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Targeted delivery of hygromycin B using reconstituted Sendai viral envelopes lacking hemagglutinin-neuraminidase

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Hygromycin B was encapsulated in reconstituted Sendai viral envelopes containing only the fusion (F) protein (F-virosomes). Incubation of loaded F-virosomes with cultured HepG2 cells resulted in fusion mediated delivery of hygromycin B to the cell cytoplasm, as was inferred from inhibition of DNA synthesis. Binding of the F-virosomes to HepG2 cells was mediated by the interaction of terminal β-galactose residues of fusion protein with asialoglycoprotein receptor on HepG2 cells, subsequently leading to fusion between the two membranes. The cytotoxic effect of hygromycin B enclosed in F-virosomes was comparable with that of F,HN-virosomes containing both hemagglutinin-neuraminidase (HN) and F protein and F,HN_{red}-virosomes containing HN whose disulfide bonds were irreversibly reduced (HN_{red}). Hygromycin B loaded fusogenic liposomes were prepared by coreconstituting the viral envelope containing only fusion protein with exogenous lipids. These fusogenic liposomes were found to be more active than F-virosomes at the same fusion protein concentrations.

Sendai; Reconstitution; Targeting; Fusion; Hygromycin B

1. INTRODUCTION

A major problem in the delivery of drugs and other macromolecules into the cells is crossing the permeability barrier imposed by the plasma membrane. Numerous carriers have been employed in the search for an effective delivery system that may allow the transfer of their contents into the cytoplasm of specific cells [1,2]. In the last few years, closed lipid bilayer vesicles (liposomes) have been extensively used as biological carriers for introducing biologically active molecules into living cells. It appears, however, that liposomes, like other particulate matter, are taken up by endocytosis and hence do not efficiently introduce their contents into the cytoplasm [3].

One of the most promising approaches for this purpose involves the use of reconstituted Sendai viral envelopes (RSVE). Fusion of loaded RSVE is promoted by the viral envelope glycoproteins, which results in microinjection of RSVE contents into the cell cytoplasm [1]. Binding of RSVE to cell membrane receptors is mediated by the viral HN glycoprotein, whereas for the induction of envelope–cell fusion the F protein is essential [1]. During recent years efforts have been made to construct a targeted RSVE. It has been shown that specific antibodies or ligands against cell surface antigens or receptors can substitute for the viral binding protein to mediate functional binding between the viral envelopes and cell membranes [1]. We have recently shown that HN protein is not essential for mediating the viral fusion process [4]. A Sendai mutant deficient in HN protein (ts271) has been shown to infect HepG2 cells in culture [5]. F is a glycoprotein and contains terminal galactose moieties [6] which can specifically recognise asialoglycoprotein receptor on the membrane of HepG2 cells [4,5].

In the present work, we have entrapped hygromycin B (an aminoglycoside antibiotic active against both prokaryotic and eukaryotic cells) within HN depleted RSVE (F-virosomes). We show that these loaded F-virosomes can effectively fuse with HepG2 cell membranes and microinject the encapsulated antibiotic in the cell cytoplasm. We further show the enhancement in the biological activity of hygromycin B when entrapped in fusogenic liposomes containing only the F protein of Sendai virus.

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Abbreviations: ASGP-R, asialoglycoprotein receptor; D-MEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate buffered saline; Tx100, Triton X-100; DTT, dithiothreitol; ID_{50}, inhibitory dose; RBC, red blood cell; WGA, wheat germ agglutinin; poly(U), polyuridylic acid.

2. MATERIALS AND METHODS

2.1. *Materials*

Hygromycin B, DTT, WGA and cholesterol were purchased from Sigma Chemical Co., USA. Egg phosphatidylcholine was obtained from Avanti Polar Lipids, USA. Rabbit reticulocyte lysate was procured from Promega Corp., USA. [^{14}C]Phenylalanine and [^{3}H]thymidine were from NEN Research Laboratories, USA. Poly(U) was obtained from Boehringer Mannheim, Germany. Triton X-100 was

available from Aldrich, USA. Bio-Beads (SM-2) was purchased from Bio-Rad, USA. Exoglycosidase (*M. mercenaria*, hard-shelled clam) was a kind gift from Prof. Subhash C. Basu, Notre Dame, IN, USA. All other reagents used were of analytical grade.

2.2. *Virus*

Sendai Virus was isolated and its activity was determined as previously described [7].

2.3. *Cells*

HepG2 cells (human hepatoblastoma cell line from ATCC, USA) were grown in monolayers at 37°C, 5% CO_2 in D-MEM (Gibco, USA) containing 4 mM L-glutamine, 25 mM HEPES, 110 mg/l sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate in 75 cm^2 plastic bottles (Nunc, USA).

2.4. *Construction of loaded virosomes and fusogenic liposomes*

F-Virosomes were prepared following the method of Tomasi and Loyter [8] with modifications [4]. Triton X-100 solubilised fraction of the virus was mixed with hygromycin B (0.25 M final concentration in buffer containing 10 mM Tris-HCl, 150 mM NaCl, 2 mM Ca^{2+}, 2 mM Mg^{2+}, pH 7.4) and reconstituted by stepwise addition of SM-2 Biobeads as described earlier [4]. This resulted in the formation of hygromycin B loaded reconstituted Sendai viral envelopes containing only F protein. The unentrapped hygromycin B was removed by extensive dialysis against the same buffer followed by centrifugation at 100,000 × *g* for 1 h. Loaded F,HN-virosomes were prepared by directly solubilising Sendai virus (20 mg protein) with 40 mg Triton X-100 and then treated as above. F,HN_{red}-virosomes with entrapped hygromycin B were prepared by treating F,HN-virosomes with 3 mM DTT at 37°C for 2 h as described earlier [4]. Fusogenicity of these vesicles was measured by their ability to cause hemolysis in the presence of wheat germ agglutinin (WGA) [4]. The protein in loaded F-, F,HN-, F,HN_{red}-virosomes and fusogenic liposomes was estimated by the method of Markwell et al. [9]. Stability was checked in terms of leakage of hygromycin B from these virosomes. Heat treated virosomes were prepared by incubating the virosomes at 56°C for 20 min [10]. Degalactosylated virosomes were prepared by treating F-virosomes with clam exoglycosidase following the protocol described by Ghosh et al. [11]. Blank virosomes were prepared by reconstituting Triton X-100 solubilised fraction in the absence of hygromycin B.

Fusogenic liposomes (large unilamellar) were prepared following the method of Ozawa and Asano [12] with minor modifications. In brief, Triton solubilised extract (as described above) was added to a film of egg phosphatidylcholin e (PC) (5 μmol) and cholesterol (5 μmol) (made under N_2 gas) and incubated for 1 h at 20°C with gentle rocking. The remaining steps were the same as for the preparation of F-virosomes. Activity of fusogenic liposomes was also checked by hemolysis of mouse RBC in the presence of WGA [4].

The loaded virosomes and liposomes were lysed with Triton X-100 (0.1%, v/v) and the entrapped hygromycin B was measured by its ability to inhibit poly(U)-directed polyphenylalanine synthesis using rabbit reticulocyte lysate translation system [13]. The standard curve with free hygromycin B was prepared at known concentrations. Intact loaded virosomes were also used in this assay as a control to eliminate the contribution of surface adsorbed hygromycin B, if any. The effect of Tx100 on the in vitro translation was also checked.

2.5. *Fusion mediated microinjection of hygromycin B in HepG2 cells by virosomes and fusogenic liposomes*

Interaction of various virosomes containing hygromycin B with HepG2 cells was determined by following the inhibition of DNA synthesis [14]. Cells were placed in 24-well tissue culture plates at 2×10^5 cells per well in 2 ml culture medium and allowed to grow for 24 h in a CO_2 incubator. The monolayer cultures were washed twice with 2 ml medium with and without serum and incubated with different concentrations of virosomal hygromycin B for 18 h at 37°C in 5% CO_2. The cells were then washed with 2 ml D-PBS twice and were cultured with [^{3}H]thymidine (0.2 μCi) for 6 h. DNA synthesis was followed by measuring [^{3}H]thymidine incorporation into cellular DNA. Kinetics of cytotoxicity of hygromycin B entrapped in various virosome preparations was checked on HepG2 cells by incubating cells with fixed amount of virosomes for various time periods followed by [^{3}H]thymidine addition. Loaded heat-treated virosomes, trypsinized virosomes [1] and black virosomes together with free hygromycin B were used as controls. After incubation with [^{3}H]thymidine, cells were fixed with 5% chilled TCA. Fixed cells were finally dissolved in 0.5 N NaOH and the radioactivity associated was measured by liquid scintillation counter as described by Stubblefield et al. [14]. Specific interaction between the terminal galactose moiety of F protein and ASGP-R on the membrane of HepG2 cells was checked by determining the cytotoxicity of hygromycin B encapsulated in degalactosylated F-virosomes.

Cytotoxicity of loaded F-virosomes and fusogenic liposomes was compared by incubating these vesicles with HepG2 cells at 37°C for 12 h and determining the inhibition of DNA synthesis caused by each of them as described above.

3. RESULTS

3.1. *Characterization of loaded fusogenic virosomes and liposomes*

Vesicle preparations were checked for the purity of the envelope glycoprotein based on SDS-PAGE analysis as described earlier [4]. Hygromycin B encapsulated in the vesicles was measured by its ability to inhibit poly(U)-directed polyphenylalanine synthesis. The entrapment was calculated as μg hygromycin B associated per μg of F protein in vesicles (Table I). The ratio of F to HN in F,HN-virosomes and F,HN_{red}-virosomes was found to be 1:1 (w:w) in all preparations [4]. As compared to F-virosomes and F,HN-virosomes, fusogenic liposomes were found to enclose about 10-fold more of hygromycin B (Table I). F-, F, HN-, F,HN_{red}-, and degalactosylated virosomes and fusogenic liposomes were found to be active in causing lysis of mouse erythrocytes in the presence of WGA. However, heat-treated and trypsinized vesicles were found to be inactive. No significant leakage of hygromycin B was observed during heat treatment, trypsinization or degalactosylation

Table I

Entrapment of hygromycin B in various virosomes and liposomes

Virosomes	Hygromycin B (μg/μg F protein)
F-virosomes	0.98 ± 0.11
F,HN-virosomes	1.00 ± 0.14
Heat-treated virosomes	0.97 ± 0.11
Fusogenic liposomes	10.32 ± 1.04

Hygromycin B was measured by its ability to inhibit polyphenylalanine synthesis in vitro. Virosomes containing hygromycin B were solubilized with Triton X-100 (0.1%, v/v). Aliquots from the solubilized extract were incubated with rabbit reticulocyte lysate in reaction buffer (10 mM Tris-HCl, 10 mM magnesium acetate, 100 mM NH_4Cl and 1 mM DTT, pH 7.8) supplemented with [^{14}C]phenylalanine and poly(U) for 1 h at 37°C. Radioactivity was then measured as described earlier [13]. The amount of hygromycin B entrapped in vesicles was then calculated from a standard curve plotted using free hygromycin B at known concentrations. Intact loaded vesicles failed to inhibit polyphenylalanine synthesis.

of virosomes. Moreover, leakage was found to be less than 5% after a 20 h incubation at 37°C. Intact loaded virosomes and liposomes were unable to inhibit polyphenylalanine synthesis. Moreover, Triton X-100 used for lysis also had no effect. Using ^{125}I-labelled virosomes, it was found that heat-treated F-virosomes were able to bind to HepG2 cells to an extent similar to untreated F-virosomes. Degalactosylated F-virosomes, however, were unable to bind to the cultured cells due to the loss of recognition by ASGP-R owing to removal of terminal galactose residues (data not shown). Thus, heat- treated F-virosomes were used as nonfusogenic targeted liposomes whereas degalactosylated F-virosomes behaved as nontargeted fusogenic virosomes in this study.

3.2. *Interaction of various virosomes with HepG2 cells: fusion mediated microinjection of hygromycin B*

The cytotoxic activity of hygromycin B enclosed in F-, F,HN- and F,HN$_{red}$-virosomes was evaluated on HepG2 cell line and was assessed by measuring the inhibition of DNA synthesis in these cells. Fig. 1 shows the dose–response curve of drug toxicity for HepG2 cells incubated for 18 h with varying amounts of loaded F-virosomes (Fig. 1A) or with F,HN- and F,HN$_{red}$-virosomes (Fig. 1B). The drug encapsulated in all these virosomes was highly toxic and the toxicity increased with the increasing amount of virosomes incubated with the cells. The drug encapsulated in F,HN-virosomes displayed higher toxicity (ID_{50} = 0.001 μg) as compared to F- or F,HN$_{red}$-virosomes (ID_{50} = 0.01 μg). As is evident from Fig. 1, the inhibition of DNA synthesis occurred only as a result of introduction of hygromycin B to the cells through loaded virosomes. Addition of free hygromycin B either alone or mixed with empty virosomes displayed low cytotoxic activity, i.e. about 25–35% (Fig. 1A and B).

Kinetic studies revealed that inhibition of DNA synthesis in cells was progressive and 50% inhibition was achieved after 6 h of incubation with loaded F- and F,HN$_{red}$-virosomes (Fig. 2A and B). However, the kinetics of inhibition was comparatively much faster with F,HN-virosomes, i.e. 50% inhibition was obtained within an hour (Fig. 2B). After long incubation with all these virosomes (18 h), most of the cells in the culture were not viable. However, empty virosomes exhibited very low cytotoxicity as shown in Fig. 1. Loaded heat-treated F-virosomes and F,HN-virosomes behaved as targeted nonfusogenic proteoliposomes and were mildly toxic with only 30–35% inhibitory activity observed even after 18 h of incubation. Free drug and drug mixed with blank virosomes responded similarly as that of heat-treated virosomes (Fig. 2A). Trypsinized virosomes showed similar inhibition profile. This supported the view that introduction of hygromycin B via loaded virosomes into cultured cells was mediated by a virus–cell fusion process and that an active fusion protein is

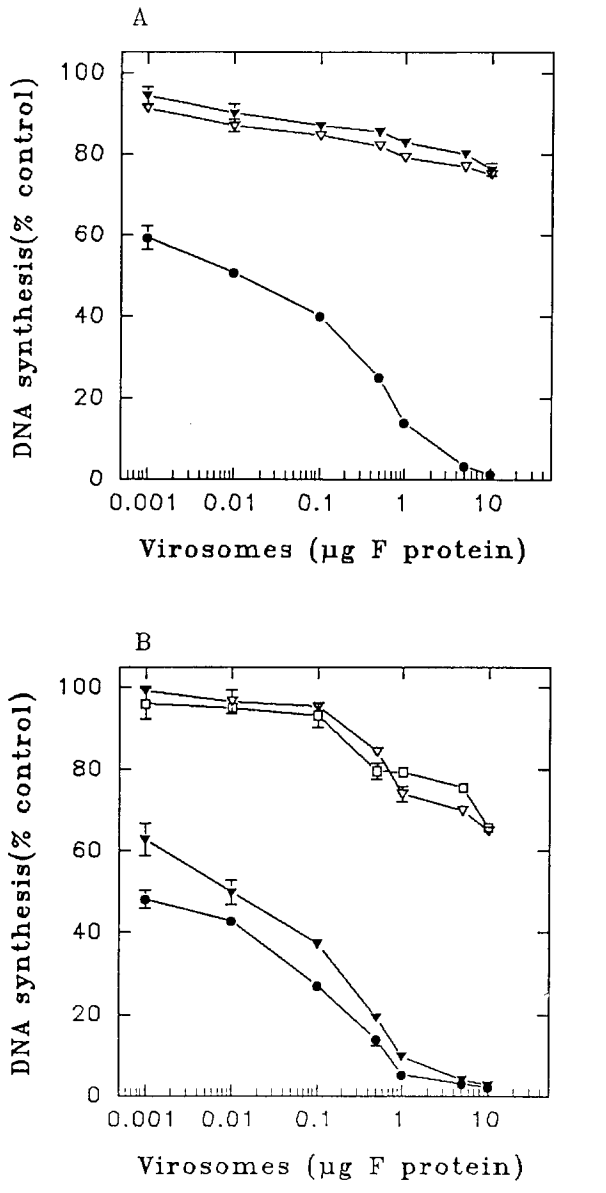

Fig. 1. Dose–response curve of cytotoxic activity of free or encapsulated hygromycin B measured by DNA synthesis inhibition assay. HepG2 cells (2×10^5) were placed in culture plates for 24 h at 37°C in 5% CO_2 followed by washing with culture medium. Cells were then incubated in 2 ml culture medium for 18 h at 37°C with various amounts of (A) free drug (closed triangles), free drug and empty F-virosomes (open triangles) or drug encapsulated in F-virosomes (closed circles) (B) free drug and empty F,HN-virosomes (open triangles), free drug and empty F,HN$_{red}$-virosomes (open squares), drug encapsulated in F,HN-virosomes (closed circles) or F,HN$_{red}$-virosomes (closed triangles). Amount of virosomes is expressed in terms of F protein associated with virosomes. The corresponding amount of free drug was taken as described in Table I. After incubation and washing with D-PBS, 0.2 μCi of [^{3}H]thymidine was added in 1 ml culture media and DNA synthesis was followed for 6 h. Cells were then fixed and radioactivity measured as described earlier [14]. Results are expressed as percent of DNA synthesis activity of cells incubated without free or enclosed drug. Each point is average of two independent experiments.

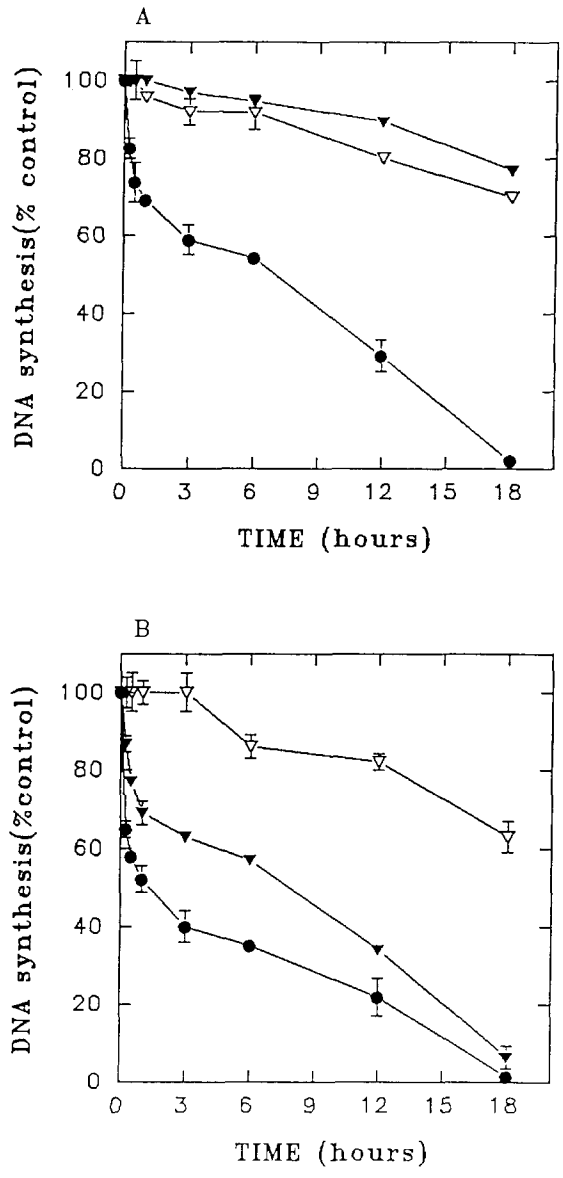

Fig. 2. Inhibition of DNA synthesis in HepG2 cells by free or encapsulated hygromycin B: kinetic study. HepG2 cells (2×10^5) were placed in culture plates for 24 h at 37°C. Cells were then incubated for various time periods at 37°C with 5 μg of (A) free drug (closed triangles), loaded heat treated F-virosomes (open triangles) or loaded F-virosomes (closed circles) (B) loaded heat-treated F,HN-virosomes (open triangles), loaded F,HN_{red}-virosomes (closed triangles) or loaded F,HN-virosomes (closed circles). Cells were then washed with D-PBS and incubated with [^{3}H]thymidine for 6 h. Radioactivity associated with cells was then measured as described in Fig. 1. Trypsinized virosomes showed similar behaviour as that of free drug.

required for this process. To prove that F-virosomes interact specifically with HepG2 cells, F protein in loaded F-virosomes was degalactosylated with clam exoglycosidase. Fig. 3 clearly indicates that nontargeted fusogenic F-virosomes were ineffective in causing inhibition of DNA synthesis and thus binding and fusion of virosomes with HepG2 cells is solely mediated by F protein leading to microinjection of hygromycin B.

3.3. *Fusion mediated microinjection of hygromycin B with fusogenic liposomes containing Sendai viral F protein*

A comparative study of the toxicity of the drug entrapped both in fusogenic liposomes and F-virosomes was carried out. Fig. 4 shows about a 3–4-fold enhancement in the drug toxicity when it was enclosed in the fusogenic liposomes. This effect may be due to a combination of the high trapping efficiency of liposomes and the fusogenic property of viral F protein. Heat-treated fusogenic liposomes exhibited significantly lower cytotoxicity, indicating again that microinjection of hygromycin B is due to F protein mediated fusion process with HepG2 cells. The presence or absence of 10% fetal bovine serum in the medium during incubation of cells and virosomes/fusogenic liposomes did not exhibit any significant effect on the toxicity of the drug.

4. DISCUSSION

The results presented in this work clearly demonstrate the efficiency of reconstituted Sendai viral envelopes lacking hemagglutinin-neuraminidase in introducing their contents into cultured cells. Hygromycin B was entrapped in F-virosomes to study its targeted cytosolic delivery in HepG2 cells. Hygromycin B is a membrane impermeant broad spectrum antibiotic, able to kill cells by blocking the translocation of phenylalanine tRNA and subsequently inhibiting protein and DNA synthesis only in cell-free systems or in the presence of an ionophore [15]. We used a DNA synthesis inhibition assay as an index of cytotoxic activity of hygromycin B. A human hepatoma cell line, HepG2,

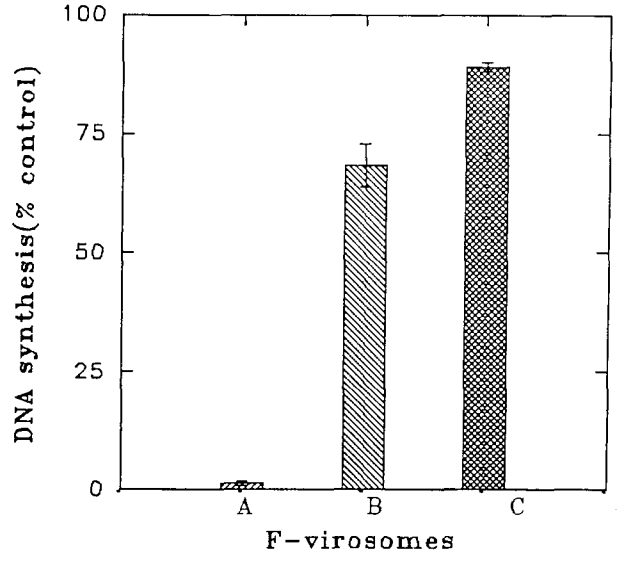

Fig. 3. Specificity of interaction of F protein with HepG2 cells. Cells (2×10^5) were grown for 24 h at 37°C and incubated for 18 h at 37°C with 10 μg of hygromycin B containing, F-virosomes (A), heat treated F-virosomes (B) or degalactosylated F-virosomes (C). DNA synthesis was then measured as in Fig. 1.

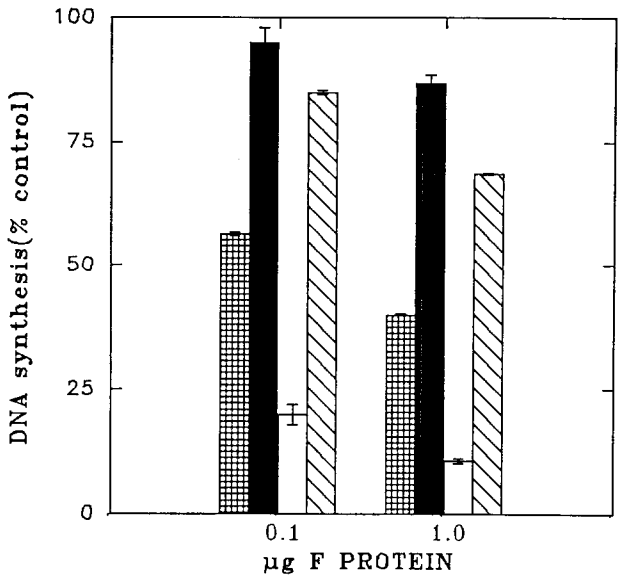

Fig. 4. Comparison of the toxicities of drug encapsulated in F-virosomes and fusogenic liposomes for HepG2 cells. Cells (2×10^5) were placed in culture plates 24 h before assaying and further incubated for 12 h at 37°C with hygromycin B containing: F-virosomes (cross-hatched), heat-treated F-virosomes (solid), fusogenic liposomes (blank), or heat-treated fusogenic liposomes (left-hatched). The *x*-axis represents the amount of F protein associated with virosomes and liposomes. Cytotoxicity was then measured as in Fig. 1.

having a large number of functional asialoglycoprotein receptors on the cell surface [16] is known to bind and fuse with F-virosomes [4]. This specific interaction was further confirmed by the finding that the hygromycin B entrapped in degalactosylated F-virosomes exhibited negligible cytotoxicity. Incubation of loaded F-virosomes with HepG2 cells led to a strong inhibition of DNA synthesis indicating that the trapped drug was transferred to these cells by the fusion of F-virosomes with the recipient cell membranes. Support for the view that introduction of the antibiotic indeed occurred by F protein mediated fusion was obtained from the experiments showing that loaded nonfusogenic virosomes (heat-treated or trypsinized virosomes) caused very little inhibition of DNA synthesis in the cells. Heat treatment of virosomes is known to abolish the fusogenicity of the F protein [10] without altering its galactose mediated binding to the ASGP-R of HepG2 cells [4]. Hence, the reduction of cytotoxicity of the heat-treated virosomes may be explained by their receptor mediated endocytotic uptake and the probable degradation of the aminoglycoside structure of the drug by lysosomal hydrolases.

A great deal of work has also been reported to generate specificity on Sendai viral envelopes for fusion-mediated microinjection of several biologically active molecules to appropriate target cells in vitro [1]. The fusion of these engineered viral envelopes depends to a large extent on the presence and involvement of HN protein in addition to the specific attachment molecules. We report here for the first time the dual role (in binding and fusion) of F protein in fusion mediated delivery of hygromycin B to HepG2 cells. We have compared the cytotoxicity of hygromycin B loaded in F-, F,HN- and F,HN_{red}-virosomes. F,HN_{red}-virosomes have been shown earlier to exhibit a slightly faster rate of lysis of RBC than that of F-virosomes only in presence of wheat germ agglutinin [4]. Reduced HN spans the virosomal membrane but is unable to bind to the sialic acid moiety of target cells [4]. Both dose dependence and kinetics of cytotoxic pattern of F- and F,HN_{red}-virosomes were found to be quite similar confirming our earlier studies on hemolysis. On the other hand, F,HN-virosomes showed 10-fold more activity than F- and F,HN_{red}-virosomes as far as the ID_{50} values were concerned. Moreover, the kinetic studies revealed that F,HN-virosomes were about 1.5-fold more active than that of F- and F,HN_{red}-virosomes in the initial (until about 6 h) phase of interaction with target cells. The enhancement of the cytotoxicity of F,HN-virosomes can be explained on the basis of attachment contributed by both F and HN protein to their respective receptors on the membrane of HepG2 cells, as reported earlier using a fluorescence probe-based membrane fusion assay [4]. Transmembrane disposition of inactive HN protein (HN_{red}) did not exhibit any significant contribution to the cytotoxicity of virosomes. It is important to note here that the final extent of inhibition caused by all these virosomes were about the same. The contribution of and absolute requirement for HN protein in the fusion process, described by Gitman et al. with erythrocytes [17] and hepatoma tissue culture cells [18] was not prominent in our experiments with HepG2 cells. We also observed a 3–4-fold enhancement of cytotoxicity of fusogenic liposomes as compared to that of F-virosomes. This may be explained by the-10-fold more drug entrapped in liposomes with same F protein content. The efficacy of this targeted fusion mediated microinjection process can be expressed as amplification factor (AF) which can be be defined as the ratio of the free drug concentration to that of the targeted drug, measured at the same cytotoxicity. The AF calculated in our virosomal systems are more than 10^4. Fusogenic liposomes containing Sendai viral envelope proteins have been recently used to target this antibiotic to leukemic T cells in vitro [19]. In this study, a specific monoclonal antibody against target cell plasma membrane antigen has been employed to mediate functional binding between fusogenic vesicles and target cell membrane. Using these fusogenic immunoliposomes, AF values ranging from 10^2–10^3 have been reported.

The terminal sugar residues of the F protein may offer suitable sites to generate further specificity against various cell surface antigens and receptors. 'Trojan horse' strategies involving virosomes and fusogenic li-

posomes in the field of gene therapy and drug delivery are already in the limelight of modern biotechnology [20]. We are currently engaged in evaluating the potential of F-virosomes and fusogenic vesicles as targeted biological carriers in vivo.

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