Targeting influenza virosomes to ovarian carcinoma cells

Enrico Mastrobattista[a,*], Pieter Schoen[b,1], Jan Wilschut[b], Daan J.A. Crommelin[a], Gert Storm[a]

[a] *Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands*

[b] *Department of Medical Microbiology, Molecular Virology Section, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

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Abstract Reconstituted influenza virus envelopes (virosomes) containing the viral hemagglutinin (HA) have attracted attention as delivery vesicles 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. This was achieved by incorporating poly(ethylene glycol) (PEG)-derivatized lipids into the virosome membrane. This PEG layer serves as shield to prevent interaction of HA with ubiquitous sialic acid residues and as spatial anchor for antibody attachment. Coupling of Fab′ fragments of mAb 323/A3 (anti-epithelial glycoprotein-2) to the distal ends of PEG lipids resulted in specific binding of virosomes to OVCAR-3 cells. These antibody-redirected virosomes fused with membranes of OVCAR-3 cells in a pH-dependent fashion. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Influenza virus; Hemagglutinin; Virosome; Membrane fusion; Targeting; Cellular delivery; Poly(ethylene glycol)

1. Introduction

The cytosol of cells is an important but relatively inaccessible compartment for many therapeutic macromolecules. Due to unfavorable physicochemical characteristics, in particular large size, these molecules often show restricted ability to pass through cell membranes. There is a strong need for a carrier that can deliver these often membrane-impermeable macromolecules into the cytosol of target cells. Although liposomes have been widely used for the delivery of therapeutic compounds to target cells, liposome-mediated delivery of membrane-impermeable macromolecular agents into the cytosol remains 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 main destination 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 is present as 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, influenza virosomes have a tropism for sialic acid-bearing cells. Since sialic acid residues are present 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, binding of influenza virosomes to its natural receptor (i.e. sialic acid residues) has to be prevented, and second, specific homing devices (e.g. antibodies) have to be introduced. Despite these modifications fusogenic activity must be preserved. Although binding and fusion 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

*Corresponding author. Fax: (31)-30-2517839.
E-mail address: e.mastrobattista@pharm.uu.nl (E. Mastrobattista).

[1] Current address: HaemoProbe BV, L.J. Zielstraweg 1, 9713 GX Groningen, The Netherlands.

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ends of surface-exposed PEG molecules, antigen-specific binding of influenza virosomes to ovarian carcinoma cells with full retention of fusogenic activity was obtained.

2. Materials and methods

2.1. 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 *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-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, UK). All other chemicals were from the highest grade available.

2.2. 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 BRL, Grand Island, NY, USA) 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).

2.3. 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 μmol of phospholipid was pelleted by ultracentrifugation for 1 h at 100 000×*g* 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 100 000×*g* for 30 min. The amount of pyrPC relative to the total amount of phospholipid was 10%, the amount of PEG-DSPE varied from 0 to 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 h on a shaking device (1400 rpm) at room temperature while protected from light. Subsequently, two batches of 25 mg of fresh Bio-Beads were added to the mixture within a time interval of 30 min while shaking was continued. For targeting purposes, Fab′ fragments of mAb 323/A3 were irreversibly 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 onto a discontinuous sucrose gradient (10–40–50–60%) and centrifuged at 100 000×*g* for 90 min at 4°C. The virosomes sedimented onto the 40% sucrose band were collected and dialyzed overnight against 1 l of HBS at 4°C.

2.4. Preparation of reconstituted membrane vesicles from erythrocytes and ovarian carcinoma cells

Erythrocyte membrane vesicles (erythrocyte ghosts) were prepared according to the method of Steck et al. [23]. Membrane vesicles composed of the reconstituted membranes of OVCAR-3 cells were prepared essentially as described before [24]. A total amount of 1×10^7 OVCAR-3 cells were collected from culture flasks and washed twice with 10 ml of PBS by consecutive centrifugation (200×*g*, 5 min) and resuspension steps. Pelleted cells were resuspended in 5 ml of hypotonic buffer (10 mM Tris–HCl; 1 mM $MgCl_2$; 1 mM KCl; 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.3) and subjected to four freeze/thaw cycles with liquid nitrogen and lukewarm water, respectively. The resulting suspension was vigorously shaken for 5 min and centrifuged at 1000×*g* 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). Pooled supernatants were centrifuged at 100 000×*g* 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 (700×*g*; 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 membrane vesicles were collected and analyzed for size distribution, phospholipid and protein contents.

2.5. 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 membrane vesicles at a final concentration of 50 μM or 200μM 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 a LS50B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) 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.

2.6. Hemagglutination (HA) assay

The hemagglutination assay was carried out essentially as described [25]. 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-well 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% (volume of pelleted erythrocytes) 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 overnight after which the occurrence of agglutination was screened by visual inspection.

2.7. 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 2×10^5 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 Dickinson, Mountain View, CA, USA).

3. Results

3.1. Effect of surface-exposed PEG on sialic acid binding

The influence of the presence of PEG molecules exposed on the surface of virosomes on the capacity to agglutinate human red blood cells was determined with a hemagglutination test [25]. 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. The presence of PEG molecules exposed to the surface of virosomes may inhibit HA-mediated sialic acid binding. Indeed,

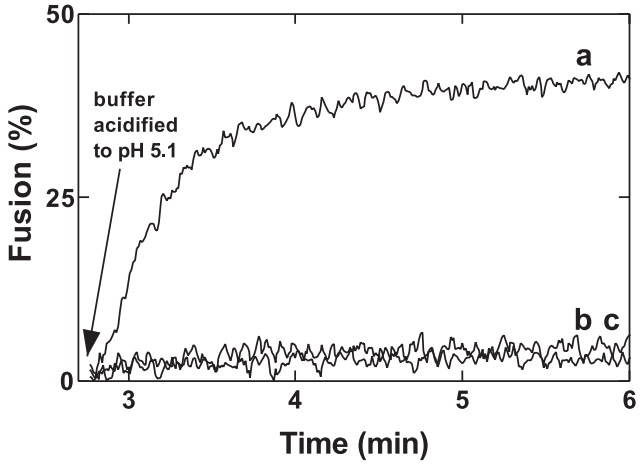

Fig. 1. Effect of surface-exposed PEG on fusion of influenza virosomes with erythrocyte ghosts. Erythrocyte ghosts (50 μM phospholipid) were added to pyrPC-labeled virosomes (2.5 μM phospholipid). At the time point indicated with the arrow, the medium was acidified to pH 5.1. Pyrene excimer fluorescence 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.

binding of influenza virosomes to red blood cells negatively correlated with the molecular weight and density of surface-exposed PEG (Table 1). 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.

3.2. Effect of surface-exposed PEG on membrane fusion

As PEG molecules anchored to the surface of virosomes 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. Inclusion of PEG reduced the degree of lipid mixing between erythrocyte ghosts and PEG virosomes (Fig. 1). At PEG densities that fully block hemagglutination (8 mol% PEG-5000-DSPE and 20 mol% PEG-2000-DSPE, see Table 1), the rate of lipid mixing was drastically reduced compared to virosomes without membrane-coupled PEG.

3.3. 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. Influenza virosomes without PEG bound to both A2780 (EGP-2 negative) and OVCAR-3 (EGP-2 positive) cells (Fig. 2), 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) inhibited the binding to both types of tumor cells. 323/A3-PEG virosomes only bound to OVCAR-3 cells, expressing EGP-2 (to which the conjugated antibody is directed), and not to A2780 cells, which lack this cell surface receptor. Thus, by conjugating Fab′ fragments to the distal ends of PEG chains present on the virosome surface, influenza virosomes could be specifically redirected to OVCAR-3 cells.

3.4. Fusogenic activity of redirected influenza virosomes

To determine whether binding of 323/A3-PEG virosomes via antigen/antibody interaction results in fusion comparable to 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 with reconstituted membranes of OVCAR-3 cells (referred to as OVCAR-3 membrane vesicles) was measured. 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. Therefore, membrane vesicles were prepared from crude membrane extracts of OVCAR-3 cells according to the method of Bergers et al. [24]. These reconstituted cell membranes 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 membrane vesicles upon lowering the pH of the incubation medium to 5.1. As expected, plain virosomes induced lipid mixing with OVCAR-3 membrane vesicles. The extent of lipid mixing was lower than that 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 eryth-

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

Virosome preparation	Dilution	2	4	8	16	32	64	128	256	512	1024	2048	4096
	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-2000	E	+	+	+	+	+	±	−	−	−	−	−	−
13.5% PEG-2000	F	±	±	−	−	−	−	−	−	−	−	−	−
20mol% PEG-2000	G	−	−	−	−	−	−	−	−	−	−	−	−
No virosomes	H	−	−	−	−	−	−	−	−	−	−	−	−

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 h at room temperature to allow sedimentation of non-agglutinated red blood cells, the occurrence of agglutination was visually scored. + indicate wells positive for agglutination; − indicate wells negative for agglutination and ± indicate wells where agglutination is observed together with sedimentation of red blood cells.

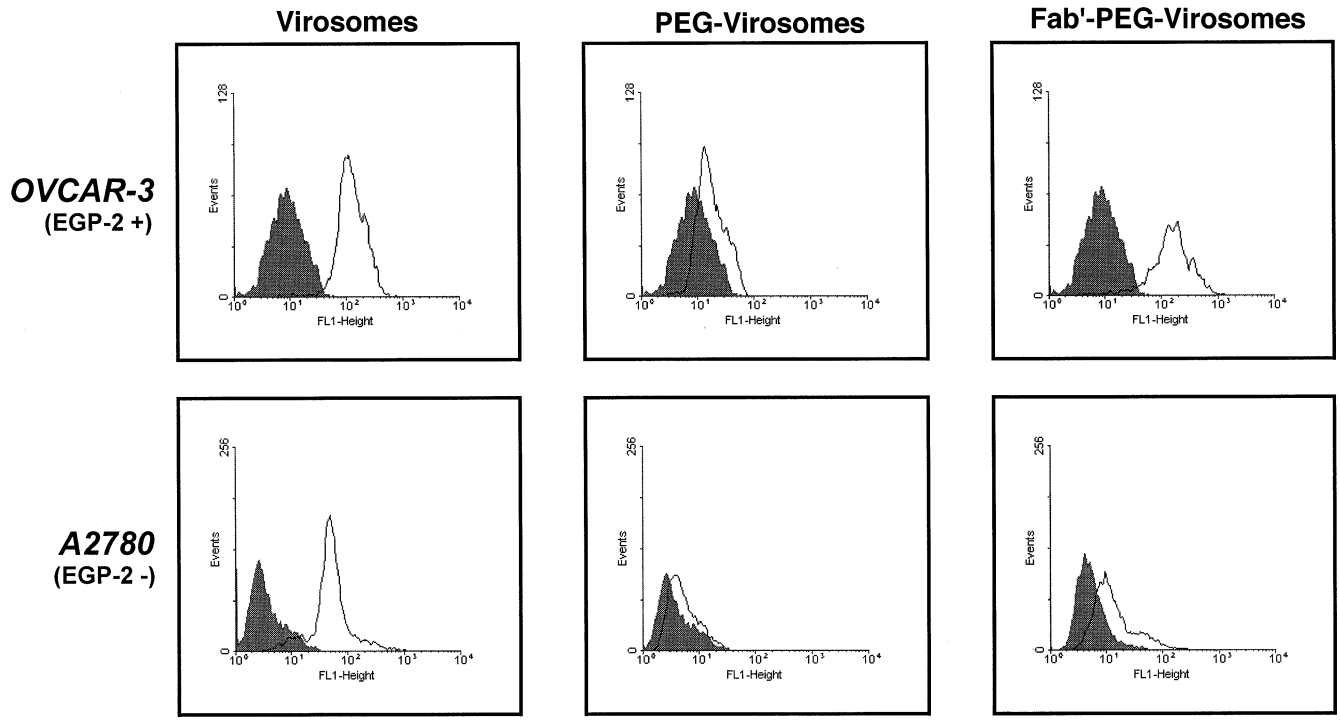

Fig. 2. Virosome binding to OVCAR-3 cells (expressing high levels of the target receptor EGP-2) and A2780 cells (no EGP-2 expression). Virosomes, PEG-2000 virosomes (prepared with 20 mol% PEG-2000-DSPE) and 323/A3 Fab′-PEG-2000 virosomes (prepared with 20% PEG-2000-DSPE), all labeled with 0.1% NBD-PE, were incubated with either OVCAR-3 cells or A2780 cells (1×10^6 cells/ml) for 1 h at 4°C at a concentration of 10 μM phospholipid. Unbound virosomes were removed by two centrifugal wash steps before cells were analyzed by flow cytometry. Histograms in black show the fluorescence intensity of 3000 cells incubated in buffer. White overlays show the fluorescence intensity of 3000 cells after incubation with the indicated virosome preparation.

rocyte membranes are a rich source of sialic acid-bearing glycolipids and glycoproteins. When 323/A3-PEG virosomes were added to OVCAR-3 membrane vesicles 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 membrane vesicles for 1 h at room temperature, the extent of lipid mixing was as high as with unmodified virosomes (Fig. 3A). Treatment of OVCAR-3 membrane vesicles 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 (Fig. 3B).

4. 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 [26–29]. 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 [30,31]. 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 [26] also result in loss of fusogenic activity of the HA protein. In this study we have chosen for 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. [32], 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 from interacting 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 ends of the PEG chains in order to obtain specific binding to target cells without destruction of the HA protein. 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 [33]. 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 [34]. Considering the dimensions of the PEG layer and the HA protein (13.5 nm) one would expect that only PEG-5000 would be able to steri-

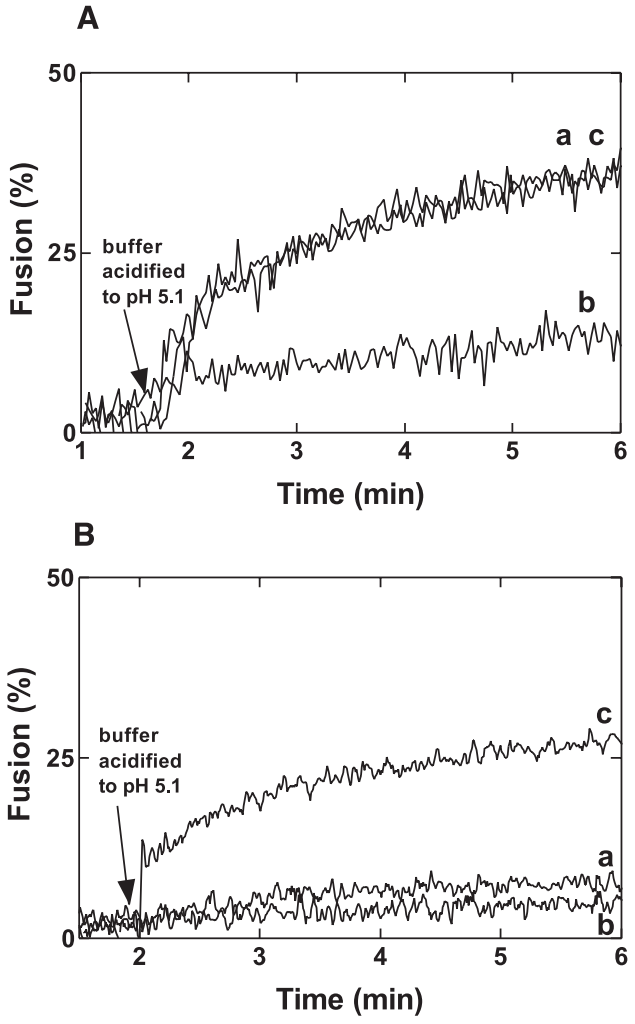

Fig. 3. Fusion of influenza virosomes with OVCAR-3 membrane vesicles (A) or OVCAR-3 membrane vesicles treated with neuraminidase to remove sialic acid residues (B). OVCAR-3 membrane vesicles (200 μM phospholipid) were added to pyrPC-labeled virosomes (2.5 μM phospholipid). At the time point indicated with an arrow, the medium was acidified to pH 5.1, and fusion was monitored as previously described (see legend to Fig. 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).

cally 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. High densities of surface-grafted PEG cause the PEG chains to stretch out (i.e. brush formation) [35]. So, it may well be possible that the PEG chains have a fully-stretched conformation in our PEG-2000 virosomes. With a fully-stretched conformation, which has a theoretical dimension of 15.8 nm (45 units of ethylene glycol of 3.5 Å each), the HA protein would be shielded. 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 [36].

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 [37]. Indeed, we observed that 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 and 40%) at the highest PEG lipid concentration used (20 mol% PEG-2000) compared to virosome preparations without PEG lipids (recovery between 70 and 80%). This indicates that solubilization of components from the viral envelope (lipids, spike proteins) into mixed micelles has occurred in the preparation with the highest concentration of PEG lipids. 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 functions of the HA binding to sialic acid residues are to initiate receptor-mediated endocytosis of the virosome particles and 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. Not only should it allow specific binding of virosomes 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 [38,39].

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 [40,41]. 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.

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