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Effect of surface charge and density of distearylphosphatidylethanolamine-mPEG-2000 (DSPE-mPEG-2000) on the cytotoxicity of liposome-entrapped ricin: Effect of lysosomotropic agents

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Abstract

Ricin was encapsulated in various liposomes having neutral, negatively and positively charged and different density of DSPE-mPEG-2000 on the surface and cytotoxicity of ricin entrapped in these different charged liposomal formulations was studied in CHO pro$^-$ cells and compared with free ricin with a view to develop an optimum delivery system for ricin *in vivo*. It was observed that the cytotoxicity of ricin entrapped in various charged liposomes was significantly dependent on the charge on the surface of liposomes. The maximum cytotoxicity of ricin was observed when it was delivered through negatively charged liposomes. Monensin enhances the cytotoxicity of ricin entrapped in various charged liposomes and the extent of enhancement of the cytotoxicity is significantly dependent on the charge on the surface of liposomes. Maximum potentiation (213.14-fold) of cytotoxicity of ricin was observed when it was delivered through positively charged liposomes followed by negatively charged (83.36-fold) and neutral (71.30-fold) liposomes, respectively. Studies on the kinetics of inhibition of protein synthesis by ricin entrapped in various charged liposomes revealed that lag period of inhibition of protein synthesis is significantly lengthened following delivery through various charged liposomes. However, in the presence of monensin, the lag period was reduced. There is a marginal variation in the cytotoxicity of ricin entrapped in various charged liposomes after incorporation of 5 mol% of DSPE-mPEG-2000 on the surface. However, there is a significant variation in the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes in CHO pro$^-$ cells following incorporation of 5 mol% DSPE-mPEG-2000 on the surface. Studies on the effect of variation of density of DSPE-mPEG-2000 on the surface of various charged liposomes on the enhancement of cytotoxicity of entrapped ricin by monensin in CHO pro$^-$ cells showed that the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes is significantly dependent on the density of DSPE-mPEG-2000 on their surface. It was also observed that the efficacies of monensin on the enhancement of cytotoxicity of ricin entrapped in various charged PEG-liposomes in CHO pro$^-$ cells was highly related to their amount of cell-association. The present study has clearly shown that by suitable alteration of liposomal lipid composition, charge and density of hydrophilicity it would be possible to direct liposomal ricin to specific cells for their selective elimination in combination with monensin.

Keywords: Ammonium chloride; CHO cells; Golgi apparatus; Liposomal ricin; Monensin; Ricin cytotoxicity

1. Introduction

Ricin is a heterodimeric cytotoxic protein inhibits the synthesis of protein in eukaryotic cells by inactivation of 60S ribosomal subunit. This is due to the cleavage of specific adenine residue from 28S rRNA mediated by the *N*-glycosidase activity of A-chain (Endo and Tsurugi, 1987; Endo et al., 1987). The toxin enters into mammalian cells by endocytosis after binding to the galactose residues on cell surface through the B-chain (Sandvig and van Deurs, 1996). After endocytosis, a part of ricin is transported to the Golgi apparatus (Gonatas et al., 1975; van Deurs et al., 1986) mainly to the *trans*-Golgi network (TGN) (van Deurs et al., 1988) and reaches the endoplasmic reticulum (ER) (Wales et al., 1993; Wesche et al., 1999), before the release of A-chain into the cytoplasm. However the use of ricin as an

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anticancer agent is limited due to the non-specific nature of the B-chain. Hence, attempts have been made by several investigators to prepare immunotoxins (IT) and hormonotoxins by cross linking the ricin A-chain with monoclonal antibodies (MAb) directed against tumor cell-specific antigens (Thrush et al., 1996; Pastan and Kreitman, 1998) and hormones whose receptors are over expressed specifically on tumor cell surface (Singh et al., 1989). These heteroconjugates are tumor cell-specific but are less efficacious due to the sub-optimal entry of A-chain into the cytoplasm, in the absence of facilitation afforded by B-chain (Olsnes and Sandvig, 1985).

Negatively charged and antibody tagged liposomes have been reported to enter into mammalian cells by endocytosis and reach an acidic pH compartment (endosome) (Straubinger et al., 1983; Allen et al., 1995). These liposomes have been employed to deliver ricin and ricin A-chain in tumor and ricin-resistant normal mammalian cells (Dimitriadis and Butters, 1979; Gardas and Macpherson, 1979; Watanabe and Osawa, 1987). However, the cytotoxicity of ricin is significantly reduced following its entrapment in liposomes (Dimitriadis and Butters, 1979). The lysosomotropic agent NH_4Cl and the carboxylic ionophore monensin are known to enhance the cytotoxicity of ricin and ricin A-chain-based IT (Casellas et al., 1984; Carriere et al., 1985; Ghosh et al., 1985; Ghosh and Wu, 1988). Very recently, we have shown for the first time that monensin and NH_4Cl significantly enhanced the cytotoxicity of ricin entrapped in negatively charged liposomes composed of soya phosphatidylcholine (Bharadwaj et al., 2006). In the present investigation, we have extended our initial observations on liposome–mediated delivery of ricin and have examined the effect of surface charge and density of DSPE-mPEG-2000 on the cytotoxicity of entrapped ricin in CHO pro$^-$ cells and compared with free ricin with a view to develop an optimum delivery system for ricin *in vivo*. In addition we have also examined the effect of NH_4Cl and monensin on the cytotoxicity of ricin entrapped in these liposomes and provide evidence bearing the mechanism of enhancement of cytotoxicity of ricin entrapped in various liposomal formulation.

2. Materials and methods

Cholesterol and L-α-phosphatidic acid (PA), stearylamine (SA), lactoperoxidase, monensin, penicillin-G (potassium salt), streptomycin sulphate, proline, glutamine, 2.5% trypsin in HBSS, DMSO, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soya phosphatidylcholine (SPC) was procured as phospholipon 90 from Natterman, Germany. DSPE-mPEG-2000 (Distearoyl phosphatidylethanolamine polyethylene glycol) was purchased from Matreya, Inc. (PA, USA). Fetal calf serum (FCS) was obtained from HyClone (Utah, USA). Sepharose CL6B, Sephacryl S-300 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Ammonium chloride (NH_4Cl) was of ExcelaR grade from Qualigens Laboratories (Bombay, India).

Powdered RPMI-1640 (deficient) medium was obtained from Sigma Chemical Co. (St. Louis, MO, USA) RPMI-1640 (complete) medium purchased from Invitrogen Corporation (New York, USA) DMEM media purchased from HyClone (Utah, USA). [^{3}H] leucine (117 Ci/mmol) and Na ^{125}I (17.4 Ci/mg iodine) were obtained from Perkin-Elmer life and Analytical Sciences (MA, USA).

2.1. Cells

CHO pro$^-$ cell line, auxotrophic for proline, was generously provided by Dr. Henry C. Wu (Uniformed Services University of the Health Sciences, Bethesda, MD, USA). It was maintained in RPMI-1640 or DMEM. The culture media were supplemented with 10% FCS, proline, glutamine, HEPES and $NaHCO_3$, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were then grown in a humidified incubator at 37 °C, 5% CO_2 and 95% air atmosphere.

2.2. Purification of ricin

Ricin was purified from the seeds of *Ricinus communis* by affinity chromatography on cross-linked guar gum column following the procedure published by Appukuttan et al. (1977), followed by gel permeation chromatography on Sephacryl S-300.

2.3. Radioiodination of ricin

Ricin was radiolabeled with Na ^{125}I by lactoperoxidase according to the method reported earlier (Madan and Ghosh, 1992).

2.4. Entrapment of ricin in various liposomes

Liposomes composed of soya phosphatidyl choline and cholesterol in a molar ratio of 55:45 were prepared by hand shaken method. Briefly, the lipids (40 μmol total lipids) were dissolved in chloroform in a 100 ml round bottom flask. The chloroform was evaporated to dryness at 37 °C, under reduced pressure by using rotary evaporator (Wheaton). The thin film so formed, was desiccated for 1 h, followed by hydration with 1 ml PBS (20 mM, pH 7.4), containing ricin (3 mg/ml) and trace amounts of ^{125}I-ricin as aqueous phase marker. The round bottom flask containing liposomes suspension was stored, under N_2 atmosphere to avoid lipid oxidation, at 4 °C for overnight for complete hydration. The following day, liposomes were sonicated in a bath type sonicator (Branson) at 25 °C for 30 min in 10 min batches to avoid the heat generation. Negatively and positively charged liposomes containing ricin were prepared exactly as described above only 10 mol% either phosphatidic acid (PA) or stearylamine (SA) were added during the preparation of lipid film. Sterically stabilized liposomes containing ricin were prepared as described above by adding various (1–7.5 mol%) of DSPE-mPEG-2000 during the preparation of lipid film.

The liposomal ricin was separated from free ricin by affinity chromatography on Sepharose CL 6B column pre-equilibrated with PBS (20 mM, pH 7.4) at 25 °C. Briefly, 1 ml liposomal suspension (40 μmol total lipid in 1 ml) was loaded on Sepharose CL 6B column (1 × 43 cm). The column was eluted first, with

one bed volume of PBS, followed by, one bed volume of 0.1 M lactose (in PBS). The presence of liposomes in the fractions thus obtained was determined by checking the turbidity at 430 nm and measuring the radioactivity in each fraction in LKB Mini Gamma Counter. It was observed that elution of the column with PBS gave only one peak corresponding to liposomal ricin as both the turbidity and the radioactivity was maximum in the peak. The fractions containing liposomal ricin were pooled and extruded ten times through 100 nm polycarbonate membrane. Traces of free ricin were separated by ultracentrifugation at 340,000 × *g* for 1 h, three times. It was found that 2.5–5.5% of the total amount of ricin added during the preparation of liposomes was associated with the liposomal fraction. The rest, 97.5–94.5% of free ricin bound to the column was subsequently eluted with 0.1 M lactose. The mean diameter of the liposomes measured from the volume distribution curves produced by particle analyzer was found to be 140 ± 40 nm. Zeta Potentials of various liposome preparations were measured at 25 °C in PBS, pH7.4 using Malvern 3000 HS Zetasizer (Malvern Instruments, Worcestershire, UK).

2.5. Evaluation of cytotoxicity of ricin in free and liposomal form in CHO pro$^-$ cells

The cytotoxicity of ricin in free and liposomal form was determined from the observed inhibition of [^{3}H] leucine incorporation into protein in cell cultures exposed to various concentrations of the toxin. Cells were plated in 24-well plates at a cell density of 8×10^5 cells/well in 1 ml RPMI-1640 medium containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml), 24 h prior to the experiment. The monolayer cultures were washed twice with 1 ml DBSS and incubated in 0.9 ml serum free RPMI-1640 medium containing penicillin and streptomycin for 1 h at 37 °C. Cells were then incubated with different concentrations of ricin in free and different liposomal form for 4 h at 37 °C, followed by washing with DBSS twice and incubated with 0.9 ml leucine free medium for 1 h at 37 °C. After 1 h of preincubation with leucine free medium, 100 μl [^{3}H] leucine (0.5 μCi/ml) was added to the cells and the monolayer cultures were further incubated for 1 h at 37 °C. The monolayers were then fixed with 3% (w/v) perchloric acid and 0.5% (w/v) phosphotungstic acid twice, washed with DBSS twice and dissolved in 0.5 ml of 0.5 N NaOH. A 50 μl aliquot of the solubilized cell extract was transferred to a scintillation vial containing 5 ml scintillation cocktail, neutralized with 25 μl 1 N HCl and counted in an LKB 1209 Rack beta liquid scintillation counter. For the determination of the effect of monensin and NH_4Cl, cells were preincubated with these agents for 1 h, followed by their incubation with ricin.

2.6. Concentration-dependent binding and internalization of ^{125}I-ricin in free and various liposome-entrapped form

Binding of ^{125}I-ricin in free or different liposomal form was assayed at 4 °C as a function of concentration of ^{125}I-ricin in free or different liposomal form. The cells at a density of 8×10^5 cells/well were plated in 24-well plate, 24 h before the experiment. Monolayers were washed twice with DBSS and incubated with RPMI-1640 medium with or without 50 nM monensin at 4 °C/37 °C for 1 h. This pre-incubation was followed by the 4 h of incubation with varying concentrations of ^{125}I-ricin in free and different liposomal form at 4 °C for binding and at 37 °C for internalization. Cells were then washed thrice with ice cold DBSS and solubilized with 0.1N NaOH. The cell associated radioactivity was determined with the help of 1275 LKB Minigamma counter.

2.7. Kinetics of release, intracellular level and degradation of intracellular ricin endocytosed in free or encapsulated in different charged liposomal form

Cells at a density of 8×10^5 cells/well in a 24-well tissue culture plate were seeded 24 h prior to the experiment. Monolayers were washed twice with DBSS and incubated with serum free RPMI-1640 medium with or without 50 nM monensin for 1 h at 37 °C. After this preincubation, ^{125}I-ricin in free and different charged liposomal form were added to each well and incubated at 37 °C for 2 h. After this, the surface bound ^{125}I-ricin was removed by washing the cells with 0.1 M lactose thrice. A set of cells in duplicate wells was dissolved with 0.1 N NaOH to determine the amount of internalized ^{125}I-ricin at 2 h. The cells in the remaining wells were further incubated with fresh RPMI-1640 medium without toxin but containing 1 mM lactose at various time intervals of 5, 15, 30, 60 and 120 min at 37 °C. After the different time intervals, the culture medium of each well was collected separately and the cells were solubilized with 0.5 ml of 0.1N NaOH. The amount of 10% TCA soluble and precipitable radioactivity, released in culture medium and that associated with cells was determined with respect to the control.

2.8. Kinetics of inhibition of protein synthesis by ricin in free and various charged liposomal form: effect of monensin

The cells at the density of 8×10^5 cells/(ml well) were plated in 24-well plates, 20–24 h prior to the experiment. The monolayers cultures were washed with DBSS and incubated with FCS free medium containing ricin in free form (150 ng/ml) or liposomal (10 μg/ml) form with or without monensin, for various time intervals at 37 °C. After different time intervals, the cells were washed twice with DBSS and incubated with leucine free medium containing [^{3}H] leucine with or without monensin for 30 min. The inhibition of protein synthesis was measured as described earlier in Section 2.5.

3. Results

3.1. Cytotoxicity of ricin in free and entrapped in various charged liposomes in CHO pro$^-$ cells

In order to ascertain weather variation in the charge on the liposomal surface has any effect on the cytotoxicity of entrapped ricin, ricin was encapsulated in neutral, negatively and positively charged liposomes and the cytotoxicity of ricin entrapped in these different charged liposomal formulations was studied

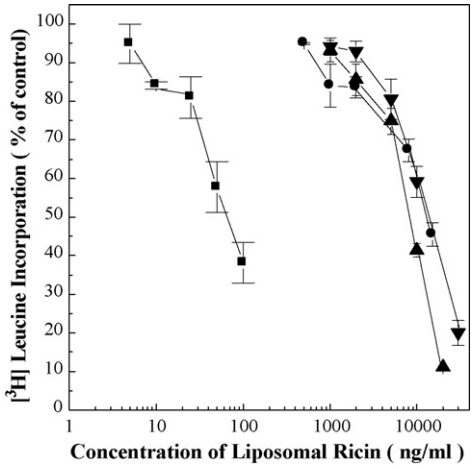

Fig. 1. Cytotoxicity of ricin in free and entrapped in various charged liposomes in CHO pro$^-$ cells. 8×10^5 cells were plated 24 h prior to the experiment. The monolayers were washed twice with DBSS and incubated in serum free RPMI-1640 medium containing different concentration of (■) free ricin; (●) neutral liposomal ricin; (▲) negatively charged liposomal ricin; (▼) positively charged liposomal ricin (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)) for 4 h at 37 °C. Following incubation, the inhibition of protein synthesis was measured as described in Section 2. Each point represents the mean ($n = 3$).

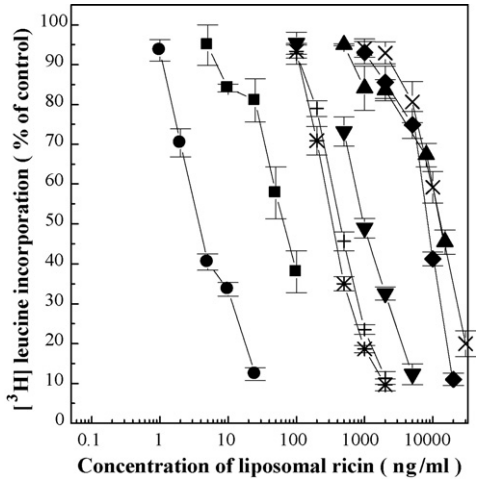

Fig. 2. Effect of NH_4Cl on the cytotoxicity of ricin in free and encapsulated in different charged liposomal formulations in CHO pro$^-$ cells. The monolayer cultures were preincubated with NH_4Cl (20 mM) for 1 h followed by incubation with different concentration of ricin in free (without (■) and with NH_4Cl (●)) neutral (without (▲) and with NH_4Cl (▼)) negatively charged (without (□) and with NH_4Cl (+)) and positively charged (without (×) and with NH_4Cl (✳)) liposomal form (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)) for 4 h at 37 °C. The inhibition of protein synthesis was determined by measuring the incorporation of [^{3}H] leucine into proteins as described in Section 2. The results are expressed as percentage of incorporation of [^{3}H] leucine into proteins in untreated cells, i.e., the control. All the points represent mean ($n = 3$).

in CHO pro$^-$ cells. The cytotoxic activity of ricin in free and liposomal form in CHO pro$^-$ cells inferred from the incorporation of [^{3}H] leucine during the protein synthesis. It was observed that the cytotoxicity of ricin entrapped in all the formulations, i.e., positively charged, negatively charged and neutral liposomes were significantly reduced as compared to free ricin (Fig. 1). It was observed that extent of reduction of cytotoxicity of ricin entrapped in positively and neutral liposomes are very similar. The cytotoxicity of ricin entrapped in positively charge and neutral liposomes was reduced to 192- and 195-fold, respectively. The reduction of the cytotoxicity of ricin entrapped in negatively charged liposomes (122-fold) was found to be minimal as compared to the positively charged and neutral liposomes.

3.2. *Effect of NH_4Cl on the cytotoxicity of ricin in free and encapsulated in different charged liposomal formulations in CHO pro$^-$ cells*

In order to evaluate whether variation in the charge on the surface of liposomes has any effect on the ricin cytotoxicity enhancing potency of NH_4Cl, the cytotoxicity of ricin entrapped in different charged liposomes was examined in the presence of NH_4Cl (20 mM). As can be seen in the Fig. 2, the enhancement of cytotoxicity of ricin entrapped in the various charged liposomes is significantly dependent on the charge on the surface of liposomes. The maximum potentiation of the cytotoxicity (37.94-fold) was observed when ricin was delivered through positively charged liposomes followed by negatively charged (18.6-fold) and neutral (13.73) liposomes. On the other hand, NH_4Cl enhances the cytotoxicity of free ricin by 20.47-fold.

3.3. *Effect of monensin on the cytotoxicity of ricin in free form and entrapped in various charged liposomes in CHO pro$^-$ cells*

It has been reported by several investigators that after endocytosis, most of the ricin is either exocytosed to extracellular medium or transported through the endosomes to lysosomes, while a fraction is transported to the *trans*-Golgi network (TGN) (van Deurs et al., 1986; van Deurs et al., 1988; Sandvig and van Deurs, 1999). Retrograde transport of ricin from TGN to ER via Golgi stack is essential for its optimum release in the cytosol and expression of its cytotoxicity (Wales et al., 1993 and Wesche et al., 1999). Monensin, a carboxylic ionophore, is a well-known disrupter/inhibitor of intracellular protein trafficking through Golgi apparatus due to its ability to alter the morphology of these organelles (Tartakoff et al., 1978; Tartakoff, 1983; Mollenhauer et al., 1990). Consequently, the intracellular trafficking of proteins of both intracellular and extracellular origin that transit through Golgi stack, are altered/affected by monensin. As a result monensin significantly enhances the cytotoxicity of ricin and ricin-based immunotoxins in cultured cells (Casellas et al., 1984; Carriere et al., 1985; Ghosh et al., 1985; Bharadwaj et al., 2006). Wesche et al., have demonstrated that monensin induced enhancement of cytotoxicity of ricin is due to enhanced transport of ricin from cell surface to Golgi apparatus. Recently this laboratory has shown that monensin significantly enhances the cytotoxicity of ricin entrapped in negatively charged liposomes composed of soya phosphatidylcholine (SPC), cholesterol and phosphatidic acid in CHO pro$^-$ cells (Bharadwaj et al., 2006).

In order to ascertain whether variation in the charge on the surface of liposomes has any effect on the ricin cytotoxic-

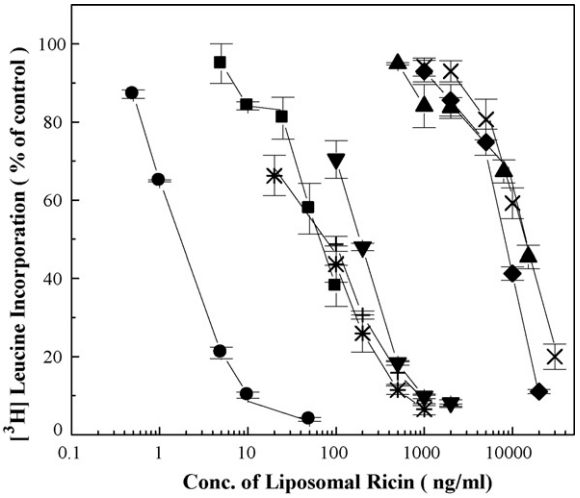

Fig. 3. Effect of monensin on the cytotoxicity of ricin in free form and entrapped in different changed liposomal form in CHO pro$^-$ cells. The monolayer cultures were preincubated without or with monensin (50 nM) for 1 h followed by incubation with different concentration of ricin in free [without (■) and with monensin (●)], neutral [(without (▲) and with monensin (▼)], negatively charged [without (□) and with monensin (+)] and positively charged [without (×) and with monensin (✱)] liposomal form (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)) for 4 h at 37 °C. The inhibition of protein synthesis was determined by measuring the incorporation of [^{3}H] leucine into proteins as described in Section 2. The results are expressed as percentage of incorporation of [^{3}H] leucine into proteins in untreated cells, i.e., the control. All the points represent mean ($n = 3$).

ity enhancing potency of monensin, the modulatory influence of monensin on the enhancement of the cytotoxicity of ricin entrapped in different charged liposomes was examined. Results of these studies are presented in Fig. 3. As can be seen in the figure, monensin significantly potentiates the cytotoxicity of ricin entrapped in various charged liposomes. At 50 nM monensin enhances the cytotoxicity of free ricin by 38.93-fold. On the other hand, the enhancement of cytotoxicity of ricin entrapped in various charged liposomes is highly dependent on the charge on the surface of liposomes. Maximum potentiation (213.14-fold) of cytotoxicity of ricin was observed when ricin was entrapped in positively charged liposomes composed of SPC, cholesterol and stearylamine. The potentiation of cytotoxicity of ricin was found to be 71.30- and 83.36-fold, respectively, when entrapped in neutral liposome composed of SPC, cholesterol and negative charged liposomes composed of SPC, cholesterol and phosphatidic acid.

3.4. Binding and internalization of ricin in free form and entrapped in different charged liposomal formulations at 4 and 37 °C in CHO pro$^-$ cells

It is known that first step in the expression of cytotoxicity of ricin involves binding of ricin to the cells surface galactose residues followed by internalization (Olsnes and Sandvig, 1985). In order to determine whether the reduction of cytotoxicity of ricin entrapped in different charged liposomes as compared to free ricin is due to the difference in binding and internalization, we examined the binding and internalization of free and different charged liposomal ricin in CHO pro$^-$ cells at 4 °C as well as 37 °C. As can be seen in Table 2, the binding of ^{125}I-ricin in free form is significantly higher as compared to ricin entrapped in different charged liposomes at 4 °C. At 4 h, 6% of free ricin is found to be associated with CHO pro$^-$ cells. 77% of this bound ricin could be released by washing with 0.1 M lactose indicating galactose specific binding. On the other hand, 0.76, 0.78 and 1.8% of neutral, negatively charged, positively charged liposomal ricin associated with cells. These results clearly show that the binding is significantly reduced following entrapment of ricin into different charged liposomes. The cell associated different charged liposomal ricin could not be released by washing with lactose suggesting that the liposomal ricin binds with the cells other than the galactose-mediated binding.

The specific uptake of ^{125}I-ricin, determined by subtracting the binding at 4 °C from the total internalization at 37 °C, from positively charged liposomes was significantly higher than that from negatively charged and neutral liposomes (Table 2). When 8×10^5 cells were incubated with 5000 ng/ml ^{125}I-ricin encapsulated in differently charged liposomes, a significantly higher concentration of specific uptake of ^{125}I-ricin was observed when delivered through positively charged liposomes (100.14 ng), fol-

Table 1
Size, zeta potential and % of entrapment of ricin of various liposomal formulations

Liposome formulations	mol% of DSPE-mPEG-2000	Size (nm ± S.E.)	Zeta potential (±S.E.)	% Entrapment (±S.E.)
PC + Chol	–	141 ± 28	−6.19 ± 0.56	3.49 ± 0.85
PC + Chol + DSPE-mPEG-2000	1	136 ± 13	−7.37 ± 0.46	4.11 ± 0.90
PC + Chol + DSPE-mPEG-2000	2.5	115 ± 23	−5.83 ± 0.67	4.75 ± 0.65
PC + Chol + DSPE-mPEG-2000	5	123 ± 11	−4.54 ± 0.48	3.39 ± 1.20
PC + Chol + DSPE-mPEG-2000	7.5	140 ± 12	−3.89 ± 0.62	4.42 ± 0.45
PC + Chol + PA	–	110 ± 12	−22.26 ± 1.15	2.75 ± 0.65
PC + Chol + PA + DSPE-mPEG-2000	1	96 ± 10	−6.20 ± 0.69	2.91 ± 0.71
PC + Chol + PA + DSPE-mPEG-2000	2.5	152 ± 23	−3.40 ± 0.42	2.79 ± 0.55
PC + Chol + PA + DSPE-mPEG-2000	5	94 ± 15	−2.08 ± 0.53	2.52 ± 0.54
PC + Chol + PA + DSPE-mPEG-2000	7.5	90 ± 11	−1.32 ± 0.09	3.23 ± 0.35
PC + Chol + SA	–	165 ± 16	7.09 ± 0.39	4.31 ± 1.10
PC + Chol + SA + DSPE-mPEG-2000	1	142 ± 22	2.13 ± 0.46	5.34 ± 1.10
PC + Chol + SA + DSPE-mPEG-2000	2.5	145 ± 18	−0.84 ± 0.13	4.97 ± 0.64
PC + Chol + SA + DSPE-mPEG-2000	5	160 ± 22	−1.82 ± 0.16	4.53 ± 0.84
PC + Chol + SA + DSPE-mPEG-2000	7.5	171 ± 16	−1.56 ± 0.11	3.91 ± 1.20

Table 2
Binding and internalization of ^{125}I-ricin in free form and entrapped in different charged liposomal formulations at 4 and 37 °C in CHO pro$^-$ cells

Ricin in different formulations	Total cell-associated ricin at 4 °C (ng/8 × 10^5 cells/well)		Total cell-associated ricin at 37 °C (ng/8 × 10^5 cells/well)		Difference in binding at 37–4 °C
	Buffer	Lactose	Buffer	Lactose	
Free ricin	55.15 ± 2.38	11.51 ± 1.57	172.16 ± 6.08	93.4 ± 4.67	117.01 ± 8.40
Neutral liposomal ricin	35.05 ± 1.37	34.70 ± 1.46	61.6 ± 2.08	60 ± 3.0	26.55 ± 0.71
Negatively charged liposomes	34.71 ± 5.8	32.4 ± 1.54	84.37 ± 2.81	96.4 ± 4.28	49.66 ± 2.99
Positively charged liposomes	98.65 ± 5.8	67.0 ± 6.6	198.79 ± 9.23	186.54 ± 8.94	100.14 ± 3.58

The 8 × 10^5 cells/(ml well) were plated 24 h prior to the experiment. The monolayer cultures were preincubated with or without 50 nM monensin for 1 h at 4 °C/37 °C followed by the incubation with (1 μg/(ml well)) free ricin and differently charged liposomal ricin (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)) with or without monensin, for 4 h at 4 °C/37 °C. After incubation cells were washed thrice with ice-cold DBSS/50 mM lactose. The binding of liposomal ricin is expressed as cell-associated liposomal ricin after 4 h incubation. All the points represent mean (n = 3).

lowed by negatively charged (49.66 ng) and neutral liposomes (26.55 ng). On the other hand, when cells were incubated with 1000 ng/ml ^{125}I-ricin in free form, 117.01 ng of ricin was internalized.

3.5. Degradation and exocytosis of intracellular free and various charged liposomal ricin in CHO pro$^-$ cells

The present study has demonstrated that the degree of enhancement of the cytotoxicity of ricin in various charged liposomes by monensin is significantly higher as compared to free ricin. This study also has demonstrated that the enhancement of cytotoxicity of ricin entrapped in various charged liposomes is highly dependent on the charge on the surface of the liposomes. In order to ascertained whether the differential enhancing potency of monensin on the cytotoxicity of ricin in various charged liposomes as compared to free ricin is due to the variation in degradation and exocytosis of intracellular ricin endocytosed in free and various charged liposomal form, the kinetics of release of intact ricin into extracellular medium and the extent of degradation of intracellular ricin endocytosed in free form and in various charged liposomal form was examined. The effect of monensin, at a concentration that enhances the cytotoxicity of ricin in free and various liposomal forms, on the release, intracellular level and degradation of ^{125}I-ricin was also studied.

Fig. 4 shows the intracellular level and rate of release of intracellular ^{125}I-ricin as well as the amount of released labeled materials, which is TCA soluble at 37 °C after 2 h of internalization of ricin in free form and entrapped in various charged liposomes. It can be seen that within 2 h, 65.74% of intracellular ricin was released into the extracellular medium. It was also observed that 70.9% of the total free ricin released was found to be in the TCA insoluble form and only 12.06% was in the TCA soluble form, i.e., in degraded form. Only 23% of total endocytosed ricin remains intracellular after 2 h of endocytosis and 70.36% of intracellular ricin was in intact form and 5% was in degraded form. On the other hand, the release of intracellular ricin delivered through various charged liposomes is significantly reduced. Only 38.53, 32.50 and 29.0% of intracellular ricin delivered through neutral, negatively charged and positively charged liposomes are released in the extracellular

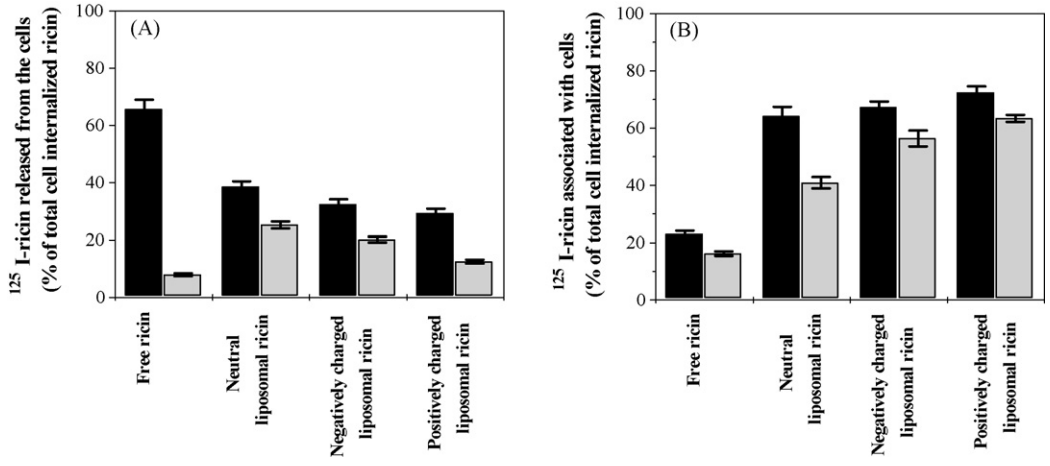

Fig. 4. Kinetics of release, intracellular level and degradation of intracellular ricin endocytosed in free or entrapped in various charged liposomal form in CHO pro$^-$ cells. The monolayer cultures were preincubated with or without 50 nM monensin for 1 h at 37 °C. Following the preincubation monolayers were treated with ricin in free (200 ng/^{125}I-ricin/well) or differently charged liposomal ricin (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)) for 2 h at 37 °C. After incubation cells were washed twice with lactose (0.1 M). The total cell-associated radioactivity was determined in duplicate wells, while the cells in remaining wells were further incubated with medium containing 1 mM lactose for 2 h. The total (■) and 10% TCA soluble (▒) radioactivity released in the medium (A), total (■) and 10% TCA soluble (▒) radioactivity associated with the cells (B) was determined as described in Section 2. The values are expressed as percent of total cell-associated radioactivity after 2 h preincubation of cell cultures with the toxin.

medium. It was also observed that most of the intracellular ricin delivered through neutral and negatively charged liposomes released in the extracellular medium in the degraded form. 65.45 and 62.0% of the total ricin released was found to be in the TCA soluble form, i.e., degraded form and only 16.40 and 19.7% was in the TCA insoluble form. 64 and 67.0% of total ricin endocytosed in neutral and negatively charged liposomal form remains intracellularly after 2 h of endocytosis and most of this ricin is in intact form. On the other hand, the major portion of ricin (53. 57%) released from intracellular ricin delivered through positively charged liposomal form was found to be in TCA insoluble form. The amount of degraded form of toxin released was found to be much lower as compared to ricin released from intracellular ricin endocytosed in neutral and negatively charged liposomal form. 42.6% of the total ricin released was found to be in the TCA soluble form, i.e., in degraded form as compared to 63.5 and 62% degradation observed in case of ricin endocytosed in neutral and negatively charged form. Interestingly, the intracellular level of intact ricin was found to be maximum after 2 h of internalization, when it was delivered through positively charged liposomes as compared to neutral and negatively charged liposomes. 72.4% of total ricin endocytosed in positively charged liposomal form remains intracellular after 2 h of endocytosis and 87.5% of this toxin was found to be in intact form and 13% was in degraded form. Monensin had no measurable effect on the intracellular level, release into extracellular medium and degradation of ^{125}I-ricin.

3.6. Effect of monensin on the kinetics of inhibition of protein synthesis by ricin in free and different charged liposomal form in CHO pro$^-$ cells

It has been reported by several investigators previously that the lag period (the time interval between the binding of ricin and onset of inhibition of protein synthesis) is shortened by treatment of cells with monensin and lysosomotropic amines (Casellas et al., 1984; Carriere et al., 1985; Ghosh et al., 1985; Ghosh and Wu, 1988; Madan and Ghosh, 1992). Recently, it has been reported from this laboratory that monensin significantly reduces the lag phase of intoxication of ricin entrapped in negatively charged liposomes (Bharadwaj et al., 2006). In order to evaluate whether the variation of charge on the liposomal surface

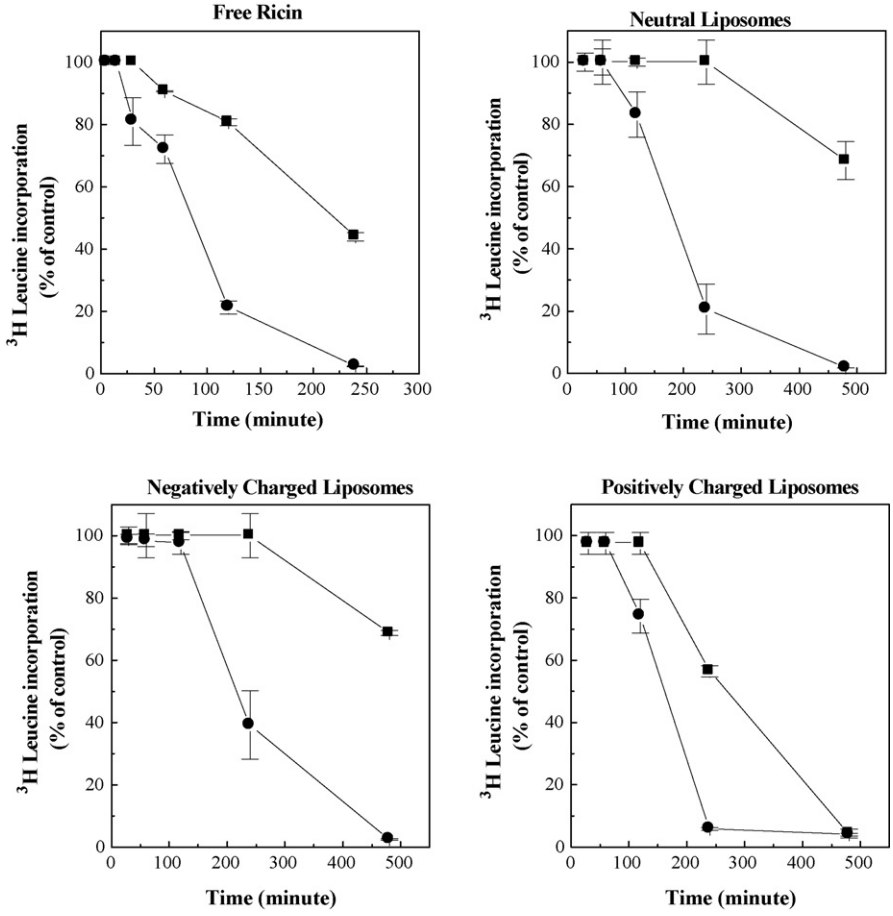

Fig. 5. Kinetics of inhibition of protein synthesis by ricin in free and different charged liposomal form in presence and absence of monensin in CHO pro$^-$ cells. The cell 8×10^5 cells/well were plated 24 h prior to the toxin treatment. The monolayers were washed twice with DBSS and preincubated in serum free RPMI-1640 medium with (●) or without 50 nM monensin (■) for 1 h, followed by the incubation with either free ricin (150 ng/ml) or liposomal ricin (10 μg/ml) liposomal ricin differently charged liposomal ricin (5 μg ^{125}I-ricin/0.4–0.8 μmol of phospholipids/(ml well)), for various times. After indicated times, the cells were washed twice with DBSS and labeled with [^{3}H] leucine for 1 h. The inhibition of protein synthesis was then measured by [^{3}H] leucine incorporation into proteins as described in Section 2. All the points represent the mean ($n = 3$).

has any effect on the lag period of intoxication by ricin encapsulated in various charged liposomal formulations, the kinetics of protein synthesis inhibition by free ricin and different charged liposomal ricin in the presence and absence of monensin was examined. As shown in Fig. 5 when cells were treated with 150 ng/ml free ricin a lag period of 45 min was observed with t_{50} (time required to achieve 50% reduction in protein synthesis) of 200 min. It was observed that the lag period of inhibition of protein synthesis by ricin is significantly increased following delivery through different charged liposomes. At 10 μg/ml of various charged liposomal ricin, a lag phase of 4 h was observed for neutral and negatively charged liposomes, on the other hand, a lag phase of 2 h was observed for positively charged liposomes. Monensin (50 nm) reduced the lag period of free ricin from 45 to 15 min (i.e., three-fold reduction of the lag period), however, in the presence of monensin, the lag period of neutral and negatively charged liposomal ricin was reduced from 4 to 1 h (four-fold reduction of lag period) and 2 h (two-fold reduction of lag period), respectively. The lag period of positively charged liposomal ricin was reduced from 2 to 1 h (two-fold reduction of lag period). These results implied that monensin causes an enhanced and efficient release of ricin A-chain from liposomal ricin located in an intracellular compartment into the cytosol leading to the rapid onset of the inhibition of protein synthesis.

3.7. Effect of incorporation of 5 mol% of DSPE-mPEG-2000 on the surface of various charged liposomes on the cytotoxicity of entrapped ricin in CHO pro⁻ cells: effect of monensin

Liposomes prepared with soya phosphatidylcholine, cholesterol and having either negative or positive charge, which is called conventional liposomes, are very unstable in the circulation. These liposomes are rapidly removed from the circulation, due to their opsonization with plasma proteins, by the cells of the reticuloendothelial system (RES), mainly liver, spleen and bone marrow, following intravenous injection. To overcome this problem, new generations of liposomes, which have prolonged circulation time in the blood, have been developed. These liposomes are called stealth or sterically stabilized liposomes and very stable in the circulation for a prolonged period of time. The development of liposomes with prolonged circulation time in the blood has been a major breakthrough in the field of drug delivery. These liposomes are constructed by inclusion of a specific amphiphile; such as monosialoganglioside (Gabizon and Papahadjopoulos, 1988) and polyethylene glycol (Klibanov et al., 1990; Blume and Cevc, 1990), which prevents adsorption of serum proteins (opsonins) on the surface of liposomes, thereby their affinity towards RES is significantly reduced. It has been reported that the sterically stabilized liposomes of 70–200 nm size containing 5–7.5 mol% of DSPE-mPEG with 2000 Da molecular weight (chain length) exhibit maximum circulatory life (Zalipsky et al., 1994). These liposomes are also preferentially accumulated in solid tumors (Papahadjopoulos et al., 1991). However no work has been done so far using these liposomes for the delivery of polypeptide toxins into tumor cells. Therefore attempts have been made to encapsulate ricin into various charged liposomes containing 5 mol% DSPE-mPEG-2000 on the surface and study the cytotoxicity of ricin in this formulation in CHO pro⁻ cells and the effect of monensin on the enhancement of cytotoxicity was also evaluated. The results of this study are shown in Fig. 6.

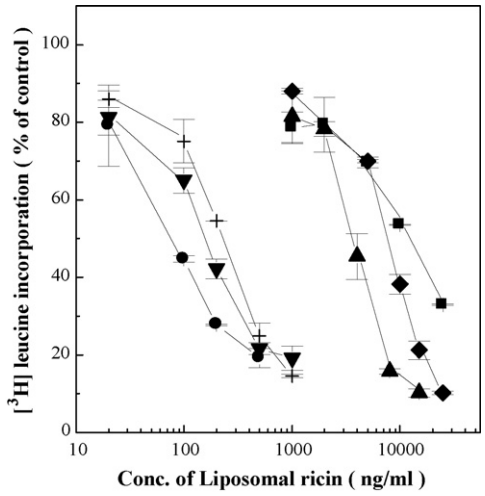

Fig. 6. Effect of Incorporation of 5 mol% of DSPE-mPEG-2000 on the surface of various charged liposomes on the cytotoxicity of entrapped ricin in CHO pro⁻ cells: effect of monensin. The monolayer cultures were preincubated with monensin, after preincubation the cells were treated with varying concentrations of 5 mol% PEGylated liposomal ricin having different charge with monensin [(●) neutral; (▼) negative; (+) positively charged] or without monensin [(■) neutral; (▲) negatively charged; (□) positively charged] (5 μg ^{125}I-ricin/0.6–0.9 μmol of phospholipids/(ml well)) for 4 h at 37 °C. The inhibition of protein synthesis was determined as described in Section 2. The results are expressed as percentage of incorporation of [^{3}H] leucine into proteins by untreated cells, i.e., the control. All the points represent mean ($n = 3$).

As can be seen in Fig. 6 that there is marginal variation in the cytotoxicity of ricin entrapped in various charged liposomes after incorporation of 5 mol% of DSPE-mPEG-2000 on their surface as compared to conventional liposomes in CHO pro⁻ cells. The ID_{50} of ricin was found to be 13.27, 8.33, and 13.07 μg/ml when entrapped in neutral, negatively and positively charged conventional liposomes. These values were reduced to 12.01, 3.66 and 7.88 μg/ml, respectively, following incorporation of 5 mol% DSPE-mPEG-2000 on their surface. However, there is a significant variation in the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes following incorporation of 5 mol% DSPE-mPEG-2000 on the surface. It was observed that the enhancement of cytotoxicity of ricin entrapped in neutral liposomes in the presence of monensin (71.30-fold) is further enhanced (161.35-fold) following PEGylation with 5 mol% DSPE-mPEG-2000. On the other hand, the enhancement of cytotoxicity of ricin entrapped in negatively and positively charged liposomes in the presence of monensin (83.36- and 213.14-fold, respectively) is drastically reduced (23.22- and 33.67-fold) following PEGylation with 5 mol% DSPE-mPEG-2000. These results clearly show that liposomal ricin cytotoxicity enhancing potency of monensin in CHO pro⁻ cells is not only dependent on the charge on the surface of liposomes but also on the composition of lipid formulations.

3.8. Binding of ^{125}I-ricin entrapped in sterically stabilized liposomes having 5 mol% DSPE-mPEG-2000 on the surface in CHO pro$^-$ cells at 4 °C

In order to ascertain whether the variation in cytotoxicity of ricin and differential potentiation ability of monensin on the cytotoxicity of ricin entrapped in various charged liposomes having 5 mol% DSPE-mPEG-2000 on the surface is due to the difference in binding, binding of ricin entrapped in different charged liposomal formulations having 5 mol% DSPE-mPEG-2000 was examined in CHO pro$^-$ cells at 4 °C. There is significant attenuation in binding of ^{125}I-ricin encapsulated in various charged liposomes following incorporation of 5 mol% DSPE-mPEG-2000 on the surface as compared to the conventional liposomal formulation. The maximum attenuation in binding (85.26%) was observed when 5 mol% DSPE-mPEG-2000 was incorporated in positively charged liposomes followed by 71.27 and 47.13% in negatively charged and neutral liposomes. Monensin has no significant effect on the binding.

3.9. Effect of incorporation of different concentration of DSPE-mPEG-2000 on the surface of various charged liposomes on the cytotoxicity of entrapped ricin in CHO pro$^-$ cells: effect of monensin

The result presented in Fig. 6 clearly show that the modulatory influence exercised by monensin on the cytotoxicity of ricin entrapped in various charged liposomes is significantly dependent on the presence of 5 mol% DSPE-mPEG-2000 on their surface. This raises an interesting question as to whether incorporation of different density of DSPE-mPEG-2000 on the surface of various charged liposomes has any effect on the cytotoxicity of ricin entrapped in these liposomes? What will be the modulatory influence of monensin on the cytotoxicity of ricin entrapped in these liposomal formulations? To answer these questions, ricin was encapsulated into various charged liposomes having different density of DSPE-mPEG-2000 on their surface. The cytotoxicity of ricin in these liposomal formulations was examined in CHO pro$^-$ cells. The modulatory influence exercised by monensin on the cytotoxicity of ricin entrapped in these liposomal formulations was also evaluated. The results of these studies are presented below.

Table 3 shows the effect of density of DSPE-mPEG-2000 on the surface of various charged liposomes on the cytotoxicity of entrapped ricin. As can be seen in the table the ID_{50} of ricin entrapped in conventional neutral, negatively charged and positively charged liposomes was found to be 13.27, 8.33 and 13.07 μg/ml, respectively. There is a marginal variation in the cytotoxicity of ricin entrapped in neutral liposomes following incorporation of different density of DSPE-mPEG-2000. On the other hand, the cytotoxicity of ricin (ID_{50}) entrapped in negatively charged and positively charged liposomes was reduced to 3.63 and 6.60 g/ml, respectively when 5 mol% and 2.5% of DSPE-mPEG-2000 was incorporated on the surface of liposomes.

Table 3
Effect of incorporation of different concentration of DSPE-mPEG-2000 on the surface of various charged liposomes on the cytotoxicity of entrapped ricin in CHO pro$^-$ cells

Mol % of DSPE mPEG-2000 in liposome	ID_{50} of ricin (μg/ml) in								
	Neutral liposomes			Negatively charged liposomes			Positively charged liposomes		
	Without monensin	With monensin	Fold	Without monensin	With monensin	Fold	Without monensin	With monensin	Fold
0	13.27 ± 0.6	0.18 ± 0.02	71.30 ± 4.92	8.33 ± 0.06	0.1 ± 0.007	83.36 ± 5.25	13.07 ± 1.9	0.06 ± 0.01	213.14 ± 4.79
1	17.65 ± 3.6	0.15 ± 0.006	120.14 ± 21.72	10.22 ± 0.23	0.05 ± 0.001	204.00 ± 0.05	17.01 ± 0.60	0.20 ± 0.06	84.27 ± 25.10
2.5	10.06 ± 2.5	0.06 ± 0.008	171.23 ± 19.71	7.61 ± 0.55	0.04 ± 0.006	168.86 ± 15.18	6.60 ± 0.80	0.05 ± 0.001	120.06 ± 13.86
5	12.01 ± 0.1	0.07 ± 0.005	161.35 ± 10.89	3.63 ± 0.43	0.15 ± 0.01	23.22 ± 1.26	7.88 ± 0.80	0.23 ± 0.005	33.67 ± 2.73
7.5	11.93 ± 0.2	0.55 ± 0.13	21.77 ± 5.09	12.51 ± 4.0	0.37 ± 0.08	33.53 ± 3.69	11.58 ± 0.12	0.55 ± 0.005	40.23 ± 0.03

Effect of monensin: 8×10^5 cells were plated 24 h prior to the experiment. The monolayer cultures were preincubated with or without 50 nM monensin for 1 h at 37 °C followed by the incubation with varying concentrations of different charged PEGylated liposomal ricin (5 μg ^{125}I-ricin/0.25–0.96 μmol of phospholipids/(ml well)) for 4 h at 37 °C. The inhibition of protein synthesis was determined by measuring the incorporation of [^{3}H] leucine into proteins as described in Section 2. The results are expressed as percentage of incorporation of ^{3}H leucine into proteins in untreated cells, i.e., the control. All the points are mean ± S.D. for three independent experiments.

However there is a significant variation in the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes having different density of DSPE-mPEG-2000 in CHO pro$^-$ cells. The potentiation ability of monensin on the cytotoxicity of ricin entrapped in neutral liposomes increases with the increasing density of DSPE-mPEG-2000 on the surface of the liposomes. The maximum potentiation of cytotoxicity of ricin (171.23-fold) was observed when the density of DSPE-mPEG-2000 on the surface of the liposomes was 2.5 mol%. The ID_{50} of ricin entrapped in conventional liposomes in presence of monensin was found to be 0.19 μg/ml. This value was further reduced to 0.15 μg/ml and 0.06 μg/ml when ricin was encapsulated to neutral liposomes having 1 and 2.5 mol% DSPE-mPEG-2000 on the surface. The ID_{50} was increased thereafter by increasing the density of DSPE-mPEG-2000. The ID_{50} of ricin was found to be 0.074 and 0.547 μg/ml when density of DSPE-mPEG-2000 increased to 5 and 7.5 mol%, respectively.

The maximum potentiation (204-fold) of cytotoxicity of ricin entrapped in negatively charged liposomes was observed when 1 mol% DSPE-mPEG-2000 was present on the surface. The ID_{50} of ricin was found to increase thereafter by increasing the density of DSPE-mPEG-2000 on the surface. The cytotoxicity of ricin was reduced to 168.9-, 23.2- and 33.3-fold when the density of DSPE-mPEG-2000 was increase to 2.5, 5.0 and 7.5 mol%, respectively.

In contrast to the observation of neutral and negatively charged liposomes, incorporation of DSPE-mPEG-2000 on the surface of positively charged liposomes reduces the potentiation of cytotoxicity of entrapped ricin by monensin. The maximum potentiation (213-fold) of cytotoxicity of ricin entrapped in positively charged liposomes by monensin was observed only when there was no DSPE-mPEG-2000 on the surface. However there is a significant variation in the potentiation of the cytotoxicity of entrapped ricin by monensin following incorporation of different density of DSPE-mPEG-2000 on the surface. Enhancing potency of monensin on the cytotoxicity of PEGylated positively charged liposomes entrapped ricin is significantly dependent on the density of DSPE-mPEG-2000. The maximum potentiation (120-fold) of cytotoxicity of ricin entrapped in PEGylated positively charged liposomes was observed when 2.5 mol% DSPE-mPEG-2000 was present on the surface. The ID_{50} of ricin was found to increase thereafter with increasing the density of DSPE-mPEG-2000 on the surface. The cytotoxicity of ricin was reduced to 33.67- and 40.23-fold when the density of DSPE-mPEG-2000 was increased to 5.0 and 7.5 mol%, respectively.

3.10. Binding of ^{125}I-ricin entrapped in differently charged liposomes having different density of DSPE-mPEG-2000 on the surface at 4 °C in CHO pro$^-$ cells

In order to evaluate whether the differential efficacy exhibited by monensin on the enhancement of the cytotoxicity of ricin entrapped in various charged liposomes having different density of DSPE-mPEG-2000 on the surface is due to the difference in binding, the binding at 4 °C of different charged liposomal ricin having different density of DSPE-mPEG-2000 on the surface in CHO pro$^-$ cells was examined.

Table 4 shows the effect of incorporation of different density of DSPE-mPEG-2000 on the surface of various charged liposomes on the binding of entrapped ^{125}I-ricin to CHO pro$^-$ cells at 4 °C. As shown in the table, when the cells were treated with 5000 ng/ml liposomal ^{125}I-ricin, the binding of ricin encapsulated in conventional neutral liposomal form was found to be maximum (35.05 ng/8 × 10^5 cells) and it reduced to nearly 18.0 ng/8 × 10^5 cells when 1 mol% with DSPE-mPEG-2000 was incorporated on the surface of the liposomes. However no significant variation of binding of neutral liposomal ricin to CHO pro$^-$ cells was observed following an increase the density of DSPE-mPEG-2000 from 1 to 5 mol% on the surface of liposomes. The binding was further reduced to 13.0 ng/8 × 10^5 cells when 7.5 mol% DSPE-mPEG-2000 was incorporated on the surface of liposomes.

The binding of conventional negatively charged liposomal ricin was found to be very similar to that observed with neutral liposomes and gradually decreases with the increasing concentration of the DSPE-mPEG-2000 on the liposomal surface. When the cells were treated with 5000 ng/ml liposomal ^{125}I-ricin, the maximum binding 34.71 ng/8 × 10^5 cells was observed with liposomes without DSPE-mPEG-2000 on the surface. This value decreased to 16.46, 13.38, 9.97 and 7.83 ng/8 × 10^5 cells

Table 4
Binding of ricin entrapped in different charged PEGylated liposomes having different density of DSPE-mPEG-2000 at 4 °C in CHO pro$^-$ cells

mol% of DSPE mPEG-2000 in liposome	Cell-associated liposomal ricin (ng/8 × 10^5 cells/well)					
	Neutral liposomes		Negatively charged liposomes		Positively charged liposomes	
	Without monensin	With monensin	Without monensin	With monensin	Without monensin	With monensin
0	35.05 ± 1.75	36.60 ± 1.83	34.71 ± 1.37	34.98 ± 1.45	98.65 ± 5.8	105.82 ± 5.2
1	18.50 ± 2.29	21.55 ± 1.07	16.46 ± 1.23	15.89 ± 0.97	29.45 ± 1.93	27.82 ± 1.93
2.5	18.26 ± 1.56	21.66 ± 1.83	13.38 ± 0.96	10.95 ± 0.57	13.94 ± 0.75	15.0 ± 0.75
5	18.53 ± 1.29	22.03 ± 1.51	9.97 ± 0.85	11.81 ± 1.10	14.54 ± 0.85	11.79 ± 0.85
7.5	13.00 ± 0.06	14.32 ± 1.1	7.83 ± 0.93	7.83 ± 0.74	9.03 ± 0.65	11.10 ± 0.65

The 8 × 10^5 cells/(ml well) were plated 24 h prior to the experiment. The monolayer cultures were preincubated with or without 50 nM monensin for 1 h at 4 °C followed by the incubation with 5 μg ^{125}I-ricin/0.25–0.96 μmol of phospholipids/(ml well) liposomal ricin with or without monensin, for 4 h at 4 °C. After incubation cells were washed thrice with ice cold DBSS. The binding of liposomal ricin is expressed as cell-associated liposomal ricin after 4 h incubation. All the points represent mean ± S.D. (n = 3).

when 1, 2.5, 5 and 7.5 mol% of DSPE-mPEG-2000 was incorporated on the surface of negatively charged liposomal ricin.

In contrast, the binding of ^{125}I-ricin entrapped in conventional positively charged liposomes without DSPE-mPEG-2000 on the surface to the CHO pro$^-$ cells was maximum as compared to neutral and negatively charged liposomes. When 8×10^5 cells/ml were incubated with 5000 ng/ml conventional positively charged liposomal ^{125}I-ricin, 98.65 ng ^{125}I-ricin was found to be associated with cells. On the other hand, 35.05 ng and 34.71 ng ^{125}I-ricin were associated with cells when incubated with ^{125}I-ricin entrapped in neutral and negatively charged liposomes. A significant reduction (3.3-fold) in binding of ^{125}I-ricin encapsulated in positively charged liposomes to CHO pro$^-$ cells was observed following an incorporation of 1 mol% DSPE-mPEG-2000 on the liposomal surface. This value further reduced to 13.94 ng and 9.03 ng/8×10^5 cells when 2.5 and 7.5 mol% of DSPE-mPEG-2000 were incorporated on the surface of the liposomes. No significant variation in the binding of ^{125}I-ricin encapsulated in positively charged liposomes was observed when the density of DSPE-mPEG-2000 was increased from 2.5 to 5 mol% on the surface of the positively charged liposomes (Table 4).

4. Discussion

In the present investigation, the ricin was encapsulated in neutral, negatively and positively charged liposomes and the cytotoxicity of ricin entrapped in these different charged liposomal formulations was studied in Chinese hamster ovary cells (CHO pro$^-$) and compared with free ricin with a view to develop an optimum delivery system for ricin *in vivo*. The results of the current study showed that cytotoxicities of ricin entrapped in all the formulations, i.e., neutral, negatively and positively charged liposomes are significantly reduced in CHO pro$^-$ cells. These results support earlier observation that there is a marked reduction in the cytotoxicity of ricin following its encapsulation in liposomes (Dimitriadis and Butters, 1979; Bharadwaj et al., 2006). However, the extent of reduction in cytotoxicity is dependent on the charge on the surface of the liposomes. The extent of reduction of the cytotoxicity of ricin entrapped in negatively charged liposomes was found to be minimum (122-fold) followed by positively charged (192-fold) and neutral liposomes (195-fold) as compared to free ricin. This result is not unexpected as it has been reported by several investigators that interaction of liposomes with mammalian cells is highly dependent on several factors like lipid composition, size, charge, bilayer fluidity, hyrophilicity of the liposomes and cell types. For examples, Hsu and Juliano (1982) found that peritoneal macrophages take up negatively charged vesicles more readily than positively charged or neutral vesicles (Hsu and Juliano, 1982). Heath et al. (1985) found that in CV1-P (African green monkey kidney cell line), negatively charged liposomes associate more effectively and deliver their contents more efficiently than neutral liposomes.

Binding and internalization studies revealed that there is a significant reduction in binding and uptake of ricin following its encapsulation into various charged liposomes in CHO pro$^-$ cells. It was also observed that there is a significant variation in the binding and uptake of ricin entrapped in various charged liposomes with CHO pro$^-$ cells. These results are in agreement with earlier observation by several investigators that interaction of liposomes with mammalian cells is highly dependent on several factors like lipid composition, size, charge, bilayer fluidity, hyrophilicity of the liposomes and cell types (Maggio et al., 1976; Du et al., 1997). These studies revealed that free ricin enter into mammalian cells by galactose-mediated endocytic pathways while liposomal ricin are taken up by mammalian cells by an alternative route bypassing normal galactose mediated endocytic pathway. This route may be less efficient in transporting ricin across mammalian cell membrane as well as release of ricin into cytosol from endocytic vesicles. This may be one of the possible basis for the reduction of cytotoxicity of ricin following encapsulation into various charged liposomes.

It was also observed that among the various liposomal compositions tested for delivery of ricin, ricin exhibit maximum cytotoxicity in CHO pro$^-$ cells when delivered through negatively charged liposomes. On the other hand, binding and internalization studies revealed that the maximum uptake of ricin into these cells was observed when it was delivered through positively charged liposomes. The reason for this discrepant result is not very clear. However, these results are in agreement with earlier report by Miller et al. (1998) that HeLa cells, a human ovarian carcinoma cell line, endocytosed positively charged liposomes to greater extent than either neutral or negatively charged liposomes. Different types of molecules are endocytosed by cells and enter through a coated pit/coated vesicle pathway. The fate of endocytosed molecules can vary from rapid recycling to the cell surface to progression through a variety of intracellular compartments depending on the receptor- ligand system present in the endosome (Steinman et al., 1983). It has been reported that the negatively charged liposomes endocytosed into cytoplasm following its binding through different sites on the cell surface than do positively charged and neutral liposomes (Fraley et al., 1981). Therefore it is reasonable to speculate that the intracellular routing of various charged liposomes is different and it is possible that the mechanism of release of ricin encapsulated in neutral, negatively and positively charged liposomes from endosome into cytosol also may be different.

This report has demonstrated that the cytotoxicity of ricin entrapped in various charged liposome is significantly enhanced by both NH_4Cl (20 mM) and monensin (50 nm). NH_4Cl and monensin (>1 μm) are known to inactivate lysosomal enzyme by raising intralysosomal pH thereby prevent degradation of ricin and immunotoxins resulting in the enhancement of the cytotoxicities of ricin and ricin A-chain based immunotoxins (Casellas et al., 1984). It has also been reported that in the presence of either of these substances IT accumulated in an enlarged endocytic vesicles and entry into the lysosomes was slowed down and concluded that intense slowing down in the speed of IT transportation into lysosomes and the functional modification of these organelles help to increase the efficacy of ITs in the presence of potentiators (Carriere et al., 1985). The maximum enhancement of the cytotoxicity of ricin was observed when it was delivered through positively charged liposomes. The enhancement of cytotoxicity of ricin entrapped in various charged liposomes

by NH_4Cl is not entirely unexpected as it has been reported by several investigators that negatively charged liposomes enter into mammalian cells by endocytosis and liposomal contents encounter the acidic pH in the endosomes (Straubinger et al., 1983). It has also been demonstrated that NH_4Cl and chloroquine inhibit significantly the degradation of liposomal protein. Similarly it was shown earlier by us (Bharadwaj et al., 2006) that NH_4Cl inhibit significantly the degradation of liposomal ricin suggesting that NH_4Cl either slowed down the transfer of liposomal ricin to lysosomes or prevents its degradation by inactivating the lysosomal enzymes possibly by increasing the intralysosomal pH. Consequently, there would be an enhanced possibility of escape of the liposome-encapsulated ricin into cytoplasm, either by fusion of liposomes directly with the phagolysosomal membrane or by some non-specific breakdown of these membranes. This may be the basis for the enhancement of the cytotoxicity of liposomal ricin by NH_4Cl. All these results indicate that ricin entrapped in various charged liposomes enter into mammalian cells by endocytosis and encounter acidic pH in the endosomes, however, neutral or alkaline pH is optimal for its release into the cytosol. Stearylamine is a weak base, and may act as a lysosomotropic agent. The mechanism of maximum enhancement of cytotoxicity of ricin by NH_4Cl when delivered through positively charged liposomes may due to synergistic action of stearylamine and NH_4Cl in disrupting function of lysosomes.

A dramatic enhancement of cytotoxicity of ricin entrapped in various charged liposomes by monensin (50 nM) was observed suggesting that Golgi apparatus is involved in liposomal ricin intoxication process as in native ricin. It appears therefore that transport of ricin entrapped in all the formulations of liposomes from endosome to Golgi apparatus is essential for its release into the cytosol and optimal expression of its cytotoxicity. However, the extent of enhancement of cytotoxicity of ricin entrapped in various charged liposomes by monensin is found to be highly dependent on the charge on the surface of liposomes and significantly higher as compared to free ricin. It appears therefore, that the rate of transport of ricin entrapped in various charged liposomes to Golgi apparatus in presence of monensin is different. Consequently, there is a significant variation in the enhancement of the cytotoxicity of ricin entrapped in various charged liposomes by monensin. The mechanism by which ricin entrapped in various charged liposomes routes through the Golgi apparatus is not obvious, however, role of ricin B-chain cannot be ruled out. Further Straubinger et al. (1983) have reported that the presence of liposomes in vesicle in the *trans*-Golgi region after endocytosis.

Studies on the extent of exocytosis and degradation of intracellular ricin delivered through various charged liposomes revealed that the amount of intracellular ricin released, either in intact or degraded form, into extracellular medium is significantly depends on the charge of the liposomes used for its delivery. However, extent of exocytosis of intracellular ricin delivered through various charged liposomes is significantly lower as compared to free ricin. The maximum amount (38.53%) of intracellular ricin released into extracellular medium when it is delivered through neutral liposomes followed by negatively (32.5%) and positively (29%) charged liposomes. On the other hand, 65.74% of free ricin is released into extracellular medium. Consequently, the intracellular level of ricin delivered through various charged liposomes was found to be significantly higher (1.7–2.3-fold) as compared to free ricin. It was also noted that the amount of ricin release in degraded form is dependent on the surface charge of the liposomes used for its delivery. The amount of ricin released in degraded form is minimum when delivered through positively charged liposomes (42.6%) followed by negatively charged (62.%) and neutral (65.45%) liposomes. The reduced rate of exocytosis, degradation and higher cell-associated ricin when delivered through positively charged liposomes as compared to negatively charged and neutral liposomes can account for higher intracellular level and greater extent of enhancement of cytotoxicity of ricin by monensin when delivered through positively charged liposomes. The observed higher intracellular level of ricin when delivered through various charged liposomes for a longer period of time as compared to free ricin may allow more efficient ricin A-chain release from liposomal ricin located in an intracellular compartment into the cytosol under the influence of monensin. This may explain why monensin brings about a higher degree of enhancement of cytotoxicity of ricin when delivered through various charged liposomes as compared to free ricin.

The mechanism of differential release of intracellular ^{125}I-ricin into extracellular medium and into cytosol internalized through various charged liposomes as compared to free ricin is not obvious. However it is known that the endosomal system is involved in sorting and routing of ligands and receptors and recycling of membranes to the cell surface. These sorting and routing events at the level of endosomal system is significantly dependent on the nature of ligands and receptors (Mukherjee et al., 1997). Although different types of molecules are endocytosed by cells and enter through a coated pit/coated vesicle pathway, the fate of endocytosed molecules can vary from rapid recycling to the cell surface to progression through a variety of intracellular compartments depending on the receptor–ligand system present in the endosome (Steinman et al., 1983; Brown and Goldstein, 1979). Further van Deurs et al. (1986) have reported that the valency of the ligands influences routing and sorting events at the level of endosomal system or ligand conjugates. They have shown that native ricin and ricin-HRP monoconjugates after endocytosis reached vesicular and tubular vesicular elements as well as Golgi apparatus, on the other hand, ricin–colloidal gold conjugates which is polyvalent (2–4 ricin per gold particle) reached vesicular and tubular elements only but did not reach the Golgi elements. However they have not studied the effect of monensin on the cytotoxicity of polyvalent ricin–colloidal gold conjugates.

Ricin enters into mammalian cells by galactose-mediated clathrin-dependent coated as well clathrin-independent uncoated endocytic pathway (Sandvig and van Deurs, 1999) and reaches the vesicular and tubular vesicular portions of the endosomal system. It has been reported that 5% of the internalized ricin is rapidly transported from endosome to TGN and only 1% of the internalized ricin is released into cytosol (van Deurs et al., 1988). It was also shown that transport of ricin from endosome to TGN and retrograde transport from TGN to

ER through Golgi stack is essential for the expression of its cytotoxicity (van Deurs et al., 1986; van Deurs et al., 1988; Wesche et al., 1999). Only that part of ricin, which is retrograde transported through Golgi stack is responsible for its toxicity. The TGN is an intracellular site where the endocytic and secretory pathway meet. The accelerated transport of ricin from endosome to TGN, the organelles responsible for exocytosis, and subsequent release of a fraction of it into cytosol may be responsible for rapid release of ^{125}I-ricin into extracellular medium. On the other hand, negatively charged liposomes enter into mammalian cells by clathrin-dependent endocytic pathway following its adsorption on the cell surface through unknown receptor (Straubinger et al., 1983). Endocytosed liposomal markers remain in a large intracellular endocytic vesicle for a longer period of time and mainly transported to lysosomes for degradation (Dijkstra et al., 1985) and part of it may be slowly transported to TGN (Straubinger et al., 1983). It is also possible that liposomal ricin having more than one ricin molecule per vesicle behaves like a multivalent ligand as ricin–colloidal gold conjugate (van Deurs et al., 1986) and did not reach Golgi apparatus which is prerequisite for expression its cytotoxicity leading to reduced toxicity and exocytosis. However, no work has been done to determine the amount of time spent by various charged liposomes in these vesicles and amount of liposomal markers transported to TGN depending on the charge of the liposomes. Therefore, it is reasonable to speculate that the time spent by various charged liposomes in these vesicles may be different. Consequently, the extent of intracellular ^{125}I-ricin, present in endocytic vesicles encapsulated in various charged liposomes, transported to TGN and released into extracellular medium may be different. As a result, the intracellular level of ricin delivered through various liposomes will vary. It appears, therefore that rate of variation in the transport of ricin into TGN from various charged liposomal form present into intracellular compartment is the limiting step in the toxicity difference between ricin encapsulated in various charged liposomes and free ricin.

Recently, it has been reported from this laboratory that monensin significantly reduces the lag phase of intoxication by ricin entrapped in negatively charged liposomes (Bharadwaj et al., 2006). In order to evaluate whether variation in the charge on the liposomal surface has any effect on the lag period of intoxication by ricin encapsulated in various charged liposomal formulations, the kinetics of protein synthesis inhibition by free ricin and ricin entrapped in various charged liposomes in the presence and absence of monensin was examined. It was observed that lag period of inhibition of protein synthesis by ricin is significantly lengthened following delivery through various charged liposomes. The extent of lengthening of lag period of ricin action is dependent on the surface charge of the vehicle. The maximum lengthening of lag period (4 h) was observed when ricin was delivered through neutral and negatively charged liposomes followed by positively charged liposomes (2 h) as compared to 45 min observed for free ricin. These results suggest that the rate of release of ricin A-chain from ricin encapsulated in various charged liposomes located in an intracellular compartment into cytosol are different. Monensin (50 nM) reduced the lag period of free ricin from 45 to 15 min (three-fold reduction of lag period). On the other hand, the reduction of lag period of inhibition of protein synthesis by ricin encapsulated in various charged liposomes in the presence of monensin is significantly dependent on the charge on the surface of the vehicle. The maximum reduction of lag phase by monensin was observed when ricin was delivered through negatively charged liposomes (four-fold) followed by positive charged liposomes (two-fold). A lag period of 2 h was observed, even in the presence of monensin (50 nm), when ricin was delivered through neutral liposomes. The mechanism of variation in the reduction of lag phase of inhibition of protein synthesis by ricin encapsulated in various charged liposomes by monensin is not very clear. However it is known that the monensin induced enhancement of cytotoxicity of ricin is due to enhanced transport of ricin from cell surface to Golgi apparatus (Wesche et al., 1999) and decreased transfer of ricin to the lysosomes (Carriere et al., 1985). Therefore, it is reasonable to speculate that monensin reduces the lag period of various liposomal ricin actions by enhancing the transport of ricin from intracellular compartment to TGN and decrease transfer of liposomal ricin to the lysosomes. This rate of transport of ricin encapsulated in various charged liposomes to TGN and lysosomes is dependent on the surface charge of the liposomes leading to variation in the reduction in lag phase of ricin action.

A significant reduction in the lag period of various charged liposomal ricin action by monensin suggests a more rapid and efficient release of ricin from intracellular compartment into the cytosol. This result is consistent with our earlier observation (Ghosh et al., 1985; Ghosh and Wu, 1988; Madan and Ghosh, 1992) and those reported by other investigators (Carriere et al., 1985) that pretreatment of cells with carboxylic ionophore results in a reduction of lag period in the inhibition of protein synthesis by ricin and immunotoxins.

A number of investigators (Miller et al., 1998; Du et al., 1997) have reported that incorporation of 5 mol% PEG-PE on the surface of liposomes inhibit adsorption of liposomes to the surface of cells and subsequent endocytosis of liposomes. Therefore, it is expected that the cytotoxicity of ricin entrapped in various charged liposomes will be reduced by incorporation of 5 mol% of DEPE-mPEG-2000 on their surface. In contrast, it was observed that there is a marginal enhancement of the cytotoxicity of ricin entrapped in various charged liposomes after incorporation of 5 mol% DSPE-mPEG-2000 on the surface in CHO pro$^-$ cells (Fig. 6). On the other hand, binding studies revealed that the overall amount of cell associated various charged PEG-liposomes examined was significantly attenuated relative to conventional liposomes. The mechanism of these discrepant results is not well understood. However, polyalcohols like polyethylene glycols, glycerols are known to alter membrane structure and hydration properties as well as membrane fusion (Maggio et al., 1976) and endocytosis (Norberg, 1970). In this respect, it should be noted that Szoka et al. (1981) have suggested that polyethylene glycol of molecular weight 1500–4000 treatment stimulates the cellular uptake of glycolipid-containing liposomes (in the presence of lectins) via their fusion with the plasma membrane. Therefore, it seems likely that polyethylene

glycol-2000 used for the preparation of various charged PEG-liposomes may stimulate the cellular uptake of liposomes by increasing the frequency of fusion or endocytosis. Similarly, Fraley et al. (1981) have reported the enhanced intracellular delivery of liposomes encapsulated DNA, RNA and fluorescent dye into mammalian cells by treatment with glycerol.

However, there is a significant variation in the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes following incorporation of 5 mol% DSPE-mPEG-2000 in CHO pro$^-$ cells. The enhancement of cytotoxicity of ricin entrapped in neutral liposomes in the presence of monensin is further enhanced from 71.30- to 161.35-fold following PEGylation. On the other hand, the cytotoxicity of ricin entrapped in negatively and positively charged liposomes in the presence of monensin is reduced from 83.36- and 213.14-fold to 23.22- and 33.67-fold, respectively (Fig. 6). Binding studies revealed that the overall amount of cell-associated various charged PEG-liposomes examined was attenuated relative to conventional liposomes. However, the extent of attenuation of cell-associated liposomes was greatest for positively (85.26%) and negatively (71.27%) charged PEG-liposomes, on the other, hand only 47.13% neutral PEG-liposomes binding was attenuated. These results clearly indicate that there is a direct correlation between the efficacies of monensin on the enhancement of cytotoxicity of ricin entrapped in various charged PEG-liposomes with their amount of cell-association. The decreased potency of monensin on the enhancement of cytotoxicity of ricin entrapped in negatively and positively charged PEG-liposomes could be accounted for their decreased association with the cells.

Studies on the effect of variation of density of DEPE-mPEG-2000 on the surface of various charged liposomes on the enhancement of cytotoxicity of entrapped ricin by monensin in CHO pro$^-$ cells showed that the enhancing potency of monensin on the cytotoxicity of ricin entrapped in various charged liposomes is significantly dependent on the density of DEPE-mPEG-2000 on their surface (Table 3). The maximum potentiation of cytotoxicity of ricin (171.23-fold) entrapped in neutral liposomes by monensin was observed when the density of DSPE-mPEG-2000 on the surface was 2.5 mol%. On the other hand, the maximum potentiation of cytotoxicity of ricin (204-fold) entrapped in negatively charged liposome was observed with 1 mol% DSPE-mPEG-2000 on the surface. In contrast to the observation of neutral and negatively charged liposomes, incorporation of any density between 1 and 7.5 mol% of DSPE-mPEG-2000 on the surface of positively charged liposomes reduced the potentiation of cytotoxicity of entrapped ricin by monensin relative to conventional liposomes. Comparison of binding of various charged liposomes having different density of DSPE-mPEG-2000 on their surface revealed that there is a significant variation in the binding of liposomes depending on the charge. The efficacies of monensin on the enhancement of cytotoxicity of ricin entrapped in various charged PEG-liposomes in CHO pro$^-$ cells was found to be highly related with their amount of cell-association. These results further confirm that there is a direct correlation between the efficacies of monensin on the enhancement of cytotoxicity of ricin entrapped in various charged PEG-liposomes with their amount of cell-association.

Binding studies revealed that the overall amount of cell-associated various charged liposomes having different density of PEG was significantly attenuated relative to conventional liposomes. In contrast, the enhancement of cytotoxicity of ricin entrapped in neutral and negatively charged liposomes by monensin was found to be maximum when 2.5 and 1 mol% DSPE-mPEG-2000, respectively, was incorporated on their surface. The attachment of PEG on the surface of liposomes neutralizes the electrical properties of all types of liposomes (neutral, negative and positive) (Table 1). However no correlation was observed between the electrical properties of liposomes and the enhancement of the cytotoxicity of ricin entrapped in various charged PEGylated liposomes by monensin. The mechanism of enhanced potentiation of cytotoxicity of ricin entrapped in neutral and negatively charged liposomes by incorporation of DSPE-mPEG-2000 on their surface by monensin is not clear, but it is reasonable to speculate that polyethylene glycol may stimulate some intracellular fusion events leading to enhanced possibility of escape of the liposomes-encapsulated ricin into the cytoplasm in the presence of monensin.

The present study has clearly shown that by suitable alteration of liposomal lipid composition, charge and density of hyrophilicity, it would be possible to direct liposomal ricin to specific cells for their selective elimination in combination with liposomal monensin. Liposomes as a delivery vehicle for ricin have an advantage over monoclonal antibody for cell-specific delivery of toxins to tumor cells. For example, it is constituted from natural lipids, which are non-immunogenic and its structure could be altered with variety of ligands for malignant cell-specific delivery. Furthermore, ricin in the aqueous compartment of liposomes is shielded from cytotoxicity and adverse immunological reaction in the circulation; consequently intact ricin can be delivered through liposomes. The present study has added to our contention that by suitable alteration of liposomal lipid composition, charge, density of hydrophilicity and tailoring of liposomal surface with appropriate ligand it would be possible to direct liposomal ricin to specific cells or tissues for their selective elimination in combination with monensin. However, the hydrophobic nature of monensin has made it difficult to administer it in optimum doses under *in vivo* condition in order to realize its full potential in the enhancement of toxicities of ricin and immunotoxins. We have shown for the first time that liposome can be used as a delivery vehicle of monensin under both *in vitro* and *in vivo* conditions (Madan and Ghosh, 1992; Vasandani et al., 1992). Subsequently, it has been reported that liposomal monensin is very effective in the treatment of tumor in combination with immunotoxins (Singh et al., 1994) Thus, liposomal ricin in combination with liposomal monensin may have potential application for selective elimination of malignant cells.

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