Doxorubicin is an anthracycline antibiotic that is extensively used in anticancer therapy. An important dose-limiting toxicity for the drug is acute and chronic cardiomyopathy (1). Liposomal encapsulation of doxorubicin leads to decreased clearance, increased area under the time versus concentration curve (AUC), and other altered pharmacokinetic properties (2). A long circulating (Stealth), slow release formulation of doxorubicin (Doxil/Caelyx) has been approved for clinical use in the treatment of Kaposi’s sarcoma, ovarian cancer, and breast cancer (3–5). Liposomal formulations of doxorubicin that are in clinical use (i.e., Doxil/Caelyx and Myocet) result in reductions in cardiac toxicity because liposome encapsulation reduces doxorubicin levels in the heart (6, 7). Liposomal delivery of doxorubicin also leads to greater accumulation of the drug in solid tumors via the enhanced permeability and retention effect (8).

We have shown previously that nontargeted formulations of liposomal doxorubicin, having slow rates of drug release, showed superior therapeutic effects in the treatment of orthotopic models of murine breast cancer than liposomes with more rapid drug release rates (9). In these experiments, liposomes with intermediate drug release rates resulted in some unexpected deaths in mice, identified tentatively as due to gastrointestinal toxicities by pathologic examination. Variations in toxicity with different formulations of doxorubicin has also been observed by other researchers (10). We hypothesize that it may be possible to overcome toxicities associated with intermediate doxorubicin release rates by antibody-mediated targeting of the liposomes.

A suitable animal model for these studies is anti-CD19-targeted liposomal doxorubicin for the treatment of xenograft models of human B lymphoma (11, 12). B cells, including B lymphoma cells, such as the Namalwa cell line, express the CD19 antigen on their cell surface (13). Binding of anti-CD19-targeted liposomes to the target cells triggers the rapid receptor-mediated internalization of the liposome-drug package into the target cell interior; the binding and internalization of the liposomes to B cells increases their rate of clearance from...
the circulation (14). This mechanism works well for drugs like doxorubicin that escape degradation by lysosomal enzymes. Free doxorubicin has a large volume of distribution (15) and liposomal doxorubicin that is released more rapidly into the circulation may lead to redistribution of the released drug to sensitive tissues like the heart or gastrointestinal tract, resulting in toxicities (16). For targeted liposomes, rapid binding and internalization of the liposomal doxorubicin by malignant B cells in circulation may, we hypothesize, reduce the amount of free drug released into circulation, particularly for liposomes having more rapid release rates. This would in turn reduce the redistribution of doxorubicin to sensitive tissues, leading to reductions in drug toxicities for these formulations.

Liposomal formulations were prepared with lipids having several different drug release profiles. The pharmacokinetics of the lipid and the drug components of the liposomal formulations were determined in vitro and in vivo, and the therapeutic activity of the nontargeted versus anti-CD19-targeted formulations was compared in severe combined immunodeficient (SCID) mice inoculated with human B-cell lymphoma (Namalwa) cells.

Materials and Methods

Materials. Dioleoyl phosphatidylcholine (DOPC), dipalmitoyl phosphatidylcholine (DPPC), palmitoyl-myristoyl phosphatidylcholine (PMPC), palmitoyl-oleyl phosphatidylcholine (POPC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Methoxy-polyethylene glycol (mPEG; Mr 2,000) covalently linked via a carbamate bond to diestearoyl phosphatidylethanolamine (DSPE), hydrogenated soy phosphatidylcholine (HSPC), and doxorubicin were generous gifts from ALZA Pharmaceuticals, Inc. (Mountain View, CA). Maleimide-derivatized PEG-DSPE was custom synthesized by Shearwater Polymers, Inc. (Huntsville, AL). Nucleopore polycarbonate membranes (pore sizes, 0.2, 0.1, and 0.08 μm) were purchased from Northern Lipids (Vancouver, British Columbia, Canada). RPMI 1640 (without phenol red), penicillin-streptomycin, fetal bovine serum, and adult bovine serum (ABS) were purchased from Life Technologies, Inc. (Burlington, ON, Canada). 2-Iminoethanol (Truat's reagent) was obtained from Sigma Chemical Co. (St. Louis, MO). Slide-a-Lyzer dialysis cassettes (M, cutoff of 10,000) and buoys were purchased from Pierce (Rockford, IL). The Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Sephadex G-50. Sepharose CL-4B, aqueous cationic scintillant, and [14C]doxorubicin (1.85-2.29 GBq/mmol) were purchased from Amersham Pharmacia Biotech (Baie d’Urfe, Quebec, Canada). Cholesterol hexadecylether ([14C]CHE; 1.48-2.22 TBq/mmol), Solvable, and Ultima Gold were purchased from Perkin-Elmer Biosciences (Boston, MA). All other chemicals were of analytic grade purity or the highest available purity.

Animals, cell culture, and antibodies. Female 6- to 8-week-old CB.17 SCID mice were purchased from Taconic Farms (Germantown, NY) and housed in the virus antigen–free unit of the Health Sciences Laboratory Animal Services, University of Alberta (Alberta, Edmonton, Canada). The Health Sciences Animal Policy and Welfare Committee of the University of Alberta approved all experiments, which were in accordance with the Guide to the Care and Use of Experimental Animals set forth by the Canadian Council on Animal Care.

The human Burkitt’s lymphoma cell line Namalwa (ATCC CRL 1432) was purchased from American Type Culture Collection (Manassas, VA). Suspension cultures of the cells were grown at 37°C in a humidified 5% CO2 atmosphere in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, penicillin G (50 units/mL), streptomycin sulfate (50 μg/mL), and glutamine. Only cells in the exponential phase of cell growth were used in experiments. Murine monoclonal antibody (mAb) anti-CD19 was produced from the FMC63 murine hybridoma cells (17) purified as described previously (18); [3H]doxorubicin was used as a tracer to measure coupling efficiencies and to determine the amount of mAb attached to the liposomes as described previously (11).

Preparation of liposomes. Nontargeted liposomes were prepared with various phosphatidylcholines, cholesterol, and mPEG-DSPE at a 2:1:0.1 molar ratio to phospholipid. The phosphatidylcholines were DOPC, POPC, DPPC, PMPC, and HSPC. All liposomes were prepared by hydration of thin lipid films as described previously and extruded to mean diameters in the range of 100 ± 10 nm as determined by laser light scattering (11). Doxorubicin was loaded into liposomes using the ammonium sulfate loading method (19). Targeted liposomes, composed of HSPC/cholesterol/mPEG-DSPE/maleimide-derivatized PEG-DSPE at a 2:1:0.9:0.02 molar ratio were prepared by a direct coupling technique. Anti-CD19 mAb was coupled to the terminus of the maleimide-derivatized PEG-DSPE at 2.0001 (lipid/protein) molar ratio using the coupling procedure described previously (14). Briefly, mAb (10 mg/mL) was incubated with 2-iminoethanol in O2-free HEPES-buffered saline (pH 8.0) at a ratio of 20:1 mol/mol for 1 hour at room temperature to thiolate the amino groups. At the end of the incubation, the sample was chromatographed on a Sephadex G-50 column, equilibrated with O2-free HEPES-buffered saline (pH 7.4), and immediately incubated with liposomes in an O2-free environment overnight with continuous stirring. To reduce loss of contents during coupling, targeted formulations of liposomes containing DOPC, POPC, DPPC, or PMPC (i.e., those with higher doxorubicin release rates) were prepared using the postinsertion method (20). All liposomes were radiolabeled with [14C]CHE as a lipid tracer. To assess coupling efficiency of the antibodies, a trace amount of [14C]doxorubicin was added to the unlabeled antibody before thiolation (11). For animal experiments, liposomes were exchanged into pyrogen-free HEPES-buffered saline by chromatography down Sephadex CL-4B columns and were sterile filtered before injection. Sephadex CL-4B column chromatography also served to remove any uncoupled mAb from the liposomes.

Antibody-coupled micelles for the postinsertion method were prepared from maleimide-derivatized PEG-DSPE and mPEG-DSPE at a 4:1 molar ratio. The dried lipid films were hydrated immediately before coupling to a concentration of 10 mmol/L in deoxygenated 25 mmol/L HEPES (pH 7.4) by heating in a 65°C water bath. Antibodies were coupled to the PEG termini of micelles as described previously (18). After overnight coupling, the micelles were incubated with preformed liposomes at a molar ratio of 0.05:1 (micelle lipid/liposomal lipid) for 1 hour at 60°C. Following transfer, the liposome/micelle mixture was cooled and chromatographed over a Sepharose CL-4B column and equilibrated with pyrogen-free HEPES-buffered saline (pH 7.4) to separate the immunoliposomes from PEG micelles and free antibody.

Leakage assays, pharmacokinetics, and biodistribution. The release rate of doxorubicin from the various liposomal formulations was determined in vitro in 50% ABS. Liposomes (0.5 mmol/L) containing trace amounts of [14C]doxorubicin were diluted in ABS (1:1 by volume), loaded in a dialysis cassette (M, cutoff of 10,000), and dialyzed against 50% ABS in HEPES-buffered saline at 37°C. At different time points, aliquots were counted for [14C]doxorubicin. Results are expressed as t1/2 (time in which 50% of drug leak out from liposomes).

For determining in vivo leakage rates and for pharmacokinetic and biodistribution experiments, liposomes were prepared using [3H]CHE as a lipid label and [14C]doxorubicin as a drug label. Naive SCID mice (three mice per time point) were injected with liposomal formulations of doxorubicin at 3 mg/kg doxorubicin, the same drug dose used in the therapeutic studies. At selected time points, mice were euthanized by cervical dislocation. Whole blood (100 μL) was collected via cardiac puncture with a heparinized syringe. The liver, kidney, spleen, and heart were dissected out, weighed, and then dissolved in Solvable (1.0 mL). For blood, an equal volume of Solvable (1.0 mL) was added to the whole blood. The tissues were then digested for 3 hours at 50°C, with the exception of blood, which was digested for 1 hour. After the vials had cooled to room temperature, 50 μL of 200 mmol/L EDTA were added and the samples were bleached with 1,200 μL of 30% (v/v)
Results

In vitro and in vivo doxorubicin release characteristics, pharmacokinetics, and biodistribution. Studies evaluating in vitro drug release, by dialysis at 37°C against 50% ABS, from the various formulations of liposomal doxorubicin are shown in Table 1. Liposomes prepared from phosphatidylcholines with unsaturated or shorter fatty acyl chains exhibited increased drug leakage rates (decreased $t_{1/2}$) compared with liposomes with longer chain, saturated fatty acyl chains. $t_{1/2}$s in vitro ranged from 11.2 to 85.5 hours with the rank order HSPC > DPPC > PMPC > POPC > DOPC.

In vivo pharmacokinetics and biodistribution for the various liposome formulations were determined in naive SCID mice as a function of time after injection of 3 mg/kg doxorubicin/kg mouse weight in the form of liposomal doxorubicin (10.4-13.6 mg/kg phospholipid). Radiolabeling of both the drug ([14C]doxorubicin) and the liposomal lipid ([3H]CHE) also allowed in vivo doxorubicin release rates to be determined from the ratios of drug/lipid at each time point, normalized to 1.0 at time 0 (Fig. 1). Clearance of lipid from blood was similar for all formulations, whereas clearance of doxorubicin from blood increased with increased rates of doxorubicin release, with doxorubicin in DOPC liposomes having the most rapid clearance (Fig. 2). The $t_{1/2}$S for doxorubicin release in vivo, calculated from the normalized lipid/drug ratios, ranged from 1.9 to 315 hours (Table 1) and, with the exception of DOPC-containing liposomes, were slower than the in vitro release rates. The rank order of release was also similar to the in vitro results, again with the exception of DOPC-containing liposomes. The $t_{1/2}$S for the clearance of liposomal lipid from blood were similar for all of the formulations, ranging from 15.7 to 20.6 hours, with a tendency for slightly faster clearance rates for liposomes that were composed of unsaturated phospholipids (e.g., DOPC; Table 1). The $t_{1/2}$S for doxorubicin clearance from blood ranged from 7.6 to 19.2 hours and had the same rank order as the in vivo $t_{1/2}$S for drug release. The in vivo results show that

<table>
<thead>
<tr>
<th>Table 1. Pharmacokinetic variables of doxorubicin liposomes</th>
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<tbody>
<tr>
<td>Phospholipid component in doxorubicin-loaded liposomes</td>
</tr>
<tr>
<td>HSPC</td>
</tr>
<tr>
<td>Doxorubicin release in vitro ($t_{1/2}$, h)</td>
</tr>
<tr>
<td>Doxorubicin release in vivo ($t_{1/2}$, h)</td>
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<tr>
<td>Phospholipid clearance ($t_{1/2}$, h)</td>
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<tr>
<td>Doxorubicin clearance ($t_{1/2}$, h)</td>
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<tr>
<td>Phospholipid AUC (blood, mg h/kg)</td>
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<tr>
<td>Doxorubicin AUC (blood, mg h/kg)</td>
</tr>
<tr>
<td>Phospholipid AUC (heart, mg h/kg)</td>
</tr>
</tbody>
</table>

NOTE: Doxorubicin liposomes composed of phosphatidylcholine/cholesterol/mPEG-DSP (2:1:0.1), where phosphatidylcholine was HSPC, DOPC, POPC, PMPC, or DPPC, were radiolabeled with [3H]CHE and loaded with [14C]doxorubicin. In vitro doxorubicin release rates were determined in ABS. In vivo release rates were determined from the lipid/drug ratios after injecting naive SCID mice (three mice per time point) with 3 mg/kg liposomal doxorubicin. At selected time points, mice were euthanized, whole blood or tissues were analyzed for radioactivity, and in vitro leakage and pharmacokinetic variables were calculated.

H$_2$O$_2$. Ultima Gold (5 mL) was added to the samples and they were left overnight in the dark at room temperature. The samples were counted in a Beckman LS 6500 liquid scintillation counter for $^3$H and $^{14}$C counts. Results are expressed as percentage of injected doxorubicin or lipid concentration present in blood or organs at each time point. To provide an estimate of drug leakage in vivo, the ratio of $[^{14}]$C:doxorubicin drug counts to $[^{1}]$H:CHE lipid counts was normalized to 1 at time 0. The rate of decrease of the drug/lipid ratio at subsequent time points indicates the rate of release of the drug from the liposomes. Pharmacokinetic variables for blood were calculated in WinNonLin version 4.1 (Pharsight, Cary, NC) using model 201 (i.v. bolus input) for blood and model 200 (extravascular input) for the heart.

In vivo survival experiments. Namalwa cells ($5 \times 10^6$) in 0.2 mL PBS were injected i.v. in the tail vein of SCID mice (8-18 mice per group). Treatments were given at 24 hours after tumor inoculation as a single bolus i.v. dose of 3 mg/kg doxorubicin (20 µmol/kg phospholipid; 40-60 µg CD19/µmol phospholipid) as free doxorubicin, untargeted doxorubicin liposomes, or anti-CD19 doxorubicin liposomes. Mice were monitored daily and euthanized when they lost weight and looked scruffy or when they developed hind leg paralysis.

Fig. 1. Drug/lipid ratios in mice injected with dual-labeled liposomes. Doxorubicin-loaded liposomes composed of phosphatidylcholine/cholesterol/mPEG-DSP (2:1:0.1), where the phosphatidylcholine component was HSPC, DPPC, PMPC, POPC, or DOPC, were radiolabeled with $[^{3}]$H:CHE and $[^{14}]$C:doxorubicin. Naive SCID mice (three mice per time point) received tail vein injections of 3 mg/kg doxorubicin and 10.4 to 13.6 mg/kg phospholipid. At selected time points, mice were euthanized, whole blood was analyzed for radioactivity, and the percentage of injected phospholipid and doxorubicin in blood was determined ($n = 3$). Drug/lipid ratios were normalized to 1.0 at the time of injection and the data were used to calculate the in vivo doxorubicin release rates (Table 1).

Fig. 2. Doxorubicin release in vivo, calculated from the normalized lipid/drug ratios, ranged from 1.9 to 315 hours (Table 1) and, with the exception of DOPC-containing liposomes, were slower than the in vitro release rates. The rank order of release was also similar to the in vitro results, again with the exception of DOPC-containing liposomes. The $t_{1/2}$S for the clearance of liposomal lipid from blood were similar for all of the formulations, ranging from 15.7 to 20.6 hours, with a tendency for slightly faster clearance rates for liposomes that were composed of unsaturated phospholipids (e.g., DOPC; Table 1). The $t_{1/2}$S for doxorubicin clearance from blood ranged from 7.6 to 19.2 hours and had the same rank order as the in vivo $t_{1/2}$S for drug release. The in vivo results show that
when the liposomes have higher release rates drug-depleted liposomes are in the circulation.

The AUC<sub>0-48 h</sub> varied very little for the lipid component of the various formulations (182-318 mg h/kg), with the lowest AUC<sub>0-48 h</sub> found in liposomes composed of DOPC. The plasma AUC for the drug component decreased as the release rate of doxorubicin from the formulations increased (Table 1). HSPC liposomes had the highest doxorubicin AUC (60 mg h/kg), whereas DOPC liposomes had the lowest AUC (19.5 mg h/kg).

The biodistribution of liposomes in naive SCID mice showed that the concentration of lipid in the liver was very similar for all formulations and reached maximal levels at 24 hours for all formulations, except DOPC, which was still increasing at 48 hours (Fig. 3). HSPC liposomes had the highest doxorubicin AUC (60 mg h/kg), whereas DOPC liposomes had the lowest AUC (19.5 mg h/kg).

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liposomes, so in this study mice receiving this treatment were carefully observed (9). The premature deaths in the PMPC and DPPC groups were not related to lipid toxicity because drug-free formulations of PMPC and DPPC liposomes showed no toxicity, nor did empty liposomes given together with free doxorubicin cause toxicity in the mice (Table 2).

Nontargeted formulations containing HSPC, DOPC, and POPC resulted in increased life spans that were not different than free doxorubicin. When formulations containing these phosphatidylcholines were targeted with anti-CD19 mAbs, they all resulted in a significant increase in life span \((P < 0.01)\) for DOPC and \(P < 0.0001\) for POPC and HSPC. No signs of toxicity were observed in mice receiving targeted POPC-containing formulations. For the nontargeted DPPC formulation, which caused premature deaths due to toxicity, targeting with anti-CD19 increased the survival of mice to levels that were significantly higher than free doxorubicin \((P < 0.0001)\) and no weight loss was observed. The life span increased by 3 days in mice receiving targeted versus nontargeted PMPC-containing liposomes \((P < 0.0001)\). Therefore, anti-CD19 targeting decreased the toxicity of the formulations in the order POPC > DPPC > PMPC. Anti-CD19-targeted HSPC liposomes with the slowest doxorubicin release rates resulted in the greatest increase life span \((\text{ILS})\), whereas anti-CD19-targeted DOPC liposomes with the fastest release rates resulted in the smallest \(\text{ILS}\). The \(\text{ILS}\) for targeted formulations of POPC and DPPC was similar and intermediate between the \(\text{ILS}\) for HSPC and DOPC.

### Discussion

Toxic side effects of doxorubicin include acute and chronic cardiotoxicity, myelosuppression, and gastrointestinal toxicities (21, 22). Commercial liposomal drug delivery systems, such as Doxil/Caelyx and Myocet, have improved the side effect profile of doxorubicin, particularly by reducing its acute and chronic cardiotoxicity (7, 23). Interestingly, although the doxorubicin release profile of Doxil/Caelyx and Myocet are very different, with Doxil/Caelyx having very slow release rates \((\text{in vivo})\) and Myocet fast drug release rates (24), both formulations moderate the cardiotoxicity of doxorubicin compared with conventional doxorubicin, with Doxil/Caelyx appearing to have an advantage over Myocet (25).

### Table 2. Survival of SCID mice bearing B-cell lymphoma after treatment with various formulations of targeted versus nontargeted liposomal doxorubicin

<table>
<thead>
<tr>
<th>Formulation</th>
<th>No. animals</th>
<th>Mean survival time (d)</th>
<th>ILS (%)</th>
<th>Long-term survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>18</td>
<td>26 ± 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Free doxorubicin</td>
<td>18</td>
<td>29 ± 2</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>Anti-CD19-PMPC[doxorubicin]</td>
<td>13</td>
<td>10 ± 1</td>
<td>—62</td>
<td>—</td>
</tr>
<tr>
<td>PMPC[doxorubicin]</td>
<td>13</td>
<td>7 ± 0</td>
<td>—73</td>
<td>—</td>
</tr>
<tr>
<td>Anti-CD19-DPPC[doxorubicin]</td>
<td>10</td>
<td>38 ± 6</td>
<td>46</td>
<td>—</td>
</tr>
<tr>
<td>DPPC[doxorubicin]</td>
<td>13</td>
<td>9 ± 1</td>
<td>—65</td>
<td>—</td>
</tr>
<tr>
<td>Anti-CD19-POPC[doxorubicin]</td>
<td>10</td>
<td>29 ± 3</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>Anti-CD19-DOPC[doxorubicin]</td>
<td>10</td>
<td>31 ± 3</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>DOPC[doxorubicin]</td>
<td>10</td>
<td>28 ± 2</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>Anti-CD19-HSPC[doxorubicin]</td>
<td>18</td>
<td>47 ± 15</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>HSPC[doxorubicin]</td>
<td>18</td>
<td>29 ± 3</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>PMPC (empty)</td>
<td>8</td>
<td>26 ± 0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>DPPC (empty)</td>
<td>8</td>
<td>27 ± 2</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>DPPC (empty) + free doxorubicin</td>
<td>8</td>
<td>27 ± 2</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>DPPC (empty) + free doxorubicin</td>
<td>8</td>
<td>25 ± 1</td>
<td>—4</td>
<td>—</td>
</tr>
</tbody>
</table>

**NOTE:** SCID mice (two to three replicate groups of six to eight mice per group) were injected i.v. with \(5 \times 10^6\) Namalwa cells in 0.2 mL PBS, and 24 hours post-inoculation, the animals were injected with the following treatments: saline (control); free doxorubicin; nontargeted liposomal doxorubicin composed of phosphatidylcholine/cholesterol/mPEG-DSPE (2:1:0.1), where the phosphatidylcholine component was HSPC, DPPC, PMPC, POPC, or DOPC; or anti-CD19-targeted liposomal doxorubicin composed of phosphatidylcholine/cholesterol/mPEG-DSPE/maleimide-derivatized PEG-DSPE (2:1:0.08:0.02), where the phosphatidylcholine component was the same as above. The concentration of anti-CD19 on the liposomes was between 54 and 78 A\(^2\)mAb/A\(^g\)mol phospholipid. All doxorubicin formulations were given at a doxorubicin dose of 3 mg/kg. The mean survival time, \(\text{ILS}\), and long-term survivors \((\geq 90\text{ days})\) were calculated.
We and others have observed that alterations in the composition of liposomal formulations of doxorubicin can alter the LD\textsubscript{50} of the formulations in mice (9, 10). Mayer et al. were the first to observe that the stability of liposomal doxorubicin in the circulation is an important factor in the toxicity of this drug in liposomal form. Although they did not report the half-lives for release of doxorubicin in their formulations, a correlation existed between the percentage retention of doxorubicin after 24 hours and the LD\textsubscript{50} of the formulations; formulations with more rapid release rates were more toxic. In these experiments, the formulations with the most rapid release rate, composed of egg phosphatidylcholine, was also the most toxic.

In our experiments, the rapid weight loss in the affected mice and the rapid time course of the toxicity suggest that the toxicity rather than cardiotoxicity is likely gastrointestinal. Indeed, the heart AUCs for doxorubicin (Table 1) and the doxorubicin levels as a function of time in heart (Fig. 4) do not show high heart doxorubicin levels and do not show substantial differences between the different liposomal formulations. From Table 2, it is clear that the toxicity is not mediated by the lipids in the formulations, because empty liposomes (no drug) had no toxicity in the mice. We can speculate that the intermediate doxorubicin release rates for DPPC and POPC lead to delivery of doxorubicin to gastrointestinal cells at a rate, and over a period of time, that corresponds, for example, to the turnover time for these cells. Further experimentation, focusing on determining bioavailable drug concentrations, will be needed to establish the exact mechanism for toxicity for the DPPC and POPC formulations.

Anti-CD19-targeted liposomal doxorubicin binds rapidly to target cells \textit{in vitro} and this triggers receptor-mediated internalization of the liposome-drug package into the B cells (14). Therefore, \textit{in vitro} cytotoxicity of the targeted liposomes should be somewhat independent of the drug release rate. The \textit{in vivo} situation is more complicated. At the time of treatment of the mice in our \textit{in vivo} experiments (i.e., 24 hours postinoculation), most of the B cells have left the circulation (11), so several hours will be required for the anti-CD19 liposomal doxorubicin to find and bind to the target cells. During this time, the more rapid release formulations will have lost some of their contents and drug-depleted liposomes will be internalized into the target cells after binding, which would, we hypothesize, lower the therapeutic effect for these liposomes. This hypothesis is supported by the \textit{in vivo} survival data in Table 2. HSPC-containing liposomes, which have the slowest doxorubicin release rates \textit{in vivo} (315 hours) resulted in the greatest ILS (88% ILS), with one long-term survivor. The more leaky the formulation, the less the ILS relative to HSPC-containing liposomes. The most dramatic effect was observed in DOPC-containing liposomes, where anti-CD19-mediated targeting (68 \textmu g mAb/\textmu mol phospholipid) completely eliminated the mortality seen in the nontargeted formulations. It is possible that the relatively low \textit{in vivo} release rates for doxorubicin from this formulation (125 hours) resulted in most of the drug being retained in the liposomes at the time they bound to and were internalized by the B cells.

This study shows that liposome composition, mechanism of drug uptake, and drug release rates are all important for the resulting toxicities and therapeutic effects of liposomal drugs. A wide range of therapeutic indices could be seen for various phosphatidylcholine/cholesterol liposomal formulations of doxorubicin depending on the drug release rates and whether the liposomes were targeted to internalizing antigens. Studies such as these can aid in the formulation of less toxic and more effective drug carriers.

Acknowledgments

We thank Elaine Moase for editorial assistance and helpful discussions.
References


Anti-CD19-Targeted Liposomal Doxorubicin Improves the Therapeutic Efficacy in Murine B-Cell Lymphoma and Ameliorates the Toxicity of Liposomes with Varying Drug Release Rates

Theresa M. Allen, Davis R. Mumbengegwi and Gregory J.R. Charrois


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