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Liposomes targeted via two different antibodies: Assay, B-cell binding and cytotoxicity

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Abstract

The selective toxicity of anticancer drugs can be improved with the use of antibody-targeted liposomes. We hypothesize that liposomes targeted via antibodies against two or more receptor populations will increase the apparent receptor density on the target cells, resulting in improved therapeutic affects. A fluorescent assay was developed, using the fluorophores Alexa Fluor® 350 and 532 to label monoclonal antibodies (mAb), and used to quantitate two different mAb populations coupled to the same liposome surface to within ±10% of the values obtained with radiolabeled antibody (^{125}I) tracers. The binding and uptake of targeted liposomes by B lymphoma (Namalwa) cells were examined for either individual populations of αCD19-targeted or αCD20-targeted liposomes, mixed populations (1:1) of αCD19-targeted liposomes plus αCD20-targeted liposomes, and dual-targeted liposomes, i.e., equal amount of both αCD19 and αCD20 on the same liposomes. At similar antibody densities, the binding and uptake of the dual-targeted liposomes were greater than that of either individually targeted liposomes alone, and showed additivity. At the same total lipid and antibody densities, 1:1 mixtures of individually targeted liposomes gave similar results to dual-targeted liposomes. Cytotoxicity was also improved, with DXR-loaded dual-targeted liposomes appearing to have higher cytotoxicity than 1:1 mixtures of individually targeted liposomes.
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1. Introduction

The lack of selectivity of anticancer drugs such as doxorubicin (DXR) leads to dose-limiting toxicities in vivo. Liposomal drug delivery systems have improved the selective toxicity of DXR by altering the pharmacokinetics and biodistribution of the drug. Associating drugs with liposomes enhances their localization to solid tumors through a passive targeting mechanism, which takes advantage of the enhanced vascular permeability and impaired lymphatic drainage in growing tumors; this phenomenon has been termed the enhanced permeability and retention (EPR) effect [1,2]. Several liposomal anticancer formulations have received clinical approval and many more are currently in clinical trials [3].

Currently, there is considerable interest in the use of ligand-targeted therapeutics, e.g., immunoliposomes, to increase the selectivity toxicity of anticancer drugs [4,5].

Abbreviations: DXR, doxorubicin; mAb, monoclonal antibody; SL, non-targeted long-circulating (Stealth®) liposomes; SIL, long-circulating antibody-targeted liposomes (Stealth® immunoliposomes); αCD19, anti-CD19 mAb; αCD20, anti-CD20 mAb (Rituxan); HSPC, hydrogenated soy phosphatidylcholine; CHOL, cholesterol; mPEG$_{2000}$-DSPE, methoxy poly(ethylene glycol) (M_r 2000) covalently linked to distearoylphosphatidylethanoloamine; Mal-PEG-DSPE, maleimide-derivatized PEG-DSPE; [^{3}H]CHE, Chol-[1,2-^{3}H-(*N*)]hexadecyl ether; MTT, 3-(4,5-dimethyltiazol-2-ly)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; HBS, HEPES-buffered saline; PL, phospholipid; SIL[αCD19], anti-CD19-targeted immunoliposomes; SIL[αCD20], anti-CD20-targeted immunoliposomes; SIL[αCD19]+SIL[αCD20], 1:1 mixtures of individual immunoliposomes; SIL[αCD19+αCD20]), dual-targeted immunoliposomes

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Immunoliposomes have monoclonal antibodies or antibody fragments coupled to their surface, which causes the liposomes to bind selectively to antigens or receptors that are either uniquely expressed or over-expressed on cancer cells, leading to increased drug delivery to the target cells [6–12]. Cell surface antigen density has been shown to be an important factor in the efficacy of immunoliposomal therapy [10]. The higher the antigen density, the greater the therapeutic effect of anti-HER2-targeted liposomal DXR [10]. Since, in a clinical setting, it is not possible to alter antigen densities, we hypothesized that the antigen density can be artificially increased by targeting to two or more antigen populations on target cells. This increase in the apparent antigen density will, we hypothesize, result in the delivery of more drug to the target cells, resulting in increased therapeutic effects. We further hypothesize that immunoliposomal drugs, targeted with two or more populations of antibodies on the liposomal surface, may be useful in delivering drug to a higher percentage of cells in tumor cell populations that have a heterogeneous expression of cell surface antigens. As well, the activation of two or more cell surface antigens may lead to synergistic cytotoxic responses by engaging two or more mechanisms of cell kill. We have begun to test these hypotheses in the human B-cell lymphoma cell line, Namalwa, which expresses numerous cell surface antigens, such as CD19, CD20 and CD22.

In this paper, we have devised a fluorescent assay to quantitate two or more antibodies coupled to liposomal surfaces. This was necessary since it was difficult to find two or more non-overlapping radioactive tracers that could be easily and economically used for the quantitation of multiple antibodies. In addition, the use of fluorescent tracers is safer than using radioactivity. Two different fluorophores with non-overlapping spectra, Alexa Fluor® 350 and Alexa Fluor® 532, were coupled to αCD19 and αCD20, respectively, to be used as tracers to quantitate αCD19 and/or αCD20 coupled to immunoliposomes. Subsequently, the binding and uptake of the various immunoliposomal populations was studied using the Namalwa cell line, and the cytotoxicity of DXR-loaded immunoliposomes was examined in the same cell line.

2. Methods and materials

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and methoxy poly(ethylene glycol) (M_r 2000) covalently linked to distearoylphosphatidylethanoloamine ($mPEG_{2000}$-DSPE) were generous gifts of ALZA Pharmaceuticals, Inc. (Mountain View, CA). Cholesterol (CHOL) was purchased from Avanti Polar Lipids (Alabaster, AL). Maleimide-derivatized PEG_{2000}-DSPE (Mal-PEG-DSPE) was custom synthesized by Nektar Therapeutics, Inc. (Huntsville, AL). Chol-[1,2-^{3}H-(*N*)]hexadecyl ether ([^{3}H]CHE, 1.48–2.22 TBq/mmol) and ^{125}I-NaI (185 MBq) were purchased from PerkinElmer Life Sciences (Woodbridge, Ontario, Canada). Bio-Rad Protein Assay Reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Alexa Fluor® 350 carboxylic acid succinimidyl ester and Alexa Fluor® 532 carboxylic acid succinimidyl ester were purchased from Molecular Probes (Eugene, OR). 2-iminothiolane (Traut's Reagent), polyclonal sheep IgG, and 3-(4,5-dimethyltiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-CD20 (Rituxan) was purchased from the University of Alberta Hospital Pharmacy (Edmonton, Alberta, Canada). Nuclepore polycarbonate membranes (pore sizes, 0.4, 0.2, 0.1, and 0.08 μm) were purchased from Northern Lipids (Vancouver, British Columbia, Canada). Sephadex G-50, Sepharose CL-4B and Aqueous Counting Scintillant (ACS) were purchased from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). RPMI 1640, penicillin-streptomycin, and fetal bovine serum were obtained from Invitrogen (Burlington, Ontario, Canada). All other chemicals were of the highest grade possible.

2.2. Antibodies and cell line

The murine IgG2a monoclonal αCD19 antibody (mAb) was produced from the FMC63 murine hybridoma (from Dr. H. Zola, Children's Health Research Institute, Adelaide, Australia) and purified as previously described [13]. A chimeric IgG1 monoclonal antibody, Rituxan, was used for αCD20. The human Burkitt's lymphoma cell line Namalwa (ATCC CRL 1432) was cultured in suspension in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml) and L-glutamine (0.292 mg/ml) in a humidified 37 °C incubator with a 5% CO_2 atmosphere. The cell surface expression of CD19 and CD20 by Namalwa cells was determined, as previously described, using single-color flow cytometry [14]. Briefly, the Namalwa cells (1×10^6) were stained with primary mAb (αCD19 or αCD20) followed by a secondary anti-mouse-FITC IgG. Cell-associated fluorescence was analyzed on a Becton Dickinson FACScan using Lysis II software (Becton Dickinson, San Jose, CA) [14].

2.3. Preparation of liposomes

Non-targeted PEGylated (Stealth®) liposomes (SL) for binding studies, or to be loaded with DXR for cytotoxicity studies, were composed of HSPC:CHOL:PEG-DSPE at a molar ratio of 2:1:0.10. PEGylated (Stealth) immunoliposomes (SIL) were composed of HSPC:CHOL:PEG-DSPE:-Mal-PEG-DSPE at a molar ratio of 2:1:0.08:0.02. The non-exchangeable, non-metabolized lipid tracer [^{3}H]CHE was added as a tracer to quantitate the phospholipids. A lipid mixture of chloroform stocks was prepared and dried to form a thin film using a rotovaporator; then placed in a vacuum overnight. The lipid films were hydrated at a

concentration of 10–30 mM phospholipid (PL) in HEPES-buffered saline (HBS, 25 mM HEPES, 140 mM NaCl, pH 7.4). Hydrated liposomes were extruded sequentially at 65 °C through a series of polycarbonate filters with pore sizes ranging from 0.4 μm down to 0.08 μm. Liposome diameters were determined to be 100±20 nm using a Brookhaven BI-90 particle sizer (Brookhaven Instruments, Holtsville, NY). Alternatively DXR was loaded into liposomes using an ammonium sulfate gradient [15].

2.4. Preparation and assay of immunoliposomes

Aliquots of antibodies were labeled with either Alexa Fluor® 350 carboxylic acid succinimidyl ester or Alexa Fluor® 532 carboxylic acid succinimidyl ester according to the manufacturers' instructions. Other aliquots were labeled with ^{125}I using Iodobeads purchased from Pierce Biotechnology Inc (Rockford, Illinois). Sheep IgG (used as an inexpensive antibody for developing methods), αCD19 (internalizing mAb) or αCD20 (non-internalizing mAb) were coupled to the terminus of Mal-PEG-DSPE using the coupling method previously described [16]. Trace amounts of antibodies, labeled with either of the Alexa Fluor® dyes or with ^{125}I, were added to the unlabeled antibodies prior to thiolation. Antibodies (10 mg/ml) were thiolated with Traut's reagent at a ratio of Traut's:IgG of 20:1 (mol/mol) in degassed HBS (pH 8.0) for 1 h; unreacted Traut's reagent was removed by chromatography on a Sephadex G-50 column with degassed HBS (pH 7.4). Individual thiolated antibodies or a 1:1 mixture of thiolated antibodies was immediately added to the liposomes at a 1000:1 molar ratio of phospholipid:antibody and incubated overnight at room temperature with continuous stirring. Unconjugated antibody was removed by chromatography on a Sepharose CL-4B column in HBS (pH 7.4).

The concentrations of the individual antibodies, labeled with ^{125}I, were determined from the specific activity of the radioactive tracer, following coupling to the liposomes. Concentrations of individual antibodies, or mixtures of antibodies, labeled with either of the Alexa Fluor® tracers, were determined from their fluorescence yields compared to a standard curve. Non-labeled liposomes, at a comparable lipid concentration, were added to the standards to control for liposome light scattering in the samples.

In order to determine if measurement artifacts might occur due to overlaps in their fluorescent spectra, fluorescence resonance energy transfer between antibodies labeled with Alexa Fluor® 350 (λ_{ex}: 346 nm, λ_{em}: 442 nm) and Alexa Fluor® 532 (λ_{ex}: 530 nm, λ_{em}: 554 nm) was determined. Standard curves were prepared with sheep IgG (10 mg/ml) labeled with either Alexa Fluor® 350 or Alexa Fluor® 532 or combinations of the two fluors. There were no statistical differences between the standard curves, indicating a lack of interference between the two fluors. Because fluorescence quenching depends on of the distance (R^6) between the two fluors, the possibility of fluorescence self-quenching between fluor-labeled sheep IgG was examined as a function of antibody density. Fluorescence emissions were measured on a SLM-AMINCO 8100 Series 2 Spectrometer (Spectronic Instruments Inc., Rochester NY) at densities of 50, 100, and 150 μg sheep IgG/μmol PL before and after the addition of 10% (v/v) Triton X-100 to solublize the liposomes. The results indicated no self-quenching of either fluor in this density range.

2.5. In vitro immunoliposomes binding and uptake

Binding experiments were performed at both 37 °C and 4 °C (permissive and non-permissive for internalization, respectively) as previously described [7]. Briefly, non-targeted liposomes (SL) or targeted liposomes (SIL[αCD19], SIL[αCD20], SIL[αCD19]+SIL[αCD20], or SIL[αCD19+αCD20]) were prepared with 74 kBq of [^{3}H]CHE label per μmol of PL. Liposomes were incubated with 1×10^6 Namalwa cells in the exponential growth phase at different phospholipid concentrations for 1 h at either 37 °C or 4 °C. Cells were then washed to remove unbound liposomes, and the amount of [^{3}H]CHE was determined by scintillation counting in a Beckman LS-6800 scintillation counter. Cell association (pmol PL/10^6 cells) was calculated from the specific activity of the liposomes. Specific binding was determined by subtracting the non-specific binding of SL from the total binding of SIL.

2.6. In vitro cytotoxicity studies

The cytotoxicity of DXR-loaded liposomes, either non-targeted (DXR-SL) or targeted (DXR-SIL[αCD19], DXR-SIL[αCD20], DXR-SIL[αCD19]+DXR-SIL[αCD20] or DXR-SIL[αCD19+αCD20]), was determined for Namalwa cells using the MTT dye reduction assay [17]. Briefly, 5.0×10^5 cells/well were plated in 96-well plates and incubated at 37 °C for 1 h with DXR-loaded liposome formulations. The cells were then washed and incubated for an additional 48 h before cell viability was assessed. Results are expressed as IC_{50}, which was obtained graphically using Graphpad Prism version 3.0 (GraphPad Software, Inc., San Diego CA).

2.7. Statistical analysis

Statistical comparisons were performed using two-tailed Student's *t* test with Graph Pad Instat Version 3.01 for Windows 95/NT (GraphPad Software, Inc., San Diego CA).

3. Results

Alexa Fluor® 350 and Alexa Fluor® 532 have non-overlapping excitation and emission spectra and do not

undergo fluorescence resonance energy transfer (i.e., distance-dependent interaction between the electronic excited states of two dye molecules, in which excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon) [18]. Standard curves, prepared with sheep IgG labeled with either fluor alone, or with 1:1 mixtures of sheep IgG labeled with either fluor, gave curves that were not statistically different (P>0.1), confirming that no fluorescence interference occurred between these dye pairs. Nor did either of the Alexa Fluor® dyes self-quench (i.e., fluorescence emission from one molecule absorbed by an adjacent molecule of the same fluor) when labeled antibodies were coupled to the liposomes within the range of antibody densities normally employed. Fluorescence emission signals of coupled antibodies were measured before and after the addition of the detergent Triton X-100, which solubilized the liposomes and dispersed the label. Antibody densities from 50–150 μg Ab/μmol PL resulted in fluorescence signals that were the same before and after the addition of Triton X-100 (P>0.1), confirming that self-quenching was not a problem in this density range.

3.1. Fluorescence assay for quantifying coupled antibodies

The levels of sheep IgG coupled to the liposomes were determined with sheep IgG that was labeled with Alexa Fluor® 350, Alexa Fluor® 532, or ^{125}I. The results were very similar (73.3±1.2, 73.0±2.6 or 66.3±1.2 μg Ab/μmol PL, respectively). As well, αCD20 and αCD19 were labelled with Alexa Fluor® 350 and Alexa Fluor® 532, respectively, and both populations of antibodies were coupled to liposomes at a 1:1 ratio in the presence of a trace amount of ^{125}I-labeled αCD20. The results showed that equal amounts of each antibody bound to the liposomes; the values for αCD20 and αCD19 from the fluorescent analysis were 48.5±6.5 and 51.5±6.6 μg Ab/μmol PL, respectively. This is comparable to the value for αCD20 of 45.9±2.2 μg Ab/μmol PL obtained in the same liposomes using a radiolabeled marker.

3.2. Binding and uptake of immunoliposomes

The [^{3}H]CHE counts associated with Namalwa cells at 4 °C measure the binding of antibody-targeted liposomes to

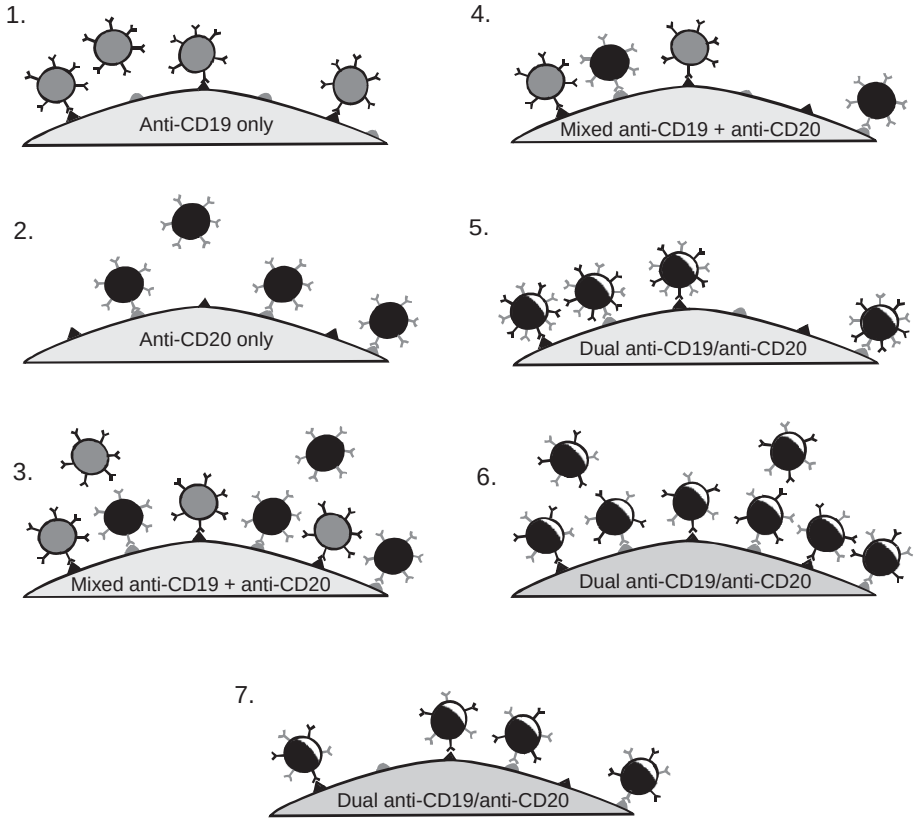

Fig. 1. Single-targeted immunoliposomes, dual-targeted immunoliposomes and combination of single-targeted immunoliposomes binding to Namalwa cells. (1) SIL[αCD19]. (2) SIL[αCD20]. (3) 50:50 mixtures of SIL[αCD19] and SIL[αCD20]. In the example, the individual antibody concentrations are the same as in 1 and 2, but the liposome concentration is double that in either 1 or 2. (4) 50:50 mixtures of SIL[αCD19] and SIL[αCD20], with the same antibody concentration, antibody density and liposome concentration as in 1 and 2. (5) SIL[αCD19+αCD20], in which the individual antibody concentrations and the total liposome concentration are the same as in 1 and 2, but the antibody density is double that in either 1 or 2. (6) SIL[αCD19+αCD20] at the same total antibody concentration and antibody density as in 1 and 2, but the total lipid concentration is double that of either 1 or 2. (7) Dual-targeted liposomes, with the same antibody concentration, antibody density and liposome concentration as in 1 and 2.

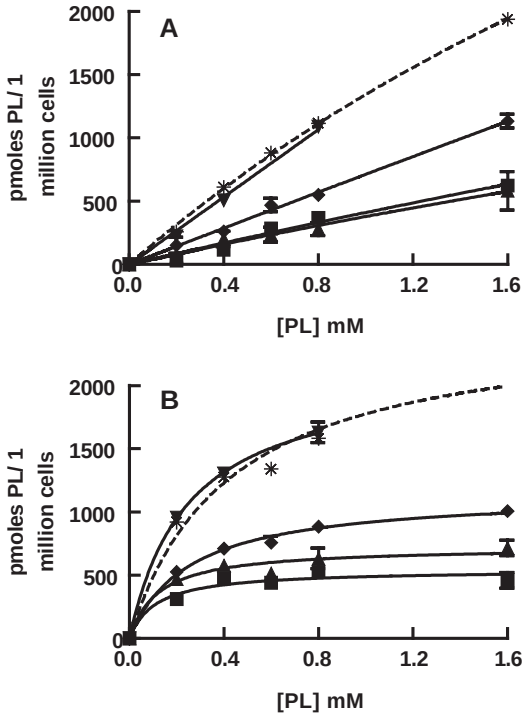

Fig. 2. Specific cell association of immunoliposomes with Namalwa cells as a function of lipid concentration and temperature. (A) 4 °C; (B) 37 °C. (▲) SIL[αCD19], 41 μg/μmol PL; (■) SIL[αCD20], 45 μg/μmol PL; (▼) 50:50 mixtures of SIL[αCD19]+SIL[αCD20]; (♦) SIL[αCD19 + αCD20], 44 μg αCD19/μmol PL and 32 μg αCD20/μmol PL (total antibody density of approximately double that of the individual immunoliposome populations as in Fig. 1-5), compared at the same total liposome concentrations. The broken line represents the theoretical line for additive effects at the same total liposome concentration (Fig. 1-4). Liposomes were labeled with [^{3}H]CHE and were composed of HSPC:CHOL:mPEG-DSPE:Mal-PEG-DSPE (2:1:0.06:0.02). They were incubated with 1×10^6 Namalwa cells for 1 h, after which the cells were washed with cold PBS to remove the unbound liposomes. Data are expressed as pmol PL/10^6 cells. Each point is an average of 3 replicates±S.D. from one representative experiment.

cell surface antigens, and at 37 °C the counts measure a combination of binding and receptor-mediated internalization of SIL[αCD19]. SIL[αCD20] are not internalized [14], so the counts for this antibody measure surface binding at both temperatures. The binding and uptake of SIL[αCD19], SIL[αCD20], SIL[αCD19]+SIL[αCD20], or SIL[αCD19+αCD20] were compared. Several factors need to be considered in designing these experiments; these include the concentration of lipid in each liposome population, the antibody density for each liposome population, the total liposome concentration and the total antibody concentration (Fig. 1). Specific cellular association was determined by subtracting the non-specific absorption of SL from the binding/uptake of SIL. Binding experiments at 4 °C showed no significant difference between SIL [αCD19] and SIL[αCD20] (Fig. 2A). At 37 °C, SIL [αCD19] showed higher binding and uptake than SIL[αCD20] did (Fig 2B); this is most likely due to receptor-mediated endocytosis and recycling of the CD19 antigen back to the cell surface where it can participate in further binding and internalization [7]. At both 4 °C and 37 °C, mixtures of two separate populations of liposomes showed an additive effect (Fig. 1-4). Dual-targeted immunoliposomes with αCD19 and αCD20 densities between 32 and 44 μg Ab/μmol PL each (total antibody density of approximately double the individual populations, as in Fig. 1-5) were subadditive (Fig. 2A,B). Another set of binding and uptake experiments was performed using SIL [αCD19+αCD20] having total antibody and liposome concentrations that were equivalent to those that would occur when individual populations of SIL[αCD19] and SIL[αCD20] were combined (Fig. 1-7). In these experiments there were no statistical differences between the binding and uptake of SIL[αCD19]+SIL[αCD20] versus that of SIL[αCD19+αCD20] (Fig. 3).

3.3. In vitro cytotoxicity

The in vitro IC_{50} values for DXR-SL, DXR-SIL [αCD19], DXR-SIL[αCD20], DXR-SIL[αCD19]+DXR-SIL[αCD20], and DXR-SIL[αCD19+αCD20] are presented in Table 1. All DXR-loaded targeted immunoliposomes were significantly more cytotoxic to Namalwa cells than DXR-SL, for a 1 h incubation time and there was a tendency for the cytotoxicity of the combination samples

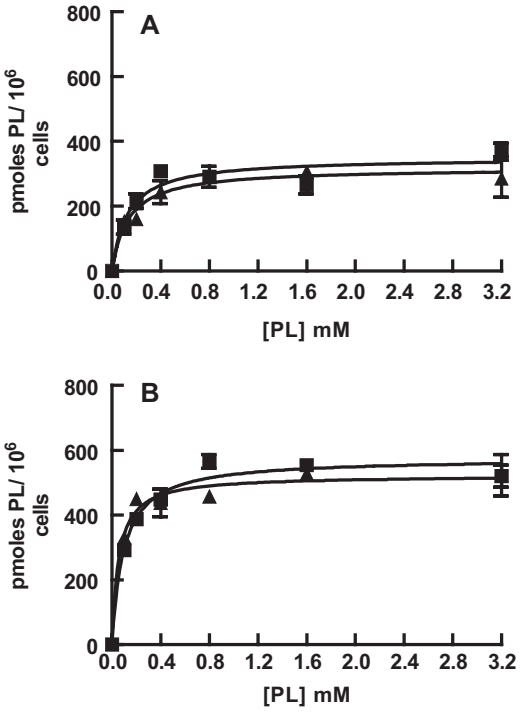

Fig. 3. Specific cell association of immunoliposomes with Namalwa cells as a function of lipid concentration and temperature. (A) 4 °C; (B) 37 °C. (■) SIL[αCD19]+SIL[αCD20], 61 μg αCD19/μmol PL and 48 μg αCD20/μmol PL; (▲) SIL[αCD19+αCD20], 38 μg αCD19/μmol PL and 30 μg αCD20/μmol PL (Fig. 1-7). The experimental protocol was as in the Fig. 2 caption.

Table 1
Cytotoxicity of liposomal doxorubicin formulations against Namalwa cells

Formulation	IC_{50}, 1 h (μM DXR)
DXR-SL	30.5
DXR-SIL[αCD19]	5.2, 3.0
DXR-SIL[αCD20]	1.9, 5.5
DXR-SIL[[αCD19]+[αCD20]	2.3
DXR-SIL[αCD19+αCD20]	2.0, 2.0

Namalwa cells (5×10^5 cells/well) were plated in 96-well plates and incubated with increasing concentrations of liposomal DXR, and various targeted-liposomal formulations for 1 h. Cells were then washed 3 times with PBS and incubated with fresh medium for a further 47 h, and a MTT assay was performed. The antibody concentrations of αCD19 and αCD20 on liposomes were as follows: DXR-SIL[αCD19], 60–65 mg mAb/mmol PL; DXR-SIL[αCD20], 39–51 mg mAb/mmol PL; DXR-SIL[αCD19], 56 mg mAb/mmol PL+DXR-SIL[αCD20], 55 mg mAb/mmol PL; DXR-SIL[αCD19+αCD20], 25–30 mg αCD19/mmol PL and 24–26 mg αCD20/mmol PL (Fig. 1-7).

to be somewhat higher (lower IC_{50}s) than that of the individual targeted populations.

4. Discussion

In this paper, we developed a fluorescence assay to quantify the different populations of antibodies coupled to the same population of liposomes. The ability to assess antibody densities on liposomes by fluorescence has advantages over radioactive labels and it also allows one to distinguish and quantify two or more antibodies on the liposomal surface. The fluorescence labels, Alexa Fluor® 350 (λ_{ex}: 346 nm, λ_{em}: 442 nm) and Alexa Fluor® 532 (λ_{ex}: 530 nm, λ_{em}: 554 nm), were carefully chosen to have non-overlapping fluorescence spectra. As well, their spectra do not overlap with the excitation and emission wavelengths of the drug DXR (λ_{ex}: 470 nm, λ_{em}: 590 nm), which was used in the cytotoxicity studies.

The ability to quantitate two different antibodies coupled to the same liposomes enabled us to test the hypothesis that the dual-targeting of liposomes would increase the binding, uptake and cytotoxicity of immunoliposomes compared to single-targeted immunoliposomes due to an increased apparent receptor density. Binding and uptake experiments showed that when dual-coupled liposomes (SIL[αCD19+αCD20]) were targeted at the same total antibody and lipid concentrations as two individual sets of single-targeted immunoliposomes, either (SIL[αCD19] or SIL[αCD20]), an additive response was obtained, which was similar in magnitude to that obtained with a 1:1 mixture of SIL[αCD19]+SIL[αCD20]. The total antibody concentration of αCD19 and αCD20 on dual-targeted liposomes was, in this instance, designed to be equivalent to that on a single antibody-targeted immunoliposome. In other words, the density of each antibody was half that of the antibody density on individual sets of single antibody-targeted immunoliposomes (Fig. 1-7 vs. 1-1 or 1-2).

Several years ago, Torchilin and colleagues reported an in vitro system using a set of multiple target antigens, fibronectin, fibrinogen and low density lipoproteins, bound to microplates [19]. The antigens were targeted using mixtures of biotinylated antibodies followed by exposure to an avidin linker and then treatment with biotinylated radiolabelled liposomes. The results demonstrated that a mixture of antibodies resulted in higher liposome binding than single antibodies at optimal concentrations [19]. Our results confirm this observation for immunoliposomes containing mixtures of antibodies, exposed to cultured B cells (this manuscript), and in an in vivo model of human B lymphoma [11].

When we investigated dual-targeted immunoliposomes with antibody densities for αCD19 and αCD20 that were the same as that on single antibody-targeted immunoliposomes (i.e., double the antibody density on individual populations of single-targeted liposomes, as in Fig. 1-5), an additive response was expected. However, we obtained sub-additive binding and uptake. At high antibody densities, there may be steric hindrance of binding. In other words, if the two different antigens were located in close proximity to each other, the binding of a liposome to one antigen might block the access of another liposome to an adjacent antigen and reduce binding via the second antibody. In addition, at higher antibody densities, the binding may become more multivalent (binding of one liposome to two or more antigens), which could reduce the numbers of liposomes binding to the two antigens. However, as discussed below, the binding of liposomes to two antigens simultaneously may lead to cell kill through additive or synergistic signaling mechanisms. These experiments show the importance of considering the antibody and liposome concentrations when interpreting the experimental results and in designing experiments. Further, the cell surface geometry of the target antigens, relative to each other, will influence the proper choice of target antigens.

In vitro cytotoxicity studies revealed that DXR-SIL[αCD19] or DXR-SIL[αCD20] have similar cytotoxic profiles after a 1 h incubation. However, the cytotoxicities for each are likely due to different mechanisms of action. The cytotoxicity of DXR-SIL[αCD20], directed against a non-internalizing antigen, is most likely due to DXR being released from liposomes bound to the cell surface and uptake of the free drug into the target cell. In vivo, drug released from these liposomes would be redistributed away from the target cells, but in the cell culture, the drug will be rapidly taken up into the cells. CD19 is an internalizing receptor, by contrast, and the cytotoxicity of DXR-SIL[αCD19] is most likely attributable to receptor-mediated endocytosis of the liposomes followed by slow breakdown of the liposomes in the lysosomal apparatus and subsequent intracellular trafficking of the released drug. In vivo, we have demonstrated that this mechanism leads to improved therapeutic effects over those seen for a non-internalizing antibody, but in vitro the cytotoxicity is equivalent [14].

Dual-targeted liposomes were slightly more cytotoxic in vitro than single-targeted liposomes.

Recently, the therapeutic effects of liposomal vincristine (VCR) or DXR, targeted with either αCD19 or αCD20 or 1:1 mixtures of each, were investigated in severe combined immunodefficient (SCID) mice injected with Namalwa cells [11]. Results showed that 1:1 mixtures of two separate populations of liposomes loaded with DXR, i.e., DXR-SIL[αCD19] and DXR-SIL[αCD20], had no enhanced therapeutic effect over DXR-SIL[αCD19] on its own. However, 1:1 mixtures of VCR-SIL[αCD19] and VCR-SIL[αCD20] had a higher cure rate in mice then either set of targeted immunoliposomes alone. In these experiments, the therapeutic effect of neither DXR-SIL[αCD19+αCD20] nor VCR-SIL[αCD19+αCD20] was examined, due to difficulties in measuring the concentrations of each antibody population in dual-targeted liposomes.

With the development of a fluorescent assay to quantitate two or more populations of antibodies on liposomes, further experiments can now be carried out. It is now possible to determine whether having two separate populations of antibodies coupled to a single population of liposomes is more advantageous than (or equivalent to) mixtures of two separate populations of liposomes, each coupled to a different antibody. This will enable us to test the hypothesis that the interaction of two different antibodies on the same liposome with two different antigen sites in close proximity on the same cell may lead to synergistic cytotoxicity, by simultaneously engaging two or more additive or synergistic mechanisms of cell kill. In an in vivo setting, dual-targeted liposomes may also behave in a manner similar to bispecific antibodies with one antibody binding to target cells while unattached antibodies recruit effector cells or molecules that can effectively kill the tumor cells. Steric hindrance is also likely to be less of a factor for dual-targeted liposomes in vivo as the greater apparent receptor density may increase the association of liposomes with cells through increased avidity in a non-static environment. In an in vivo setting, there may be added benefits of targeting two antigens by the same liposome that are impossible to predict with in vitro studies.

Survival studies comparing the therapeutic efficacy of dual-targeted immunoliposomes to single-targeted liposomes or mixtures of single-targeted liposomes are warranted. As well, survival studies, similar to those performed previously [11], could be performed with dual-targeted immunoliposomes loaded with different cytotoxic drugs. If the advantages of dual-targeted liposomes over single-targeted liposomes are slight, then consideration also has to be given to issues surrounding the manufacturing and regulatory approval of one versus the other. From the manufacturing point of view, the preparation of dual-targeted liposomes is no more complex than manufacturing single-targeted liposomes. From the therapeutic perspective, one gains the possibility of synergistic interactions with the appropriate choice of dual-targeted liposomes, but one loses the simplicity of being able to mix and match a combination of various (approved) populations of single-targeted liposomes to suit individual patient disease profiles [20]. Further experiments will be necessary to determine if there are substantial advantages of one approach over the other.

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