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Effect of monensin liposomes on the cytotoxicity of anti-My9-bR immunotoxin

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Abstract

The purpose of the study was to evaluate the utility of monensin liposomes in the enhancement of in-vitro cytotoxicity, apoptosis and in-vivo antitumour activity of anti-My9-bR immunotoxin. Monensin liposomes were prepared and studied for the enhancement of in-vitro cytotoxicity and apoptotic response of anti-My9-bR immunotoxin against both sensitive and resistant human promyelocytic leukemia HL-60 cells by MTS/PES method and acridine orange staining, respectively. Further, the in-vivo cytotoxicity enhancement of anti-My9-bR immunotoxin by monensin liposomes was studied in a survival model of severe combined immunodeficient (SCID) mice bearing intraperitoneal HL-60 tumours. The in-vitro cytotoxicity of anti-My9-bR immunotoxin was enhanced 580 fold and 4.7 fold against sensitive and resistant HL-60 cells, respectively, by monensin liposomes (5×10^{-8} M). The combination of anti-My9-bR immunotoxin (50 ng mL^{-1}) with monensin liposomes (5×10^{-8} M) produced apoptosis in 40% of cells, whereas the apoptotic response was minimal (< 10%) in anti-My9-bR immunotoxin- or monensin liposome (alone)-treated HL-60 (resistant) cells. In SCID mice bearing HL-60 tumours, anti-My9-bR immunotoxin (75 μg kg^{-1} administered intravenously every other day for a total of five courses) showed a median survival time of 20 days, which was no different than that of vehicle control- or monensin liposome-treated mice. However, anti-My9-bR immunotoxin (75 μg kg^{-1}) in combination with monensin liposomes (4 μg kg^{-1} monensin), administered every other day for a total of five courses, was found to prolong the survival of 20% of mice for more than 46 days. Our results indicate that, despite anti-My9-bR immunotoxin being ineffective in the HL-60 tumour model, its combination with monensin liposomes could improve the antitumour response.

Introduction

Antibodies linked to toxic plant or bacterial enzymes (immunotoxins) have been shown to have potential in the treatment of residual disease in blood-borne cancers (Kreitman & Pastan 1995; Kreitman 1999). The deglycosylated ricin A chain (dgA)-containing immunotoxin, IgG-RFB4-dgA (targeting CD22), was found to have some success in clinical trials (Almot et al 1993; Sausville et al 1995). A recent study with anti-B4-blocked ricin (conjugate between anti-CD19 monoclonal antibody, anti-B4 with blocked ricin) showed that it is possible to obtain durable remissions for more than 4 years in 40% of the responders in patients with indolent lymphoma by combination chemotherapy with anti-B4-blocked ricin (Longo et al 2000). However, the non-specific toxicity and immunogenicity associated with immunotoxins pose certain problems, thus impeding their wider applicability (Frankel 2002). The anti-My9-blocked ricin (anti-My9-bR immunotoxin) is an immunoconjugate of anti-My9 (CD33) antibody and a modified whole ricin that has its non-specific binding eliminated by chemical blockage of the galactose binding domains of the B-chain. The anti-My9 monoclonal antibody identifies a myeloid lineage restricted surface antigen, which appears in myeloid development at the level of the CFU-GEMM, a colony forming cell capable of giving rise to granulocytes, erythrocytes, monocytes and megakaryocytes (O'Toole et al 1998). The CD33 antigen is expressed on leukaemic cells in more than 80% of cases of acute myeloid leukaemia (AML) and chronic myelogenous leukaemia in blast crisis (Griffin et al 1984). The anti-My9-bR immunotoxin at a concentration of 10^{-8} M has been shown to produce more than four logs of cell kill, when exposed to the CD33+ myeloid leukaemia cell line HL-60 for 2 h (Roy et al 1991).

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This immunotoxin has relatively little toxicity for normal haematopoietic progenitor cells, with less than 1 log cell kill of CFU-GEMM (O'Toole et al 1998). However, phase 1 trials conducted with anti-My9-bR immunotoxin at a dose of 20–30 μg kg^{-1} per day for 5 days (a dose below that which could saturate tumour antigen binding sites) revealed severe toxicity, which led to termination of the study (O'Toole et al 1998).

The carboxylic ionophore monensin has been shown to potentiate the in-vitro cytotoxicity of immunotoxins (Griffin et al 1987; Derbyshire et al 1992; van Horssen et al 1999) and an anti-CD22 targeted cytotoxic ribonuclease (Newton et al 2001). However, the in-vivo potentiation of the immunotoxin cytotoxicity by monensin was limited (Griffin et al 1987), which was attributed to the lipophilicity and the short half-life of monensin (Griffin & Raso 1991). Delivery of monensin as linoleic acid conjugate (Griffin et al 1987) or as a lipid emulsion (Griffin & Raso 1991) showed an improvement in the in-vivo potentiation of the cytotoxicity of immunotoxins. A liposomal formulation of monensin has also been shown to enhance the in-vivo cytotoxicity of anti-Tfr immunotoxin (Griffin et al 1993). Our laboratory has been involved in the development of liposomal and nanoparticulate drug delivery systems for monensin (Ferdous et al 1998; Singh et al 1999, 2001; Shaik et al 2001a, b). We have demonstrated that monensin can be formulated into biodegradable nanoparticles (Ferdous et al 1998) and the circulation half-life improved by employing a pegylated poly (DL-lactide-co-glycolide) copolymer (Shaik et al 2001b). We have optimized the liposomal monensin formulations with respect to particle size and circulation half-life (Singh et al 1999) and monensin entrapment (Shaik et al 2001a). We have also demonstrated that monensin liposomes could be conjugated to monoclonal antibodies without any considerable loss of antibody immunoreactivity (Singh et al 1994, 2001; Shaik et al 2001a). We could also demonstrate that monensin liposomes significantly enhance the in-vitro cytotoxicity of anti-human transferrin (454-A) immunotoxin (Singh et al 1994) and anti-My9 immunotoxin (Shaik et al 2001a; Singh et al 2001) at a much lower concentration (10^{-8} M) in comparison to free monensin (10^{-6} M) to obtain the same in-vitro potentiation. The purpose of the present research work was to evaluate the monensin liposomes (Shaik et al 2001a) for their ability to enhance the in-vitro cytotoxicity and apoptotic response of anti-My9-bR immunotoxin against both sensitive and resistant HL-60 cells and also to enhance the in-vivo cytotoxicity of anti-My9-bR immunotoxin in an intraperitoneal HL-60 tumour survival model in severe combined immunodeficient (SCID) mice.

Materials and Methods

Materials

^{3}H-monensin with a specific activity of 5 Ci mmol^{-1} was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO). Distearoyl glycerophospho-ethanolamine-polyethyleneglycol 2000 (DSPE-PEG) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Other lipids and chemicals were purchased from Sigma Chemical Company (St Louis, MO). Acridine Orange Stain Dropper solution was obtained from Becton Dickinson and Company (Sparks, MD). The human promyelocytic leukaemia cell line HL-60 (doxorubicin sensitive) was obtained from American Type Culture Collection (Rockville, MD). Anti-My9-bR immunotoxin and HL-60 (doxorubicin resistant) cells were kindly provided by Dr Victor Goldmacher, Immunogen (Norwood, MO). The resistant HL-60 cells were grown in doxorubicin-supplemented medium and maintained in our laboratory as previously studied (Singh et al 1999, 2001).

Preparation and characterization of long-circulating monensin liposomes

Monensin liposomes were prepared by pH-gradient method and characterized for their particle size (BI 90 Particle sizer; Brookhaven Instruments, New York, NY) and monensin content (by scintillation counting) essentially as described elsewhere (Shaik et al 2001a).

In-vitro enhancement of the cytotoxicity of anti-My9-bR immunotoxin by monensin liposomes

The in-vitro cytotoxicity of anti-My9-bR immunotoxin alone, and in combination with monensin liposomes (5×10^{-8} M) against both sensitive and resistant HL-60 cells was assessed by Aqueous One Solution cell proliferation reagent (MTS/PES reagent; Promega, Madison, WI). The MTS/PES reagent is a simple and an efficient colorimetric method to determine viable cells in cytotoxicity assays, which would eliminate the various steps associated with MTT method. The MTS tetrazolium compound is bioreduced by metabolically active cells into a coloured formazan product that is soluble in tissue culture medium. The quantity of formazan product, as measured by its absorbance at 490 nm, is directly proportional to the number of living cells. Since, the MTS formazan product is soluble in tissue culture medium, this assay requires fewer steps than the conventional MTT assay.

Induction of apoptosis in HL-60 cells (resistant) by the combination of anti-My9-bR immunotoxin with monensin liposomes

HL-60 cells (resistant) were seeded at a density of 50 000 per mL into chamber slides (Nunc Lab-Tek) and incubated for 24 h. The cells were treated with anti-My9-bR immunotoxin (50 ng mL^{-1}), monensin liposomes (5×10^{-8} M monensin) or anti-My9-bR immunotoxin (50 ng mL^{-1}) with monensin liposomes (5×10^{-8} M monensin) for 72 h. Medium-treated cells were used as control. Cell smears were then made on glass slides, fixed with 10% buffered formalin, stained with acridine orange and finally observed with a fluorescent microscope (Olympus BX40; Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a digital camera (QImaging, Burnaby, BC, Canada). The morphological criterion used to detect apoptotic cells were: cytoplasmic blebbing and the

presence of apoptotic bodies; chromatin condensation; and cytoplasmic and nuclear shrinkage (Shiff et al 1995; Shaik et al 2002). The percent apoptosis was estimated by counting a minimum of 150 cells in randomly selected fields on each slide looking for the presence of apoptotic cells. The induction of apoptosis by the combination of anti-My9-bR immunotoxin with monensin liposomes was also studied in HL-60 (sensitive) cells in the same way as described above except that anti-My9-bR immunotoxin was used at 10 ng mL^{-1}.

Effect of monensin liposomes on the in-vivo cytotoxicity of anti-My9-bR immunotoxin in SCID mice

Female C.B-17/Icr Hsd-SCID mice (Charles River Laboratories, 6–7 weeks, 18 g) were preselected for intraperitoneal HL-60 tumours by repeated administration of HL-60 (sensitive) cells. After the third pass through the mice, the ascitic tumour cells were collected, grown in tissue culture and subsequently used for the in-vivo cytotoxicity study. The protocol followed by Shah et al (1993) for the in-vivo cytotoxicity of anti-B4-bR immunotoxin was followed with minor modification. Briefly, HL-60 cells (1.5×10^7) were injected intraperitoneally in growth medium (0.1 mL). One hour later, the mice were administered intravenously with sterile saline (control), anti-My9-bR immunotoxin (75 $\mu g\,kg^{-1}$), monensin liposomes (0.1 mL of 10^{-6} M equivalent to 4 $\mu g\,kg^{-1}$ of monensin) or monensin liposomes (0.1 mL of 10^{-6} M equivalent to 4 $\mu g\,kg^{-1}$ of monensin) with anti-My9-bR immunotoxin (75 $\mu g\,kg^{-1}$) in 0.1 mL of sterile saline. The treatments were given every other day for a total of five courses and the survival time in days was noted. The dose of anti-My9-bR immunotoxin was selected based upon the non-toxic dose for a similar type of immunotoxin, anti-B4-bR, when used in combination with monensin liposomes as determined at ImmunoGen, Inc. Each treatment group consisted of at least 10 mice. The mice were housed in pathogen-free conditions (Labgard Laboratory Animal Isolator; NuAire Inc., Plymouth, MN) and the treatments were given under Laminar Flow Biological Safety Cabinet (Labgard Class II, Type A/B3; NuAire Inc., Plymouth, MN). The mice were provided with free access to food and water. All animal use was reviewed and approved by the Florida A & M University Animal Care and Use Committee (Approval number 49/97).

Statistical analysis

Log-rank test was employed to determine the significance of the difference in the survival period in mice (Shah et al 1993). The statistical analysis was performed using GraphPad PRISM version 3.0 software (San Deigo, CA).

Results and Discussion

Characterization of monensin liposomes

Monensin liposomes were found to have a particle size of 220 ± 4.5 nm with an entrapment efficiency of 14%. The particle size of the liposomes was increased by less than 10% over a two-month period when stored at 4 °C. The leakage of monensin from liposomes has been described previously (Shaik et al 2001a). The lipid composition used in the preparation of monensin liposomes in the current study is same as used in our previous studies and the pharmacokinetics of these long-circulating monensin liposomes has already been fully established (Singh et al 1999, 2001).

Enhancement of the in-vitro cytotoxicity of anti-My9-bR immunotoxin by monensin liposomes

Figure 1 shows the in-vitro cytotoxicity profiles of anti-My9-bR immunotoxin alone and in combination with monensin liposomes (5×10^{-8} M, 0.035 $\mu g\,mL^{-1}$) against HL-60 (sensitive) cells. The concentration that produced 50% cell kill (IC50) for anti-My9-bR immunotoxin alone and in combination with monensin liposomes was found to be 2.9 ± 0.05 ng mL^{-1} and 0.005 ± 0.002 ng mL^{-1}, respectively. These results indicate a 580-fold potentiation of the in-vitro cytotoxicity of anti-My9-bR immunotoxin. We have previously shown that liposomal monensin (3.5×10^{-8} M) produces a 360-fold increase in the in-vitro cytotoxicity of anti-My9-bR immunotoxin against HL-60 (sensitive) cells (Shaik et al 2001a). This experiment was performed to determine the in-vitro cytotoxicity of the combination of anti-My9-bR immunotoxin with monensin liposomes at the presumed concentration of monensin in mice after administration of monensin liposomes at a dose of 4 $\mu g\,kg^{-1}$. Assuming a total volume of 1 mL of blood in 18 g of mice (5.5 mL/100 g body weight), the expected concentration of monensin in mice immediately after administration will be 0.07 $\mu g\,mL^{-1}$ (10×10^{-8} M). Based upon 50% clearance of monensin liposomes in 2 h in mice after intravenous administration of long-circulating monensin liposomes (Singh et al 1999), the concentration of monensin associated with liposomes will be expected to be in the range of about 5×10^{-8} M for about 4 h. Monensin liposomes (5×10^{-8} M) alone were found to have a 15% cell kill against HL-60 (sensitive) cells.

In-vitro cytotoxicity studies with 10×10^{-8} M monensin liposomes resulted in extremely high cytotoxicity for the combination of anti-My9-bR immunotoxin with monensin liposomes possibly due to the non-specific cytotoxicity of monensin liposomes. However, studies performed with monensin liposomes at 1×10^{-8} M could not show any appreciable increase in the cytotoxicity of anti-My9-bR immunotoxin (data not shown).

In the case of HL-60 (resistant) cells, the IC50 for anti-My9-bR immunotoxin was found to be 8 ± 1.4 ng mL^{-1}. However, the combination of anti-My9-bR immunotoxin with monensin liposomes (5×10^{-8} M) produced a 4.7-fold increase in the cytotoxicity of anti-My9-bR immunotoxin (IC50 was reduced to 1.7 ± 0.2 ng mL^{-1}, Figure 2). This increase in the cytotoxicity is in agreement with our previous report in which long-circulating monensin nanoparticles were shown to produce a five-fold increase in the in-vitro cytotoxicity of anti-My9-bR immunotoxin against resistant HL-60 cells (Shaik et al 2001b). We have earlier shown that liposomal monensin overcomes

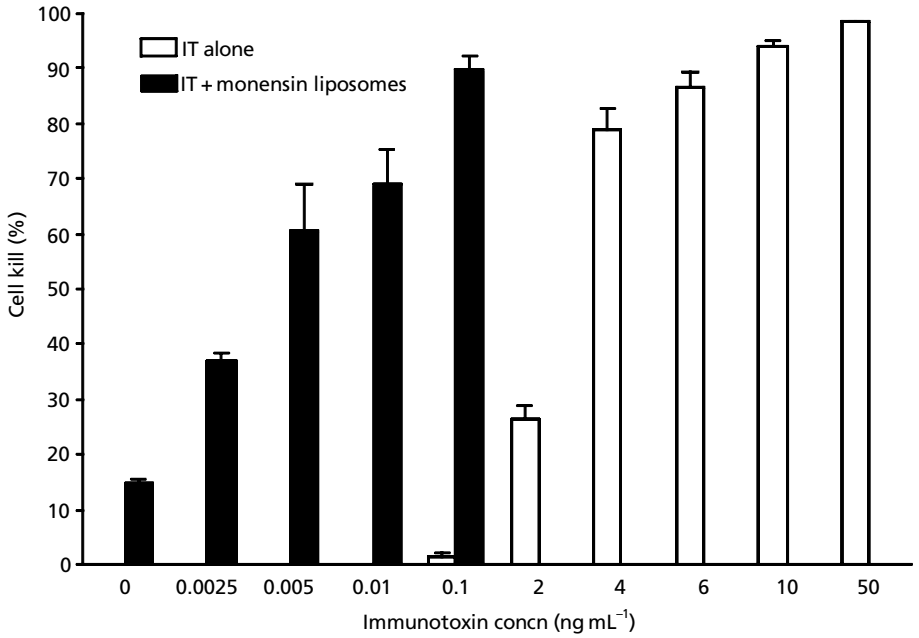

Figure 1 Enhancement of the in-vitro cytotoxicity of anti-My9-bR immunotoxin (IT) against HL-60 (sensitive) cells by monensin liposomes. The concentration of monensin employed was 5.0×10^{-8} M. Data are mean ± s.e.m. (n = 3).

doxorubicin resistance by 6 and 2400 times in resistant HL-60 and human breast tumour MCF-7 cells, respectively (Singh et al 1999). We demonstrated that liposomal monensin increases the uptake and decreases the efflux of doxorubicin in HL-60 (resistant) cells. We also observed the dilation of Golgi in HL-60 (resistant) cells treated with doxorubicin and monensin liposomes, which might have altered the intracellular distribution of doxorubicin (Singh

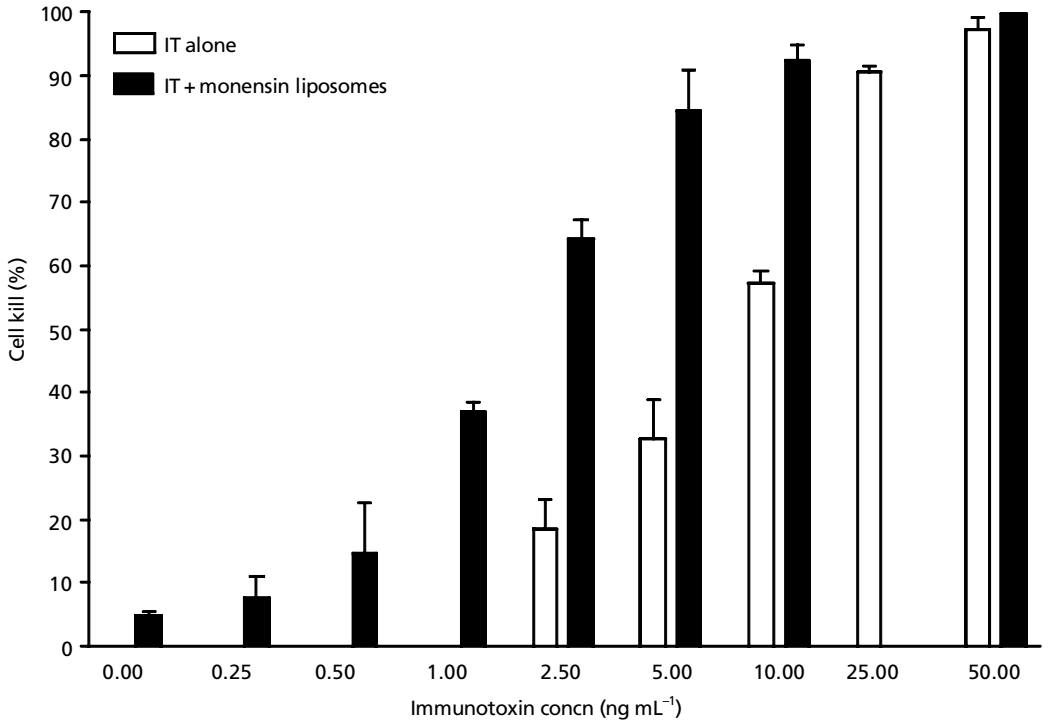

Figure 2 Enhancement of the in-vitro cytotoxicity of anti-My9-bR immunotoxin (IT) against HL-60 (resistant) cells by monensin liposomes. The concentration of monensin employed was 5.0×10^{-8} M. Data are mean ± s.e.m. (n = 3).

et al 1999). Further, we showed that monensin liposomes in combination with anti-My9-bR immunotoxin induce dilation of Golgi in HL-60 (resistant) cells (Singh et al 2001). Thus, the alteration of intracellular distribution of anti-My9-bR immunotoxin may be responsible for its increased cytotoxicity in combination with monensin liposomes. Further studies are warranted to understand the effect of monensin liposomes on the cellular kinetics of anti-My9-bR immunotoxin.

Induction of apoptosis in HL-60 cells (resistant) by the combination of anti-My9-bR immunotoxin with monensin liposomes

We observed an enhanced apoptotic response (40%) by the combination of anti-My9-bR immunotoxin (50 ng mL^{-1}) with monensin liposomes (5×10^{-8} M), whereas treatment with 50 ng mL^{-1} of anti-My9-bR immunotoxin or 5×10^{-8} M monensin liposomes alone showed negligible apoptosis (< 10%) in HL-60 (resistant) cells upon acridine orange staining. Increased apoptosis was also seen in HL-60 (sensitive) cells by the combination of anti-My9-bR immunotoxin (10 ng mL^{-1}) with monensin liposomes (5×10^{-8} M) in comparison with anti-My9-bR immunotoxin or monensin liposomes alone (data not shown). Apoptosis has been implicated in the in-vitro antiproliferative effect of several immunotoxins (Hafkemeyer et al 1998; Bolognesi et al 2000; Zhong et al 2001). However, multidrug-resistance (MDR) modifiers or P-glycoprotein (Pgp) antagonists such as ciclosporin have been used to restore the cytotoxic effect and enhance the apoptotic response of the immunoconjugate calicheamicin with anti-CD33 monoclonal antibody against MDR-positive AML cells, respectively (Naito et al 2000; Linenberg et al 2001). The enhanced in-vitro cytotoxicity and apoptotic response by the combination of liposomal monensin with immunotoxins, as observed in our study, might be exploited to improve the sensitivity in MDR-positive AML cells.

Enhancement of the in-vivo cytotoxicity of anti-My9-bR immunotoxin by monensin liposomes

Figure 3 shows the effect of different treatments on the survival of HL-60 tumour transplanted mice. It is evident from Figure 3 that the survival profile of vehicle control mice is similar to that of anti-My9-bR immunotoxin- or monensin liposome-treated mice. The median survival time of mice treated with control, anti-My9-bR immunotoxin or monensin liposomes was found to be 20 days. This indicates that anti-My9-bR immunotoxin is ineffective in prolonging the survival of mice under the experimental conditions studied. On the other hand, the median survival time in mice treated with anti-My9-bR immunotoxin and monensin liposomes was 23 days (Figure 3). Further, 2 out of 10 mice in the anti-My9-bR immunotoxin with monensin liposomes group survived for more than 46 days, which was twice the longest survival period (23 days) in the control, anti-My9-bR immunotoxin- or monensin liposome-treated groups. The median survival time in mice treated with anti-My9-bR immunotoxin with monensin liposomes was found to be significantly higher than that in control- ($P = 0.008$), monensin liposome- ($P = 0.004$) or immunotoxin-treated mice ($P = 0.01$).

Shah et al (1993) reported that anti-B4-bR immunotoxin (100 μg kg^{-1} on five consecutive days) is effective in prolonging the median survival time by at least 10 days in intraperitoneal or intravenous Namalwa tumour models in SCID mice. We have earlier found that anti-B4-bR enhances the median survival time in nude mice with HL-60 tumours by 8 days compared with untreated control mice (unpublished data). In the current study, we found that anti-My9-bR immunotoxin treatment was no different from control. Increasing the dose of anti-My9-bR

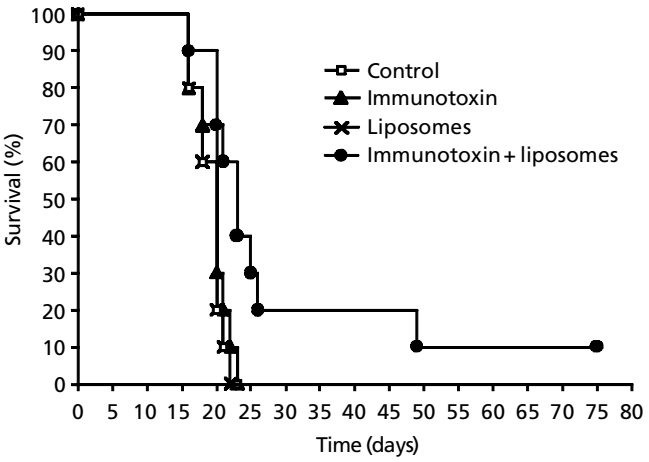

Figure 3 Effect of monensin liposomes on the survival of mice with HL-60 tumours. The mice were administered intraperitoneally with HL-60 (sensitive) cells (1.5×10^7 per mouse) and treated with vehicle (control), monensin liposomes (liposomes), anti-My9-bR immunotoxin (immunotoxin) or anti-My9-bR immunotoxin with monensin liposomes (immunotoxin + liposomes) and the survival period in days was noted. Each treatment group consisted of at least 10 mice.

immunotoxin to 100 μg kg^{-1} did not result in any increase in the median survival time of mice in our study (data not shown). Our results indicate that anti-My9-bR immunotoxin does not possess in-vivo activity, even though it was shown to have potent in-vitro cytotoxicity against HL-60 cells (Roy et al 1991; Shaik et al 2001a, b). Indeed, anti-My9-bR immunotoxin has been used for purging leukaemic cells from bone marrow harvests before re-infusing these cells in patients undergoing autologous bone marrow transplantation (O'Toole et al 1998). In this study, we explored the feasibility of enhancing the in-vivo cytotoxicity of anti-My9-bR immunotoxin by using it in combination with liposomal monensin. Even though anti-My9-bR immunotoxin by itself is ineffective in the HL-60 tumour model, its combination with monensin liposomes could produce a statistically significant increase in the median survival time. Further, this combination increased the life span by two fold in 20% of mice. Griffin et al (1993) have reported that liposomal monensin (0.3 mL of 10^{-5} M) in combination with anti-Tfr immunotoxin resulted in a long-term survival in 21% of nude mice bearing human malignant mesothelioma, H-MESO-1, as an advanced intraperitoneal xenograft. In this study, we employed fewer doses (five) of immunotoxin and monensin liposomes as opposed to a total of seven doses used by Griffin et al (1993) and the concentration of monensin in liposomes was less by one order of magnitude. Despite the differences in the type of immunotoxin, monensin liposomes, and other experimental conditions, the long-term survival proportion in our study is similar to that of Griffin et al (1993). The enhancement of the in-vivo antitumour activity of immunotoxins by monensin liposomes as observed in our study assumes significance in the light of recent data with other cytotoxicity enhancers such as chloroquine, which was found not to be effective in enhancing the in-vivo antitumour activity of CD22-recombinant ricin A in SCID mice, despite being administered continuously with an intraperitoneally implanted mini-osmotic pump (van Horssen et al 2000). This is a preliminary study and further studies are warranted to evaluate the use of liposomal monensin for the in-vivo enhancement effect of other immunotoxins in suitable animal models. In conclusion, monensin liposomes may be used for enhancing the cytotoxicity of immunotoxins for the in-vitro purging of both sensitive and resistant (MDR) AML cells from autologous bone marrow transplants. Further, liposomal monensin may also be exploited for the in-vivo potentiation of suitable immunotoxins.

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