in vitro* growing conditions the accessibility of EGP-2 for IL binding is poor. Consequently, the accessibility of target receptors should be taken into consideration when targeted drug delivery systems are tested *in vitro* and *in vivo*. Besides poor accessibility, the rate of IL internalization bound to EGP-2 on OVCAR-3 cells is slow: only 15% of cell surface-bound 323/A3-IL were internalized by OVCAR-3 cells grown in suspension after 2 hrs incubation.
The third receptor expressed on OVCAR-3 cells which has been tested for IL internalization is the EGFR. The EGFR is a receptor tyrosine kinase that is over-expressed in a wide variety of solid human cancers, including non-small cell lung, breast, head and neck, bladder and ovarium carcinomas [11-13]. For this reason the EGFR and other members of the EGFR-family have often been used as targets for site-specific drug delivery [11,12,14-16]. As demonstrated in the **Appendix to Chapter 2,** the EGFR appears to be a suitable candidate to achieve IL internalization. The EGFR is readily accessible for IL binding both on OVCAR-3 cells grown in suspension and grown as a monolayer. In addition, 425-IL binding to OVCAR-3 cells resulted in approximately 40% internalization of surface-bound IL within 2 hrs. Based on the *in vitro* results

on IL targeting to OVCAR-3 cells we can conclude that, under the conditions tested, the EGFR is the most suitable receptor to deliver immunoliposomes into the endocytic pathway of OVCAR-3 cells.

Clearly, the choice of the target receptor able to shuttle drug-laden IL into the endocytic pathway of tumor cells is critically important. The target receptor has to meet several requirements. First, its expression level on the surface of target cells must be high enough to allow binding of a sufficient amount of drug-laden IL. Second, the target receptors must be accessible for the IL. Third, the target receptor should be tumor specific or at least overexpressed on malignant cells in order to obtain tumor-selectivity. Finally, the target receptor should mediate internalization of receptor-bound liposomes. As screening of existing mAb that specifically bind to receptors for aforementioned requirements is time-consuming and laborious, the availability of a selection system that allows rapid selection of antibodies recognizing tumor cell-selective internalizing cell-surface receptors is highly needed. Recently, it was demonstrated that scFv molecules displayed on bacteriophage particles could be selected for both tumor cell-specific binding and internalization properties by selecting and enriching internalized phagemids [17,18]. This system may prove useful in rapidly selecting antibody molecules that can deliver large drug carriers like liposomes into the endocytic pathway of tumor cells as a first step in cytosolic drug delivery.

ENDOSOMAL ESCAPE BY LOW PH-INDUCED MEMBRANE FUSION

As immunoliposomes with the entrapped biotherapeutic macromolecules are liable to degradation inside lysosomes after receptor-mediated endocytosis, enormous gains in therapeutic efficacy can be anticipated when this degradative pathway is circumvented. Hence, we have exploited the low pH-induced fusion mechanism of the human influenza virus in two different ways. In the first approach reconstituted envelopes of influenza virus particles (virosomes) were used as basis to construct a targeted fusogenic liposome by conjugating antibody fragments via a PEG-spacer to their surface. In the second approach, synthetic fusion peptides that resemble the influenza virus HA2 N-terminal fusion domain were encapsulated in IL to induce membrane destabilization or membrane fusion upon release from IL exposed to the acidic environment within the endosomes.

The use of influenza virosomes (*i.e.* reconstituted envelopes of the influenza virus containing the viral fusion protein HA) guarantees efficient membrane fusion similar to the native virus, which by no means can be equaled with synthetic fusion peptides. However, as HA also contains the sialic acid binding pocket, the use of unmodified virosomes will result in undesired binding to the ubiquitous sialic acid residues present on glycoproteins and glycolipids on the surface of many cell types. To circumvent HA-mediated sialic acid binding without losing fusogenic activity, we have coupled PEG molecules to the surface of influenza virosomes, which, at high concentrations, were able to prevent binding to sialic acid residues on cells **(Chapter 3)**. In

addition to a 'steric shielding effect' provided by the PEG chains, the PEG chains served as a spacer to which antibody molecules were conjugated. In this way, influenza virosomes could be re-directed towards EGP-2 expressed on the surface of OVCAR-3 cells with preservation of fusogenic activity. This chapter represents the first report on successful antibody-mediated virosome targeting and may be used for target-cell specific delivery of drugs or antigens.

A major impediment to the use of influenza virosomes as drug carrier is that the presence of viral HA will induce immunogenic reactions, which will adversely affect the *in vivo* targeting capacity of these carriers upon repeated injection [19,20]. One option to circumvent the problem of immunogenicity may be the use of different strains of influenza virus for preparing the virosomes for separate injections. Alternatively, the presence of high amounts of PEG-molecules on the surface of virosomes may effectively prevent immune recognition by a sterical shielding effect as was recently demonstrated for PEGylated adenoviruses [21,22].

Although not the focus of this thesis, antibody-targeted influenza virosomes may be of great value in tumor vaccination protocols. In the case of vaccination, the immunogenicity of HA is highly useful for raising an immune response against the antigenic peptides of interest incorporated in the virosomal carriers. Due to their fusogenic capacity, influenza virosomes are able to introduce antigen into the MHC class I presentation pathway of antigen presenting cells [23]. Active targeting of influenza virosomes may therefore enhance antigen delivery to specific subsets of antigen presenting cells (*e.g.* dendritic cells) *in vivo*.

In an attempt to demonstrate their cytosolic delivery capacity we have encapsulated the bacterial toxin DTA inside antibody-targeted virosomes. No increased cytotoxicity of virosomal DTA over free DTA was observed (results not shown). This can be ascribed to a poor encapsulation efficiency of DTA into virosomes, which is inherent to the method of virosome preparation (detergent removal at low lipid concentrations). In addition, loss of biological activity of DTA has been reported when exposed to hydrophobic beads used to remove detergent during the preparation of virosomes [24]. Due to the low amounts of virosome-associated DTA it was impossible to determine the exact amount of incorporated DTA. It was estimated that approximately 0.1% of the bacterial toxin was associated with targeted influenza virosomes. Furthermore, as the influenza virosomes are prepared with a detergent with a low critical micelle concentration (CMC) value, a relatively high amount of residual detergent monomers can be expected to be present in the virosome membranes, which makes them permeable for DTA leakage [25].

To circumvent the problems of low encapsulation efficiency and membrane instability a novel method of virosome preparation was explored: HA proteins solubilized with short-chain PC molecules (DCPC) were post-inserted into preformed liposomes (**Chapter 4**). The potential advantage of this 'post-insertion method' is that liposome preparation techniques can be used that allow high efficiency entrapment of water-soluble substances into stable liposomes. HA could be functionally post-inserted in liposomes. However, the insertion process resulted in excessive leakage of both small (calcein) as well as large (DNA) molecules from the liposomes. Hence,

with this alternative method of virosome preparation the encapsulation of water-soluble substances in virosomes was not improved. Nonetheless, efficient entrapment of drug molecules inside virosomes prepared by the post-insertion method may be obtained by a remote loaded procedure after removal of the short-chain PC molecules by dialysis. The high CMC value of DCPC used for solubilization of HA in the post-insertion method allows efficient removal of bilayer-associated detergent by dialysis in contrast to $C_{12}E_8$ used in the traditional method of virosome preparation. As a result, the bilayers of virosomes prepared by the post-insertion method are expected to be more stable and therefore better to sustain transbilayer gradients, which may allow remote loading of these virosomes with drug molecules [26].

The use of pH-dependent fusion peptides represents another approach to obtain cytosolic delivery of biotherapeutic molecules with IL. Synthetic peptides derived from viral fusion proteins are expected to be less immunogenic than whole viral fusion proteins as they lack the major antigenic determinants. This will be particularly true when these peptides are entrapped inside liposomes. In addition to reduced immunogenicity, peptides have the advantage that they can be readily synthesized at large scale without the need for laborious purification procedures. Based on previously reported results [27], a dimerized synthetic peptide (diINF-7) resembling the N-terminal fusion domain of the influenza virus hemagglutinin HA2 subunit was used to enhance the endosomal escape of IL-entrapped compounds after endocytosis **(Chapter 5)**. Liposome-encapsulated diINF-7 peptides were able to induce release of co-encapsulated calcein from liposomes upon exposure to pH 5.2. Likely, due to the hydrophobic nature of the peptide, part of the encapsulated peptide is associated with the bilayer, which has induced the release of calcein upon membrane destabilization at low pH. A more relevant observation was that liposome-encapsulated diINF-7 peptides were able to induce release of calcein encapsulated in another set of liposomes (devoid of peptide) at low pH. This suggests that the encapsulated diINF-7 peptides destabilized the bilayers such that they facilitated their own release. Subsequently, released peptides were able to destabilize the separate calcein-containing liposomes, resulting in the observed calcein leakage. In line with these results, co-encapsulation of the fusion peptide together with diphtheria toxin A chain into immunoliposomes resulted in specific cytotoxicity towards OVCAR-3 cells whereas DTA-containing IL without co-encapsulated fusion peptides were negligibly toxic. As DTA –which lacks the cell binding domain and membrane-translocation domain– is only toxic to cells when delivered into the cytosol, the results indicate that co-encapsulation of diINF-7 into IL can mediate endosomal escape of DTA after receptor-mediated endocytosis. In conclusion, the results in chapter 5 clearly indicate that co-encapsulation of the diINF-7 fusion peptide is a good strategy to obtain cytosolic delivery of drug molecules from internalized immunoliposomes.

CELL-SPECIFIC GENE DELIVERY

Chapter 6 demonstrates that cell-specific gene delivery to ovarian carcinoma cells can be obtained by coating p(DMAEMA)-based polyplexes with an anionic lipid layer bearing conjugated antibody fragments. As the lipid coat around these so-called lipopolyplexes (LPP) efficiently shields the positive charge of polyplexes, the predominant electrostatic interaction with cell membranes, which is unwanted for cell type-specific gene delivery, could be avoided. As LPP without antibody did not show transfection, it can be concluded that the presence of a targeting ligand on the immunolipopolyplexes (ILPP) is essential. In addition, the lipid coat around the LPP provided protection of the polyplexes against destabilization by polyanions such as poly(aspartic acid) and hyaluronic acid. This is expected to be essential for *in vivo* application of LPP as naturally occurring polyanions have been shown to have detrimental effects on plain polyplexes [28]. Preliminary *in vivo* testing of the ILPP yielded results, which are in good agreement with this expectation.

The transfection efficiency of ILPP is relatively low compared to plain polyplexes under *in vitro* transfection conditions. This can be ascribed to less efficient cellular uptake of ILPP via receptor-mediated endocytosis compared to adsorption-mediated cellular uptake of plain polyplexes. Flow cytometry analysis of fluorescently labeled polyplexes and ILPP has shown that cellular association of polyplexes was much higher than ILPP. Moreover, the presence of the lipid-coat around ILPP may hamper the release of DNA into the cytosol and subsequently the nucleus of target cells. It is not well understood how DNA from p(DMAEMA)-based polyplexes can escape from the endocytic vesicles after cellular uptake. *In vitro* experiments have demonstrated that neither free p(DMAEMA) polymers nor p(DMAEMA)-based polyplexes were able to induce erythrocyte hemolysis at low pH, indicating that polymer-induced endosomal membrane disruption probably does not occur [29]. For another polymer used in non-viral gene delivery, poly(ethyleneimine) (PEI), a proton sponge mechanism has been proposed: PEI buffers the acidification in the endosomal/lysosomal vesicles resulting in osmotic swelling and subsequent burst of endosomes/lysosomes [30]. In analogy with PEI, the proton sponge hypothesis may also hold for p(DMAEMA)-based polyplexes, although no experimental evidence is available. Anyway, intracellular release of the DNA from LPP is required either in the endocytic compartment or after arrival in the cytosol. Disassembly of the lipid coat of LPP upon arrival in the acidic endosomal compartment may be improved by incorporating non-bilayer forming lipids such as dioleoylphosphatidylethanolamine (DOPE) into the lipid coat. Alternatively, synthetic fusion peptides such as those discussed in chapter 5 may be co-encapsulated into the LPP to obtain low-pH induced membrane destabilization. Clearly, additional research is needed to identify and improve the rate-limiting step in non-viral gene delivery with LPP.

CONCLUSION

In this thesis, a Trojan horse strategy with antibody-targeted liposomes has been followed to obtain cytosolic delivery of biotherapeutics to tumor cells *in vitro*. This strategy involves targeting of immunoliposomes to specific receptors on tumor cells that result in receptor-mediated uptake of the immunoliposomes. Escape of immunoliposome-entrapped biotherapeutics from within the endocytic compartment was realized by incorporating either influenza virus hemagglutinin proteins or synthetic dimeric fusion peptides resembling the fusion peptide domain of influenza virus HA into the immunoliposomes/virosomes. These molecules have membrane destabilizing and fusion activity at low pH allowing passage of liposome-entrapped drug over the endosomal membrane into the cytosol. Via the same cellular uptake route cationic polymer-condensed DNA could be specifically delivered into ovarian carcinoma cells by encapsulating polyplexes into antibody-targeted liposomes. The results demonstrate *in vitro* that liposomal delivery of biotherapeutics into the cytosol of target cells via the route of receptor-mediated endocytosis and subsequent endosomal escape is feasible and provide exciting new avenues to be explored in the future.

REFERENCE LIST

1 Bloemen, P.G.M., Henricks, P.A.J., van Bloois, L., van den Tweel, M.C., Bloem, A.C., Nijkamp, F.P., Crommelin, D.J.A., and Storm, G. (1995): **Adhesion molecules: a new target for immunoliposome-mediated drug delivery**. *FEBS LETT.*, 357:140-144.

2 Okamoto, Y., Tsurunaga, T., and Ueki, M. (1999): **Serum soluble ICAM-1 levels in the patients with cervical cancer**. *Acta Obstet.Gynecol.Scand.*, 78:60-65.

3 Regidor, P.A., Callies, R., Regidor, M., and Schindler, A.E. (1998): **Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera**. *Eur.J.Gynaecol.Oncol.*, 19:377-383.

4 Maurer, C.A., Friess, H., Kretschmann, B., Wildi, S., Muller, C., Graber, H., Schilling, M., and Buchler, M.W. (1998): **Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer**. *Int.J.Cancer*, 79:76-81.

5 Cook, G., Dumbar, M., and Franklin, I.M. (1997): **The role of adhesion molecules in multiple myeloma**. *Acta Haematol.*, 97:81-89.

6 Agrez, M.V. (1996): **Cell adhesion molecules and colon cancer**. *Aust.N.Z.J.Surg.*, 66:791-798.

7 Balzar, M., Briaire-de, B., I, Rees-Bakker, H.A., Prins, F.A., Helfrich, W., de Leij, L., Riethmuller, G., Alberti, S., Warnaar, S.O., Fleuren, G.J., and Litvinov, S.V. (2001): **Epidermal growth factor-like repeats mediate lateral and reciprocal interactions of Ep-CAM molecules in homophilic adhesions**. *Mol.Cell Biol.*, 21:2570-2580.

8 Balzar, M., Winter, M.J., de Boer, C.J., and Litvinov, S.V. (1999): **The biology of the 17-1A antigen (Ep-CAM)**. *J.Mol.Med.*, 77:699-712.

9 Litvinov, S.V., Bakker, H.A., Gourevitch, M.M., Velders, M.P., and Warnaar, S.O. (1994): **Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion**. *Cell Adhes.Commun.*, 2:417-428.

10 Litvinov, S.V., Velders, M.P., Bakker, H.A., Fleuren, G.J., and Warnaar, S.O. (1994): **Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule**. *J.Cell Biol.*, 125:437-446.

11 Davies, D.E. and Chamberlin, S.G. (1996): **Targeting the epidermal growth factor receptor for therapy of carcinomas**. *Biochem.Pharmacol.*, 51:1101-1110.

12 Lango, M.N., Shin, D.M., and Grandis, J.R. (2001): **Targeting growth factor receptors: integration of novel therapeutics in the management of head and neck cancer**. *Curr.Opin.Oncol.*, 13:168-175.

13 Wells, A. (1999): **EGF receptor**. *Int.J.Biochem.Cell Biol.*, 31:637-643.

14 Chandler, L.A., Sosnowski, B.A., McDonald, J.R., Price, J.E., Aukerman, S.L., Baird, A., Pierce, G.F., and Houston, L.L. (1998): **Targeting tumor cells via EGF receptors: selective toxicity of an HBEGF- toxin fusion protein**. *Int.J.Cancer*, 78:106-111.

15 Kikuchi, A., Sugaya, S., Ueda, H., Tanaka, K., Aramaki, Y., Hara, T., Arima, H., Tsuchiya, S., and Fuwa, T. (1996): **Efficient gene transfer to EGF receptor overexpressing cancer cells by means of EGF-labeled cationic liposomes**. *Biochem.Biophys.Res.Commun.*, 227:666-671.

16 Mendelsohn, J. (1997): **Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy**. *Clin.Cancer Res.*, 3:2703-2707.

17 Heitner, T., Moor, A., Garrison, J.L., Marks, C., Hasan, T., and Marks, J.D. (2001): **Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library**. *J.Immunol.Methods*, 248:17-30.

18 Poul, M.A., Becerril, B., Nielsen, U.B., Morisson, P., and Marks, J.D. (2000): **Selection of tumor-specific internalizing human antibodies from phage libraries**. *J.Mol.Biol.*, 301:1149-1161.

19 Ishbaeva, R.I., Guliako, A.A., Simonov, A.N., Kleppish, I., and Makhov, A.M. (1989): **Enhanced immunogenicity of influenza virus hemagglutinin when incorporated into liposomes**. *Vopr.Virusol.*, 34:237-240.

20 Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G., and Lerner, R.A. (1982): **Immunogenic structure of the influenza virus hemagglutinin**. *Cell*, 28:477-487.

21 Romanczuk, H., Galer, C.E., Zabner, J., Barsomian, G., Wadsworth, S.C., and O'Riordan, C.R. (1999): **Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice**. *Hum.Gene Ther.*, 10:2615-2626.

22 O'Riordan, C.R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S.C., Smith, A.E., and Francis, G.E. (1999): **PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*.** *Hum.Gene Ther.*, 10:1349-1358.

23 Arkema, A., Huckriede, A., Schoen, P., Wilschut, J., and Daemen, T. (2000): **Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes**. *Vaccine*, 18:1327-1333.

24 Bron, R., Ortiz, A., and Wilschut, J. (1994): **Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza virus envelopes (virosomes)**. *Biochemistry* , 33:9110-9117.

25 Stegmann, T., Morselt, H.W., Booy, F.P., van Breemen, J.F., Scherphof, G., and Wilschut, J. (1987): **Functional reconstitution of influenza virus envelopes**. *EMBO J.*, 6:2651-2659.

26 Haran, G., Cohen, R., Bar, L.K., and Barenholz, Y. (1993): **Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases**. *Biochim.Biophys.Acta*, 1151:201-215.

27 Wagner, E. (1999): **Application of membrane-active peptides for nonviral gene delivery**. *Adv.Drug.Del.Rev.*, 38:279-289.

28 van de Wetering, P., Schuurmans-Nieuwenbroek, N.M.E., Hennink, W.E., and Storm, G. (1999): **Comparative transfection studies of human ovarian carcinoma cells *in vitro*, *ex vivo* and *in vivo* with poly(2-(dimethylamino)ethylmethacrylate)-based polyplexes**. *J.Gen.Med.*, 1:156-165.

29 van de Wetering, P. **Gene delivery with cationic polymers**. 1999. Thesis. Department of pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands.

30 Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P. (1995): **A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine**. *Proc.Natl.Acad.Sci.U.S.A.*, 92:7297-7301.

NL

NEDERLANDSE SAMENVATTING VOOR LEKEN

"Leuk zo'n boekje, maar wat heeft die jongen nou eigenlijk gedaan?"

HET INFLUENZA VIRUS: VAN ZIEKTEVERWEKKER TOT FARMACON TRANSPORTEUR

Het influenza virus, de verwekker van de griep, teistert de mensheid al eeuwenlang. Tijdens de grote 'Spaanse griep' pandemie van 1918 waren naar schatting wereldwijd 1 miljard mensen besmet met het virus en stierven er binnen een periode van een jaar meer dan 20 miljoen mensen aan influenza. Ter vergelijking: het aantal slachtoffers van de Eerste Wereldoorlog van 1914-1918 is geschat op 7 miljoen. Ook tegenwoordig veroorzaken influenza virussen nog bijna jaarlijks epidemieën, ten gevolge waarvan aanzienlijk wat mensen overlijden.
Veel onderzoek is gedaan naar de manier waarop het influenza virus in staat is cellen van de gastheer binnen te dringen en te infecteren. Dit proces is uitgebeeld in Figuur 1. Het virus is

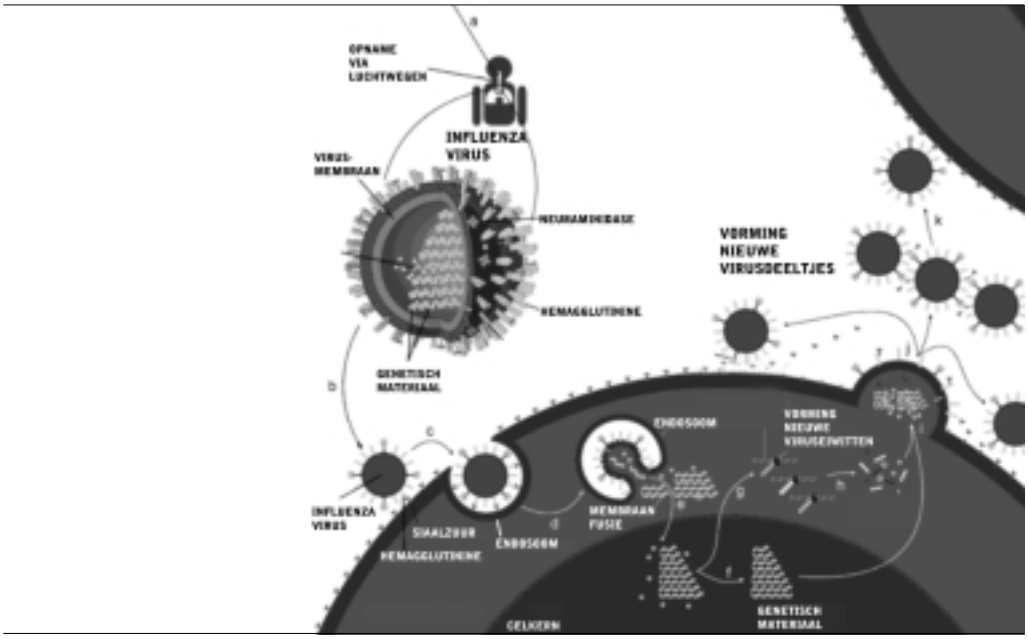

Figure 1 — Schematische weergave van de infectiecyclus van het humane influenza virus. Virusdeeltjes komen via kleine waterdruppeltjes in de luchtwegen terecht **(a)** waar ze kunnen binden aan het oppervlak van cellen die de luchtwegen bekleden **(b)**. Na binding aan siaalzuur worden de virusdeeltjes opgenomen in kleine blaasjes die afsnoeren van de celmembraan, de zogenaamde endosomen **(c)**. Doordat het milieu in de endosomen langzaam verzuurt, wordt het eiwit hemagglutinine van het virus geactiveerd waardoor het het virusmembraan laat fuseren met het membraan van het endosoom **(d)**. Het genetische materiaal van het virus komt nu vrij in het cytoplasma van de cel **(e)**. Via een ingewikkeld proces wordt de cel nu gedwongen aan de hand van deze genetische code nieuwe virale eiwitten te gaan aanmaken **(f,g,h)**. Deze virale eiwitten worden getransporteerd naar de celmembraan **(i)**, waar nieuwe virusdeeltjes afsnoeren van de celmembraan **(j)**. Deze nieuwe virusdeeltjes kunnen vervolgens weer andere cellen infecteren **(k)**.

omgeven door een membraan die is opgebouwd uit lipiden (vetten) waarin eiwitten zijn verankerd. Deze langwerpige eiwitten spelen een belangrijke rol bij het binnendringen van het virusdeeltje in het hart van de gastheercel, het cytoplasma. Eén van deze eiwitten, het hemagglutinine, zorgt ervoor dat het virusdeeltje na verspreiding via kleine waterdruppeltjes in lucht en na inademing kan hechten aan cellen die de luchtwegen bekleden (epitheelcellen). Het hemagglutinine bindt aan suikerstructuren (siaalzuren) die voorkomen op het oppervlak van epitheelcellen. Binding van virusdeeltjes aan deze suikerstructuren op de celmembraan resulteert in instulping van de celmembraan en vervolgens afsnoering van een membraan-omgeven blaasje met de virusdeeltjes daarin opgesloten. De zuurgraad binnenin deze blaasjes, de zogenaamde endosomen, neemt toe naarmate de blaasjes verder de cel in getransporteerd worden. Het zure milieu binnenin de endosomen zorgt ervoor dat het hemagglutinine van het influenza virus van vorm verandert en daardoor in staat is het virusmembraan met het endosomale membraan te laten versmelten (fuseren). Door deze membraanfusie komt het genetische materiaal (blauwdruk) van het virus vrij in het cytoplasma van de gastheercel. Via een ingewikkeld proces wordt nu de cel gedwongen de genetische code van het virus af te lezen en aan de hand van deze code viruseiwitten te gaan aanmaken. De viruseiwitten worden naar de celmembraan getransporteerd wat uiteindelijk resulteert in de afsnoering van nieuwe met membraan omgeven influenza virusdeeltjes, die vervolgens weer andere cellen kunnen infecteren. Een cel die op deze manier belaagd wordt is niet meer in staat zijn eigen functie naar behoren te vervullen en kan daardoor zelfs afsterven. Deze celdood, samen met de uitwerkingen van het afweermechanisme van de gastheer op de virusinfectie zorgen ervoor dat de geïnfecteerde persoon ziek wordt.

De manier waarop het influenza virus haar genetische materiaal met grote efficiëntie aflevert aan epitheelcellen van de gastheer kan als voorbeeld dienen voor de gerichte afgifte van geneeskrachtige stoffen (farmaca) aan kankercellen met behulp van zogenaamde dragersystemen. Het gebruik van dragersystemen voor de afgifte van farmaca in het cytoplasma van kankercellen is vaak een noodzaak: veel farmaca, met name de nieuwe generatie van door de biotechnologische industrie geproduceerde stoffen zoals eiwitten en DNA, kunnen niet uit zichzelf de celmembraan passeren om zo het cytoplasma of andere compartimenten in de cel te bereiken waar ze hun werking hebben. Deze farmaca zijn daarom afhankelijk van dragersystemen die, net als het influenza virus, in staat zijn hun inhoud te legen in het cytoplasma van een bepaalde populatie cellen (in dit geval kankercellen). Een veel gebruikt dragersysteem is het liposoom.

Liposomen zijn minuscuul kleine blaasjes gevuld met water die omgeven zijn door een membraan bestaande uit een dubbele laag (bilaag) van fosfolipiden (Figuur 2). Fosfolipiden zijn moleculen bestaande uit een hydrofiel gedeelte dat zich graag in water bevindt en een hydrofoob gedeelte dat letterlijk vertaald 'bang is voor water'. Wanneer zulke fosfolipiden in water worden gebracht zullen deze moleculen zich zo rangschikken dat de hydrofobe gedeeltes zo min mogelijk in aanraking zullen komen met water: ze vormen bilagen waarin de hydrofobe gedeeltes van de fosfolipiden naar binnen steken. Aangezien de randen van deze bilagen ook niet graag blootgesteld worden aan water zullen deze membranen van fosfolipide bilagen blaasjes vormen

en zie hier, het liposoom is gevormd. Liposomen bevatten een waterige ruimte die afgesloten is van de grote ruimte waarin zij zich bevinden. In deze waterige kern kunnen wateroplosbare farmaca ingesloten worden terwijl vetoplosbare farmaca goed in de fosfolipide bilaag ingebouwd kunnen worden. De grootte van liposomen kan variëren van 40 nanometer (er passen dan 25000 liposomen in rij op één millimeter) tot enkele micrometers (500-1000 liposomen op één millimeter). Hoewel dit erg klein lijkt, zijn liposomen vergeleken met eiwitten relatief groot waardoor ze niet in staat zijn na inspuiting de bloedbaan te verlaten. Echter, op plaatsen waar de bloedvaatwand van nature gaten vertoont of beschadigd is, zijn kleine liposomen (<150 nanometer) in staat uit de bloedbaan te treden en het betreffende weefsel binnen te dringen. Aangezien tumoren vaak van bloed voorzien worden door vaten die vele gaten vertonen, kunnen deze liposomen daar uit de bloedbaan treden en ophopen in het tumorbevattende weefsel. Deze eigenschap van liposomen wordt vaak gebruikt om celdodende stoffen selectief af te leveren aan tumoren. Door bepaalde moleculen (antilichamen) die in staat zijn zeer specifieke structuren (antigenen) op het oppervlak van kankercellen te herkennen te koppelen aan de liposomen (Figuur 2) zijn deze antilichaamgestuurde liposomen, ook wel immunoliposomen genoemd, in staat specifiek te binden aan kankercellen. De antilichaam-antigeen binding is zeer specifiek, net zoals maar één type sleutel past op een slot. Op deze manier kan een grote hoeveelheid farmacon

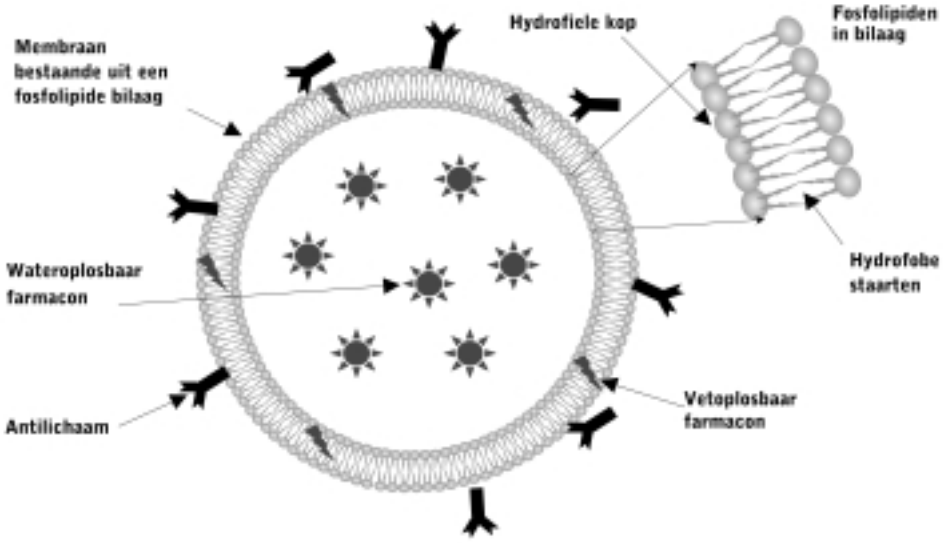

Figure 2 — Schematische weergave van een liposoom als dragersysteem van farmaca. De membraan van liposomen bestaan uit fosfolipiden die een bilaag vormen doordat ze hun hydrofiele kop naar de waterfase toe richten. De lipide membraan van liposomen omsluit een waterig compartiment waarin water-oplosbare farmaca ingesloten kunnen worden. Tevens kunnen vetoplosbare farmaca in de lipide membraan ingebouwd worden. Aan de bilaag kunnen vervolgens antilichamen gekoppeld worden die bepaalde receptoren op kankercellen herkennen en hieraan kunnen binden.

ingesloten in liposomen in de buurt van kankercellen worden gebracht. Een overzicht van de literatuur die het gebruik van immunoliposomen voor de specifieke afgifte van celdodende stoffen aan kankercellen beschrijft, is gegeven in **hoofdstuk 1** van dit proefschrift.
Het in de buurt brengen van farmaca bij kankercellen wil echter nog niet zeggen dat deze farmaca daadwerkelijk beschikbaar zijn voor de kankercellen. Ter verduidelijking: het bezorgen van de post door de postbode wil ook niet zeggen dat de geadresseerde de post meteen kan lezen. Hiervoor moet de post eerst door de geadresseerde vanuit de postbus of brievenbus binnengehaald worden, en vervolgens moet de envelop waarin de brief zich bevindt geopend worden, voordat de informatie in de brief door de geadresseerde gelezen kan worden. Net zoals de post moeten immunoliposomen met ingesloten farmaca, die met behulp van gekoppelde antilichamen 'geadresseerd' zijn aan kankercellen voor efficiënte bezorging aan het juiste adres, na binding door de desbetreffende kankercellen opgenomen worden.
Of een kankercel immunoliposomen opneemt is afhankelijk van het type kankercel en de receptor waaraan het antilichaam van het liposoom bindt. Aangezien bij de behandeling van kanker het 'farmacon' vaak bestaat uit een celdodende stof, wil men immunoliposomen die deze celdodende stof bevatten alleen laten binden aan kankercellen en niet aan gezonde cellen. Vandaar dat de keuze van receptoren op het oppervlak van kankercellen voor binding van immunoliposomen beperkt blijft tot die receptoren die uitsluitend of voornamelijk vóórkomen op het oppervlak van kankercellen. Tevens moeten de receptoren in staat zijn de gebonden immunoliposomen op te nemen. In **hoofdstuk 2** en de **appendix bij hoofdstuk 2** wordt gekeken naar 3 verschillende receptoren op kankercellen van de eierstokken en de long die mogelijkerwijs in staat zijn immunoliposomen naar binnen te sluizen. Aangetoond wordt dat een receptor die normaalgesproken groeihormonen de cel binnen brengt uitermate geschikt is om immunoliposomen de cel in te transporteren.
Opname van farmacon bevattende liposomen door kankercellen alleen is echter niet genoeg: na opname in de cel moet het liposoom zich 'openen' om zo het farmacon op de juiste plaats in de cel (in veel gevallen het cytoplasma) af te leveren, waar het zijn werking kan uitoefenen. Helaas is vaak gebleken dat cellen de immunoliposomen gebonden op het oppervlak van de cel wel binnenhalen in endosoomblaasjes, maar vervolgens de liposomen met inhoud doorsluizen naar andere blaasjes in de cel die stoffen (enzymen) bevatten welke in staat zijn de liposomen en het ingesloten farmacon af te breken. Dit is natuurlijk ongewenst omdat zo het ingesloten farmacon niet zijn werk kan uitoefenen. Terugkomend op het influenza virus zou het inbouwen van het virale eiwit hemagglutinine in farmacon bevattende immunoliposomen een oplossing zijn om de afbraak van het farmacon na opname van immunoliposomen in kankercellen te voorkomen.
Hoofdstuk 3 laat zien dat het mogelijk is liposomale dragersystemen te maken die zowel gekoppelde antilichamen (nodig voor binding en opname van de liposomen) als functionele hemagglutinine eiwitten (nodig voor membraanfusie na opname van de liposomen) bevatten. Deze zogenaamde 'fusogene' immunoliposomen zijn gemaakt door membranen van het influenza virus met daarin de hemagglutinine eiwitten op te lossen met zeep. Hierdoor wordt het virus

geïnactiveerd en kan het genetische materiaal van het influenza virus verwijderd worden zodat het zichzelf niet meer kan vermenigvuldigen. Aan de zeepoplossing, waarin zich de virusmembranen en hemagglutinine eiwitten bevinden, worden antilichamen die gekoppeld zijn aan fosfolipiden toegevoegd. Door op een speciale manier het zeep langzaam te verwijderen vormen zich automatisch liposomen omdat de fosfolipiden zich nu weer gaan rangschikken in bilagen. Deze liposomen bevatten zowel het hemagglutinine membraaneiwit als de antilichamen. Omdat deze liposomen virale eiwitten bevatten worden ze ook wel virosomen genoemd. Aangezien de methode van bereiding van het virosoom met behulp van zeep niet toelaat dat grote hoeveelheden farmaca ingebouwd kunnen worden in de waterige kern van de virosomen is in **hoofdstuk 4** gekeken naar een andere manier van virosoombereiding. Hierbij wordt getracht het eiwit hemagglutinine in te bouwen in voorgevormde liposomen waarin grote hoeveelheden farmaca zijn ingesloten. Ons onderzoek toont aan dat de hemagglutinine eiwitten inderdaad ingebouwd kunnen worden in de voorgevormde liposomen. De ingebouwde hemagglutinine eiwitten zijn tevens functioneel: ze zijn in staat in een zuur milieu de membranen van virosomen te laten fuseren met celmembranen. Echter, tijdens het inbrengen van de hemagglutinine eiwitten in voorgevormde liposomen lekt de ingesloten wateroplosbare stof (in dit geval was dit geen farmacon maar een fluorescerend stofje wat makkelijk te detecteren valt), uit de liposomen. Ondanks dit ongewenste effect zijn de gevormde liposomen fusieactief en kunnen daarom goed gebruikt worden voor de afgifte van bijvoorbeeld vetoplosbare farmaca (die wel in de liposomen blijven) aan het cytoplasma van tumorcellen.

Door het inbrengen van hele hemagglutinine eiwitten in liposomen is men eigenlijk bezig kunstmatig een virus na te bouwen met alle negatieve gevolgen van dien. Virussen worden namelijk in meer of mindere mate herkend door het afweersysteem van de gastheer. Dit zogenaamde immuunsysteem is in staat vreemde indringers (bijvoorbeeld bacteriën, virussen en vreemde cellen) in het lichaam te herkennen en deze te vernietigen. Zo worden ook de hemagglutinine eiwitten van het influenza virus herkend door het immuunsysteem waardoor het virus na verloop van tijd door het immuunsysteem kan worden vernietigd. Aangezien de dragersystemen ontwikkeld in **hoofdstuk 3 en 4** hemagglutinine eiwitten bevatten, zullen ook deze dragersystemen na inspuiting in de bloedbaan uiteindelijk herkend worden door het immuunsysteem en afgebroken worden. Vandaar dat in **hoofdstuk 5** gebruik is gemaakt van een klein gedeelte van het hemagglutinine eiwit waarvan bekend is dat het membraanfusie in zuur milieu kan induceren, maar waarvan verwacht wordt dat het veel minder goed (of helemaal niet) door het immuunsysteem van de mens herkend zal worden. Deze zogenaamde influenza fusiepeptiden zijn ingesloten in immunoliposomen samen met een celdodende stof (DTA). DTA moet in het cytoplasma van de kankercellen worden afgegeven om werkzaam te zijn. Toevoeging van DTA aan de buitenkant van de cel heeft geen enkel effect. Na binding en opname van de immunoliposomen aan eierstokkanker cellen, komen de immunoliposomen in de endosoom-blaasjes van de cel terecht. In het zure milieu van de endosoomblaasjes worden de ingesloten fusiepeptiden geactiveerd waardoor de membranen van de opgenomen liposomen fuseren met

de membranen van de endosomen om zo de DTA moleculen af te geven in het cytoplasma van de kankercellen. Aangetoond is dat alleen immunoliposomen die zowel de fusiepeptiden als de celdodende stof bevatten in staat zijn de kankercellen te doden, terwijl immunoliposomen die alleen de celdodende stof bevatten daartoe niet in staat zijn.

Het laatste hoofdstuk van dit proefschrift, **hoofdstuk 6**, gaat over de afgifte van genetisch materiaal aan kankercellen. Informatie over hoe de cel een eiwit moet bouwen (de blauwdrukken) is opgeslagen in genen. Ieder gen codeert voor een ander eiwit. In plaats van het inbrengen van geneeskrachtige eiwitten in zieke cellen om zo de cel te genezen, is het ook mogelijk het gen coderend voor deze geneeskrachtige eiwitten in te brengen. Het voordeel hiervan is dat de zieke cel die dit gen opneemt zelf in staat wordt gesteld over een lange periode het geneeskrachtige eiwit aan te maken. Wanneer men genen (DNA) met therapeutische werking in zieke cellen brengt is men bezig met gentherapie. Net als vele eiwitten is DNA door zijn omvang en lading zelf niet in staat de zieke cellen binnen te dringen. Tevens is DNA buiten en in de cel erg gevoelig voor afbraak door speciale enzymen. Het DNA moet dus beschermd en in staat gesteld worden cellen binnen te dringen. In ons laboratorium is een polymeer ontwikkeld dat in staat is het DNA te verkleinen tot een bolletje. Een polymeer is een keten van repeterende moleculen. Dit is te vergelijken met een kralenketting die is opgebouwd uit dezelfde kralen (parelketting). Het DNA vormt een complex met het polymeer aangezien de negatieve ladingen van het DNA aangetrokken worden door de positieve ladingen van het polymeer, waardoor kleine positief geladen deeltjes ontstaan, de zogenaamde polyplexen. Het DNA gecomplexeerd in deze polyplexen is goed beschermd tegen afbraak door enzymen. Tevens hechten deze polyplexen goed aan negatief geladen celmembranen doordat ze positief geladen zijn. Door deze hechting worden de polyplexen opgenomen door de cellen. Vervolgens komt het DNA vrij in de cel op een manier die niet geheel bekend is. Dit DNA kan door de zieke cel afgelezen worden om zo het therapeutische eiwit aan te maken. Het probleem van deze opname is echter dat het niet specifiek is. Alle cellen hebben negatief geladen celmembranen waardoor de polyplexen door vele cellen opgenomen zullen worden. Dit is ongewenst omdat alleen de zieke cellen de genen dienen op te nemen. Vandaar dat wij in **hoofdstuk 6** deze polyplexen hebben ingebouwd in immunoliposomen. Door polyplexen in te bouwen wordt de positieve lading van de polyplexen afgeschermd zodat ze niet meer aspecifiek aan de celmembranen kunnen 'kleven'. Vervolgens kan, met behulp van specifieke antilichamen op het oppervlak van deze immunoliposomen, specifieke binding aan kankercellen van de eierstokken bewerkstelligd worden. Aangetoond is dat op deze manier de genen gecomplexeerd in de polyplexen en ingebouwd in immunoliposomen aan kankercellen van de eierstokken worden afgegeven en niet aan andere cellen. Tevens zijn de polyplexen ingebouwd in liposomen (ook wel lipopolyplexen genoemd) beter bestand tegen ongewenste destabilisatie door negatief geladen eiwitten.

Concluderend kunnen we zeggen dat immunoliposomen met ingebouwde virale fusie-eiwitten of fusiepeptiden kunnen dienen als dragersystemen voor de bezorging van biotechnologische eiwitten en DNA in het cytoplasma van kankercellen. Deze cytoplasmatische dragersystemen zijn

echter alleen nog getest op losse cellen in kweekbakjes in het laboratorium. Toekomstig onderzoek zal moeten uitwijzen of deze dragersystemen ook hun werk kunnen doen in proefdieren en in een nog later stadium de mens.


bedankt

CURRICULUM VITAE

Enrico Mastrobattista werd op 1 maart 1973 geboren te Giesbeek. In 1991 werd het Atheneum B diploma behaald aan 'Het Rhedens Lyceum' te Rozendaal. In datzelfde jaar werd begonnen aan de studie Medische Biologie aan de faculteit Geneeskunde van de Universiteit Utrecht. Als onderdeel van deze studie werd onder begeleiding van prof. dr. J.G. van de Winkel voor 9 maanden een stage gevolgd bij de toenmalige vakgroep Experimentele Immunologie verbonden aan het Academisch Ziekenhuis te Utrecht. Tijdens deze stage werd onderzoek verricht naar de karakterisering van FcgRI (één van de receptoren voor immunoglobuline G) in getransfecteerde cellijnen. Zijn tweede onderzoeksstage bij "l'Istituto Nazionale Per La Ricerca Sul Cancro" te Genua onder begeleiding van prof.dr. S. Ferrini omvatte het genereren van zogenaamde T-bodies. Dit zijn T-cellen met een chimere T-cel receptor welke een antilichaam domein bevat voor MHC-onafhankelijke herkenning van tumorcellen. In Augustus 1996 volbracht hij de studie Medische Biologie en ontving hij de bul met het judicium "met genoegen".
Van 1997 tot 2001 was hij werkzaam als assistent in opleiding bij de disciplinegroep Biofarmacie en Farmaceutische Technologie van het Utrecht Institute for Pharmaceutical Sciences (UIPS). Het onderzoek, uitgevoerd onder begeleiding van prof. dr. G. Storm en prof. dr. D.J.A. Crommelin, had als doel het ontwikkelen van liposomale dragersystemen voor de specifieke afgifte van farmaca in het cytoplasma van tumorcellen. Tijdens het promotieonderzoek werd nauw samengewerkt met de vakgroep Medische Microbiologie, sectie moleculaire virologie te Groningen, onder begeleiding van prof. dr. J.C. Wilschut, waar hij geleerd heeft influenza virosomen te bereiden. Tevens heeft hij tijdens zijn promotieonderzoek een onderzoeksvoorstel geschreven met als titel "Liposomal compartmentalisation for *in vitro* selection and evolution of proteins."
Sinds 1 juni 2001 is hij werkzaam als junior docent/onderzoeker bij de disciplinegroep Biofarmacie en Farmaceutische Technologie van het UIPS.

PUBLICATIONS

FULL PAPERS

E. Mastrobattista, G. Storm, L. van Bloois, R. Reszka, P. G. M. Bloemen, D. J. A. Crommelin, and P. A. J. Henricks. Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells. Biochim.Biophys.Acta 1419:353-363, 1999.

E. Mastrobattista, G. A. Koning, and G. Storm. Immunoliposomes for the targeted delivery of antitumor drugs. Adv.Drug.Del.Rev. 40 (1-2):103-127, 1999.

E. Mastrobattista, R.H.G. Kapel, M.H. Eggenhuisen, P.J.M. Roholl, D.J.A. Crommelin, W.E. Hennink and G. Storm. Lipid-coated polyplexes for targeted gene delivery to ovarian carcinoma cells. Cancer Gene Ther. 2001, in press. MS#00-559.

E. Mastrobattista, J.C. Wilschut, P. Schoen, D.J.A. Crommelin, G. Storm. Targeting of influenza virosomes to ovarian carcinoma cells. Submitted.

ABSTRACTS

E. Mastrobattista, P. Schoen, J.C. Wilschut, D.J.A. Crommelin, P.A.J. Henricks, and G. Storm. Development of targeted liposomes for the intracellular delivery: cellular internalisation and introduction of fusogenic properties. J. Liposome Res. 8 (Abstract Issue): 82, 1998.

E. Mastrobattista, R.H.G. Kapel, M.H. Eggenhuisen, D.J.A. Crommelin, W.E. Hennink and G. Storm. Cell-specific gene transfer to cultured ovarian carcinoma cells with polymer-condensed DNA complexes encapsulated in targeted liposomes. J. Liposome Res. 10 (2-3) (Abstract Issue): 252, 2000.

ABBREVIATIONS

BSA	Bovine serum albumin
CD	circular dichroism
CHOL	cholesterol
CLSM	confocal laser scanning microscopy
DiD	1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine perchlorate
diINF-7	dimerized synthetic influenza virus fusion peptide
DNA	deoxyribonucleic acid
DSPE	distearoyl phosphatidylethanolamine
DT	diphtheria toxin
DTA	diphtheria toxin A chain
DTT	dithiothreitol
EGFR	epidermal growth factor receptor
EGP-2	epithelial glycoprotein-2
EPC	egg phosphatidylcholine
EPE	egg phosphatidylethanolamine
EPG	egg phosphatidylglycerol
Fab'	Antigen binding antibody fragment
FCS	fetal calf serum
FIL	fusogenic immunoliposomes
FITC	fluorescein isothiocyanate
FL	fusogenic liposomes
HA	hemagglutinin
HBS	HEPES-buffered salt solution
HEPES	4-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HyAc	hyaluronic acid
i.p.	intraperitoneal
i.v.	intravenous
ICAM-1	intercellular cell adhesion molecule-1
IF	immunofluorescence
IgG	immunoglobulin type G
IL	immunoliposomes
ILPP	immunolipopolyplexes
LM	light microscopy
LPP	lipopolyplexes
mAb	monoclonal antibody

MES	2-[*N*-morpholino]ethanesulfonic acid
MPB-PE	maleimide-4-(p-phenylbutyryl)-phosphatidylethanolamine
MWCO	molecular weight cut-off
NA	neuraminidase
OG	octyl glucoside
ONPG	o-nitrophenyl-b-D-galactopyranoside
p(Asp)	poly(aspartic acid)
p(DMAEMA)	poly(2-dimethylamino)ethylmethacrylate
PBS	phosphate-buffered salt solution
pCMVLacZ	plasmid vector containing the gene encoding for ?-galactosidase under the control of the CMV promoter
pDNA	plasmid DNA
PEG	poly(ethylene glycol)
PHEPC	partially hydrogenated egg phosphatidylcholine
PI-virosomes	Post-insertion virosomes
PL	phospholipid
PyrPC	(1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine)
Rho-PE	L-a-phosphatidylethanolamine–N-(lissamine rhodamine B sulfonyl)
SATA	*N*-succinimidyl-S-acetylthioacetate
SDS-PAGE	sodium dodecyl sulphate –poly(acrylamide) gel elecrophoresis
TL	total lipid
XTT	sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate
$\Delta\varepsilon$	difference in molar absorbance