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Research paper

Stealth monensin immunoliposomes as potentiator of immunotoxins in vitro

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Abstract

Stealth monensin liposomes (SML) were prepared using dipalmitoyl phosphatidylcholine, cholesterol, distearoyl glycerophosphoethanolamine coupled to polyethylene glycol, stearylamine, and *N*-succinimidyl pyridodithiopropionate linked to stearyl amine, in the molar ratio of 10:5:1.4:1.4:1.5. SML was conjugated to the anti-MY9 antibody by a disulfide linkage to form stealth monensin immunoliposomes (SMIL) by an already established procedure. The encapsulation concentrations of monensin in SML and SMIL were 10^{-7} and 4.9×10^{-8} M, respectively. More than 20% of monensin remained in circulation after 24 h in BALB/c mice. The ability of SML and SMIL to potentiate the effect of anti-MY9 immunotoxin (anti-MY9-IT) was tested against human leukemia HL-60 sensitive and resistant tumor cells in vitro. SML and SMIL potentiated the activity of anti-MY9-IT by 10–20 times against HL-60 sensitive tumor cell lines. However, greater potentiation of anti-MY9-IT was observed in combination with SML and SMIL against HL-60 resistant tumor cells, found to be 200 and 500 times, respectively. The potentiation of anti-MY9-IT by SMIL was more than two-fold compared with SML against both HL-60 sensitive and resistant tumor cells. Transmission electron microscopy studies conducted with HL-60 resistant cells incubated with anti-MY9-IT and monensin liposomes showed significant dilation of the golgi, which was reversible after re-incubation in fresh medium. Our studies show that SML and SMIL can be successfully used to potentiate the activity of ricin based anti-MY9-IT in vitro, and further in vivo studies will demonstrate the usefulness of this approach.

Keywords: Immunotoxin; Stealth liposomes; Stealth immunoliposomes; HL-60 tumor cells; Potentiation

1. Introduction

For many years, liposomal drug delivery systems have shown reduced toxic side-effects and increased activity as a result of changes in the pharmacokinetics and biodistribution of entrapped drug [1]. However, the in vivo use of conventional liposomes is hampered by their rapid removal from circulation by the reticuloendothelial system (RES) [2]. The circulation half-life of liposomes in vivo can be significantly prolonged by steric stabilization of the liposomes [3]. This has been achieved by the inclusion of polyethylene glycol (PEG) derivatized lipids within the bilayer of the classical liposomes [4].

Many toxic drugs may be targeted to tumor sites by conjugation with a specific antibody that has a selective affinity for tumor cells [5,6]. Recent developments of sterically stabilized or 'stealth' liposomes have caused a surge in interest in the formulation of sterically stabilized liposomes conjugated to specific monoclonal antibody (SIL). Successful SIL should have: (a), a long circulation time; (b), the ability to recognize and specifically bind to the target cells; and (c), minimum delivery of the drug to non-specific tissues. There are reports of successful formulations of different types of SIL having the above-mentioned properties [7–9].

Toxins conjugated with monoclonal antibody offer a unique opportunity to target and kill potential tumor cells [10,11]. The anti-MY9 blocked ricin conjugate was developed at ImmunoGen, Inc. (Norwood, MA). Blocked ricin is an altered ricin derivative that has its non-specific binding site inactivated by chemically blocking the galactose binding domains of the B-chain. This conjugate is designed to selectively destroy acute myeloid leukemia cells (AML) in vitro and in vivo. The monoclonal antibody (Mab), anti-MY9, reacts with an antigen (CD 33) found on clonogenic AML cells from greater than 80% of cases and does not react with normal pluripotent stem cells [12,13]. This conjugate has shown very high potency against HL-60 cells and

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an inhibitory concentration to kill at least 50% of cells (IC_{50}) of about 3×10^{-11} mol/l. However, clinical studies have shown some limitations in the potency of this conjugate. Earlier studies were conducted using other chemotherapeutic drugs, like cisplatin and vincristine, in combination with the anti-MY9 immunotoxin (anti-MY9-IT) to potentiate its activity. The anticancer drugs being used for anti-MY9-IT potentiation are non-selective, and hence, are toxic. Presently, there is a dire need for non-toxic potentiating agents which can increase the activity of anti-MY9-IT without increasing its toxicity. The use of delivery systems for ionophores like monensin is a very rational and desired approach at this time [14,15].

In the present study, we report on the formulation of long circulating stealth monensin liposomes (SML) and anti-MY9 antibody conjugated stealth monensin immunoliposomes (SMIL). The in vitro potentiating effect of the formulations with anti-MY9-IT against both sensitive and resistant human leukemia HL-60 tumor cell lines has been reported. Transmission electron microscopy (TEM) studies have also been conducted in order to understand the mechanism of action of monensin liposome formulations.

2. Materials and methods

2.1. Chemicals and drugs

Cholesterol (CH), stearylamine (SA), dipalmitoyl phosphatidylcholine (DPPC) and monensin were obtained from Sigma Chemical Co. (St. Louis, MO). Distearoylphosphatidyl ethanolamine linked to polyethylene glycol (DSPE-PEG) was obtained from Shearwater Polymers, Inc. (Huntsville, AL). *N*-succinimidyl 3,2-pyridodithiol propane (SPDP) was obtained from Pharmacia Biotech (NJ). All lipids were more than 99% pure and were used without further purification. Radioactive [^{3}H]monensin was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate and tissue culture media were purchased from Sigma Chemical Co. (St. Louis, MO). For gel electrophoresis, precast gels were obtained from Bio-Rad Laboratories (Hercules, CA); other chemicals were obtained from Novex (San Diego, CA).

2.2. IT and antibody

The anti-MY9-IT and the anti-MY9 antibody were generous gifts from Dr Victor Goldmacher, ImmunoGen, Inc. Much of the work with the anti-MY9-IT has already been published [16]. Anti-MY9 is a mouse IgG targeted against the CD33 antigen. The CD33 antigen is present on normal colony forming unit granulocyte–monocyte, on a fraction of burst forming unit erythroid, monocyte and megakaryocyte and absent from normal pluripotent cells.

2.3. Preparation of SML

Various liposome formulations were made (more than 100), and finally, a formulation was chosen, which contained DPPC, cholesterol, DSPE-PEG, SA, *N*-succinimidyl pyridodithiopropionate linked to stearyl amine (PDP-SA) and monensin in the molar ratio of 10:5:1.4:1.4:1.5:1. All the lipids (DPPC, cholesterol, DSPE-PEG, stearyl amine) and monensin were dissolved in 20 ml of a chloroform–methanol (2:1) mixture in a pear shaped flask. The solvent was evaporated in a rotary evaporator to form a thin film of lipid. Multilamellar vesicles (MLV) were formed by shaking the flask with phosphate-buffered saline (PBS) for 1 h at 60°C. The MLVs were extruded through an Extruder (Lipex Biomembranes, Vancouver, B.C., Canada), at high pressure through a series of polycarbonate membranes of gradually decreasing pore size, 0.6, 0.4, 0.2, 0.1 and 0.05 μm, which produced small unilamellar vesicles (SUV). The extrusion was carried out at 70°C. Free monensin was removed from SUVs by centrifuging the liposomes at 270 000 × *g* for 30 min and removing the supernatant. The centrifugation was repeated 3–4 times until the washings contained no free monensin.

2.4. Conjugation of the anti-MY9 antibody with stealth liposomes

Briefly, the method consists of adding anti-MY9 antibody (100 μg/ml) to *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) solution (at 1:10 molar ratio) with continuous stirring for 45 min. The mixture was initially dialyzed with PBS buffer (pH 7.4), and then with acetate buffer (pH 4.0) to remove the impurities. The antibody was then reduced with an excess amount of dithioerythritol (DTT) and passed through a Sephadex column (Pharmacia, Sweden). The absorbance of the eluted samples was recorded at 280 nm using a Beckman DU800 spectrophotometer. Samples containing the antibody were pooled together and mixed with SML and stirred overnight, which formed the SMIL. Finally, the SMIL was washed by centrifugation to remove non-entrapped monensin. Alternatively, normal mouse globulin (NMG) was used as non-specific control to conjugate it to SML with the same method as described above.

2.5. Analysis of monensin in SML and SMIL

The method reported by Singh et al [13] was used for analysis of monensin. After preparation and final washing of the SML or SMIL, 10 μl of the sample was taken and 50 μl of Tween 20 was added. The sample was vortexed for few minutes, 3 ml of scintillation fluid (Scintiverse II, Fisher) was added, and analyzed by a LKB Wallac Liquid scintillation counter.

2.5.1. Particle size analysis

The mean diameter and the standard deviation of SML and SMIL were determined by photon correlation spectro-

scopy with a BI 90 particle sizer (Brookhaven Instruments, NY). Each sample was diluted with 3 ml of filtered saline and measured at 25°C. The duration was 5000 cycles for each sample, and the minimum count rate was 50 kcps. The particle size was analyzed after the final washing. Liposomes with an average diameter of 115–150 nm were evaluated for their efficacy in all of the in vitro cytotoxic studies.

2.6. Immunofluorescence assay

HL-60 cells (2×10^6) in RPMI media were mixed with SMIL (50 μl) and incubated at 37°C for 45 min. The cells were centrifuged and washed with cold PBS 4–5 times to remove SMIL. Goat anti-mouse IgG FITC conjugate (50 μl) was added and incubated at 4°C for 30 min. The cells were washed with cold PBS buffer to remove the excess goat anti-mouse IgG FITC. Finally, 1 drop of 10% Tris-buffered glycerol was added to the cells. The cells were mounted on a glass slide and observed under a fluorescent microscope. The concentration required to stain 50% of the cells was regarded as the titer of the antibody either by itself (control) or conjugated to SML.

2.7. Analysis by gel electrophoresis

Precast gel was placed in Mini-Protean II Cell (Bio-Rad, CA). The wells were loaded with SMIL, anti-MY9 antibody, and standard protein. The buffer chamber was filled with running buffer and attached to a 200 V constant power supply for 1 h. After electrophoresis was complete, the gel was stained with Coomassie Blue R-250 stain (Novex, San Diego, CA) for half an hour. The gel was then destained, washed and dried with Gel-Dry solution (Novex, San Diego, CA). Two pieces of cellophane were placed on two sides of gel and dried overnight at room temperature.

2.8. TEM

In order to understand the mechanism of action of SML, studies were carried out at the ultrastructure level using TEM. Briefly, the method consisted of incubating HL-60 resistant cells in the presence of SML and IT for periods of 0, 10, 30, 60 and 120 min. The cells, after incubation, were washed with cold PBS thrice and then fixed overnight in 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer. The tumor cells were then post-fixed in osmium tetroxide (1% buffer, 2 h) and uranyl acetate overnight. The cells were then washed and dehydrated in a series of ethanol: 50, 75, 95, 100 and 100% (dried). The dehydrated cells were then embedded in tetra-anhydro-amino-benzaldehyde (TAAB) resin and subsequently allowed to mature in an oven at 65–70°C. Thin sections were cut with a LKB Huxley ultramicrotome and picked up on 300 mesh nickel coated grids, stained with 2% (w/v) uranyl acetate and lead citrate. The cells were then observed with a Philips electron microscope (EM 200). The control in these studies was HL-60 resistant cells incubated with IT alone and monensin solution (no liposomes). In an another experiment, the HL-60 resistant cells were incubated with SML and IT for 1 h and then washed with PBS to remove SML and IT. The cells were then incubated with fresh media for 2 h. The cells were finally washed, post-fixed, dehydrated, embedded and analyzed by TEM as mentioned above.

2.9. Determination of half-life

These studies were conducted using BALB/c mice (18–22 g) that were dosed by bolus tail vein injection. Each mouse was injected with either SML or SMIL (containing [^{3}H]monensin and 0.7 M of total lipid) into the tail vein. At regular time intervals (0, 2, 4, 6, 8, 12 and 24 h), 0.5 ml of blood was collected by heart puncture after anesthesia by halothane and the mouse was sacrificed. The blood was collected in tubes and centrifuged to separate the serum. To 0.1 ml of serum, 0.1 ml of Solvable (Packard Instruments Co., Meriden, CT) was added to decolorize the sample. Finally, 3 ml of scintillation fluid was added, and the concentration of monensin was determined by the scintillation counter. Radioactivity in the blood samples was converted into the percentage of injected dose units. Each experiment was performed in triplicate and the average values were plotted.

2.10. In vitro potentiating effect of SML and SMIL

2.10.1. The [3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] assay

Briefly, 15 000 HL-60 tumor cells (in 100 μl)/well were plated in a 96-well plate. After 24 h, various dilutions of anti-MY9-IT were added along with a constant, non-toxic amount of SML or SMIL (containing 10^{-9} M monensin). The cells were incubated in a CO_2 jacketed incubator for 2 days at 37°C. The cells were then treated with a combination of phenazine methosulfate (PMS), an electron coupling reagent and a novel tetrazolium compound (MTS). The MTS was bioreduced by cells into formazan which was soluble in tissue medium. This was accomplished by dehydrogenase enzymes present in the viable tumor cells. The quantity of formazan could be measured directly from 96-well assay plates. The absorbance of formazan was measured at 490 nm, which was directly proportional to the number of living cells in the 96-well culture plates [17]. The plates were read using a KC3 Microplate Reader (Bio-TeK Instruments, Winooski, VT). From the absorbance of the treated cells and control, IC_{50} values for the tumor cells were calculated. Similar tests were also carried out with Namalwa cells. Control cytotoxicity experiments were also performed in a similar way to see the non-specific toxicities of SML and SMIL formulations against HL-60 tumor cells.

2.10.2. [^{3}H]leucine incorporation assay

Inhibition of [^{3}H]leucine incorporation was also used to

test the cytotoxic action of IT with SML [13]. Briefly, HL-60 tumor cells (25 000) were taken in Eppendorf tubes, to which varying concentrations of IT, either alone or in combination with SML (containing 10^{-9} M monensin), were added to the cells and incubated for 16 h at 37°C. The HL-60 cells were then washed with cold PBS and [^{3}H]leucine was added (0.8 μCi/tube). Following a 2 h incubation at 37°C, radioactive leucine was washed with cold PBS. The cells were lysed with Tween 20 and the amount of [^{3}H]leucine incorporated into the cells was analyzed by the scintillation counter.

3. Results

3.1. Formulation of SML and SMIL

SML were prepared using DPPC, cholesterol, DSPE-PEG, SA, PDP-SA and monensin in the molar ratio of 10:5:1.4:1.4:1.5:1. For the purpose of analysis, 50 μCi of radioactive [^{3}H]monensin was added to the formulation. Each formulation was tested for the amount of monensin entrapped, particle size, leakage and stability. The SML was then conjugated with modified anti-MY9 antibody to form stealth immunoliposomes (SMIL). The amount of monensin entrapped and the particle size were determined for each formulation and are provided in Table 1. The SMIL had an average liposome size of 127 (±41) compared with 114 (±32) nm for SML. The slight increase in size of SMIL compared with SML can be attributed to very minor cross-linkages of small sized SML during conjugation with modified anti-MY9 antibody. In all SML and SMIL formulations, the polydispersity index was always less than 0.05, indicating a uniform size distribution. The amount of monensin entrapped in SML was 10^{-7} M. The ratio of amount of monensin entrapped to total lipids was 10 mM:19.3 M. After conjugation, the concentration of entrapped monensin in SMIL decreased to 4.9×10^{-8} M. The decrease in concentration of monensin in SMIL was due to slight leakage during conjugation and subsequent washing of the preparation.

3.2. Stability of formulations

The SML and SMIL formulations were kept at 4°C and the stability was tested over a period of time. The amount of leakage of the monensin entrapped in the liposome preparations was 20 and 28%, respectively after 4 weeks of storage. The leakage, as observed after 8 weeks, did not increase considerably and was found to be 24 and 30%, respectively. The size of the preparation did not change significantly (less than 5%) during the same period of time.

Table 1
Entrapment efficiency and particle size analysis of SML and SMIL formulations[a]

Formulation	Particle size (nm)	Monensin entrapped ($\times 10^{-8}$ M)
SML	114 (32)	10.1 (1.6)
SMIL	127 (41)	4.9 (1.9)

[a] The values were obtained from at least six batches of liposomes and the values within parentheses indicate standard deviations.

3.3. Characterization of SMIL and retention of anti-MY9 antibody activity after conjugation

More than 30% of the anti-MY9 antibody was conjugated to SML. There was a slight increase in the size of SML after conjugation, indicating very limited cross-coupling during conjugation. About 4–6 antibody molecules/liposome were conjugated by this procedure. The ratio of antibody to lipid was 1.3 nm:19.3 M. More than 90% of the immunoreactivity of the anti-MY9 antibody was retained after conjugation, as ascertained by indirect immunofluorescence assay. Fig. 1 shows the coating and subsequent capping of SMIL around HL-60 resistant cells, indicating that the immunoconjugate was tumor targeted.

3.4. Half-life of SML and SMIL

The plasma half-lives of SML and SMIL were studied in BALB/c mice by single bolus dose administration. The percentage of radioactive monensin in the liposomal formulation circulating in the blood was plotted against time and is shown in Fig. 2. The results indicate that SML and SMIL have almost similar pharmacokinetic patterns. More than 20% of SML or SMIL remained in circulation, even after 24 h of injection. The half-life of these formulations was found to be between 7 and 8 h.

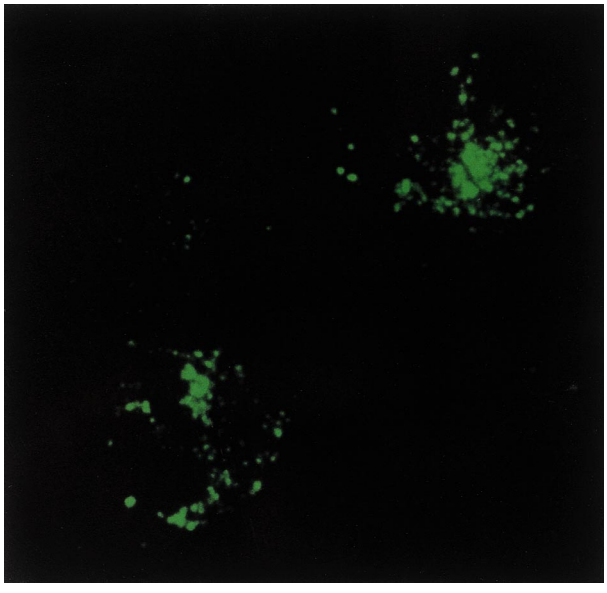

Fig. 1. Immunofluorescence assay of anti-MY9 antibody conjugated with SML after 45 min of incubation with HL-60 resistant cells. Brighter spots on the cell surface indicate capping of liposomes around the poles of HL-60 resistant cells. Also, internalization of SMIL within the cell is observed.

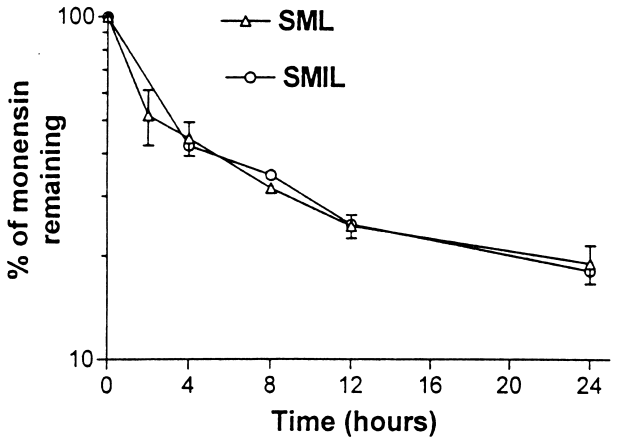

Fig. 2. Pharmacokinetics of SML and SMIL in BALB/c mice. The concentration of monensin in serum is plotted as a percentage of the injected dose. At least three experiments were performed and the average value is plotted.

3.5. TEM

In order to understand the mechanism of anti-MY9-IT potentiation, HL-60 resistant tumor cells were incubated with anti-MY9-IT and SML for various time periods and observed with TEM. Fig. 3a shows the partial dilation of golgi, as observed after 10 min of incubation with SML, and after 30 min post-incubation, the golgi was completely dilated (Fig. 3b). However, there was no damage or morphological change to other cellular organelles (e.g. mitochondria or endoplasmic reticulum) after incubation with SML. The control HL-60 resistant cells, which were not treated with SML and anti-MY9-IT, did not show any golgi dilation (Fig. 3c). In an another experiment, HL-60 resistant tumor cells were initially incubated with SML and anti-MY9-IT for 1 h, washed and then incubated with fresh RPMI media for 2 h. TEM of those cells revealed that the golgi reverted to its original shape (picture not shown). Also, HL-60 tumor cells, treated either with anti-MY9-IT or monensin solution (10^{-9} M), did not show any golgi dilation (picture not shown). It appears that the SML caused dilation of golgi, which played a significant role in potentiating the cytotoxic effect of anti-MY9-IT.

3.6. In vitro potentiation of anti-MY9-IT

The potentiation of anti-MY9-IT by SML and SMIL was tested against HL-60 sensitive and resistant tumor cell lines and compared with those of anti-MY9-IT. In all experiments, the concentration of monensin was kept at 10^{-9} M, which was non-toxic to the cells [10], and used with varying concentrations of anti-MY9-IT. The IC_{50} values for each type of tumor were determined (by MTS assay) and are shown in Table 2. For HL-60 sensitive tumor cells, the IC_{50} was 0.005 μg/ml with anti-MY9-IT (Table 2); whereas when anti-MY9-IT was combined with SML, the IC_{50} decreased to 0.0005 μg/ml. Therefore, the combination potentiated the effect of anti-MY9-IT by ten times. Against the same tumor cells, the combination of anti-MY9-IT and SMIL killed more than 85% of cells at 0.00025 μg/ml (Table 2). Therefore, the potentiation was more than 20 times against HL-60 sensitive tumor cells. The IC_{50} for anti-MY9-IT against HL-60 resistant tumor cells was found to be 0.05 μg/ml (Table 2). When anti-MY9-IT was combined with SML, the IC_{50} value decreased to 0.00025 μg/ml. The combination potentiated the effect of anti-MY9-IT by 200 times. When anti-MY9-IT was combined with

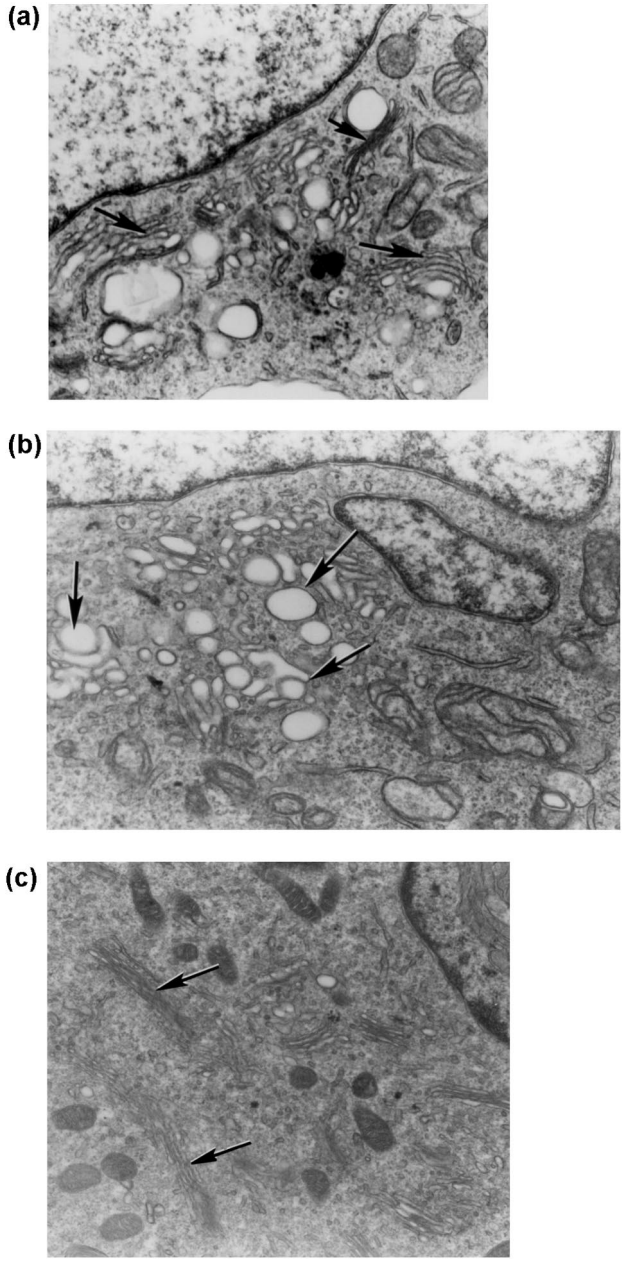

Fig. 3. TEM studies on HL-60 resistant tumor cells treated with anti-MY9-IT and SML. The cells were incubated in RPMI media at 37°C. (a) The cells after incubation for 10 min. The beginning of golgi dilation can be seen. (b) After incubation for 30 min, the golgi are completely dilated. (c) The control cells which were not treated with anti-MY9-IT or SML.

Table 2
Cytotoxicity assay of anti-MY9-IT, and combination of anti-MY9-IT with SML or SMIL against HL-60 sensitive and resistant tumor cell lines[a]

	IC_{50} (μg/ml)		
	HL-60 sensitive	HL-60 resistant	Namalwa cells
Anti-MY9-IT	0.005	0.05	NE[b]
Anti-MY9-IT + MS[c]	0.005	0.05	NE
Anti-MY9-IT + SML	0.0005	0.00025	NE
Anti-MY9-IT + SMIL	<0.00025	<0.0001	NE
Anti-MY9-IT + NMG-SML	0.00045	0.00030	NE
SML	NE	NE	NE
SMIL	NE	NE	NE

[a] The results were obtained from at least four different experiments. The concentration of monensin in SML or SMIL was 10^{-9} M.
[b] NE, no effect.
[c] MS, monensin solution containing 10^{-9} M monensin.

SMIL at 0.0001 μg/ml, it killed more than 85% of HL-60 resistant cells. The combination therapy produced a dramatic increase of potentiation by >500 times against HL-60 resistant cells. The results thus indicate that both SML and SMIL potentiate the effect of anti-MY9-IT in HL-60 sensitive and resistant tumor cell lines. The potentiation effect of SMIL was greater compared with SML. Also, the potentiation was much greater against resistant tumor cell lines. Anti-MY9-IT and the combination of immunotoxin with SML or SMIL had no inhibitory effect on Namalwa cells at the concentrations used for HL-60 sensitive or resistant tumor cell lines. NMG linked SML showed no difference in behavior in cytotoxicity than SML by itself.

IC_{50} values for HL-60 resistant cells were also determined by [^{3}H]leucine assay. Using this method, the IC_{50} for anti-MY9-IT was found to be 0.25 μg/ml, whereas the combination of SML and anti-MY9-IT had an IC_{50} value of 0.0005 μg/ml. The difference between the IC_{50} values, as determined by two methods, may be attributed to the difference between the two analytical techniques and the incubation time.

4. Discussion

The hypothesis in our studies was that SML, when conjugated to anti-MY9 monoclonal antibody, would significantly potentiate the activity of anti-MY9-IT in vitro. Our studies have clearly demonstrated that SMIL significantly potentiated the activity of anti-MY9-IT against HL-60 sensitive and resistant human tumor cell lines, indicating the possibility of these formulations to be of further use in vivo. The ricin A-chain subunits of anti-MY9-ITs kill target cells by inhibiting cellular protein synthesis by the enzymatic removal of adenine from the 28S ribosomal RNA.

This is the first study with the use of SML and SMIL for potentiation of ricin based immunotoxins. SML were successfully prepared and more than 20% of monensin remained in circulation in BALB/c mice after 24 h, indicating a significant improvement from the conventional monensin liposomes prepared earlier in this laboratory [13]. More than 100 formulations were prepared to eventually result in the most stable and optimal formulation. Our main intention was to prepare a formulation of liposomes which could be in circulation for a longer time for effective potentiation of the anti-MY9-IT. This property is most essential for in vivo studies, and we expect that our future tumor model studies will reflect that.

The method of attaching antibody to the liposomal surface is very important for targeting to the tumor cells. Many methods are available for attaching antibodies and proteins to the liposomal surface [18–23]. In the present study, we conjugated the anti-MY9 antibody to the SML by disulfide linkage using a method developed in our laboratory [13]. This method of antibody–liposome conjugation is very reproducible and can be used successfully in preparing SMIL. The SMIL formulated by our procedure showed a high degree of tumor specificity as shown by the indirect immunofluorescence assay and also by the in vitro cytotoxicity studies carried out with Namalwa (antigen negative cells) and HL-60 cells (CD 33 antigen positive cells). Namalwa cells did not respond to the potentiation of anti-MY9-IT with SMIL, indicating a high degree of immunoreactivity and specificity retained in the anti-MY9 antibody after conjugation. Furthermore, a non-specific mouse IgG (NMG) linked SMI did not show any difference in potentiation as compared with SMI for anti-MY9-IT conjugate (Table 2). These observations clearly demonstrate the tumor specificity of the SMIL to tumor cells in our studies and also the successful conjugation procedure.

Our earlier approach to increase the potency of immunotoxins was the use of conventional monensin liposomes, either alone or linked to monoclonal antibodies [12]. Subsequent studies with SML have shown that SML and SMIL could potentiate the activity of Adriamycin against a variety of human sensitive and resistant tumor cell lines. This increased activity was proposed mostly due to a higher uptake of the drug when incubated with SML [24].

Our earlier in vivo studies showed at least 30% survivors in athymic nude mice xenotransplanted with H-meso tumors

which were treated with a combination of 7D3 monoclonal antibody (against the transferrin receptor) linked to ricin A and conventional monensin liposomes [12]. The mice used in these studies had a huge tumor burden and the treatment was started 21 days post-injection of 9×10^6 H-meso cells. Another study performed with the antib4Br conjugate in combination with conventional monensin liposomes (targeted against Namalwa cells) showed at least 1 log more cell kill as compared with antib4Br alone in SCID mice xenotransplanted with Namalwa cells (i.v./i.v. tumor model). It was observed in these studies that the effectiveness of the antib4Br could have been significantly increased if SML were used (unpublished observations). Other workers have also used conventional monensin liposomes for the potentiation of ricin A, but no immunotoxin or stealth monensin preparations were used in their studies [25]. Our studies have clearly shown that not only monensin could be formulated in stealth liposomes, but the SML resulting from our studies were able to significantly potentiate the activity of anti-MY9-IT in vitro. The increased ability of SMIL to potentiate anti-MY9-IT activity as compared with SML is to be expected due to enhanced tumor targeting. In all of our studies, the amount of monensin used in either SML or SMIL was non-toxic to tumor cells.

Another interesting observation from our studies was the higher potentiation of anti-MY9-IT by SML or SMIL against resistant HL-60 cells as compared with sensitive cells. It appears that monensin somehow reverses the resistance experienced with anti-MY9-IT by some mechanisms probably dealing with decreased efflux of the ricin A from tumor cells. The increased residence time of ricin A in tumor cells enables it to be cytotoxic at lower concentrations. The amount of free monensin required for potentiating the activity of anti-MY9-IT was 10^{-6} M, which was at least 1000 times more than the amount of monensin in SML or SMIL.

An attempt was made to study the mechanism of action of monensin liposomes for the potentiation of anti-MY9-IT using TEM. The TEM studies clearly showed that the dilation of the golgi was reversible after re-incubation of tumor cells in fresh media. All of the other cell organelles, like the endoplasmic reticulum or mitochondria, were not affected by monensin. Monensin is a very dose responsive compound, and at higher doses, it has been shown to elevate the pH of cytosol [26]. We believe that the dilation of the golgi was the major mechanism which played a role in the increased activity of the anti-MY9-IT against HL-60 cells. The golgi is involved in the excretion of proteins from cells and is thought to be the main organelle involved in the removal of ricin A from cells. This can be explained by the fact that despite targeting several thousand molecules of ricin A to a tumor cell as determined by our earlier studies, there was still not enough cell kill [13]. This indicated a problem of delivery of ricin A after being cleaved from the antibody inside the cell. Other workers have also observed the dilation of the golgi with monensin which was found to be reversible [27,28]. Furthermore, we observed that for the golgi to be fully dilated, it took 30 min of incubation with the SML. All other preparations, blank liposomes and anti-MY9-IT, did not result in any effect on the golgi apparatus. The amount of free monensin required for this effect was 10^{-6} M (a difference of 1000 times as compared with SML). Hence, from the above observations, one probable mechanism which can be proposed is that the dilation of the golgi prevents the removal of ricin A from tumor cells which leads to increased availability of ricin A in the tumor cell, leading to a decreased IC_{50} value of the anti-MY9-IT.

Our studies show that monensin can be formulated in stealth liposomes which can be further conjugated to monoclonal antibodies. These preparations were able to potentiate the in vitro cytotoxicity of the anti-MY9-IT against HL-60 cells by several logs. Presently, studies are being conducted in our laboratory: (a), to study the in vivo potency of these formulations; and (b), mechanistic studies to understand the mechanism of action of monensin liposomes for resistant tumor cells.

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