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Specific binding of sterically stabilized anti-B-cell immunoliposomes and cytotoxicity of entrapped doxorubicin

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Abstract

Administration of doxorubicin (DXR) formulated in sterically stabilized liposomes, (SL) containig engrafted poly(ethylene glycol)-modified phosphatidylethanolamine (PEG-PE) on their surface, has been shown to increase the therapeutic index of the drug. A further improvement could be achieved through targeting of liposome-entrapped drug selectively to cancer cells. This paper describes the conjugation of the anti-B-cell lymphoma monoclonal antibody LL2 to the surface of DXR-loaded liposomes by use of a PEG-based heterobifunctional coupling agent. Competitive-binding ELISA of the resulting immunoliposomes (SIL) against the monoclonal anti-idiotype antibody, WN, indicated preserved immonological activity. The pH-sensitive probe, HPTS was used to study the binding of liposomes with target cells. The results showed a 3.8-fold increased cellular association of SIL compared to that of SL and an apparent internalization of SIL into low pH compartments. Addition of an excess of unconjugated free LL2 displaced about 72% of the HPTS-SIL association with cells. Experiments with ^{125}I-labeled free and SIL-bound LL2 showed approximately 50% degradation for both preparations. In vitro MTT cytotoxicity tests against neoplastic B cells gave IC_{50} values of 1.6, 2.9 and 0.35 μM for DXR-SIL, DXR-SL and free DXR, respectively. Leakage of drug from the liposomes apparently reduced the specificity of the cytotoxic action of DXR-SIL. © 2000 Published by Elsevier Science B.V.

Keywords: B cell lymphoma; Liposomal doxorubicin; Monoclonal antibody; Antibody conjugation; Immunoliposome

1. Introduction

Antibody-mediated targeting of liposomes containing entrapped anti-cancer drug is a fascinating prospect in cancer therapy. The development of sterically stabilized liposomes (SLs), containing lipid derivatives of poly(ethylene glycol) (PEG), has made targeted liposomal therapy more feasible by reducing the uptake by the mononuclear phagocyte system (MPS) and thereby prolonging circulation times. However, surface-grafted PEG chains have been shown to interfere with the binding of immunoliposomes to their cellular targets (Klibanov et al., 1991; Park et al., 1995).

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To overcome this problem new techniques have been developed for coupling of monoclonal antibodies (mAbs) at the PEG termini of SL, forming sterically stabilized immunoliposomes (SILs) (Blume et al., 1993; Park et al., 1995; Maruyama et al., 1995; Hansen et al., 1995). A logical first choice for targeting of SIL in cancer therapy seems to be hematological malignancies, where liposomes have free excess to malignant cells. In a recent paper the conjugation of the anti-CD22 pan-B-cell mAb, LL2 to long-circulating lipid emulsion drug carriers was described and the resulting complexes were found to have well preserved immunochemical activity (Lundberg et al., 1999). In this study LL2 is coupled to long-circulating liposomes and the SILs formed are evaluated against cultured cancer cells for their properties as specific drug carriers.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC), hydrogenated egg phosphatidylcholine (HEPC), distearoyl phosphatidylethanolamine (DSPE), cholesterol (CHOL), doxorubicin (DXR), 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS), carbonyl diimidazole, maleimide activated alkaline phosphatase and 2-mercaptoethanol were from Sigma Chemical (St Louis, MO). Poly(ethylene glycol)-vinylsulfone-*N*-hydroxy-succinimidyl ester (VS-PEG-NHS) was purchased from Shearwater Polymers Europe (Enschede, The Netherlands), [^{14}C]dipalmitoyl phosphatidylcholine (DPPC) and ^{125}I from Amersham International plc (Amersham, UK), and 4-methylumbelliferyl phosphate (MUP) from Koch-Light Limited (Suffolk, England). Poly(ethylene glycol)-modified phosphatidylethanolamine (PEG_{2000}-PE) was synthesized as described before (Lundberg et al., 1996) and a PEG_{2000} derivative of DSPE with a vinylsulfone group at the distal terminus of the PEG chain (DSPE-PEG-VS) by reacting 25 μmol NHS-PEG-VS with 23 μmol DSPE and 50 μmol triethylamine in chloroform for 6 h at 40°C. The product was purified by preparative silica gel TLC.

2.2. Cell lines and monoclonal antibody

The Burkitt's lymphoma cell lines obtained from American Type Culture Collection (Rockville, MD), Raji, Daudi, and Ramos, were grown as suspension cultures in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. Cells were maintained at 37°C and gassed with 5% CO_2 in air.

The murine mAb LL2, an IgG specific for epitope B of CD22 (Pawlak-Byczkowska et al., 1989; Stein et al., 1993), raised by immunization of BALB/c mice with an extract of Raji cells, and WN, a monoclonal anti-idiotype Ab to LL2 (Losman et al., 1995), were obtained from Immunomedics, (Morris Plains, NJ). The molecular weight of LL2 was measured at 154 kD by mass-analysed laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (Mass Consortium, San Diego, CA). The ^{125}I-labeling of LL2 was performed by the chloramine-T procedure. For alkaline phosphatase conjugation, the mAb was first reduced with 50 mM 2-mercaptoethanol for 10 min at 4°C in 0.2 M Tris buffer (pH 8.7) and then reacted with maleimide activated phosphatase in phosphate buffered saline (PBS) (pH 7.0) for 3 h at room temperature. Remaining maleimide groups were blocked with 1.5 mM 2-mercaptoethanol.

2.3. Preparation of liposomes

Liposomes were composed of either HEPC:CHOL: PEG_{2000}-DSPE at a 2:1:0.1 molar ratio or HEPC:CHOL:DSPE-PEG_{2000}-VS at a 2:1:0.1 molar ratio. In experiments involving HPTS-encapsulated SL the phospholipid component was EPC. Briefly, dried lipid films were hydrated in 25 mM HEPES and 140 mM NaCl buffer (pH 7.4), (containing 35 mM HPTS when appropriate) subjected to five freezing-thawing cycles and subsequent extruding through 0.1 μm polycarbonate filters. This extrusion procedure has been shown to produce primarily small unilamellar vesicles (Olson et al., 1979). For DXR-loaded SL, the drug was encapsulated by remote loading using an ammonium sulfate gradient (Bolotin et al., 1994). In short, the lipids were

hydrated in 155 mM ammonium sulfate (pH 5.5) and the external buffer was exchanged by a Sephadex G-25 column equilibrated with 123 mM sodium citrate (pH 5.5). DXR was added to the SL at a PC:DXR ratio of 1:0.2 (w/w) and incubated at 65°C for 1 h. The SL-encapsulated DXR was separated from the free drug by a Sephadex G-25 column eluted with 123 mM sodium citrate (pH 5.5). The concentration of DXR was measured by spectrofluorometry with $\lambda_{ex} = 470$ nm and $\lambda_{em} = 580$ nm. The phospholipid concentration was quantitated by [^{14}C]DPPC.

2.4. Conjugation of LL2 to liposomes

Coupling of LL2 to SLs was performed by reaction between the vinylsulfone (VS) groups at the distal PEG termini on the surface of SLs and free thiol groups on the mAb. Before the coupling reaction LL2 was reduced with 50 mM 2-mercaptoethanol for 10 min at 4°C in 0.2 M tris buffer (pH 8.7), which yields 8–10 thiol groups per mAb (Lundberg et al., 1999). The reduced mAb was separated from excess 2-mercaptoethanol by use of Sephadex G-25 spin-columns, equilibrated with 50 mM sodium acetate buffered 0.9% saline (pH 5.3). The conjugation was performed in HEPES-buffred saline (pH 7.4) for 16 h at room temperature under argon. Excess vinylsulfone groups were blocked with 2 mM 2-mercaptoethanol for 30 min, whereafter excess mAb and 2-mercaptoethanol was removed on a Sepharose CL-4B column. The SILs were collected near the void volume of the column, passed through a 0.22 μm sterile filter and stored at 4°C. Recovery of phospholipid was quantitated by counting of [^{14}C]DPPC and coupling efficiency was estimated by use of ^{125}I-labeled LL2. The number of mAb per liposome was calculated assuming a liposome diameter of 100 nm and a molecular weight of 150 kD for LL2.

2.5. ELISA assay

The immunoreactivity of the SILs were assessed by competitive binding ELISA using the anti-idiotype mAb, WN. ELISA plates were coated with WN (50 μl/well of a 10 μg/ml solution) and alkaline phosphatase-conjugated free and liposome-bound LL2 (0.4 μg/ml) was coincubated with 0.1 to 5 μg/ml of LL2. After 2 h the binding of phosphatase-conjugated LL2 was measured with MUP as substrate (0.1 mg/ml in Tris buffer, pH 8.0). The MUP hydrolysis was measured with a microtiter plate spectrofluorometer using a 356 nm excitation filter and a 450 nm emission filter. Competitive binding experiments were also performed with Ramos cells (2×10^6 cells) incubated with HPTS-loaded SIL (200 nmol PL/ml) and different concentrations of free LL2 during 6 h at 37°C. After the incubation the cells were thoroughly washed and measured for HPTS uptake.

2.6. Celluar uptake and metabolism

Liposomes containing the pH-sensitive fluorophore HPTS was used as a probe to study the cellular internalization of SL and SIL. HPTS exhibits two major fluorescence excitation maxima: a peak at 403 maximal at low pH values and a peak at 454 maximal at high pH values, while the fluorescence is independent of pH at 413 nm (isobestic point) (Daleke et al., 1990). The ratio between the fluorescence at 454 and 413 nm can be used to study the internalization of the HPTS-liposomes to intracellular acidic compartments (Straubinger et al., 1990). HPTS-liposomes were diluted to 80 μM phospholipid in HEPES buffer and added to culture dishes (4×10^6 cells) at 37°C. After incubation for 6 h the cells were washed twice with cold PBS and the fluorescence was measured in a stirred cuvette at 20°C. Peak heights were measured at 510 nm emission at the three excitation wavelengths (403, 413 and 450 nm) and corrected for appropriate background fluorescence.

Cellular uptake and catabolism of free and liposome-bound LL2 was studied with ^{125}I-labeled mAb essentially as described by Shih et al. (1994). Briefly, Raji cells were suspended in fresh medium and aliquoted into culture dishes at 1×10^6 cells per dish. Approximately 7.5×10^6 cpm of ^{125}I-labeled LL2 or SIL were added and incubated at 4°C for 2 h. After binding the cells were washed three times with cold medium to remove unbound mAb and an aliquot was taken for determination

of total cell-bound radiactivity. The rest of the cells were resuspended in fresh medium and incubated at 37°C for internalization. At different time points the supernatant was collected and counted to determine the percent of radioactivity released. After counting, the supernatant was treated with cold 10% TCA solution and centrifuged at 4000 g for 15 min to precipitate the intact mAb. The degraded components were calculated as the difference between total released and intact mAb.

2.7. Cytotoxicity assays

Comparison of the in vitro cytotoxicity of free DXR and DXR-loaded liposomal formulations was performed on Raji, Daudi and Ramos human B-cell lymphoma lines with an proliferation assay utilizing tetrazolium dye, MTT (Mosmann, 1983). Briefly, 4×10^5 cells were plated in 24-well plates and incubated with either free DXR, DXR-SL or DXR-SIL. A control experiment included free LL2 and empty liposomal formulations. Cells were incubated for 6 or 24 h at 37°C in an athmosphere of 95% humidity and 5% CO_2. At the 6 and 24 h time points, the cells were washed twice before replacing with fresh media and incubated for an additional 42 or 24 h, respectively. At the end of the incubation time, tetrazolium dye was added, the formed reduction product was spun down, dissolved in EtOH:DMSO 1:1 and read at 570 nm.

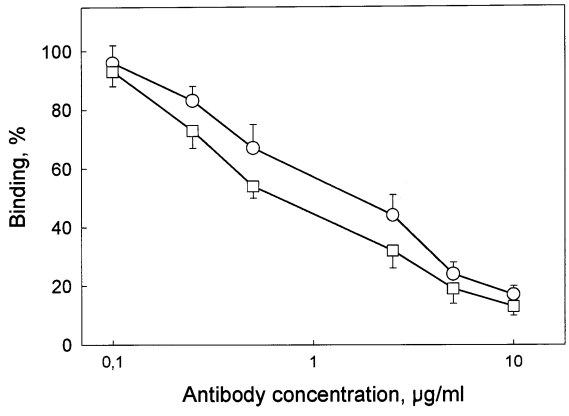

Fig. 1. Competitive binding ELISA with WN IgG-coated plates of alkaline phosphatase-conjugated free (○) and liposome-associated LL2 (□). Values are mean ± SE, $n = 3$.

3. Results and discussion

3.1. Imunoreactivity of immunoliposomes

Grafting of hydrophilic, flexible chains of poly(ethylene glycol) on the liposome surface has proven to be among the most successful methods to achieve lon-circulating properties (Blume and Cevc, 1990). Targeting of sterically stabilized liposomes containing PEG-DSPE was initially effected by attachment of antibodies in close proximity to the liposome surface (Torchilin et al., 1992). This type of immunoliposome had long-circulating properties, but their interaction with the target was reduced by the PEG chains (Klibanov et al., 1991). The present study represents a more recent approach where target-specific molecules are attached to the distal termini of liposome-grafted PEG chains, leading to targeted SILs with fully preserved target-binding capability (Park et al., 1995; Hansen et al., 1995). The immunoreactivity of the LL2-SIL was assessed by competitive binding ELISA utilizing the anti-idiotype mAb, WN. This antibody is specific for LL2 and reacts with no other antibody (Losman et al., 1995). The displacement curves in Fig. 1 show that free and liposome bound alkaline-phosphatase conjugated LL2 (conjugation level approximately 20 LL2/liposome) compete equally with unconjugated LL2 for binding on WN. Displacement experiments were also performed with HPTS-loaded SIL and Ramos cells. Fig. 2 shows a clear competition between SIL and free LL2 for binding to the cells. These findings suggests that LL2 preserves its immunoreactivity after binding to liposomes and is in line with results obtained with LL2 attached to lipid emulsions (Lundberg et al., 1999).

3.2. Cellular uptake of immunoliposomes

Liposome-encapsulated HPTS were used to study the uptake of SL and SIL by the Burkitt's lymphoma cell lines Raji, Daudi and Ramos and

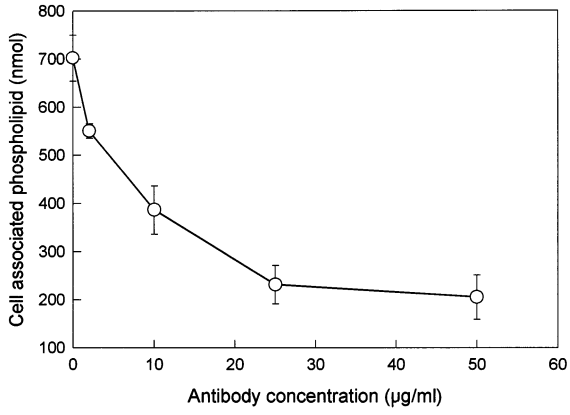

Fig. 2. Displacement curve for the uptake of HPTS-loaded LL2 immunoliposomes in the presence of different concentations of free LL2 (mean ± SE, $n = 3$).

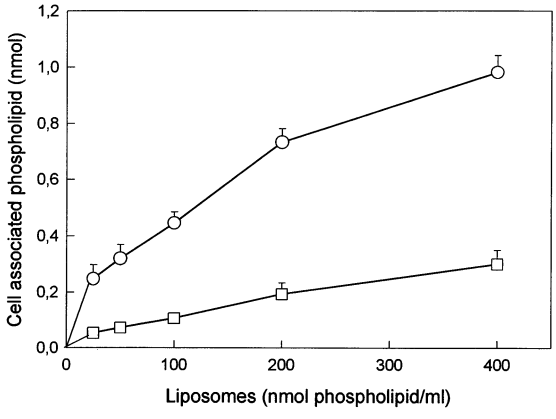

Fig. 3. Concentration-dependent cellular uptake of HPTS-containing SIL (○) and SL (□) during a 6 h incubation at 37°C (mean ± SE, $n = 4$).

with the chronic myelogenous leukemia cell line K562 as comparison.. The spectral shifts of HPTS with changes in pH make it a useful marker of the uptake and fate of the encapsulated dye (Straubinger et al., 1990). The concentration-dependent uptake of dye in SL and SIL by Raji cells is shown in Fig. 3. At low concentration of HPTS-SIL there is a sharp increase in cellular association of dye, which apparently represents binding of HPTS-SIL to the surface of the cells. Further increase in concentration up to 200 nmol/ml results in a slower linear accumulation of dye probably representing internalization of conjugates. The association of HPTS-SL to cells is much less pronounced compared to that of HPTS-SIL, and increases in a linear fashion over the entire concentration interval used. The approximately 3.8-fold increase in association of HPTS-SIL compared to that of mAb-free SL is similar to that obtained with SIL[anti-CD19] and and corresponding SL using [^{3}H]cholesteryl hexadecyl ether as label (Lopes de Menezes et al., 1998). The uptake by Ramos and Daudi cells of HPTS loaded liposomal preparations gave similar values as those with Raji cells as shown in Table 1. A considerable internalization of HPTS-SIL to low-pH compartments was suggested by measurements of the fluorescence ratio λ_{ex} 454/413 nm. Raji and Ramos cells gave values near 0.6, which corresponds to a pH value of 6.5, while the Daudi cells gave a somewhat higher value. These values can be compared with those of Kirpotin et al. (1997), who obtained an 454/413 ratio of about 0.6 for HPTS loaded anti-HER2 SIL during a 3 h incubation and Lopes de Menezes et al. (1999), who found that the pH sensed by HPTS delivered via SIL[anti-CD19] dropped to 6.1 during a 4 h incubation. The lowest 454/413 nm ratio obtainable for HPTS pinocytosed by J774 macrophages corresponded to an average pH of 6.5 (Daleke et al., 1990). SL gave values near 0.8, which corresponds to a pH value of about 7.0. The uptake values with K562 cells were very similar for SIL and SL and the 454/413 nm values were near 0.8, indicating a slow and unspecific uptake of the preparations.

Table 1
Cellular association (pmol) and λ_{ex} 454/413 nm of HPTS-SIL 9200 nm/ml) incubated with Burkitt lymphoma-cells and K562 myelogenous leukemia cells (2×10^6 cells) for 6 h at 37°C[a]

Celltype	Preparation	Cell association	λ_{ex} 454/413 nm
Raji	SIL	732 ± 11	0.59
	SL	193 ± 29	0.78
Ramos	SIL	702 ± 24	0.62
	SL	218 ± 11	0.81
Daudi	SIL	901 ± 33	0.74
	SL	121 ± 9	0.82
K562	SIL	165 ± 20	0.84
	SL	156 ± 23	0.81

[a] Values are means ± SE, $n = 4$.

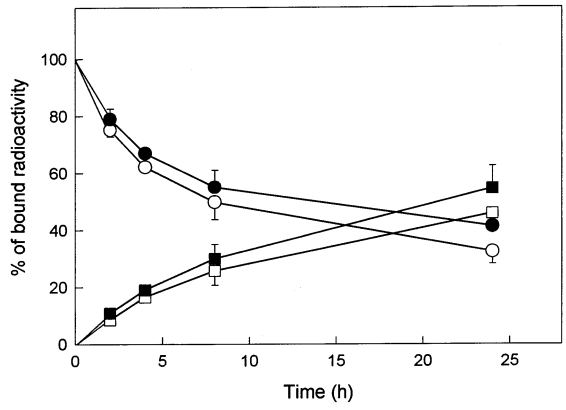

Fig. 4. Time course of degradation by Raji cells of ^{125}I-labeled LL2 antibody. Radioactivity associated with cells from free LL2 (○) and SIL-bound LL2 (●) or degraded and released into medium, free LL2 (□), SIL-bound LL2 (■), was counted and calculated as a percentage of that at zero time (mean ± SE, $n = 3$).

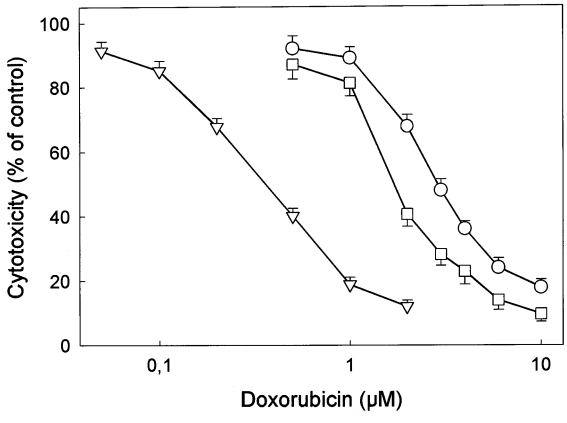

Fig. 5. Dose–response curves for DXR-SIL (□), DXR-SL (○) and free DXR (▽) against Raji cells. Cytotoxicity data (mean ± SE, $n = 4$) were obtained by the MTT method for a 6 h incubation time.

The binding and subsequent processing of SIL by the target cells were further studied by using ^{125}I-tagged LL2. The labeled free and liposome bound LL2 was allowed to bind to Raji cells for 2 h at 4°C, and the excess, unbound antibody was removed. The cells were then incubated in medium for different times and radioactivity associated with the cells or released into the supernatant was determined. In addition, the radioactivity released into the supernatant was analyzed further for the percentage that was TCA non-precipitable, which was considered degraded. As shown in Fig. 4, the radioactivity was gradually released into the supernatant. At 24 h 32 and 41% of the label remained cell-bound for free and liposome conjugated LL2, respectively, while the corresponding values for degraded antibody were 46 and 54%. The rate of the degradation of free LL2 (about 42% at 21 h) agrees very well with the value of 40% obtained by Shih et al. (1994). The extensive degradation of the antibody suggests that it must have been endocytosed and consequently degraded by the cells. These data thus support the conclusion from the HPTS study that the SIL are internalized and processed by the cells.

3.3. Cytotoxic activity

The cytotoxic activity of free and liposome encapsulated DXR was compared by a 6 h incubation followed by an additional 42 h in fresh medium. As seen from the dose–response curves in Fig. 5 DXR-SIL show higher activity than DXR-SL, but both liposomal preparations exhibited much lower activity than free DXR. The IC_{50} values were calculated to 1.6, 2.9 and 0.35 μM for DXR-SIL, DXR-SL and free DXR, respectively (DXR-SL:DXR-SIL ratio 1.8). In control experiments free LL2 and drug-free liposomal preparations displayed no significant cytotoxicity. When the incubation time was extended to 24 h the activity of DXR-SIL and DXR-SL was essentially the same (data not shown). The IC_{50} values can be compared with those obtained by Lopes de Menezes et al. (1998), with anti-CD19 DXR-SIL during a 24 h incubation, amounting to 1.4, 5.1 and 0.23 μM, respectively (DXR-SL:DXR-SIL ratio 3.6). The IC_{50} ratio of 1.8 between DXR-SL and DXR-SIL seems to be quite low bearing in mind the about 3.8-fold preference in cellular uptake of HPTS-SIL compared HPTS-SL. An explanation could be that DXR delivered by SIL was unable to reach the DNA target in the nucleus. To clarify this question the cellular uptake of DXR from SIL and SL preparations was studied. During a 6 h incubation of 10^6 cells with 5 μM solutions of DXR-SIL, DXR-SL and free DXR, they accumulated 0.27, 0.14 and 0.77 nmol

DXR, respectively. The about 2-fold uptake of DXR from SIL compared to that from SL agreeded quite well with the toxicity data showing corresponding lower value for DXR-SIL. Another explanation could be that the conjugation of LL2 to SL augmented the leakage of DXR from liposomes. To check this possibility dialysis experiments were performed at 20°C in growth medium (40 nmol DXR in 1 ml). The release of DXR measured was very similar for DXR-SL and DXR-SIL, amounting to 0.54 ± 0.07 and 0.51 ± 0.05 nmol, respectively and it could be concluded that conjugation of the mAb to SL did not increase the release of DXR. However, these values indicate a considerable leakage of DXR from both liposomal preparations in growth medium and may explain the relatively low difference in IC_{50} between DXR-SL and DXR-SIL and the fact that the cytotoxic activity of these preparations was similar after 24 h incubation. Lopes de Menezes et al. (1998) also noted a conciderable reduction in DXR-SL:DXR-SIL IC_{50} ratio (from 5.8 to 3.6) when increasing the incubation time from 1 to 24 h. In a later study by the same group (Lopes de Menezes et al., 1999) free drug was absorbed from the growth medium with the cation exchange resin Dowex-50W. This treatment increased the IC_{50} of DXR-SL approximately 5-fold and that of DXR-SIL 2-fold, also pointing at the fact that release of DXR from liposomes contribute to the drug cytotoxicity.

4. Conclusions

The development of SL with reduced uptake by the MPS system has greatly improved the utility of liposomes as targetable drug delivery systems (Woodle and Lasic, 1992). This study describes the conjugation of the internalizing mAb, LL2 to to the PEG-terminus of SL. The resulting SIL showed preserved immunoreactivity and was endocytosed faster by the cells than unconjugated SL. DXR-loaded SIL was found to be more cytotoxic to target Raji cells than corresponding SL, but to a lesser extent than expected from the uptake experiments. This discrepancy seemed to be caused by leakage of drug out of the liposomes. Liposomes with improved retention of drug or a more stable drug carrier like lipid emulsions (Lundberg et al., 1996) could thus improve the specificity of the cytotoxic action. The moderate increases in cellular association and cytotoxicity of SIL compared to nontargeted SL presented in this study are similar to those obtained by Lopes de Menezes et al. (1998). However, they obtained significantly improved therapeutic benefit in vivo with mice inplanted with B-cells, including some long-term survivors. Thus even though the gain in in vitro toxicity numbers are low useful preclinical therapy with DXR-loaded immunoliposomes against B-cell malignancies may still be possible.

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