Biochimica et Biophysica Acta, 1152 (1993) 15–25

BBAMEM 76084

Reconstituted Sendai virus envelopes as biological carriers: dual role of F protein in binding and fusion with liver cells

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(Received 29 March 1993)

Key words: Sendai virus; Virosome; Fusion; Drug delivery; Liver

We have assessed the potential of reconstituted Sendai viral envelopes containing only the fusion protein (F-virosomes) as biological carriers for the delivery of drugs and macromolecules. [^{125}I]lysozyme entrapped in F-virosome is used to study its distribution in various organs of Balb/c mouse in vivo as a function of dose and time. F-virosomes injected intravenously are rapidly cleared from circulation. A major percentage (55–60%) of vesicle contents is delivered to liver at 15 min after injection, showing thereby the liver to be the major site for the accumulation of vesicles. Uptake of virosomes by liver is found to reach a near saturation level at a dose of 0.5 mg F-protein associated with virosomes. In competition studies, the inhibitory effect of asialofetuin on the uptake of F-virosomes suggests the involvement of asialoglycoprotein receptor in its recognition by hepatic parenchymal cells. Incorporation of asialoganglioside-GM_1 in the F-virosomes enhanced the uptake by about 1.6-fold. The observed specific interaction of hepatic receptor with F-protein containing a terminal galactose moiety is further supported by degalactosylation of F-virosomes with hard-shelled clam exoglycosidase. The uptake of degalactosylated F-virosomes by liver is found to be significantly reduced. The subcellular radioactivity profile in liver cells exhibits a considerable decrease in cytosolic localisation of the degalactosylated F-virosomal contents with a concomitant increase in their accumulation in lysosomal/mitochondrial fraction as compared to the untreated virosomes. Trypsinized and heat-treated F-virosomes also reflect similar subcellular distribtuion profile as that of degalactosylated virosomes. Moreover, F-virosomes are able to interact and deliver [^{125}I]lysozyme to the HepG2 cells in culture in the presence of a potent inhibitor of endocytotic process. These results indicate the involvement of specific binding of F-proteins with hepatic receptors followed by their fusion with the membrane of liver cells in the delivery of [^{125}I]lysozyme. The findings reported here open up the possibility of using F-virosomes with defined specificity as fusogenic vehicles for efficient delivery of drugs and biologically active macromolecules both in vivo and in vitro.

Introduction

In recent years, there have been many attempts to introduce impermeant molecules into living cells using liposomes both in vivo and in vitro [1]. Numerous studies have been carried out with the inclusion of a suitable ligand-receptor in the vesicle membrane to achieve target specificity [2]. In particular, liposomes carrying terminal galactose moieties have been shown to be directed to hepatic parenchymal cells containing the galactose-specific asialoglycoprotein receptor in vivo [3]. A galactose-specific receptor on Kupffer cells has also been characterized [4]. Liposomes with galactose-containing structures on their surface are taken up by both types of liver cells in vivo through receptor-mediated endocytosis [5].

The efficiency of liposomes as biological carriers in vivo depends to a large extent on the recipient cells to internalize loaded liposomes via endocytosis and the ability of lysosomal enzymes to disrupt the liposomal membrane for releasing its contents [6]. However, this mode of drug delivery has the disadvantage arising from excessive inactivation or degradation of targeted macromolecules by various hydrolases of the lysosome

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Part of this work has been published as an abstract at the 4th National Symposium on Liposome Research, 1–4 March 1992, University of Delhi South Campus, New Delhi, India.

Abbreviations: WGA, wheat-germ agglutinin; asialoGM_1, asialoganglioside-GM_1; HRP, horseradish peroxidase; Tx100, Triton X-100; DTT, dithiothreitol; RCA_1, *Ricinus communis* agglutinin; ASGP-R, asialoglycoprotein receptor; Gal, D-galactose; Fuc, L-fucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; DMEM, Dulbecco's modified Eagle's medium; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; ATCC, American type culture collection; DPBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; ASOR, asialoorosomucoid; EDTA, ethylenediaminetetraacetate (disodium salt).

at low pH. This route of liposome internalization is beneficial for targeting its contents to lysosomes in case of lysosomal storage diseases [7]. Liposomal drug delivery can also be useful in designing a targeting approach for chemotherapeutic agents against certain parasitic infections where the parasites reside within the lysosomes of the host cells [8]. However, delivery of biologically active molecules to extralysosomal sites demands the construction of delivery vehicles that can bypass the lysosomal route.

It is therefore conceivable that the vehicles of choice for this purpose would be the fusogenic vesicles whose contents can be delivered directly into the cytosol. Cytoplasmic delivery of encapsulated macromolecules has been reported using pH-sensitive liposomes. Although there has been some success, there is a need to improve the efficiency of liposome-cell fusion [1]. The reconstituted Sendai virus envelopes (virosomes) on the other hand have been successfully used to achieve this goal of cytosolic delivery to various cells in culture [9]. Sendai virus, an enveloped animal virus contains two glycoproteins (HN, hemagglutinin-neuraminidase and F, fusion factor) in the outer leaflet of the lipid bilayer [10]. Hemagglutinin-neuraminidase binds to the sialic acid residues of the membrane sialoglycoproteins and sialoglycolipids followed by the F-protein-mediated fusion of the viral envelope with the host cell plasma membrane at neutral pH [11]. As a result, the viral nucleocapsid gets transferred to the cytosol of the target cell. The behaviour of virosomes is similar to that of the intact virus in reference to their binding and fusion with the target cells [12]. In the targeting of virosomal contents to desired cell types in vitro, the envelope has been modified by replacing the HN mediated binding with specific antibodies or ligands against cell surface antigens or receptors, respectively [9]. These engineered virosomes are also effective in binding and fusing with respective target cells in vitro. Recently, it has been clearly demonstrated that HN-mediated binding can be replaced by suitable attachment factors [13,14]. The F protein is a glycoprotein and has terminal biantennary galactose moieties [15] which can bind specifically to the asialoglycoprotein receptor on the membrane of HepG2 (human liver hepatoblastoma) cells in culture [13]. The biantennary disposition of galactose moieties is also known to promote clustering leading to a pronounced increase in the affinity towards cell-surface receptors [16]. Moreover, a temperature sensitive mutant (ts271) of Sendai virus lacking the HN protein has been reported to promote binding and fusion of HepG2 cells [13]. Recently, we have also confirmed that F-virosomes (devoid of HN protein) prepared from intact Sendai virus can efficiently bind and fuse with HepG2 cells though the presence of HN enhances only the initial rate of virosomes-cell fusion [17].

In this report, we have exploited the Sendai viral fusion protein/asialoglycoprotein receptor interaction in targeting [^{125}I]lysozyme (entrapped in F-virosomes) to liver cells of Balb/c mouse in vivo. Our work demonstrates the feasibility of constructing targeted fusogenic lipid vesicles for cytosolic delivery of entrapped material in vivo using only the F protein of Sendai virus. To our knowledge this is the first systematic study on the use of viral fusion protein in the delivery of a model drug under in vivo conditions.

Materials and Methods

Reagents

Asialofetuin, asialoganglioside-GM_1, lysozyme (chicken egg white), dithiothreitol, wheat-germ agglutinin (WGA), phenylmethylsulfonyl fluoride (PMSF), sodium azide, EDTA, Mannan (*Saccharomyces cerevisiae*) and diaminobenzidine were purchased from Sigma (St. Louis, MO, USA). Bio-Beads (SM-2) and Protein A-HRP conjugate were obtained from Bio-Rad (Richmond, CA, USA). Triton X-100 was procured from Aldrich (Milwaukee, WI, USA). Exoglycosidase (*M. mercenaria*, hard-shelled clam) was a kind gift from Prof. Subhash C. Basu (Notre Dame, IN, USA). RCA_1 and anti-ricin antibody were available from Ms V.M. Vasandani and Dr. V.K. Chaudhary, respectively, of our department. Anti-lysozyme antibody was raised in rabbit. Uranyl acetate was obtained from E.M. Sciences. Carrier-free Na^{125}I was supplied by BARC (Bombay, India). Balb/c mice were purchased from NIN (Hyderabad, India). [^{14}C]Dipalmitoylphosphatidylcholine and ^{3}H-labelled Tx100 came from NEN. DMEM, DPBS, Fetal bovine serum and Penicillin/Streptomycin were obtained from Gibco (Grand Island, NY, USA). All other reagents used were of analytical grade.

Virus

Sendai virus was grown in the allantoic sac of 10–11-day-old embryonated chicken eggs. The virus was harvested and purified according to the published procedure [11]. Purified virus was resuspended in PBS (10 mM Na_2HPO_4, 150 mM NaCl (pH 7.4)). The viral yield was estimated in terms of protein by the method of Markwell et al. [18] and its activity was checked by agglutination and lysis of mouse red blood cells [9]. Aliquots of virus were stored at −70°C.

Cells

HepG2 cells (Human hepatoblastoma cell line) were obtained from ATCC (Rockville, MD, USA) and were grown at 37°C, 5% CO_2 in DMEM containing 4 mM L-glutamine, 25 mM Hepes, 110 mg/l sodium pyruvate, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate in 75-cm^2 plastic bottles

(Coster, MA, USA). Single-cell suspensions were obtained by treatment of the cell monolayer with 5 mM EDTA in DPBS [19]. Fresh red blood cells were prepared from healthy Swiss albino mice stock (University of Delhi South Campus).

Radioiodination of lysozyme

Lysozyme was radiolabelled using the chloramine-T method, using $Na^{125}I$ as a source of iodine [20]. The iodinated protein was separated from free iodine by gel filtration through a Sephadex G-25 column, pre-equilibrated with PBS. The specific activity of labelled protein was calculated from TCA-precipitable counts and expressed as cpm/mg protein.

Preparation of fusogenic F-virosomes

Reconstituted Sendai virus envelopes containing only the F protein (F-virosomes) were prepared as reported by Tomasi and Loyter [21] with minor modifications [17]. A pellet of Sendai virus particles (20 mg protein) was suspended in 4 ml of buffer A (150 mM NaCl, 20 mM Tris-HCl (pH 8.4)) containing 3 mM DTT and was incubated at 37°C for 4 h. It was followed by dialysis at 4°C for 16 h against three changes of 2 litre of buffer B (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM Ca^{2+}, 2 mM Mg^{2+}). The viral suspension was centrifuged at $100\,000 \times g$ for 1 h at 4°C and the pellet obtained was resuspended in 2 ml buffer B containing 40 mg Tx100. After incubating at 20°C for 1 h, the suspension was centrifuged to remove detergent insoluble substances (reduced HN protein and nucleocapsid). From the clear supernatant, detergent was removed by stepwise addition of SM2 Bio-Beads with constant rocking. Initially the supernatant was incubated with 320 mg methanol washed SM2 Bio-Beads at 4°C. After 2 h, an additional 320 mg SM2 Bio-Beads were added and incubation continued at 20°C for 2 h. To the mixture 320 mg SM2 Bio-Beads were further added and incubation terminated after 2 h at 20°C. The turbid suspension was separated from Bio-Beads using a 26-gauge needle and centrifuged at $100\,000 \times g$ for 1 h at 4°C. Protein concentration in virosome sample was determined by the method of Markwell et al. [18]. The pellet containing F-virosomes (0.8–1 mg F protein) was resuspended in 1 ml of buffer B and stored at 4°C. The total phospholipids in the virosomes were calculated from the lipid phosphorous analysis by the modified method of Fiske and SubbaRow [22]. [^{14}C]Dipalmitoylphosphatidylcholine and ^{3}H-labelled Tx100 were used to determine the phospholipid recovery and presence of residual detergent in the F-virosomal preparation, respectively [23]. Before use, the virosomes were passed through a 26-gauge needle 20-times and filtered through 0.22 μM Millipore type Millex GV filter to remove large aggregates. SDS-PAGE was used to check the purity of the F-virosomes [24]. Heat-treated virosomes were prepared by incubating F-virosomes at 56°C for 20 min [25]. F-virosomes were stained with 1% aqueous solution of uranyl acetate and viewed under an electron microscope [26].

Entrapment of ^{125}I-labelled lysozyme in F-virosomes

Following removal of detergent insoluble viral components, as described above, ^{125}I-labelled lysozyme (200 μg, $4 \cdot 10^7$ cpm/mg protein) was added to the detergent soluble fraction. The subsequent steps and removal of Tx100 were exactly as described above for reconstitution of unloaded Sendai viral envelopes. The unentrapped protein was separated by repeated centrifugation at $100\,000 \times g$ for 1 h at 4°C. Radioactivity associated with virosomes was estimated using a gamma counter (1275, Minigamma counter, LKB, Wallac). Entrapment was calculated as the amount of [^{125}I]lysozyme associated per mg of F protein. Membrane intercalation of lysozyme was checked by treating the virosomes with trypsin [27]. Stability of the loaded virosomes was assessed by measuring the leakage of entrapped [^{125}I]lysozyme both at 37°C and 4°C for 8 h and 7 days, respectively. Leakage was also examined in the presence of fresh mouse plasma at 37°C for various time periods. The entrapped [^{125}I]lysozyme served as virosomal aqueous marker in this study.

Incorporation of asialoganglioside-GM_1 in F-virosomes

Asialoganglioside-GM_1 was solubilised in chloroform/methanol (2:1 (v/v)). An aliquot was taken in a glass test tube and the solvent evaporated under N_2 gas to make a thin film. The film was dessicated under vacuum for 1 h. To this thin film, a detergent-soluble extract of DTT-reduced Sendai virus (as described in the preparation of F-virosomes) was added and incubated at 20°C for 1 h. The rest of the steps were exactly the same as described for the preparation of F-virosomes. Activity of the asialoGM_1-containing F-virosomes was estimated in terms of hemolysis of mouse red blood cells in the presence of WGA as described [17,21].

Degalactosylation of F-virosomes

Virosomes were treated with exoglycosidase having both α- and β-galactosidase activity [28]. To a 1-mg virosome suspension in 0.15 M sodium acetate buffer (pH 5.0), 200 μg of exoglycosidase was added followed by an incubation for 5 h at 37°C. Virosomes were washed by centrifugation as described above and suspended in buffer B. Virosomes loaded with [^{125}I]lysozyme were also treated in the same way without any significant loss of radioactivity. Fusogenicity of these treated virosomes (degalactosylated virosomes) were checked as described earlier [17]. Degalactosylation of F protein in virosomes was examined by Western blotting [29]. Both treated and control virosomes were run

on 10% SDS-PAGE and subsequently transferred by electroblotting onto a nitrocellulose membrane. After treating with 0.05% Tween-20 in PBS, the membrane was incubated with RCA_1, which is known to bind to terminal galactose residues of glycoproteins [30]. Excess RCA_1 was removed by washing and further processed sequentially with anti-ricin antibody, protein A-HRP conjugate, diaminobenzidine and H_2O_2 to visualize the protein bands.

Animal experiments

Throughout this study male Balb/c mice (30–35 g body weight) were used. Virosomes loaded with [^{125}I]lysozyme were injected into the tail vein of mouse in a volume of 0.2 ml buffer B (0.2 mg F protein). With each 0.2 ml virosomes injected the amount of [^{125}I]lysozyme-associated radioactivity was $(8–10) \cdot 10^4$ cpm. Blood and organ samples from a group of three mice were collected at various time points after injection to study the distribution profile of the virosomes. As a control, free [^{125}I]lysozyme ($8 \cdot 10^4$ cpm) alone and mixed with virosomes (0.2 mg) were injected. Trypsin treated, degalactosylated and heat-treated virosomes were also used as the controls. In the competition experiments, asialofetuin alone (3.5 mg/0.2 ml Buffer B) and mannan, either alone (3.5 mg/0.2 ml buffer B), or together were injected intravenously about 2 min prior to the injection of virosomes. At the desired time, mice were anaesthesized with chloroform, dissected and blood was collected with heparin from heart. The total radioactivity in the blood was estimated by considering that the total volume of blood was 7.3% of the body weight [31]. The entire liver, kidneys, lungs and spleen of each mouse were digested in 2–5 ml 30% KOH overnight. Aliquots of the digested samples were counted for radioactivity.

Subcellular fractiontion of liver

Subcellular fractionation was carried out according to a published procedure [32]. After removal the liver from each mouse was washed thoroughly with 0.9% NaCl solution and blotted with filter paper. The whole liver was placed in 10 ml 0.01 M Tris-HCl buffer (pH 7.4), containing 0.25 M sucrose and then dispersed in a homogeniser of the Potter-Elvehjem type at 4°C with 15 up and down strokes. The separation of nuclear, lysosomal/mitochondrial and plasma membrane fractions were carried out by differential centrifugation at $600 \times g$, $15\,000 \times g$ and $100\,000 \times g$ at 4°C for 10, 30 and 60 min, respectively. The nuclear fraction was washed twice with the isotonic homogenising buffer and washings were added to the original supernatant before separation of the lysosomal/mitochondrial fraction. After the separation of plasma membrane, the cytosolic fraction was obtained. The fractions were further characterized by marker enzyme analysis [33]. Radioactivity in each fraction was expressed as percent of total radioactivity present in the liver homogenate before differential centrifugation.

Kinetics of fusion-mediated delivery of [^{125}I]lysozyme into HepG2 cells: effect of heat treatment and metabolic inhibitor

Single-cell suspensions of HepG2 cells were made as described earlier [17,19] and resuspended in DPBS containing 2 mM Ca^{2+} and 2 mM Mg^{2+} to obtain $1.5 \cdot 10^6$ cells/ml. HepG2 cells ($1.5 \cdot 10^6$) were incubated with loaded F-virosomes (200 μg F protein) at 37°C for various time periods. The virosome-cell mixtures were washed three times with cold DPBS and finally incubated for 2 min at 4°C in DPBS containing 5 mM EDTA (EDTA stripping). EDTA stripping could completely remove the cell surface bound (unfused) virosomes [19]. The cell pellet after EDTA stripping was solubilized in extraction buffer (10 mM Tris-HCl, 150 mM NaCl (pH 7.4) containing 1% Tx100 and 1 mM PMSF) and the internalized radioactivity was determined by a gamma counter. Sodium azide is known to be an efficient inhibitor of endocytotic process [19]. Fusion-mediated delivery of [^{125}I]lysozyme by F- and heat-treated F-virosomes to HepG2 cells was also studied in the presence of 20 mM azide.

Results

Characterization of F-virosomes

Based on SDS-PAGE analysis in the presence of β-mercaptoethanol, the F-virosomes were found to be free of any detectable contamination by other proteins (Fig. 1). The F_2 fragment could not be distinguished

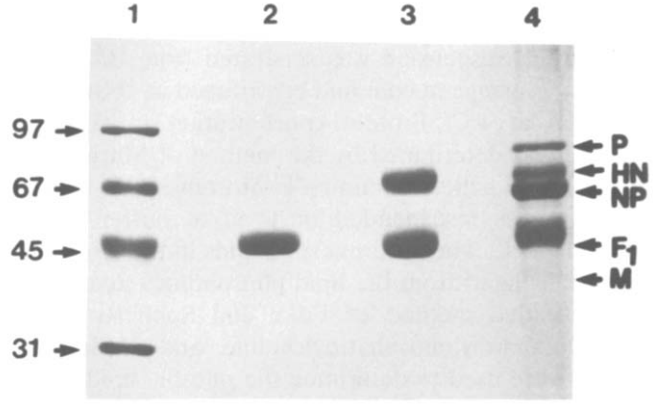

Fig. 1. Electrophoretic analysis of the F-virosomes. Molecular weight markers (Bio-Rad, low-molecular-weight range, 10 μg, lane 1), F-virosomes (20 μg, lane 2), F,HN-virosomes (40 μg, lane 3) and Sendai virus (60 μg, lane 4) were visualized by staining with Coomassie blue after separation by SDS-PAGE on a 10% (w/v) polyacrylamide gel. Protein samples were prepared in the buffer containing 1% SDS and 5% β-mercaptoethanol.

from the bromophenol blue marker dye in a 10% polyacrylamide separating gel [23]. F-virosomes were expressed throughout this study in terms of amount of F protein. About 2–3 μg of [^{125}I]lysozyme per 0.2 mg of F protein was found to be entrapped inside F-virosomes. From each mg of intact virus particles about 50 μg of F proteins were recovered, which is consistent with that reported earlier [14,21]. The phospholipid to protein ratio was estimated to be 2.1 (w/w) from the analysis of protein and lipid phosphate. Using [^{14}C]dipalmitoylphosphatidylcholine more than 95% of the total lipid was recovered after the reconstitution procedure. The amount of detergent left in the F-virosome preparation was very low, only 0.005% (w/v) as determined from the recovery of ^{3}H-labelled Tx100. The entrapped lysozyme was found to be retained inside F-virosomes for a period of 60 min at 37°C in the presence of fresh mouse plasma without any significant leakage. During heat treatment of F-virosomes, no leakage was noticed. Moreover, the F-virosome-associated [^{125}I]lysozyme was completely trypsin resistant, thereby indicating that it was trapped inside rather than adsorbed or intercalated within the virosomal membrane. F-virosomes preparation with entrapped lysozyme could also be stored at 4°C for about 10 days without any considerable leakage and loss of fusogenicity. The F glycoprotein contains Galβ1 → 4 (Fucα1 → 3)GlcNAc residues in the exposed terminal position of the complex type sugar chains [15]. This terminal galactose can be recognised by *Ricinus communis* agglutinin [30]. An exoglycosidase from a hard-shelled clam has recently been characterized which can cleave the terminal galactose residues from a glycoprotein substrate [28]. The F-virosomes were digested with exoglycosidase with a view to prepare its degalactosylated counterpart. Western blot analysis using RCA_1 and antiricin antibody confirmed the removal of substantial amount of terminal galactose from the F protein by the β-galactosidase present in the exoglycosidase (Fig. 2). The degalactosylated F-virosomes and asialoGM_1 containing virosomes were found to be active in hemolysing red blood cells in presence of WGA. Heat-treated F-virosomes were earlier reported to be fusion inactive, but retained the galactose-specific binding to HepG2 cells [17]. It is established that hemolytic property of F protein in F-virosomes is directly related to its membrane fusion potential [23]. Electron microscopy of a negatively stained preparation of F-virosomes revealed their spherical shape with size varying between 100 nm and 200 nm in diameter (Fig. 3).

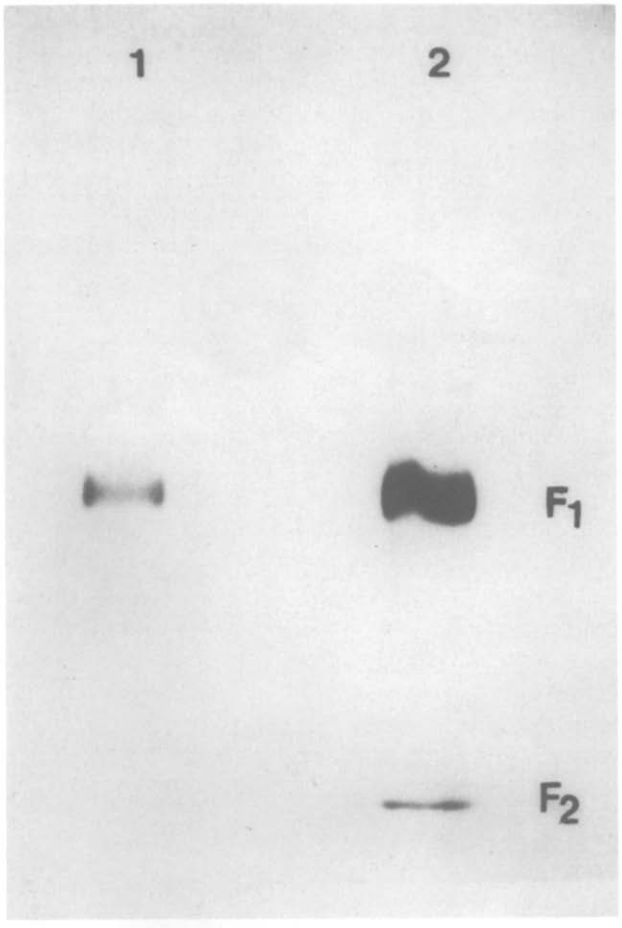

Fig. 2. Western blot of F-virosomes and degalactosylated F-virosomes. Virosomes were treated with exoglycosidase for 5 h (as described in text). 200 ng of treated (lane 1) and untreated (lane 2) virosome samples were first separated on a 10% (w/v) polyacrylamide gel as described in Fig. 1 and then transferred onto a nitrocellulose membrane by electroblotting. The blot was developed as described in Materials and Methods. Under these conditions the F_2 fragment was found to be completely degalactosylated (absence of F_2 band in lane 1).

Homing of F-virosomes in various tissues

The distribution profile of [^{125}I]lysozyme-entrapped F-virosomes in various organs and tissues was studied (Table I). Within an hour of injection through the tail vein the F-virosomes were found to be rapidly cleared from the blood circulation (Fig. 4). The amount of degalactosylated F-virosomes retained in the circulation was found to be 1.8-fold higher than that of F-virosomes at 15 min (Fig. 4). More than 80% of the injected virosomes were cleared from the circulation within 5 min after the injection as compared to 70% removal of degalactosylated virosomes during the same period. The rate of uptake of F-virosomes by the liver paralleled its clearance from the plasma. The radioactivity associated with the liver was found to be maximum (more than 50%) at 15 min after injection and subsequently declined (Fig. 5). Spleen and kidneys did not show any marked uptake. However, a significant uptake by lungs (about 10%) was observed at 15 min post-injection (Table I).

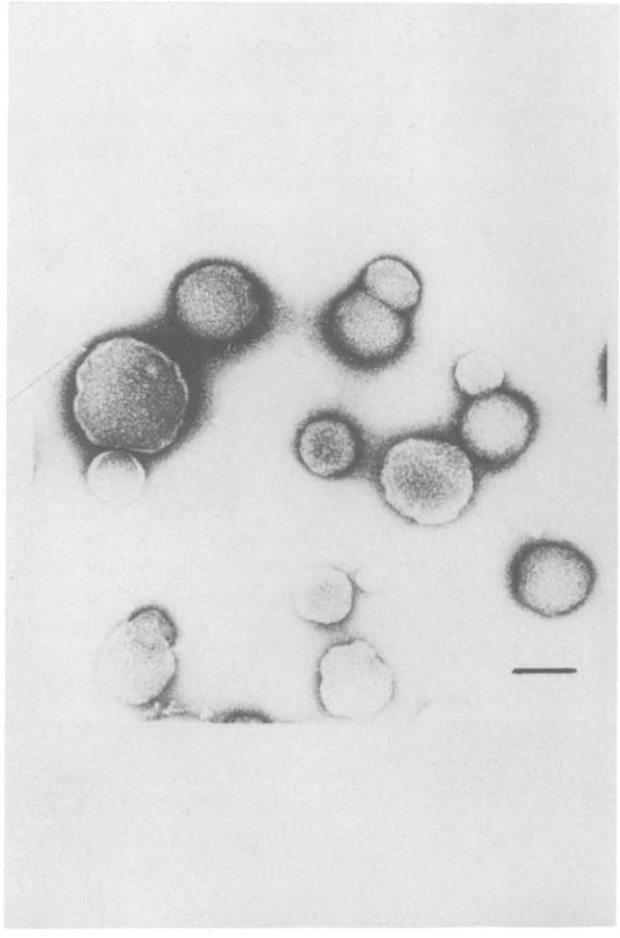

Fig. 3. Electron micrograph of F-virosomes. About 25 μl of the F-virosome suspension was put onto a carbon-coated grid (400 mesh) and allowed to adsorb for about 2 min. Excess suspension was drained out using a 25-μl capillary tube. The samples were stained with 1% aqueous uranyl acetate solution for 2 min for negative staining. The micrographs were taken in Philips EM 410 transmission electron microscope operated at 60 kV (×45500). Bar equals 100 nm.

Dynamics of uptake of [^{125}I]lysozyme-entrapped F-virosomes by liver

Dose-dependent uptake of F-virosomes by liver reached a saturation level at 0.5 mg F-virosomes. Al-

TABLE I

Kinetics of uptake of F-virosomes by various tissues

Balb/c mice were injected into tail vein with [^{125}I]lysozyme (2–3 μg lysozyme, (8–10)·10^4 cpm) entrapped in F-virosomes (0.2 mg F protein in 0.2 ml buffer B). Groups of three animals were killed at each time point and tissue samples were taken to measure the radioactivity. Results are expressed as the mean percent of the administered radioactivity in three animals ± S.D.

Tissue	% of administered radioactivity ± S.D. at various time points post-injection			
	5 min	15 min	30 min	60 min
Lung	9.3 ± 0.6	10.4 ± 0.8	6.7 ± 1.1	6.3 ± 0.3
Spleen	1.4 ± 0.1	1.0 ± 0.1	1.2 ± 0.5	1.0 ± 0.0
Kidney	1.2 ± 0.2	1.4 ± 0.1	1.9 ± 0.2	0.8 ± 0.0

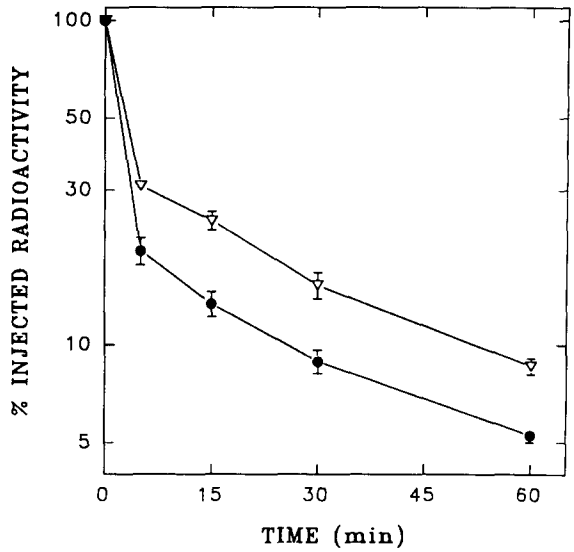

Fig. 4. Clearance of F-virosomes and degalactosylated F-virosomes from blood. Balb/c mice (30–35 g body weight) were injected intravenously with virosomes (0.2 mg F protein in 0.2 ml buffer B) containing ^{125}I-labelled lysozyme (2–3 μg lysozymes, (8–10)·10^4 cpm). At various time points, radioactivity was measured in the blood and was expressed as percent of total injected radioacitivity per total volume of blood. Each point represents the mean value (± S.D.) from three animals. F-virosomes (●); degalactosylated F-virosomes (▽).

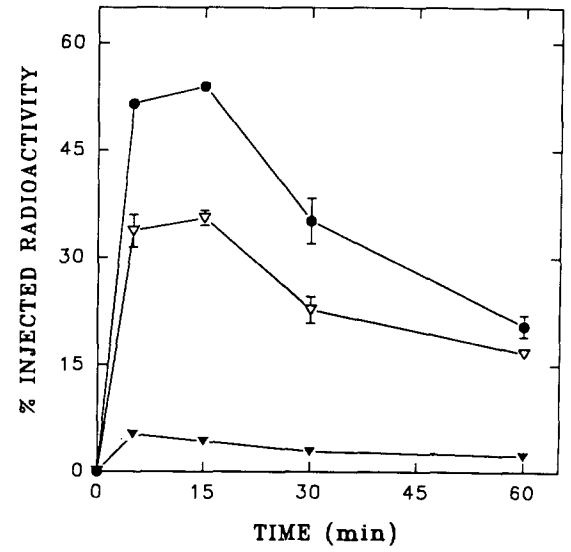

Fig. 5. Rate of uptake of F-virosomes and degalactosylated F-virosomes by liver. Virosome preparations (0.2 mg F protein in 0.2 ml buffer B containing [^{125}I]lysozyme, (8–10)·10^4 cpm) were injected intravenously. At various time points the livers were removed and washed thoroughly with PBS. After blotting with filter paper, the livers were digested in 5 ml of 30% KOH overnight. An aliquot was taken to measure the radioactivity. The results were expressed as percent of total injected radioactivity associated with total liver. Each point represents the mean from three animals (± S.D.). As a control, free [^{125}I]lysozyme (8·10^4 cpm) mixed with F-virosomes (0.2 mg in 0.2 ml buffer B) was injected and treated in the same way as above. F-virosomes (●); degalactosylated F-virosomes (▽); [^{125}I]lysozyme + F-virosomes (▼).

most half of the maximum uptake was obtained with 0.2 mg of F-virosomes. A dose of 0.2 mg of F-virosomes was used throughout these studies. The kinetics of uptake of virosomes by liver is shown in Fig. 5. At 15 min post-injection of F-virosomes, about 55% of the injected radioactivity was associated with liver, which gradually declined to nearly 20% after 60 min of injection. Only 35% of the injected radioactivity was associated with liver after 15 min of the injection of degalactosylated F-virosomes. A significant inhibition (about 41%) of the uptake by liver was noticed in case of degalactosylated virosomes (Table II). The influence of terminal galactose residues of F protein in determining the rapid uptake of virosomes was further confirmed by competition experiments with asialofetuin. When asialofetuin was injected prior to the introduction of F-virosomes, about 34% inhibition in the uptake was observed (Table II). Furthermore, asialofetuin could also inhibit the uptake of heat-treated virosomes. Maximum inhibition (62%) of uptake was observed in case of trypsin-treated F-virosomes thereby indicating the role of intact F protein in targeting the virosomes to liver cells. Trypsin treatment of intact Sendai virus and F,HN-virosomes is known to cleave the F protein and subsequently abolish the fusion activity [9,17]. On the other hand, mannan (a homo-polymer of α-mannose) completely failed to inhibit the uptake of virosomes by the liver. When asialofetuin was injected prior to the introduction of degalactosylated virosomes, the extent of inhibition was found to be further increased by about 8% (Table II). This could be probably due to an incomplete removal of β-galactose residues from virosomes by the clam exoglycosidase (as visualized in the Western blot, Fig. 2). No significant uptake by the liver was obtained when a mixture of virosomes and [^{125}I]lysozyme were injected (Fig. 5). In order to enhance the galactose-mediated uptake of F-virosomes by liver cells, asialoGM$_1$ was incorporated into the virosomal membrane with various mol% of total viral phospholipids. The radioactivity associated with the liver was found to be related to the amount of asialoGM$_1$ present in the virosomal membrane. The uptake of the asialoGM$_1$ containing F-virosomes by liver increased with the increasing amount of asialoGM$_1$ and was found to be maximum at 6 mol% (Fig. 6).

TABLE II

Effect of various treatments on the uptake of F-virosomes by liver 15 min post-injection

Balb/c mice were injected with 3.5 mg asialofetuin or asialofetuin and mannan together (3.5 mg each) in 0.2 ml buffer B, 2 min before the injection of F-virosomes (0.2 mg F protein in 0.2 ml buffer B) containing [^{125}I]lysozyme ((8–10)·10^4 cpm) through tail vein. Effect of prior injection of asialofetuin (3.5 mg) on the uptake of degalactosylated virosomes and heat-treated virosomes, both containing 0.2 mg F protein in 0.2 ml buffer B was checked. The liver-associated radioactivity in each case was measured as described in Fig. 5. Percent inhibition is calculated considering uptake of untreated F-virosomes as 100%.

Experiment	% uptake by liver	% inhibition of uptake by liver
F-virosomes	54.2±0.3	–
Trypsin-treated F-virosomes	20.5±1.5	62.0
Degalactosylated F-virosomes	32.0±1.1	41.0
Heat-treated F-virosomes	51.5±0.8	–
F-virosomes + asialofetuin	36.2±1.6	33.3
F-virosomes + asialofetuin + mannan	36.2±0.9	33.3
Degalactosylated F-virosomes + asialofetuin	27.2±1.0	49.9
Heat-treated F-virosomes + asialofetuin	30.1±0.8	41.7

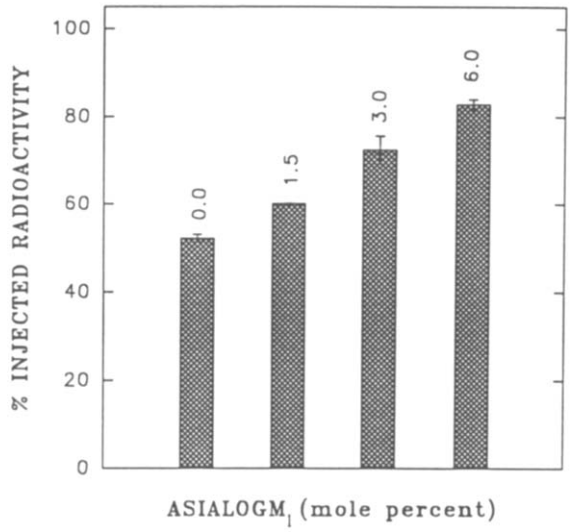

Fig. 6. Effect of incorporation of asialoganglioside-GM$_1$ on the uptake of F-virosomes by liver 15 min post-injection. Mice were injected intravenously with F-virosomes (0.2 mg F protein in 0.2 ml buffer B containing [^{125}I]lysozyme, (8–10)·10^4 cpm) containing various mol% of asialoGM$_1$. Rest of the procedure was same as described in Fig. 5. Values are mean percent±S.D. of the injected radioactivity. Each group contained three animals.

Subcellular localization of [^{125}I]lysozyme in liver cells delivered through F-virosomes

Table III shows the subcellular localization of virosome entrapped [^{125}I]lysozyme 15 min after injection under various conditions. About 30% of the total radioactivity associated with the liver was recovered in lysosomal/mitochondrial fraction. The cytosolic fraction contained about 45% of the radioactivity. 9–14% of the radioactivity was found to be localised in the membrane and nuclear fractions. The ratio of ^{125}I in the cytosolic and lysosomal/mitochondrial fractions may be viewed as an indicator of the fusion activity of the virosomes due to F proteins. AsialoGM$_1$ containing F-virosomes (6 mol%) and F-virosomes revealed a ratio of about 1.4 and 1.5, respectively. Inclusion of varying amounts (mol%) of asialoGM$_1$ in the F-virosomes did

TABLE III

Distribution pattern of radioactivity over subcellular fractions of the liver 15 min post-injection of various types of F-virosomes

Mice were injected through tail vein with different virosomal preparations (0.2 mg F protein in 0.2 ml buffer B with [^{125}I]lysozyme ((8–10)·10^4 cpm)). Subcellular fractionation was carried out as described in the text. Fractions were assayed for radioactivity. Percent recovery of radioactivity (±S.D.) in each fraction is calculated taking total liver uptake as 100%. Each group contained three animals. Varying amounts of asialoGM$_1$ (mol%) in F-virosomes showed similar subcellular distribution profile.

Virosome	% recovery of radioactivity ± S.D.			
	Nuclear	Lysosomal/ mitochondrial	Cytosolic	Membrane
F-virosomes	13.2±0.6	30.4±2.9	44.4±3.2	8.0±0.3
Degalactosylated F-virosomes	11.5±1.0	54.4±1.2	19.1±1.3	9.4±0.9
AsialoGM$_1$ F-virosomes	14.2±1.2	30.9±0.2	41.8±1.1	10.6±0.9
Heat-treated F-virosomes	13.2±0.8	59.0±0.1	12.2±0.7	10.6±0.6
Trypsin-treated F-virosomes	12.5±0.7	58.5±0.2	12.0±0.7	8.5±0.5

not seem to have any effect on fusion scenario as is evident from the ratio of radioactivity in the cytosolic and lysosomal/mitochondrial fractions. The exoglycosidase and heat-treated virosomes showed a ratio of 0.4 and 0.2, respectively. This is about 4–8-fold less than that with untreated virosomes. In other words, about 57% and 72% decrease in cytosolic radioactivity was observed in case of exoglycosidase and heat-treated F-virosomes, respectively, when compared with the untreated virosomes. Trypsin-treated virosomes showed a similar distribution pattern as that of heat-treated virosomes. These findings can be explained by taking into consideration the loss of fusion activity of heat-treated virosomes, lack of attachment to the target cells in the case of degalactosylated virosomes, and loss of both these properties in the case of trypsinized virosomes. All these virosomes are supposed to be taken up by liver cells by endocytosis/phagocytosis, leading to their localization in lysosomal fraction. It is interesting to note that in the case of F-virosomes and asialoGM$_1$ containing F-virosomes more than 88% of the radioactivity in cytosolic fractions could be recovered as TCA-precipitable counts, thereby indicating the presence of intact [^{125}I]lysozyme. Structural integrity of the [^{125}I]lysozyme is further supported by the fact that more than 90% of the cytosolic radioactivity was precipitable by a specific antibody against lysozyme. On the other hand, in case of heat-treated virosomes (with and without asialoGM$_1$) negligible amount of radioactivity in cytosolic fractions was precipitable both by TCA and the antibody. To indicate the purity of the subcellular fractions we have followed the marker enzyme analysis (Table IV) following the method of Bijsterbosch et al. [33].

TABLE IV

Analysis of marker enzymes over subcellular fractions

The subcellular fractions obtained in Table III were subjected to marker enzyme analysis. The amount of the marker enzymes were expressed in terms of specific activity as described earlier [33].

Enzyme	Specific activity (μmol/min per mg total protein)			
	Nuclear	Lysosomal/ mitochondrial	Cytosolic	Membrane
Acid phosphatase	0.013	0.25	0.02	No activity
Lactate dehydrogenase	No activity	0.09	2.7	No activity

Evidence for cytosolic delivery of [^{125}I]lysozyme into HepG2 cells by F-virosomes

It is well-known that endocytosis (not the binding) of asialoorosomucoid (a potent ligand of asialoglycoprotein receptor of HepG2 cells membrane) by HepG2 cells is totally blocked in the presence of 10–12 mM azide at 37°C [19]. HN-deficient mutants of Sendai virus (ts271) and F-virosomes have been shown to bind specifically with ASGP-R of HepG2 cells through the terminal galactose moiety of the F protein [13,17]. This galactose-mediated binding of F-virosomes leads to efficient fusion of the viral envelope with HepG2 cells

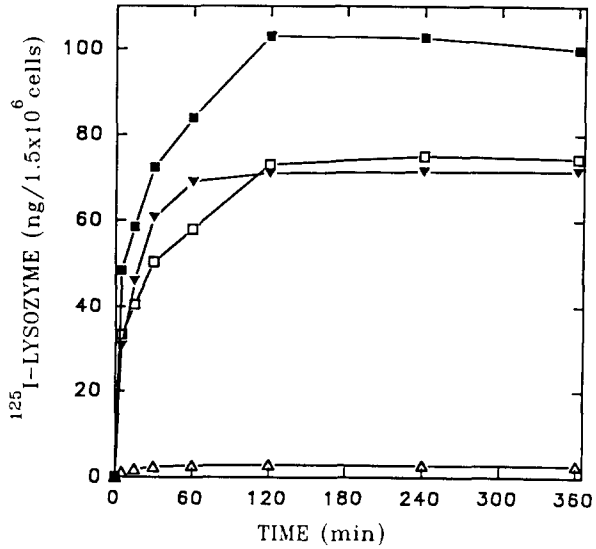

Fig. 7. Kinetics of delivery of [^{125}I]lysozyme to HepG2 cells through F-virosomes. HepG2 cells (1.5·10^6) were incubated with F- and heat-treated F-virosomes (200 μg F protein) for various times at 37°C in the presence or absence of 20 mM azide. At the indicated times, cells were analyzed for cell associated [^{125}I]lysozyme as described in the text. The figures represent the mean of duplicate determinations. (■), F-virosomes (−azide); (□), F-virosomes (+azide); (▼), heat-treated F-virosomes (−azide); (△), heat-treated F-virosomes (+azide).

at the plasma membrane level [17]. Thus, internalization of the marker due to fusion can be distinguished from endocytosis in the presence of azide (Fig. 7). About 70% of total cell associated radioactivity (EDTA resistant) is retained in the presence of azide, thereby demonstrating that introduction of lysozyme mainly occurs by fusion mode. The initial rate of uptake of lysozyme through heat-treated F-virosomes (in the absence of azide) was found to be similar to that of F-virosomes both in presence and absence of azide. On the other hand, heat-treated F-virosomes completely failed to deliver lysozyme to HepG2 cells in the presence of azide.

Discussion

Our objective here has been to design a targeted fusogenic vehicle for in vivo application using F protein of Sendai virus. We have used F-virosomes (reconstituted Sendai virus envelopes) loaded with [^{125}I]lysozyme as a radioactive marker for this purpose. The binding role of HN is shown to be replaced by the terminal galactose residues on F protein which are found to be specifically recognised by ASGP-R on the membrane of liver cells [34]. The salient feature of this work is that virosomes are found to selectively deliver their contents into liver cells in vivo through a membrane-fusion-mediated process. This is manifested in the observed localization of significant amount of the TCA and antibody-precipitable radioactivity in the cytosolic fraction of the liver homogenate. The fusion-mediated cytosolic delivery is also inferred from the internalization of the marker in HepG2 cells in the presence of azide.

The difference in the clearance pattern observed between F-virosomes and degalactosylated virosomes (Fig. 4) is found to be less than that reported in case of sugar and non-sugar-coated liposomes [3]. This may be due to an incomplete removal of galactose residues from the F protein (Fig. 2) or due to the differences in the lipid composition of the virosomes and liposomes. On the other hand, more than 53% of the radioactivity is found to be associated with the liver (Fig. 5). This is consistent with an earlier report of an in vivo distribution of ^{125}I-labelled reconstituted Sendai virus envelope in mice [35]. This indicates towards a specific interaction of the F protein with the liver cells. The membrane composition of virosomes as manifested in the phospholipid/cholesterol profile is quite similar to that of the host cell membrane, with little or no detectable amount of gangliosides of any type [36]. In contrast to a negligible uptake by spleen and kidney, a significant uptake by lungs may be attributed to the presence of 14–20% phosphatidylserine out of total phospholipids in the virosomal membrane or to a unique lipid composition of the virosomes [37].

The kinetics of uptake of the virosomes in the liver (Fig. 5) is fairly comparable to that of galactosylated liposomes as reported earlier [3]. Furthermore, substantial decrease in uptake of trypsinized and degalactosylated F-virosomes by liver is observed at 15 min after injection. Presumably this may be due to an interaction of the terminal galactose of F protein with ASGP-R of liver cells, which is a major protein on the blood-facing surface of the hepatocyte plasma membrane. Such an interaction is known to ensure a tight binding with terminal galactose of asialoglycoproteins and ASGP-R [38]. AsialoGM$_1$ containing F-virosomes exhibit an enhanced uptake by liver after 15 min of injection (Fig. 6). It is known that asialoganglioside liposomes are more efficiently taken up by the liver through the specific interaction with ASGP-R present on the cell surface [5]. The galactose specific binding of virosome to receptor (ASGP-R) on liver cells is confirmed by the inhibition of its uptake by asialofetuin (Table II). Mannan completely fails to inhibit uptake of virosomes by the liver. From these results, it seems reasonable to assume that the galactose moiety of F protein is responsible for the binding of virosomes to ASGP-R of liver parenchymal cells as was also observed in vitro with HepG2 cells [13,17]. It is further supported by the observation that more than 80% of asialofetuin is taken up by ASGP-R of parenchymal cells in vivo [39]. Particle size of 200–250 nm in diameter are reported to pass through the fenestrations of the liver endothelium [40]. However, we can not rule out the possibility of uptake of virosomes by Kupffer cells [41].

To assess the fusion potential of liver-associated virosomes, we have followed the subcellular distribution of radioactivity in total liver cells. Both the parenchymal and Kupffer cells are known to have galactose receptors on their membrane. Sendai virosomes after fusing with the target cells deliver their contents to cytosol [42]. Hence, the amount of radioactivity in cytosol particularly the ratio of cytosolic and lysosomal/mitochondrial counts offers a convenient parameter to assess the fusion potential of virosomes with liver cells. F protein loses its potential to bring about fusion after heat treatment at 56°C for 20 min [17,25]. However, it is interesting to note that even upon heat treatment of F-virosomes, galactose specific recognition is not lost [17]. This is also conceivable from the uptake of heat-treated F-virosomes by the liver and the ability of asialofetuin to inhibit it (Table II). In essence, heat-treated F-virosomes behave as proteoliposomes and are effectively endocytosed like other liganded liposomes [1,3]. Although 9–14% of the radioactivity is found to be localized in the nuclear and membrane fraction, a significantly higher amount of radioactivity is associated with cytosolic fraction in case of F-virosomes and asialoGM$_1$ containing F-virosomes.

The fusion mode of delivery of F-virosomes becomes more apparent when the ratio of radioactivity in cytosolic and lysosomal/mitochondrial fractions is compared to that of controls (degalactosylated, heat-treated and trypsin-treated F-virosomes, Table III). The TCA- and antibody-precipitable radioactivity in the cytosolic fractions is consistent with the structural integrity of [^{125}I]lysozyme, which again supports our contention regarding the delivery of F-virosomes by a fusion mode. It is important to note that in case of F-virosomes and asialoGM$_1$ containing F-virosomes, the amount of radioactivity associated with lysosomal/mitochondrial fraction (about 30%) may be due to both receptor-mediated endocytotic and phagocytotic uptake by the liver cells without membrane fusion. Partial loss of fusogenicity of virosomal F proteins during reconsitution and under in vivo conditions may also be accountable for this. This view is further supported by our in vitro studies using HepG2 cells as the target where more than 70% of the [^{125}I]lysozyme is found to be localized in cytosolic fraction. Conversely, more than 80% radioactivity is found to be associated with the lysosomal/mitochondrial fraction following the interaction of the HepG2 cells with heat-treated F-virosomes (data not shown). It is also interesting to note here that about 70% of the internalized radioactivity by HepG2 cells is through fusion mode (Fig. 7). However, heat-treated virosomes showed negligible uptake by HepG2 cells in the presence of 20 mM azide. Hence, the uptake through heat-treated virosomes is considered to be solely due to endocytosis. The heat-treated virosomes are envisaged to be taken up by liver cells in vivo by a combination of receptor-mediated endocytosis and phagocytosis while most of the trypsin-treated and degalactosylated virosomes are taken up by Kupffer cells through phagocytosis [41].

The fusion of Sendai virus with the target cells is characterized by two distinct phenomena, viz., binding and fusion [11]. In this particular case both the binding and the fusion processes are mediated by F protein in virosomes. The density of the F protein in the virosomal membrane therefore might be a key factor in controlling the fusion process. In case of Influenza haemagglutinin (HA) it is known that a threshold number of envelope protein need to be expressed on the cell membrane to induce cell fusion [43]. Both the binding and fusion functions are attributed to the HA molecules in the Influenza envelope. We may correlate the molecular behaviour of HA expressed in cells and F in virosomes. Studies are in progress to construct fusogenic liposomes with varying density of F proteins to test this phenomenon in case of liposome cell-fusion. The fusion potential of F containing immunoliposomes has been found to be highly effective in targeting a cytotoxic drug (Hygromycin B) to leukemic T cells in culture [14]. The high trapping efficiency of liposomes combined with the fusogenic property of F protein offers a versatile system for an efficient introduction of biologically-active molecules into the cellular cytosol both in vivo and in vitro, bypassing the lysosomal degradation pathway. Using this approach, chimerasome and proteoliposome-mediated gene transfer has already been reported both in vitro and in vivo [44]. In other in vivo studies, 'liposome-red blood cell ghost–Sendai virus complexes' have been successfully used to introduce and express foreign DNA into the nuclei of adult rat liver cells. Using this delivery system human insulin gene is reported to be transiently expressed in intact rat liver [45,46]. However, these highly promising in vivo studies on gene delivery lack cell type specificity. The 'Trojan horse' strategy of reconstituted Sendai virus envelope in the field of drug delivery and gene therapy is already in the limelight of modern biotechnology [47]. We are currently engaged in a critical evaluation of the efficieny of F-virosomes and fusogenic liposomes with defined specificities to deliver various biodegradable cytotoxic agents into selected cell types both in vitro and in vivo.

Acknowledgements

We thank Profs. B.K. Bachhawat and U.N. Singh for helpful discussions and careful consideration of the manuscript. We are grateful to Prof. Subhash C. Basu for his generous gift of exoglycosidase and many valuable suggestions. We do appreciate the help of Dr. Nirupam Roy Choudhury, Department of Biotechnology, Jawahar Lal Nehru University, New Delhi, for making the electronmicrograph of virosomes. Constructive criticism from Dr. V.K. Chaudhary, Mr. P. Chowdhury and expert technical assistance of Mr. Sandeep K. Tyagi are gratefully acknowledged. We also thank Mr. Rajiv Chawla, J.T.P.A., for his skillful help in preparation of the manuscript. This work was supported by Department of Science and Technology, Council of Scientific and Industrial Research and Department of Atomic Energy, Government of India.

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