Fusion of reconstituted influenza virus envelopes with liposomes mediated by streptavidin/biotin interactions

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Received 13 June 1996

Abstract Reconstituted influenza virus envelopes (virosomes) containing the viral hemagglutinin (HA) represent an efficient fusogenic cellular delivery system. By interaction of HA with its natural receptors, sialylated lipids (gangliosides) or proteins, virosomes bind to cells and, following endocytic uptake, deliver their contents to the cytosol through fusion from within acidic endosomes. Here, we show that binding to sialic acid is not necessary for fusion. In the presence of streptavidin, virosomes containing a biotinylated lipid fused with liposomes lacking sialic acid if these liposomes also had a biotinylated lipid in their membranes. Moreover, fusion characteristics corresponded well with fusion of virosomes with ganglioside-containing liposomes.

Key words: Influenza virus; Hemagglutinin; Virosome; Membrane fusion; Targeting; Cellular delivery; Carrier system

1. Introduction

The plasma membrane receptor for the influenza virus envelope glycoprotein hemagglutinin (HA) is formed by sialylated lipids (gangliosides) or proteins [1]. Receptor binding of HA mediates cellular uptake of the virus through receptor-mediated endocytosis, directing the virus to acidic endosomes. At the mildly acidic pH within endosomes, HA mediates merging of the viral with the endosomal membrane [2–4], resulting in the release of the viral genome into the cell cytosol.

One of the key issues in biological and pharmacological research is how to bring about efficient and selective cytoplasmic delivery of normally impermeant substances. We have previously shown that reconstituted influenza virus envelopes (virosomes) can be used to deliver proteins to the cytosol of target cells [5–7]. Although virosome-mediated delivery was efficient, because of the ubiquitous nature of target sialic acid residues it is not expected that macromolecules may be introduced into the cytosol of a selected set of target cells. Clearly, no specificity is possible if cytoplasmic delivery is dependent on the sialic-acid-binding characteristics of the HA molecules.

In this study we investigated whether fusion of virosomes with target membranes lacking sialic acid residues is possible with an alternative mode of virosome-target membrane binding. The latter was provided by the high-affinity interaction between streptavidin and biotin. As model target membranes we used liposomes with HA-binding gangliosides or without gangliosides but containing biotin-phosphatidylethanolamine (biotinPE). Virosomes, labeled with pyrene-phosphatidylcholine (pyrPC), were induced to fuse with these liposomes. Fusion was monitored by the decrease of pyrene excimer fluorescence [8]. In this system, in the absence of target gangliosides, virosomes containing biotinPE fused specifically at acid pH with biotinPE-containing liposomes. This depended on binding at neutral pH in the presence of streptavidin. These results show that fusion of virosomes can be directed towards specific target membranes, which provides support for the further development of virosomes as an efficient and selective cellular delivery system.

2. Materials and methods

2.1. Materials

Octaetheleneglycol monododecyl ether ($C_{12}E_8$) and the hydrophobic resin Bio-Beads SM-2 (bead size 300–1180, 180 μm) were obtained from Fluka Chemie AG (Buchs, Switzerland) and Bio-Rad (Hercules, CA, USA), respectively. Prior to use Bio-Beads were conditioned with methanol and washed with 5.0 mM HEPES (pH 7.4) containing 0.15 M NaCl (HBS). PyrPC (1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine) was from Molecular Probes (Eugene, OR, USA). DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and cholesterol were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). BiotinPE, the triethylammonium salt of *N*-(6-((biotinoyl)amino)hexanoyl)dipalmitoyl-L-α-phosphatidylethanolamine, and D-biotin were purchased from Pierce (Rockford, IL, USA). Gangliosides (type III, purified from bovine brain), and streptavidin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), fraction V, was from Life Technologies Ltd. (Paisley, UK). All other chemicals were of the highest grade available.

2.2. Virus, virosomes and liposomes

The X47 recombinant strain of influenza virus, carrying the HA of influenza A/Victoria/3/75, was grown and purified as described elsewhere [8]. Virosomes were prepared and purified by sucrose density centrifugation as described previously [5,9]. To incorporate biotinPE or pyrPC in the virosome membrane, $C_{12}E_8$-solubilized virus was added to either pyrPC or a mixture of pyrPC and biotinPE, after removal of the viral nucleocapsid by ultracentrifugation. The amounts of pyrPC and biotinPE, relative to the total amount of phospholipid, were 14 and 3.0%, respectively.

Liposomes were prepared from dry films of 2.5 μmol total lipid, by rehydration with 0.5 ml HBS, 10 cycles of freezing and thawing, and

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Abbreviations: HA, hemagglutinin; PE, phosphatidylethanolamine; biotinPE, biotin-derivatized PE; PC, phosphatidylcholine; pyrPC, pyrene-labeled PC; $C_{12}E_8$, octaetheleneglycol monododecyl ether; HBS, HEPES-buffered saline; DOPC, dioleoylPC; DOPE, dioleoylPE; BSA, bovine serum albumin; R18, octadecylrhodamine

PII S0014-5793(96)00682-5

subsequent extrusion [10] through a Nucleopore polycarbonate membrane with a pore size of 0.2 μm (Costar Co., Cambridge, MA, USA), using a mini-extruder (Avanti Polar Lipids, Inc.). The liposomes were composed of DOPC, DOPE, cholesterol, biotinPE and ganglioside in the following molar ratios: 40:20:40:0:0, 40:15:40:5:0 and 40:20:35:0:5. The phospholipid contents of virosomes and liposomes were determined by phosphate analysis [11] after digestion of the lipids [12].

2.3. Fluorescence measurement of membrane fusion

Membrane fusion measurements were carried out with an SPF-500C spectrofluorometer (SLM Instruments Inc., Urbana, IL, USA) equipped with a thermostated cuvette holder and a magnetic stirring device. Virosomes (at a final concentration of 1.0 μM phospholipid) and streptavidin were added to a quartz microcuvette containing HBS/BSA (HBS supplemented with 1 mg/ml BSA). After 30 s liposomes were added. After an additional 2 min incubation period the medium was acidified to pH 5.1 by the addition of 25 μl of 0.10 M acetic acid, 0.10 M 2-[*N*-morpholino]ethanesulfonic acid (pH 4.1). The final volume was 0.7 ml. Fusion was continuously monitored at 37°C by the decrease of pyrene excimer fluorescence, at excitation and emission wavelengths of 345 nm (bandpass 0.5 nm) and 490 nm (bandpass 20 nm), respectively. A 475 nm cut-off filter was placed in the emission beam. Background fluorescence was assessed at infinite dilution of pyrPC, which was obtained by adding 35 μl of 0.20 M $C_{12}E_8$. Relative fluorescence was obtained by calculating $100\times(E_0-E_\infty)/(E_0-E_\infty)$, where E represents the excimer fluorescence intensity during fusion, and E_0 and E_∞ represent, respectively, the intensities at 490 nm at time zero and after the addition of $C_{12}E_8$, both corrected for dilution effects. After acidification of virosomes alone the pyrene excimer fluorescence declined slowly, at a rate of approximately 0.01%/s. All fusion curves were corrected for this value.

3. Results and discussion

3.1. Fusion of influenza virosomes with liposomes: effect of pre-fusion binding

Fig. 1 (curve a) shows that virosomes fuse slowly with DOPC/DOPE/cholesterol liposomes without gangliosides. This slow fusion can be accounted for by hydrophobic membrane association of the HA fusion peptides [13,14] that are exposed upon acidification [15]. The possibility of specifically targeting influenza virosomes was investigated by co-reconstituting biotinPE in the virosomes and measuring fusion of these virosomes with liposomes lacking gangliosides but containing biotinPE, in the presence of streptavidin (Fig. 1, curve b). After a 2 min incubation period at pH 7.4, acidification of the medium resulted in rapid fusion, with an initial rate of fluorescence change which was 40 times higher than the rate obtained in the absence of biotinPE in the target liposomes. In fact, fusion of biotinPE-containing virosomes with biotinPE-containing liposomes in the presence of streptavidin resembled fusion of the same virosomes with ganglioside-bearing liposomes (Fig. 1, curves b and c, respectively). At neutral pH (in the presence of streptavidin) no change of fluorescence above background was noted. In the absence of streptavidin (at pH 5.1) fusion with biotinPE-containing liposomes was similar to fusion obtained in the absence of gangliosides or biotinPE in the target liposomes (see Fig. 1, curve a).

The rates of fusion of these and control experiments are summarized in Fig. 2. Virosomes lacking biotinPE fused to a very limited extent with liposomes lacking gangliosides. The presence of streptavidin had no effect on the rates of fusion of these virosomes with any of the target liposomes, including ganglioside-containing liposomes. In the absence of streptavidin the rates of fusion of biotinPE-containing virosomes with any of the liposomes were essentially the same as those of unmodified virosomes. Thus, the presence of biotinPE in the virosomal membrane by itself did not affect the rate of membrane fusion. The only fusion reaction which was affected by the presence of streptavidin was that of biotinPE-containing virosomes with biotinPE-containing liposomes, demonstrating that biotinPE-containing virosomes can be specifically targeted to biotinPE-containing membranes in the presence of streptavidin. Importantly, the rate of virosome fusion with biotinPE-containing liposomes in the presence of streptavidin ($2.3\pm0.4\%$ (mean ± S.D.) fluorescence/s) was very similar to that of ganglioside-containing liposomes ($3.3\pm0.4\%$ fluorescence/s). Thus, the rapid fusion of influenza virus and virosomes with membranes carrying sialic-acid-containing receptors can be mimicked by using streptavidin/biotin-mediated targeting to membranes without a binding moiety for the virosomal HA.

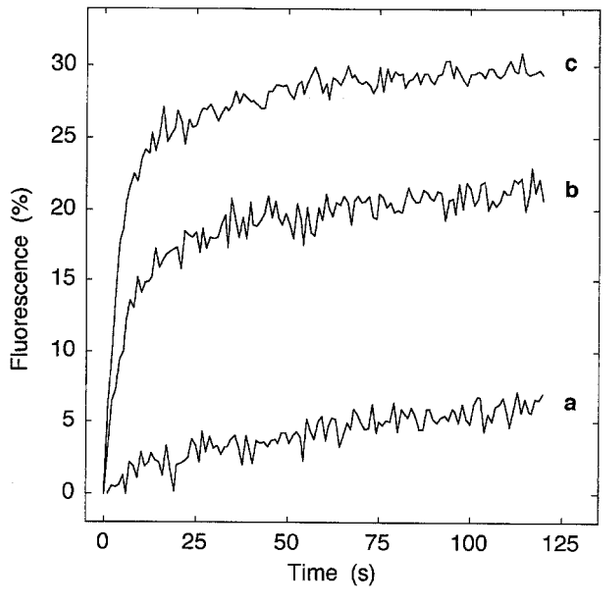

Fig. 1. Fusion of influenza virosomes with liposomes. Liposomes (20 μM lipid) were added to pyrPC-labeled virosomes containing biotinPE (1.0 μM phospholipid), and the medium was acidified to pH 5.1. Pyrene excimer fluorescence was continuously monitored at 37°C. The decrease of fluorescence was expressed relative to the difference between the initial fluorescence and the fluorescence at infinite pyrPC dilution. The latter value represents complete (100%) fusion. Curves a, b and c were obtained with liposomes without gangliosides or biotinPE, liposomes with biotinPE and liposomes with gangliosides, respectively. In all cases 15 nM streptavidin was present.

3.2. Kinetics and specificity of streptavidin/biotin-mediated fusion

The rates of fusion were determined over a range of liposome concentrations (Fig. 3). The kinetics of fusion after streptavidin/biotin-mediated targeting were almost identical to the kinetics of fusion after ganglioside-mediated pre-fusion binding. Fusion of virosomes with liposomes without gangliosides or biotinPE was about one order of magnitude slower over the whole range of liposome concentrations.

Virosome targeting to liposomes with streptavidin/biotin depended on the streptavidin concentration, with an optimum in the rate of fusion at 10–15 nM streptavidin under the experimental conditions employed (Fig. 4). Virosomes were used at a concentration of 30 nM biotin. Assuming that biotinPE is equally distributed over the inner and outer membrane leaf-

lets, half of the biotin should have been accessible to streptavidin. Since streptavidin has four biotin-binding sites, the optimum rate of fusion corresponded to an equimolar ratio of streptavidin to virosomal biotin.

A further demonstration of specificity came from the observation that streptavidin-induced fusion of biotinPE-containing virosomes and liposomes was reduced to background levels in the presence of excess fluid-phase biotin (data not shown). Thus, streptavidin/biotin-mediated targeting of virosomes to liposomes specifically depended on simultaneous interaction of streptavidin with virosomal and liposomal biotin. The virosome and liposome membranes which fuse in this study are separated by the diameter of the streptavidin molecule, which is about 4.5 nm [16]. The interesting relationship between this distance and the extent of fusion may be investigated further by the use of lipophilic biotin ligands with spacers of different lengths.

3.3. *Streptavidin/biotin-mediated HA-dependent fusion: Implications for the fusion mechanism and the development of fusogenic delivery systems*

In this study we have demonstrated the feasibility of streptavidin/biotin-mediated targeting to permit HA-dependent fusion of virosomes with liposomes containing no binding moiety other than biotinPE. These observations have implications for the mechanism of HA-mediated fusion. Sialic-acid-containing receptors, such as gangliosides, have been shown to enhance the rate [14,17] and extent [17] of influenza virus fusion in liposomal model systems, by increasing prefusion binding of the virus to the target membrane at neutral pH. In the absence of gangliosides, fusion is also observed, but the rates and extents are relatively low [14,17] (see also Fig. 1). In addition to enhancing the extent of prefusion binding, it has been concluded that interaction of HA with gangliosides also facilitates correct insertion of the HA fusion peptides into the target membrane [14,18]. This may result in higher rates of membrane fusion and, at temperatures below 37°C, in a detectable reduction of lag times preceding the actual onset of fusion [14].

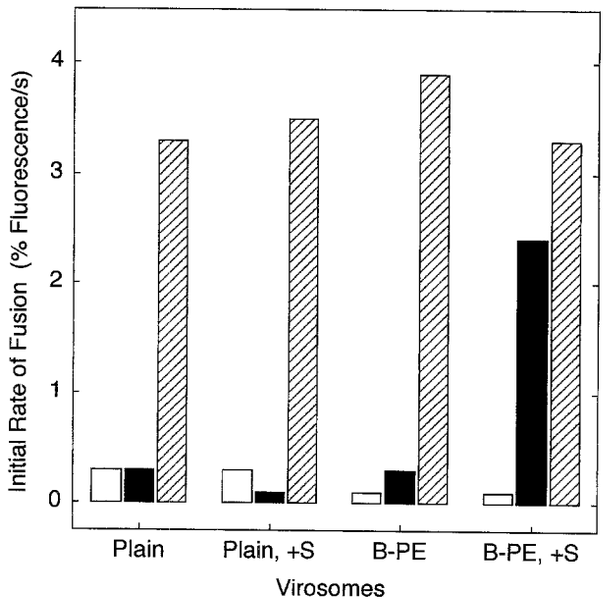

Fig. 2. Initial rates of fusion of virosomes with liposomes. Fusion was measured as in Fig. 1. Fusion rates were determined from the slopes of the tangents to the initial parts of the curves. Plain and biotinPE-containing virosomes (B-PE) were tested. Liposome preparations were without gangliosides or biotinPE (open bars), with biotinPE (solid bars) or with gangliosides (hatched bars). The abbreviation +S indicates the use of streptavidin (15 nM).

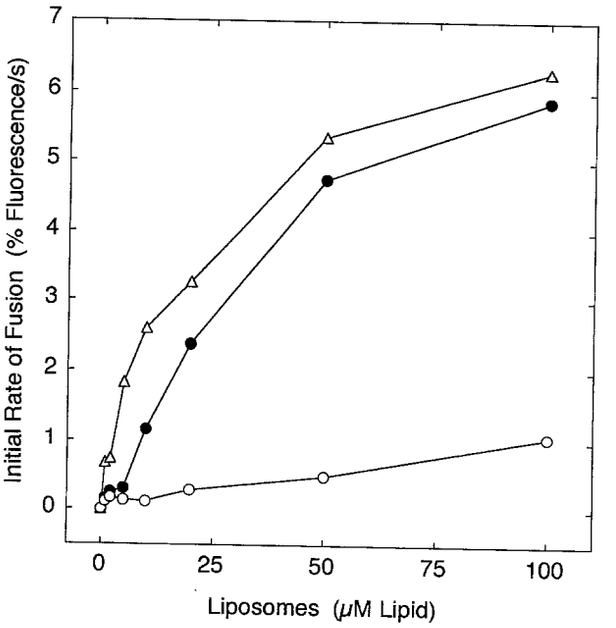

Fig. 3. Effect of liposome concentration on the initial rates of fusion. The data were obtained as in Fig. 2 with liposomes lacking gangliosides or biotinPE (○), and with liposomes containing biotinPE (●) or gangliosides (△).

A greater importance of the role of target sialic acid residues in fusion has been postulated by others. In a study of fusion between influenza virus and planar lipid bilayers it was proposed that binding of HA to a sialic-acid-containing receptor may fundamentally alter the course of the conformational change in HA, to direct the fusion peptides to the target membrane [19]. In the present study streptavidin/biotin-mediated targeting resulted in fusion kinetics very similar to those observed with virosomes and liposomes containing gangliosides (Fig. 3). Thus, the notion that the interaction between HA and a sialic-acid-containing receptor has a fundamental effect on the fusion process does not seem tenable. Rather, our present findings support previous conclusions of Stegmann et al. [14,17] that gangliosides do not influence the low-pH-dependent conformational change of HA or the characteristics of the membrane merger itself.

It is well established that influenza virus fuses avidly with erythrocyte ghosts. Pre-treatment of the ghosts with neuraminidase almost completely inhibits this fusion reaction [20]. Likewise, in our hands virosomes failed to show any decrease of excimer fluorescence upon incubation with neuraminidase-treated erythrocyte ghosts at acidic pH and 37°C (P. Schoen, unpublished results). Removal of sialic-acid-containing receptors from human T lymphocytic leukemia cells by neuraminidase treatment [18], or from L929 cells (P. Schoen, unpublished results), inhibits binding of influenza virus by 60–70%. Moreover, in the case of L929 cells, the virosome particles which did bind (at 4°C) showed essentially no change of pyrene excimer fluorescence during a subsequent 1 h incubation at 37°C. Apparently, these virosomes were not bound to sites

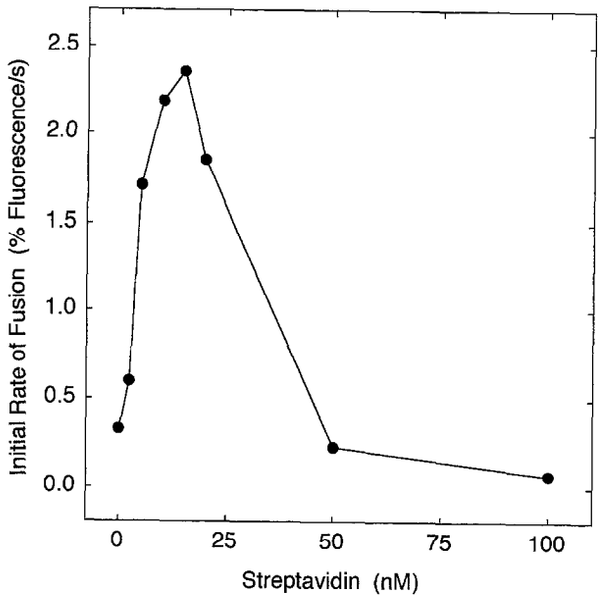

Fig. 4. Initial rates of fusion of biotinPE-containing virosomes with biotinPE-containing liposomes (20 μM) as function of streptavidin concentration.

allowing functional receptor-mediated endocytosis and subsequent acidic-pH-mediated fusion from within the endosomes.

Thus, the delivery of virosomal contents can in principle be specifically targeted, when the normal HA-dependent binding to target cells can be inhibited [21,22]. Here we used the high-affinity streptavidin/biotin system as targeting device, but there is no reason why other modes of targeting should not be applicable as well. This has promising implications for development of virosomes as efficient and selective delivery systems.

Acknowledgements: We thank Solvay Duphar BV (Weesp, The Netherlands) for ample supply of influenza virus. This study was supported by Inex Pharmaceuticals Corp. (Vancouver, Canada), by the Netherlands Organization for Scientific Research (NWO), under the auspices of the Chemical Foundation (SON) and the Technology Foundation (STW), by the Association pour la Recherche sur le Cancer, by the Ligue National Française contre le Cancer and by institutional grants from the Centre National de la Recherche Scientifique (CNRS) and the Institut National de la Santé et de la Recherche Médicale (INSERM).

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