The potential of reconstituted Sendai viral envelopes containing only the fusion protein (F-virosomes) was evaluated for a targeted cytosolic delivery of lysozyme to human hepatoblastoma cells (HepG2) in culture. 125I-Lysozyme loaded into F-virosomes was used to monitor its fusion-mediated transfer to the HepG2 cells. Using fusion assay based on the transfer of water soluble probe, we have demonstrated the existence of aqueous connection between F-virosomes and target cells. Target specificity of the F-virosomes was ensured by the strong interaction between terminal β-galactose moiety of F protein and the asialoglycoprotein receptor on the membrane of HepG2 cells. Incubation of the loaded F-virosomes with cells resulted in fusion-mediated injection, as inferred from the ability of cells to internalize lysozyme in the presence of azide (an inhibitor of the endocytotic process). Binding as well as fusion of the F-virosomes to HepG2 cells was solely mediated by the F protein. Introduction of 125I-lysozyme into the HepG2 cells was confirmed by selective accumulation of acid and antibody-precipitable radioactivity in the cytosolic compartment. The structural integrity of the internalized lysozyme was also assessed. The potential usefulness of F-virosomes with defined specificities as biological carriers for both in vitro and in vivo cytosolic delivery of macromolecules and drugs has been established.

Sendai, an enveloped animal virus, contains two glycoproteins (hemagglutinin neuraminidase and fusion factor (F)) in the outer leaflet of its lipid bilayer (1). Entry of virus particles into cells involves recognition of specific cell surface receptors followed by membrane fusion resulting in injection of viral nucleocapsid into the intracellular space (2). The hemagglutinin neuraminidase protein is endowed with the ability to recognize the receptor and is a sialidase. F protein is absolutely required for the induction of virus-cell fusion (3). Reconstituted Sendai viral envelopes (fusion factor and hemagglutinin neuraminidase virosomes) are known to fuse efficiently with the plasma membrane of target cells and have served as excellent carriers for fusion-mediated microinjection of macromolecules such as DNA, RNA, toxins, and polypeptides into viable cultured cells (4, 5). Reconstituted Sendai viral envelopes-mediated delivery utilizes the binding property of hemagglutinin neuraminidase 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 (6). In the last few years, numerous attempts have been made to construct targeted vehicles by coupling specific ligands/antibodies to phospholipid liposomes (7, 8). Since liposomes are internalized in the recipient cells primarily via receptor-mediated endocytosis and/or phagocytosis (9), the delivery of biologically active molecules to extralysosomal sites demands the construction of vehicles that can bypass the lysosomal route.

Fusogenic vesicles such as reconstituted Sendai viral envelopes offer a powerful tool for introduction of macromolecules directly into the cytosol of target cells (10). In the selective delivery of virosomal contents to desired cell types in vitro, the envelope has been modified by replacing the hemagglutinin neuraminidase-mediated binding with specific antibodies or ligands against cell surface antigens or receptors (4, 11-15). It has also been demonstrated that hemagglutinin neuraminidase of Sendai virus has little or no role to play in F protein-mediated membrane (virosome/fusogenic liposome) fusion (15, 16). A Sendai mutant deficient in hemagglutinin neuraminidase protein has been shown to infect HepG2 cells, a line that expresses abundant asialoglycoprotein receptor (ASGPR) in culture (17). Using an assay based on the dequenching of a lipid probe incorporated in the virosomal membrane, we have also confirmed that, although hemagglutinin neuraminidase enhances F protein-mediated membrane fusion of virosomes with target cells, it is not essential for the fusion process per se (18). However, the information regarding the core connection between virosomes and target cells is yet to be established. F is a glycoprotein containing biantennary chains with terminal galactose moieties (19). The β-galactose-terminated glycoproteins are known to be specifically recognized by the ASGPR on the HepG2 cell's plasma membrane with high affinity (Kd = 7 x 10^-9 M) (20). In the present study, we have prepared hemagglutinin neuraminidase-depleted Sendai viral envelopes (F-virosomes) loaded with 125I-lysozyme. We demonstrate that F-virosomes can specifically mediate the transfer of 125I-lysozyme into the cytosol of HepG2 cells through a natural receptor/ligand interaction. We report for the first time the delivery of aqueous probe from virosomes to target cells in a systematic and quantitative fashion. The efficacy of this delivery system with respect to antigen presentation and gene therapy is discussed.

MATERIALS AND METHODS
Lysozyme (chicken egg white, IUB 3.2.1.17), trypsin (type III), Clostridium perfringens neuraminidase (Type V), dithiothreitol,
wheat germ agglutinin, diaminobenzidine, phenylmethylsulfonyl fluoride, sodium azide, EDTA (dissodium salt), and Nigrosin were purchased from Sigma. Bio-Beads (SM2) were obtained from Bio-Rad. Triton X-100 was from Aldrich. Exoglycosidase (Mercenaria mercenaria, hard shelled clam) was a kind gift from Prof. Subhash Basu (University of Delhi South Campus, New Delhi) and Carrier-free Na131I was supplied by Bhabha Atomic Research Centre, Bombay, India. DMEM, DPBS, Fetal bovine serum and Triton X-100 was from Aldrich. Exoglycosidase (Mercenaria mercenaria) were also of the highest grade commercially available.

The virus was harvested and purified as described earlier (21). Purified virus was resuspended in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4). Viral yield was estimated in terms of protein according to Markwell et al. (22), and its activity was checked by agglutination and lysis of mouse red blood cells (18). Aliquots of the virus were stored at -70°C.

Cells—HepG2 cells (human hepatoblastoma cell line) were obtained from American Type Culture Collection and were used as described earlier (18). CHO cells (provided by Dr. P. C. Ghosh, University of Delhi South Campus, New Delhi) were grown at 37°C, 5% CO2 in DMEM. Single cell suspensions were obtained by treatment of the cultures with trypsin (23). Monolayer cells were washed thrice with DPBS. Fresh red blood cells were prepared from healthy Swiss albino mice.

Radioiodination of Lysozyme—Lysozyme was radiolabeled by chloramine-T method, using Na131I as the iodine source (24). The specific activity of labeled protein was calculated from the trichloroacetic acid-precipitable counts and expressed as cpm/mg protein.

Double Labeling of Lysozyme—Lysozyme was biotinylated using NHS-LC-biotin and the biotin-conjugate streptavidin was obtained from ICN Biomedicals, Inc. NHS-LC-biotin and the biotin-lysozyme conjugate was further radiiodinated as described above. Biotinylation was carried out for 4 h at room temperature in boro buffer (0.1 mM borate, pH 8.8). The reaction was stopped by addition of 1 M NH4Cl (25). Biotinylated samples were extensively washed with PBS. The biotin-labeled protein was characterized by Western blotting (26) using streptavidin-HRP conjugate as well as by autoradiography.

Preparation of Loaded F-virosomes—F-virosomes were prepared as described earlier (18). In brief, Triton X-100-solubilized fraction of the virus was mixed either with 100 μg of 125I-lysozyme or double-labeled lysozyme (5 x 105 cpm/mg protein) and reconstituted by stepwise addition of SM2 Bio-Beads in a ratio of 1:8 (detergent:beads, w/w) for a period of 6 h. The unentrapped protein was separated by repeated centrifugation at 100,000 x g for 1 h at 4°C. Radioactivity associated with the supernatant was estimated in a 1275 Gamma Counter (LKB, Wallac). The entrapped 125I-lysozyme served as virosonal aqueous marker. Intercalation of lysozyme into the membrane was checked by trypsin digestion of the virosomes (27). Heat-treated and degalactosylated virosomes were used as controls. Heat inactivation of virosomes was carried out by incubation at 56°C for 20 min (28). Heat-inactivated virosomes were prepared by treating F-virosomes with exoglycosidase having both a- and b-galactosidase activities, for 5 h at 37°C (29). Trypsinization of F-virosomes was carried out as described by Loyter et al. (5). Stability of the virosomes was ascertained by their ability to bring about lysis of mouse red blood cells (18). After washing and EDTA stripping, subcellular fractions and whole cell preparations were incubated with cells (9 x 106) for 20 min at 37°C with and without azide. After EDTA stripping, cells were resuspended in 6 ml of DPBS and distributed in six tubes for incubation at 37°C. At various time points, tubes were removed and kept in ice. The cells were washed with DPBS at 4°C and solubilized in the extraction buffer. The trichloroacetic acid-insoluble radioactivity was determined as described above. Degradation was monitored by the decrease in trichloroacetic acid-insoluble radioactivity and compared with that of control F protein and F-virosomes.

Autoradiography and Western Blot Analysis of Microinjected Lysozyme—Cell-associated 125I-lysozyme and double-labeled (biotin-, 125I-) lysozyme were extracted as described above and analyzed by SDS-PAGE in a 12.5% separating gel (55). The gels were fixed, dried, and stained (36).

The virosome-cell mixtures were washed with cold DPBS thrice and finally incubated at 4°C for 2 min in DPBS containing 5 mM EDTA (stripping) to remove the cell surface-bound (unbound) virosomes. The cell pellet after EDTA stripping was solubilized in the extraction buffer (10 mM Tris-Cl, 150 mM NaCl, pH 7.4, containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) and the cell-associated radioactivity was determined. Degradation products of endogenous 125I-lysozymes were identified by SDS-PAGE and the cell pellet in accordance with the method described by Goldstein and Brown (31).

Effect of Specific Ligand and Metabolic Inhibitor on the Binding and Fusion of F-virosomes with HepG2 Cells—Binding assays were performed by incubating virosomes with cells in DPBS containing Ca2+ and Mg2+ at 4°C for a period of 60 min followed by three washings at 4°C with the same buffer. Cell pellet was solubilized in extraction buffer and cell-associated radioactivity (bound but not internalized) was measured in a y counter. Nonspecific binding was determined by treating the virosome/HepG2 complex with 5 mM EDTA, which is known to dissociate the ASGP-R-ligand interaction (23). In another control experiment, the F-virosome/HepG2 cell complex was subjected to neuraminidase treatment before incubating with 5 mM EDTA to check the binding due to any residual hemagglutinin neuraminidase protein in the virosomal preparation. Binding and fusion assays were carried out in the presence of asialoorosomucoid, a potent ligand of ASGP-R on the plasma membrane of liver cells (23). HepG2 cells (1.5 x 106) were preincubated with varying amounts of asialoorosomucoid at 4°C for 30 min followed by a further incubation with loaded F-virosomes (200 μg of F protein) at 4°C for 1 h. After washing, the reaction mixture was kept at 4°C for 15 min. Internalized radioactivity (EDTA-resistant) was measured in the cell pellet after the fusogenic-mediated transfer. Interaction of trypsinized and degalactosylated F-virosomes with HepG2 cells was carried out similarly. Sodium azide is known to be an efficient inhibitor of endocytic process (23). Binding and fusion of F-virosomes and heat-treated F-virosomes with HepG2 cells were studied in the presence of varying concentrations of azide. Interaction of F-virosomes with CHO cells that lack the ASGP-R receptor (32) was also studied and compared with that of HepG2 cells to demonstrate the specific binding of terminal galactose of F protein to this receptor. The F-virosomes (untreated or heat-treated, 200 μg of F protein in each case) were incubated with HepG2 cells (1.5 x 106) at 37°C for 30 min. After washing three times with DPBS and EDTA stripping, the cells were subjected to subfractionation following a published procedure (33). In brief the cells were resuspended in the isotonic homogenizing buffer (0.01 M Tris-Cl, pH 7.4, containing 0.25 mM sucrose) and then dispersed in a Potter-Elvehjem type homogenizer at 4°C. The isolation of nuclear, lysosomal/mitochondrial, and plasma membrane fractions was carried out by differential centrifugation at 800, 15,000, and 100,000 x g at 4°C for 10, 30, and 60 min, respectively. The supernatant obtained after the final centrifugation (100,000 x g). Subcellular fractions were further characterized by marker enzyme analysis (34). Radioactivity associated with each fraction was analyzed and expressed as percent of total amount of radioactivity associated with the whole fraction obtained by trichloroacetic acid precipitation.
and autoradiographed. In a parallel experiment, Western blotting (26) of the double-labeled lysozyme was carried out with HRP-labeled streptavidin.

RESULTS

Characterization of Loaded F-virosomes—The purity of the virosomal preparations was checked by SDS-PAGE analysis as shown earlier (18). The F glycoprotein contains Gal[1→4](Fuca1→3)GlcNAc sequence at the termini of the glycan chains (19), which can be recognized by Ricinus communis agglutinin I (29). Western blot analysis using RCA1 and anti-ricin antibody confirmed the removal of substantial amount of terminal galactose from the F protein by the β-galactosidase activity associated with the exoglycosidase as described earlier (29). Virosome associated 125I-lysozyme was trypsin-resistant, indicating thereby that it was entrapped rather than adsorbed or intercalated within the virosomal membrane. The encapsulated 125I-lysozyme was found to remain intact during the fusion conditions employed throughout. No detectable leakage of the marker was observed from untreated, heat-treated, or trypsinized, and degalactosylated F-virosomes over a period of 10 h at 37°C after entrapment. F-virosomes and degalactosylated F-virosomes were found to be fusogenic as observed by their ability in hemolyzing red blood cells, while the heat-treated and trypsinized F-virosomes were fusion-inactive. Electron microscopy of negatively stained preparations of virosomes revealed their spherical shape with size varying between 100 and 200 nm in diameter (18).

Specificity of Interaction of F-virosomes with ASGP-R of HepG2 Cells—The binding of F-virosomes as inferred from the amount of 125I-lysozyme associated with HepG2 cells at 4°C followed a dose-dependent saturation type curve (Fig. 1, inset). This interaction was completely (>98%) abolished in the presence of 5 mM EDTA. The binding of F-virosomes to the cells was strongly (>90%) inhibited in the presence of 40 μg/ml ASOR (Table I). Similarly degalactosylated F-virosomes failed to bind and subsequently fuse with HepG2 cells (Fig. 2). As much as 96% reduction in the internalization of lysozyme was observed with degalactosylated F-virosomes. Trypsinized virosomes showed a similar behavior. Further-

![Figure 1](image1.png)

**Fig. 1.** Dose-dependent internalization of 125I-lysozyme into HepG2 cells delivered through F-virosomes. Different amounts of F-virosomes (in terms of F protein) were incubated with HepG2 cells (1.5 × 10^6) in DPBS (containing Ca^2+; Mg^2+) at 4°C for 1 h followed by washing and further incubation for 15 min at 37°C. 125I-Lysozyme associated with the cells was determined after "EDTA stripping" as described under "Materials and Methods." Each point represents mean (±S.D.) of three independent determinations. Inset shows the amount of lysozyme (average of duplicate determinations) associated with the cells at 4°C with and without EDTA stripping.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>125I-Lysozyme (ng/1.5 × 10^6 HepG2 cells)</th>
<th>125I-Lysozyme (ng/1.5 × 10^6 CHO cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-virosomes (37°C, 15 min)</td>
<td>57.05</td>
<td>2.20</td>
</tr>
<tr>
<td>F-virosomes (+20 μg/ml ASOR, 37°C, 15 min)</td>
<td>6.30</td>
<td>2.80</td>
</tr>
<tr>
<td>F-virosomes (+40 μg/ml ASOR, 37°C, 15 min)</td>
<td>2.80</td>
<td>2.80</td>
</tr>
<tr>
<td>F-virosomes (4°C, 1 h)</td>
<td>108.35</td>
<td>3.00</td>
</tr>
<tr>
<td>F-virosomes (+40 μg/ml ASOR, 4°C, 1 h)</td>
<td>10.40</td>
<td>10.40</td>
</tr>
<tr>
<td>F-virosomes (heat) (4°C, 1 h)</td>
<td>107.20</td>
<td>107.20</td>
</tr>
<tr>
<td>F-virosomes (heat) (+40 μg/ml ASOR, 4°C, 1 h)</td>
<td>10.50</td>
<td>10.50</td>
</tr>
</tbody>
</table>

* — not detected.

![Figure 2](image2.png)

**Fig. 2.** Effect of degalactosylation of F-virosomes on internalization of 125I-lysozyme into HepG2 cells. Degalactosylated (vertical lines) and untreated F-virosomes (cross-hatched) (with varying amounts of F protein) were incubated with HepG2 cells (1.5 × 10^6) for 15 min at 37°C. Cell-associated radioactivity was measured as described under "Materials and Methods." The values are mean of duplicate determinations. Trypsinized virosomes behaved similarly as degalactosylated virosomes.

more, trypsinized HepG2 cells failed to bind to F-virosomes. The amount of CHO cell-associated 125I-lysozyme was negligibly small as compared to that observed with HepG2 cells (Table I).

Internalization of 125I-lysozyme into HepG2 Cells Delivered through F-virosomes: Fusion Versus Endocytosis—Uptake of 125I-lysozyme by cells at 37°C was examined as a function of dose of F-virosomes. Fig. 1 shows a saturation type curve representing EDTA resistant cell-associated lysozyme. Half of the maximum cell-associated radioactivity was obtained with about 200 μg of F-virosomes and was employed in all subsequent experiments. Both F-virosomes and heat-treated F-virosomes were able to bind to HepG2 cells to the same extents at 4°C (Table I). This suggests that heat denaturation of F protein has no effect on its recognition by ASGP-R. However, heat treatment of F protein renders it fusion-inactive, and hence heat-treated virosomes behaved as liganded
liposomes. It is well known that endocytosis is almost totally blocked in the presence of 10–12 mM azide at 37 °C (23). Thus internalization of the marker due to fusion can be discerned from endocytosis in the presence of azide. Fig. 3 shows the effect of azide on the interaction of F-virosomes with cells. About 32% reduction in internalization was observed in the presence of 10 mM azide and remained constant thereafter. However, no effect on binding was observed. The observed internalization of lysozyme in the presence of 10 mM azide was apparently due to an effective fusion of virosomes with HepG2 cells induced by F protein at the plasma membrane level. Comparing the uptake with F-virosomes (without azide) the maximum internalization by fusion mode was found to be about 70% (Fig. 3A). However, in case of heat-treated F-virosomes the uptake was negligible in the presence of 10 mM azide at 37 °C (23). Thus about 73% of the total cell-associated radioactivity was recovered in the cytosolic fraction using F-virosomes as a marker. Furthermore, the recovered cytosolic radioactivity could be precipitated (>98%) by 10% trichloroacetic acid and anti-lysozyme antibodies. On the contrary, 90% of the radioactivity was recovered in lysosomal/mitochondrial fraction with a

![Fig. 3. Effect of azide on internalization of 125I-lysozyme into HepG2 cells delivered through F-virosomes.](image)

**Fig. 3.** Effect of azide on internalization of 125I-lysozyme into HepG2 cells delivered through F-virosomes. F-virosomes (200 μg of F protein) were incubated with HepG2 cells (1.5 × 10^6/ml) containing various concentrations of sodium azide either at 37°C for 15 min (A) or 4°C for 1 h (B). Specific binding at 4°C was assessed as described under "Materials and Methods." Internalization was determined as cell-associated 125I-lysozyme. Each value is a mean of duplicate determinations.

Thus, the uptake was negligible in the presence of 10 mM azide at 37 °C. An approximately linear increase in the amount of EDTA-resistant internalization was found to be remarkably faster (4-5-fold higher than that observed in case of F-virosomes, Table III). This value agreed with the receptor-mediated endocytosis of F-virosomes. There was no detectable degradation of 125I-lysozyme encapsulated in virosomes in the absence of HepG2 cells when maintained under identical conditions. It is important to note that most of the intracellular radioactivity due to 125I-lysozyme (>98%) could be recovered in trichloroacetic acid-insoluble fractions and immunoprecipitates. A qualitative assessment of the integrity of 125I- and biotinylated 125I-lysozyme associated with the cells at various time points was done by autoradiography (Fig. 4B) and Western blotting (data not shown), respectively.

**Evidence for Fusion-mediated Microinjection of Lysozyme into HepG2 Cells by F-virosomes**—As stated above, about 70% of total amount of radioactivity (EDTA-resistant) associated with the cells was retained in the presence of azide, indicating thereby the vital role played by the fusion mode in the delivery of lysozyme by F-virosomes. Table III shows the distribution of radioactivity among various organelles of HepG2 cells. About 73% of the total cell-associated radioactivity was recovered in the cytosolic fraction using F-virosomes as a carrier. Furthermore, the recovered cytosolic radioactivity could be precipitated (>98%) by 10% trichloroacetic acid and anti-lysozyme antibodies. On the contrary, 90% of the radioactivity was recovered in lysosomal/mitochondrial fraction with a

![Fig. 4. A. kinetics of accumulation of 125I-lysozyme into HepG2 cells delivered through virosomes.](image)

**Fig. 4.** A. kinetics of accumulation of 125I-lysozyme into HepG2 cells delivered through virosomes. HepG2 cells (1.5 × 10^6) were incubated with F-virosomes and heat-treated F-virosomes (200 μg of F protein) for varying times at 37 °C in the presence or absence of 10 mM azide. At the indicated time points, cells were analyzed for cell-associated 125I-lysozyme as described under "Materials and Methods." The figures represent the mean of duplicate determinations of cell-associated 125I-lysozyme as described under "Materials and Methods." The figures represent the mean of duplicate determinations of cell-associated 125I-lysozyme as described under "Materials and Methods."
concomitant decrease (96%) in the cytosolic radioactivity in case of heat-treated F-virosomes. These results strongly support the fusion-mediated delivery of lysozyme by F-virosomes. Heat-treated F-virosomes being non-fusogenic are likely to be taken up by endocytosis leading to their accumulation and subsequent degradation in lysosomes.

Degradation of Proteins Microinjected into HepG2 Cells by F-virosomes—Fig. 5 shows the rate of degradation of $^{125}$I-lysozyme and biotinylated $^{125}$I-lysozyme into HepG2 cells over a period of 6 h. As expected, the trichloroacetic acid-precipitable radioactivity associated with the cells decreased progressively, indicating the degradation of injected proteins to acid-soluble products. The rate of degradation of $^{125}$I-lysozyme was found to be slightly higher (~5%) than that of biotin-labeled $^{125}$I-lysozyme. Strong inhibition of degradation was observed in the presence of 10 mM azide. A qualitative follow-up of the integrity of lysozyme and biotinylated lysozyme was demonstrated by autoradiography and Western blotting, respectively (data not shown).

**DISCUSSION**

We have presented evidence to demonstrate the potential of F-virosomes in the fusion-mediated delivery of entrapped aqueous markers to the cytosol of human hepatoblastoma cells (HepG2). F, the only glycoprotein of these virosomes, contains terminal galactose moieties (19), which constitute a unique ligand for recognition by ASGP-R on the membrane of HepG2 cells. As a result of this specific attachment, the F-virosomes were found to be slightly higher (4%) than that of biotin-labeled $^{125}$I-lysozyme. Heat-treated F-virosomes were found to be competence higher (~5%) than that of biotin-labeled $^{125}$I-lysozyme. Strong inhibition of degradation was observed in the presence of 10 mM azide. A qualitative follow-up of the integrity of lysozyme and biotinylated lysozyme was demonstrated by autoradiography and Western blotting, respectively (data not shown).

**TABLE II**

Kinetics of degradation of $^{125}$I-lysozyme in HepG2 cells delivered through virosomes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Media Cells</th>
<th>Media Cells</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
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<tr>
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<tr>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>360</td>
<td></td>
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</tr>
</tbody>
</table>

**TABLE III**

Distribution pattern of microinjected $^{125}$I-lysozyme over subcellular fractions of HepG2 cells

The distribution pattern of microinjected $^{125}$I-lysozyme over subcellular fractions of HepG2 cells is shown in Table III. The results demonstrate the specific binding of F-virosomes to the HepG2 cells.

**TABLE IV**

Recovery of radioactivity (± S.D.)

<table>
<thead>
<tr>
<th>Virosomes</th>
<th>Recovery of radioactivity (± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear (%)</td>
</tr>
<tr>
<td>F-virosomes</td>
<td>12.0 ± 0.92</td>
</tr>
<tr>
<td>Heat-treated F-virosomes</td>
<td>5.0 ± 0.16</td>
</tr>
</tbody>
</table>
protein-mediated fusion. Hemagglutinin neuraminidase is the carbohydrate moiety of the sialoglycoconjugates of the target cells (6). The intact virus and the hemagglutinin neuraminidase-containing virosomes can be completely detached from target cells (after binding at 4 °C) by treating with exogenous neuraminidase (41). Following the same protocol, we failed to dissociate the F-virosome-HepG2 complex with neuraminidase, whereas 5 mM EDTA could almost completely (>98%) detach the F-virosomes from HepG2 cells (Fig. 1, inset).

The evidence in support of fusion-mediated delivery of the entrapped markers at various time points comes from the integrity of the 125I-lysozyme as revealed by autoradiography (Fig. 4B) and Western blot analysis. We present further evidence of fusion by comparing the kinetics of degradation of internalized markers in HepG2 cells delivered by native and heat-treated virosomes (Table II). Degradation kinetics corresponding to heat-treated virosomes appears similar to that of 125I-ASOR in HepG2 cells (23). In the studies on subcellular localization of trichloroacetic acid-insoluble and antibody-precipitable radioactivity (intact 125I-lysozyme), more than 72% of the internalized marker (EDTA-resistant) is found to be associated with the cytosolic fraction (Table III). This is in contrast to heat-treated F-virosomes where most of the radioactivity (>88%) is retained in lysosomal/mitochondrial compartment. This constitutes the most convincing evidence in support of cytosolic delivery mediated by F-virosomes and is consistent with an earlier report of microinjection of 125I-bovine serum albumin to rat hepatocytes (primary culture) through erythrocyte ghosts and uv-inactivated Sendai virus (42). Both self and non-self proteins are degraded by a well characterized ubiquitin-dependent protease complex in the cytosol (43). This degradation process is dependent on metabolic energy. About 12-20% of the internalized marker is found to be degraded after 6 h of post-fusion incubation at 37 °C (Fig. 5), which is comparable to the degradation rate of lysozyme microinjected into various cell lines by other methods (44). We further checked the effect of biotinylation on the degradation of the marker protein. Biotinylated 125I-lysozyme show reduced susceptibility to degradation at 6 h post-fusion when compared to non-biotinylated 125I-lysozyme (Fig. 5), which is in agreement with the proposed mechanisms of ubiquitin-dependent proteolysis (44). On the other hand, practically no degradation is noticed when the microinjected cells are incubated in presence of 10 mM azide. Autoradiograph and Western blot also exhibit integrity and stability of the internalized markers. This again provide a substantive proof to the fusion-mediated cytosolic delivery.

The fusion potential of F-virosomes and fusogenic liposomes has been found to be highly effective in the targeting of a cytotoxic drug (hygromycin B) to the HepG2 cells in culture (45). 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 “liposome-red blood cell ghost-Sendai virus complexes” have been used to introduce and transiently express insulin gene in rat liver in vivo (46, 47). The F-virosomes by virtue of their dual property may be a promising tool for selective targeting and cytosolic delivery of bioactive molecules to liver cells in vivo. Our recent studies using mouse model system show the ability of F-virosomes to deliver 55-60% of the entrapped marker to the cytosol of liver cells (29). The “Trojan Horse” strategy of reconstituted Sendai virus envelope in the field of drug delivery and gene therapy both in vitro and in vivo is already in the limelight of modern biotechnology (10). The carbohydrate moiety of F protein may present a unique site for attaching antibodies, hormones, and other suitable ligands to generate specificity on F-virosomal membrane and fusogenic liposomes for both in vitro and in vivo applications. This method of cytosolic delivery may also be useful to study the degradation and mechanism of processing of various antigens as far as the class I mode of

FIG. 5. Degradation profile of 125I-lysozyme and double-labeled (biotin, 125I-) lysozyme in HepG2 cells delivered through F-virosomes. HepG2 cells (9 x 10^6) were microinjected with 125I-lysozyme (circles) and double-labeled lysozyme (triangles) by F-virosomes (1.2 mg of F protein) for 30 min at 37 °C. Cells were EDTA-stripped, resuspended in 6 ml of cold DPBS (+Ca++, +Mg++), distributed among six tubes, and incubated at 37 °C. At time indicated, tubes were removed and degradation was checked by determining remaining cell-associated radioactivity as described under “Materials and Methods” and expressed as a percentage of control (kept at 4 °C and taken as 0 min). Effect of azide on degradation of injected proteins (125I- and double-labeled) was also checked by incubating cells for various times in the presence of 10 mM azide (squares). The values are average of two independent determinations.
presentation is concerned (48). Although pH-sensitive liposomes have been used for the processing and presentation of entrapped soluble antigen in a major histocompatibility complex class I-dependent manner, the efficiency of this cytosolic delivery system is yet to be improved (49). Work is under way to study the mechanism of class I mode of antigen presentation using F-virosomes as a delivery vehicle. Study to understand the molecular mechanism of fusion of viral envelope with membrane of target cells induced by F protein is also in progress. Nonetheless, the molecular behavior of F protein in the viral envelope is found to be analogous to that of influenza hemagglutinin expressed in fibroblast cell line in inducing fusion with red blood cells (50).

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REFERENCES