Improved Outcome When B-Cell Lymphoma Is Treated with Combinations of Immunoliposomal Anticancer Drugs Targeted to Both the CD19 and CD20 Epitopes

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ABSTRACT

Purpose: **We have reported previously that successful immunoliposomal drug therapy with liposomal doxorubicin (DXR) against xenograft B-lymphoma models required targeting against an internalizing B-cell antigen, CD19 (P. Sapra and T. M. Allen. Cancer Res 2002;62:7190–4.). Here we compare targeting of immunoliposomal formulations of DXR with vincristine (VCR) targeted against CD19 *versus* a noninternalizing (CD20) epitope. We also examine the effect of targeting immunoliposomes with antibody combinations in an attempt to increase the total number of binding sites (apparent antigen density) at the target cell surface.**

Experimental Design: **Cell association of immunoliposomes (CD19-targeted, CD20-targeted, or combinations of the two) with human B-cell lymphoma (Namalwa) cells were studied using radiolabeled liposomes. Therapeutic efficacy of the same formulations was determined in a severe combined immunodeficient murine model.**

Results: **Therapeutic results in severe combined immunodeficient mice bearing Namalwa cells administered anti-CD20-targeted liposomal DXR were barely improved over those found for nontargeted liposomal DXR or free DXR but, surprisingly, administration of anti-CD20-targeted liposomal VCR resulted in a significantly improved therapeutic outcome compared with nontargeted liposomal VCR, free VCR, or anti-CD20-targeted liposomal DXR.**

Treatment of murine B lymphoma with single injections of combinations of anti-CD19- and anti-CD20-targeted liposomal VCR led to cures in 70% of mice. However, mice injected with similar combinations of liposomal DXR did not have improved survival rates over anti-CD19-targeted liposomal DXR by itself.

Conclusions: **The success of immunoliposomal therapy in combination regimens varies with the type of encapsulated drug and the nature of the target epitopes.**

INTRODUCTION

Ligand-mediated targeting of liposomal anticancer drugs to antigens that are either expressed selectively, or are overexpressed, on the surface of tumor cells is increasingly being recognized as an effective strategy for increasing the therapeutic effectiveness of anticancer drugs (1–7). However, low antigen density and/or heterogeneous expression of the target antigen at the cell surface may limit the effectiveness of targeted therapeutics (7–10).

Evidence has been presented that high antigen density on target cells improves the therapeutic effectiveness of targeted, relative to nontargeted, liposomal drugs by increasing the amount of drug delivered to the target cells (5). In addition, for antibody-targeted liposomal (immunoliposomal) drugs, antibodies are presented in a multivalent fashion at the liposome surface, and high antigen densities may facilitate the simultaneous engagement of multiple antigenic sites, which can initiate signal transduction mechanisms and lead to increased cell killing. Because increasing the density of a single population of receptors at the cell surface is impractical for *in vivo* applications, a possible approach to increasing the total targeted population of receptors at the cell surface would be to use a mixture of immunoliposomal drugs, with the immunoliposomes in the mixture being targeted against different populations of receptors. We hypothesize that combinations of immunoliposomal drugs, targeted against different populations of cell surface antigens, will lead to a higher apparent antigen density on the target cells, the delivery of more drug to the cells, and greater cell killing.

Antibody-based therapies, including immunoliposomes, target and kill individual cells that express specific cell-surface antigens; tumor cells that have little or no target antigen may be killed by a bystander effect or may escape cytotoxic effects (7). The antigen-negative cells can result in emergence of resistant cell populations and disease relapse (11). Using mixtures of immunoliposomes should facilitate delivery of drugs to a higher percentage of tumor cells in populations of cells that have heterogeneous expression of receptors. Several studies have shown significantly improved therapeutic responses when using combinations of immunotoxins compared with single immunotoxin therapy in animal cancer models (12–17).

Immunoliposomal therapy has, to date, only targeted individual antigens on tumor cells surfaces; combination immunoliposomal therapy has yet to evaluated. In this study, we examined the therapeutic efficacy, in xenograft models of human B lymphoma, of combination regimens of immunoliposomes, loaded with either doxorubicin (DXR) or vincristine (VCR), targeted to two different epitopes, CD19 (internalizing) and

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CD20 (noninternalizing or very slow internalizing; Refs. 1, 18, 19). We show that this strategy resulted in an improved therapeutic outcome for VCR-loaded liposomes relative to DXR-loaded liposomes.

Internalization of the liposome-drug package is generally accepted to be a requirement for successful immunoliposomal drug therapy (1, 20, 21). It has been shown recently that DXR-loaded immunoliposomes required internalization into Namalwa cells to improve the therapeutic responses over those obtained with nontargeted liposomes (1). Here we report, in the same animal model, the unexpected finding that mice injected with VCR-loaded immunoliposomes targeted via anti-CD20 (Rituxan; mouse:human chimeric IgG_1 anti-CD20 antibody; αCD20), had significantly increased survival rates relative to nontargeted liposomes. Indeed, survival rates for αCD20-targeted immunoliposomal VCR were comparable with those found in mice injected with anti-CD19 (anti-CD19 murine monoclonal antibody IgG_{2a}; αCD19)-targeted immunoliposomal VCR.

MATERIALS AND METHODS

Materials. Egg sphingomyelin (SM) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). Hydrogenated soy phosphatidylcholine (HSPC) and methoxypolyethylene glycol (molecular weight 2000), covalently linked via a carbamate bond to distearoylphosphatidylethanolamine (mPEG-DSPE; Ref. 22), were generous gifts from ALZA Pharmaceuticals, Inc. (Mountain View, CA). Maleimide-derivatized PEG_{2000}-DSPE (Mal-PEG-DSPE) was custom synthesized by Shearwater Polymers, Inc. (Huntsville, AL), according to a protocol described previously (23). Nuclepore polycarbonate membranes (pore sizes: 0.2, 0.1, and 0.08 μm) were purchased from Northern Lipids (Vancouver, British Columbia, Canada). 2-Iminothiolane (Traut's reagent) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 (without phenol red), penicillin-streptomycin, and fetal bovine serum were obtained from Life Technologies, Inc. (Burlington, Ontario, Canada). Bio-Rad Protein Assay Reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Sephadex G-50, Sepharose CL-4B, and aqueous counting scintillant were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). Cholesterol-[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 Mandel Scientific (Mississauga, Ontario, Canada). Centrisart concentrators (molecular weight cutoff of 100,000) were obtained from Sartorius (Goettingen, Germany). All of the other chemicals were of analytical grade purity.

Animals, Antibodies, and Cell Lines. Six-to-8-week-old female CB17 severe compromised immunodeficient 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. All of the experiments were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta.

The murine monoclonal antibody (mAb) αCD19 (IgG2a) was produced from the FMC63 murine hybridoma (24) and purified as described previously (25). Rituxan, a chimeric whole mAb (IgG1) used as a source of αCD20, was purchased from the University of Alberta Hospital Pharmacy. The human Burkitt's lymphoma cell line, Namalwa (American Type Culture Collection CRL 1432), was purchased from American Type Culture Collection (Rockville, MD) and cultured in suspension in a humidified 37°C incubator with a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml). Only cells in the exponential phase of cell growth were used for experiments.

Preparation of Liposomes. Nontargeted Stealth liposomes (SL), to be loaded with DXR for therapeutic studies or radiolabeled with [^{3}H]CHE for cell association studies, were composed of HSPC:Chol:mPEG-DSPE at a 2:1:0.1 molar ratio (HSPC-SL) and targeted Stealth immunoliposomes (SIL) were composed of HSPC:Chol:mPEG-DSPE:Mal-PEG-DSPE at a 2:1:0.08:0.02 molar ratio (HSPC-SIL). Liposomes were prepared by hydration of thin films, as described previously, and were extruded to mean diameters in the range of 100 ± 10 nm (2). DXR was loaded into liposomes using the ammonium sulfate loading method (26).

Nontargeted liposomes, to be loaded with VCR for therapeutic studies or radiolabeled with [^{3}H]CHE for cell association studies, were composed of SM:Chol:mPEG-DSPE at a 55:40:5 molar ratio (SM-SL), and targeted liposomes were composed of SM:Chol:mPEG-DSPE:Mal-PEG-DSPE at a 55:40:4:1 molar ratio (SM-SIL). Liposomes were prepared by hydration of a lipid film in 300 mM citrate buffer (pH 4.0) as described previously (27) and were extruded to a mean diameter of 120 ± 10 nm. VCR was loaded using the transmembrane pH gradient-dependent procedure (28).

Liposomes for the *in vitro* cell association studies were radiolabeled with [^{3}H]CHE], a nonmetabolizable, nonexchangeable radioactive tracer, and were prepared by hydrating the lipid films in HEPES-buffered saline [25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid and 140 mM NaCl (pH 7.4)] followed by extrusion, as above.

Coupling of mAbs to Liposomes. αCD19 or αCD20 mAbs were coupled to the terminus of the Mal-PEG-DSPE included in HSPC-SIL or SM-SIL at 2000:1 (lipid:protein) molar ratios, using a coupling procedure described previously (23). Briefly, mAb (10 mg/ml) was incubated with 2-iminothiolane in O_2-free HEPES-buffered saline (pH 8.0) at a ratio of 20:1 (mol/mol) for 1 h at room temperature to thiolate the amino groups. At the end of the incubation, the excess reagent was removed by chromatography on a Sephadex G-50 column, equilibrated with O_2-free HEPES-buffered saline (pH 7.4), and the thiolated mAb was immediately incubated overnight with liposomes in an O_2-free environment with continuous stirring. The SIL were separated from any uncoupled mAb over a Sephadex CL-4B column equilibrated with HEPES-buffered saline (pH 7.4). To assess coupling efficiency of the mAbs, a trace amount of ^{125}I-labeled αCD19 or αCD20 was added to the unlabeled mAb before thiolation. Antibody coupling is expressed as μg mAb/μmol phospholipid (PL). A coupling efficiency of 80–90% for either antibody could routinely be achieved by this procedure, and particular attention was taken to

Table 1 Survival times of SCID mice after immunoliposomal DXR[a] treatments

SCID mice (5–7/group) were injected i.v. with 5 million Namalwa cells in 0.2 ml PBS. After 24 h they were injected i.v. in the tail vein with a single bolus dose of 3 mg/kg as free DXR or liposomal DXR. Liposomes were composed of HSPC:Chol:mPEG-DSPE (2:1:0.1) or HSPC:Chol:mPEG-DSPE:Mal-PEG-DSPE (2:1:0.08:0.02). DXR-loaded liposomes targeted with mAbs had 76 μg αCD19/μmol PL (40 molecules of αCD19/liposome) or 70 μg αCD20/μmol PL (37 molecules of αCD20/liposome), respectively. Free mAbs were dosed at 15 μg of αCD19 or 13 μg of αCD20 per mouse, which corresponds to the total amount of mAb received on immunoliposomes. Empty liposomes had 36 molecules of αCD19/liposome or 33 molecules of αCD20/liposome. The combination groups of free mAbs, empty or DXR-loaded liposomes had both components in equal parts. The combination of DXR-loaded αCD19 and αCD20-targeted liposomes was dosed at a total DXR dose of 3 mg/kg, and a total dose of 14 μg free mAbs per mouse.

Group	Mean survival time ± SD (days)	Percentage increase life span	Long-term survivors (>150 days)
Control (saline)	27.6 ± 0.5		0/5
free DXR	31.3 ± 2.7	13	0/5
DXR-HSPC-SL	28.6 ± 1.0	4	0/5
HSPC-SIL (αCD20)	30.5 ± 1.0	11	0/6
HSPC-SIL (αCD19)	31.5 ± 2.4	14	0/6
HSPC-SIL (αCD20) + HSPC-SIL (αCD19)	33.2 ± 2.0	20	0/6
αCD20	34.3 ± 1.1	24	0/6
αCD19	34.8 ± 1.0	26	0/6
αCD20 + αCD19	40.7 ± 2.0	47	0/6
DXR-HSPC-SL + αCD20	33.0 ± 1.3	20	0/6
DXR-HSPC-SL + αCD19	39.5 ± 1.0	43	0/6
DXR-HSPC-SIL (αCD20)	34.3 ± 4.2	24	0/7
DXR-HSPC-SIL (αCD19)	45.7 ± 4.7	66	0/7
DXR-HSPC-SIL (αCD20) + DXR-HSPC-SIL (αCD19)	48.6 ± 4.4	76	0/7

[a] DXR, doxorubicin; HSPC, hydrogenated soy phosphatidylcholine; Chol, cholesterol; mPEG-DSPE, methoxy (polyethylene glycol)$_{2000}$ distearoylphosphatidylethanolamine; Mal-PEG-DSPE, maleimide-derivatized PEG$_{2000}$-DSPE; mAb, monoclonal antibody; PL, phospholipid; SIL, Stealth immunoliposomes; SL, Stealth liposomes.

ensure that similar antibody densities (within ±10%) occurred at the surface of either type of immunoliposome.

Cell Association of Immunoliposomes. *In vitro* cell association of immunoliposomes labeled with [^{3}H]CHE was determined, as described previously, at both 37°C and 4°C, *i.e.*, permissive and nonpermissive temperatures for endocytosis, respectively (29). Briefly, liposomes {SM-SL, HSPC-SL, SIL composed of SM:Chol:mPEG-DSPE:Mal-PEG-DSPE conjugated to αCD19 [SM-SIL(αCD19)], SIL composed of HSPC:Chol:mPEG-DSPE:Mal-PEG-DSPE conjugated to αCD19 [HSPC-SIL(αCD19)], SIL composed of SM:Chol:mPEG-DSPE:Mal-PEG-DSPE conjugated to αCD20 [SM-SIL(αCD20)], or SIL composed of HSPC:Chol:mPEG-DSPE:Mal-PEG-DSPE conjugated to αCD20 [HSPC-SIL(αCD20)]} were radiolabeled with [^{3}H]CHE and incubated in sterile tubes with 1 million Namalwa cells at PL concentrations ranging from 0.1 mM to 1.6 mM PL (in triplicate) for 1 h. For evaluating cell association of combinations of immunoliposomes, HSPC-SIL(αCD19) and HSPC-SIL(αCD20) or SM-SIL(αCD19) and SM-SIL(αCD20) were mixed at a 1:1 ratio before incubation with the Namalwa cells. Cells were then washed twice with cold PBS to remove unbound liposomes, and the amount of [^{3}H]CHE counts associated with cells was determined by scintillation counting (Beckman LS-6800 Scintillation Counter). These data were transformed into pmol PL uptake per 10^6 cells (cell association) using the specific activity of the liposomes. Specific cell association of either SIL[αCD19] or SIL[αCD20] was determined by subtracting cell association for nontargeted liposomes (nonspecific association) from the total cell association of the respective immunoliposome.

***In Vivo* Survival Experiments.** Severe compromised immunodeficient mice (5–7/group) were injected i.v. via the tail vein with 5 million Namalwa cells in 0.2 ml PBS. After 24 h, mice received a single bolus i.v. dose of 3 mg DXR/kg as free DXR or DXR-loaded liposomes [DXR-HSPC-SL, DXR-HSPC-SIL(αCD19), or DXR-HSPC-SIL(αCD20)]. Alternatively, mice were injected with 0.66 mg VCR/kg as free VCR or VCR-loaded liposomes [VCR-SM-SL, VCR-SM-SIL(αCD19), or VCR-SM-SIL(αCD20)]. The antibody density on drug-loaded immunoliposomes was used to calculate appropriate doses of either free mAb or empty (no drug) immunoliposomes that were used as experimental controls. Where combinations of two drug-loaded immunoliposomal formulations, two free mAbs, or two empty immunoliposomal formulations were administered, the total dose was composed of equal parts of each therapy. Mice were monitored daily for up to 150 days and were euthanized when they developed hind leg paralysis. Mice surviving to 150 days were determined to be long-term survivors and were subsequently sacrificed and subjected to gross pathological examination.

Statistical Analysis. Comparisons of cell association and survival studies reported in Table 1 were done using one-way ANOVA with InStat software (Version 3.0; GraphPad, San Diego, CA). The Tukey post-test was used to compare means. Survival studies reported in Table 2 were analyzed using Kaplan-Meier plots with GraphPad Prism software. Differences were considered significant at a $P < 0.05$.

RESULTS AND DISCUSSION

In Vitro Cell Association

In vitro cell association studies were performed on Namalwa cells with either SIL(αCD19) or SIL(αCD20). The term cell association reflects a combination of three processes: (*a*) specific bind-

Table 2 Survival times of SCID mice after immunoliposomal VCR[a] treatments

SCID mice (6–7/group) were injected i.v. with 5 million Namalwa cells in 0.2 ml PBS. After 24 h they were injected i.v. in the tail vein with a single bolus dose of 0.66 mg/kg as free VCR or liposomal VCR. Liposomes were composed of SM:Chol:mPEG-DSPE (55:40:5) or SM:Chol:mPEG-DSPE:Mal-PEG-DSPE (55:40:4:1). Liposomes targeted with mAbs had 66 μg αCD19/μmol PL (34 molecules of αCD19/liposome) or 52 μg αCD20/μmol PL (27 molecules of αCD20/liposome, respectively). The combination group had VCR-SM-SIL (αCD19) and VCR-SM-SIL (αCD20; total dose = 0.66 mg/kg) in equal parts.

Group	Mean survival time ± SD (days)	Percentage increase life span	Long-term survivors (>150 days)
Control (saline)	25.4 ± 0.5		0/6
Free VCR	38.7 ± 4.0	53	0/6
VCR-SM-SL	31.9 ± 3.0	26	0/7
VCR-SM-SL + αCD20	44.7 ± 5.9	76	0/6
VCR-SM-SL + αCD19	62.3 ± 8.8	145	0/6
VCR-SM-SIL (αCD20)	49.0 ± 4.6	93	2/7
VCR-SM-SIL (αCD19)	66.0 ± 13.1	160	3/7
VCR-SM-SIL (αCD20) + VCR-SM-SIL (αCD19)	77.0, 91.0	203, 258	5/7

[a] VCR, vincristine; SM, egg sphingomyelin; Chol, cholesterol; mPEG-DSPE, distearoyl phosphatidyl; Mal-PEG-DSPE, maleimide-derivatized PEG_{2000}-DSPE; mAb, monoclonal antibody; PL, phospholipid; SIL, Stealth immunoliposomes.

ing of SIL to the CD19 or CD20 cell surface antigens; (*b*) internalization (if any) of the SIL into the cell interior and recycling of the antigen back to the cell surface with possible repetition of the process; and (*c*) nonspecific binding of the immunoliposomes to the cell surface.

The levels of cell association of either SIL(αCD19) or SIL(αCD20) with Namalwa cells were significantly higher than seen for nontargeted SLs at either 37°C or 4°C (Fig. 1*A*) at all of the phospholipid concentrations. In addition, the levels of cell association of SIL(αCD19) with cells were significantly higher at 37°C than at 4°C (nonpermissive for endocytosis; Fig. 1*A*). This was likely due to the binding of SIL to Namalwa cells via the pan B-cell differentiation antigen, CD19, followed by receptor-mediated endocytosis and recycling of the epitope back

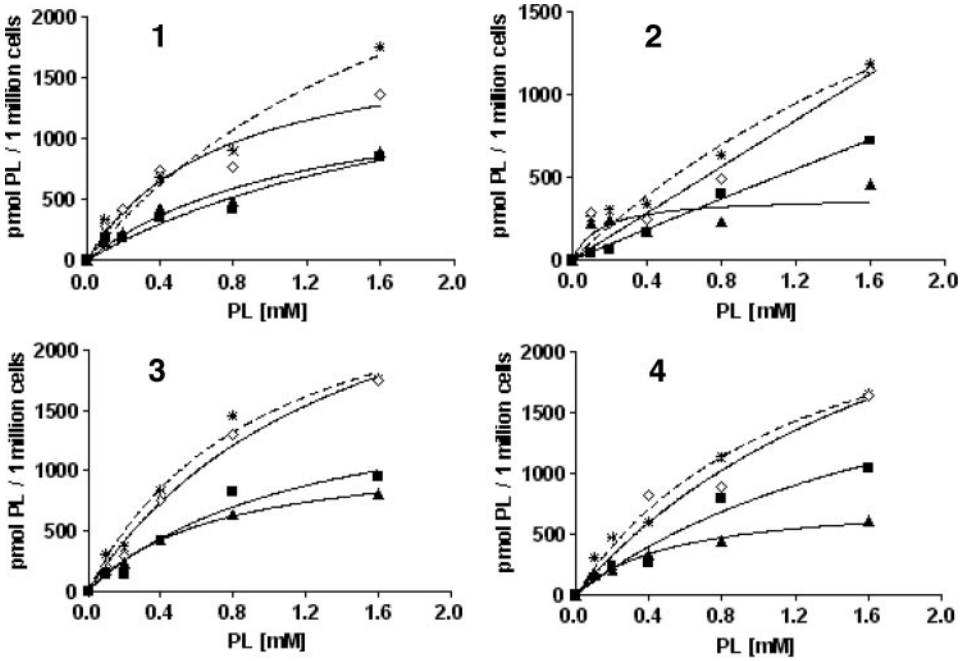

Fig. 1 *In vitro* cell association of liposomes with Namalwa cells as a function of concentration at 37°C (*1* and *3*) or 4°C (*2* and *4*). Nontargeted Stealth liposomes (●); SIL(αCD19) (▲); SIL(αCD20) (■); Targeted combination of SIL(αCD19) + SIL(αCD20) (◇). *1* and *2*, liposomes were composed of Hydrogenated soy phosphatidylcholine (HSPC):cholesterol (Chol):distearoylphosphatidylethanolamine (mPEG-DSPE):maleimide-derivatized PEG_{2000}-DSPE (Mal-PEG-DSPE; 2:1:0.1) or HSPC:Chol:mPEG-DSPE:Mal-PEG-DSPE (2:1:0.08:0.02). *3* and *4*, liposomes were composed of egg sphingomyelin (SM):Chol:mPEG-DSPE (55:40:5) or SM:Chol:mPEG-DSPE:Mal-PEG-DSPE (55:40:4:1). Liposomes were labeled with [^{3}H]CHE and incubated with 1 million Namalwa cells for 1 h after which the cells were washed with cold PBS to remove the unbound liposomes. The concentration of monoclonal antibodies on HSPC-SILs was 64 μg αCD19/μmol phospholipid (PL) or 54 μg αCD20/μmol PL and that on SM-SILs was 59 μg αCD19/μmol PL or 56 μg αCD20/μmol PL. Data are expressed as pmol PL/1 million cells. Each point is an average of three replicates from one representative experiment; *bars*, ± SD.

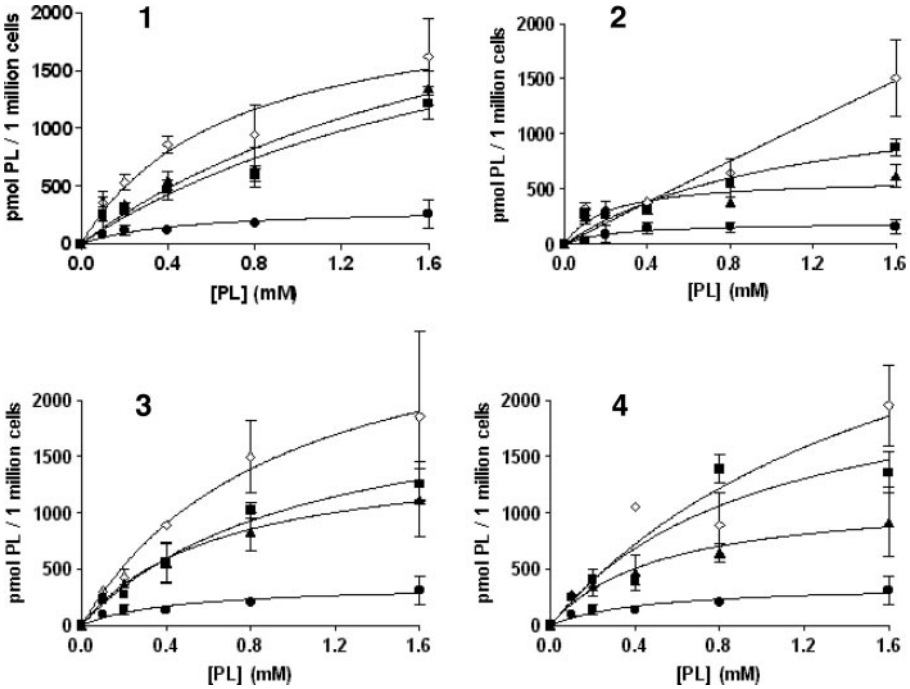

Fig. 2 Specific cell association of immunoliposomes with Namalwa cells as a function of concentration at 37°C (*1* and *3*) or 4°C (*2* and *4*). SIL(αCD19) (▲); SIL(αCD20) (■); Targeted combination of SIL(αCD19) + SIL(αCD20) (◇). *, calculated sum of cell association of SIL(αCD19) and SIL(αCD20). *1* and *2*, liposomes were composed of hydrogenated soy phosphatidylcholine:cholesterol (Chol): distearoylphosphatidylethanolamine (mPEG-DSPE; 2:1:0.1) or hydrogenated soy phosphatidylcholine:Chol: mPEG-DSPE:maleimide-derivatized PEG_{2000}-DSPE (2:1:0.08:0.02). *3* and *4*, liposomes were composed of SM:Chol: mPEG-DSPE (55:40:5) or SM:Chol: mPEG-DSPE:maleimide-derivatized PEG_{2000}-DSPE (55:40:4:1). Data are expressed as pmol phospholipid/1 million cells. Each point is an average of three replicates from one representative experiment; *bars*, ± SD.

to the cell surface where it is available to participate in additional binding and internalization events (2, 18, 30, 31). No significant differences were observed in the levels of cell association of SIL(αCD20) with cells at 37°C *versus* 4°C, which is consistent with its poor ability to internalize immunoliposomes (1). Also, using confocal fluorescence microscopy, we have shown previously that SIL(αCD19) were rapidly internalized into Namalwa cells at 37°C whereas SIL(αCD20) were not internalized at this temperature. At 4°C, both SIL(αCD19) and SIL(αCD20) were largely found on the cell surface consistent with the poor ability of CD20 epitope to internalize (1, 18, 19).

Specific cell association (targeted minus nontargeted) is given in Fig. 1*B*. As expected, the levels of cell association of 1:1 combinations of SIL(αCD19) and SIL(αCD20) were higher than those seen for either of the individual immunoliposome formulations at all of the PL concentrations and appeared to be additive. This was observed for both SM and HSPC liposomes at either 37°C or 4°C (Fig. 2). Only at one concentration (1.6 mM PL), at 37°C, did the cell association of the combination of HSPC-SIL(αCD19) and HSPC-SIL(αCD20) appear to be subadditive.

In Vivo Survival Studies

DXR-Containing Liposomes. The therapeutic effectiveness of DXR-loaded immunoliposomes targeted via either αCD19 or αCD20 was evaluated individually or in combination in severe compromised immunodeficient mice bearing Namalwa cells (Table 1). Mice treated with DXR-containing liposomes targeted via either αCD19 or αCD20 had significantly longer survival times than mice that received no treatment (control; $P < 0.01$). DXR-HSPC-SL or free DXR were not therapeutically better than control groups ($P > 0.05$). These results confirm previous observations that antibody-mediated delivery of liposomal drugs to antigens expressed on the surface of B cells can increase the therapeutic effectiveness of anticancer drugs.

A single injection of free DXR was not an effective treatment, in part because the drug has large volume of distribution, leading to low plasma drug levels. The release rate of DXR from liposomes is slow, so nontargeted liposomal DXR maintains high levels of entrapped (*i.e.*, nonbioavailable) drug in circulation for long periods of time (32). However, the entrapped drug is not delivered efficiently to B cells, because nontargeted liposomes are not bound to and internalized by these cells. This makes it unlikely that therapeutically relevant intracellular concentrations of drug are achieved for the nontargeted liposomes, explaining their lack of significant therapeutic effects.

As seen in Table 1, treatment of tumor-bearing mice with DXR-HSPC-SIL(αCD19) significantly increased their survival relative to DXR-HSPC-SIL(αCD20; $P < 0.001$), DXR-HSPC-SL ($P < 0.001$), or free DXR ($P < 0.001$). No significant difference was observed between mice treated with DXR-HSPC-SIL(αCD20) and free DXR ($P > 0.05$). Mice treated with DXR-HSPC-SIL(αCD20) had survival times that were marginally better than DXR-HSPC-SL ($P < 0.05$).

As reported previously, immunoliposomes targeted via αCD19 are rapidly internalized into Namalwa cells in contrast to immunoliposomes targeted via αCD20, which are not internalized or are internalized very slowly (1). Internalization of the αCD19-targeted formulations is the likely explanation of the improved results seen for DXR-HSPC-SIL(αCD19) relative to DXR-HSPC-SIL(αCD20). Receptor-mediated intracellular delivery of DXR-HSPC-SIL(αCD19) and subsequent release of drugs inside the cells leads to high intracellular drug concentra-

tions. We have published previously evidence that breakdown of the drug-liposome package by lysosomal and endosomal enzymes, followed by release of drug into the cytoplasm, is responsible for cytotoxic effects of DXR-HSPC-SIL(αCD19; Refs. 33–35). In contrast, DXR-HSPC-SIL(αCD20) will bind to the surface of target cells and, over time, release drug to the extracellular medium. The processes of diffusion and redistribution of the drug away from the cell surface *in vivo* competes with entry of the drug into the cell by passive diffusion and leads, we hypothesize, to low intracellular drug concentrations and inferior therapeutic results.

Another explanation for improved results seen with DXR-HSPC-SIL(αCD19) over DXR-HSPC-SIL(αCD20) may lie in the more homogeneous distribution of the CD19 epitope in Namalwa cell populations compared with the CD20 epitope (coefficient of variation for CD20 = 78, CD19 = 45, unstained cells = 44; Ref. 1). A more homogeneous expression of the CD19 epitope on the target cells would lead to a higher overall level of cell killing for the DXR-HSPC-SIL(αCD19) relative to DXR-HSPC-SIL(αCD20), which would not be expected to be effective against cells that had low or no expression of CD20.

In this study, the combination of DXR-HSPC-SIL(αCD19) and DXR-HSPC-SIL(αCD20) did not significantly improve the therapeutic effectiveness over that seen with DXR-HSPC-SIL(αCD19) alone ($P > 0.05$; Table 1). Not surprisingly, because DXR-HSPC-SIL(αCD20) alone had little or no therapeutic effect, this combination was more effective than DXR-HSPC-SIL(αCD20) alone ($P < 0.0005$). Mice injected with a combination of DXR-HSPC-SIL(αCD19) and DXR-HSPC-SIL(αCD19) had significantly improved survival time over mice injected with a combination of free mAbs ($P < 0.005$) or a combination of drug-free immunoliposomes ($P < 0.0005$).

Mice injected with either free αCD19 or αCD20 had significantly longer survival times than untreated controls ($P < 0.01$; Table 1). It was interesting to observe that free αCD19 and free αCD20 given in combination were significantly better than individual mAbs ($P < 0.0001$); this could be due to the higher total binding of the two populations of mAbs on the cell surface, *i.e.,* higher apparent antigen density, possibly leading to increases in signal transduction mechanisms.

Unlike treatments with comparable amounts of free αCD19 or αCD20, injection of mice with drug-free liposomes, conjugated to either αCD19 or αCD20, did not improve the survival times of mice compared with untreated controls (Table 1). Drug-free immunoliposomes, despite the multivalent display of mAbs at the liposome surface, may be less effective than similar concentration of free mAbs, because the orientation of the bound antibodies with respect to the liposome surface might shield the Fc segment and hinder cell killing via mechanisms involving complement-dependent cytotoxicity and/or antibody-dependent cell-mediated cytotoxicity. Alternatively, different cellular processing pathways for the free mAbs *versus* the immunoliposomes may account for the different effects of each.

Free αCD19, but not free αCD20, improved the anticancer effect of nontargeted liposomal DXR (Table 1). The DXR-HSPC-SL + free αCD19 combination was therapeutically more effective than control treatments, free DXR, free αCD19, or DXR-HSPC-SL ($P < 0.001$). However, targeting the liposomes via αCD19 [DXR-HSPC-SIL(αCD19)] was significantly more effective than giving nontargeted liposomes plus free antibody ($P < 0.01$). The combination of DXR-HSPC-SL and free αCD20 was not therapeutically better than control treatments, free DXR, or DXR-HSPC-SL ($P > 0.05$). DXR-HSPC-SIL(αCD20) was also not better than the combination of DXR-HSPC-SL and free αCD20 ($P < 0.05$).

VCR-Containing Liposomes. We performed a separate study to evaluate the therapeutic effects of VCR-containing liposomes conjugated to either αCD19 or αCD20, used individually or in combination (Table 2). An unexpected observation was that treatment with VCR-SM-SIL(αCD20) increased the survival of mice to a significantly greater extent than either VCR-SM-SL or free VCR ($P < 0.005$) and was not significantly different from VCR-SM-SIL(αCD19; $P > 0.05$). In this study, there was a tendency for a higher cure rate in mice treated with a combination of VCR-SM-SIL(αCD19) and VCR-SM-SIL(αCD20), relative to either treatment alone, although this did not reach statistical significance ($P > 0.05$). Five of 7 mice injected with the combination were long-term survivors (>150 days), with no evidence of tumor on gross pathological examination, and the remaining 2 mice had significantly improved life spans. The high cure rate was probably due to an additive effect of the individual therapies (Table 2).

An explanation for the improved therapeutics of VCR-SM-SIL(αCD20), relative to DXR-HSPC-SIL(αCD20), may lie in the faster rate of release from liposomes of VCR relative to DXR (32). Because free VCR has better therapeutic activity than free DXR in this therapeutic model ($P < 0.005$; Table 1 *versus* Table 2), the faster release of VCR from VCR-SM-SIL(αCD20) may lead to higher extracellular concentrations of drug at the cell surface and higher cytotoxic levels of drug being delivered internally to the cells, relative to DXR-HSPC-SIL(αCD20).

As observed for DXR-containing liposomes, VCR-containing liposomes had increased cytotoxic effects when combined with either free αCD19 or αCD20 mAbs (Table 2). Therapy with the combination of nontargeted liposomal VCR and either free αCD20 or free αCD19 was significantly better than controls ($P < 0.005$), therapy with free VCR ($P < 0.05$), or with VCR-SM-SL ($P < 0.005$). Targeting the immunoliposomal VCR via either αCD20 or αCD19 [*i.e.,* VCR-SM-SIL(αCD19) or VCR-SM-SIL(αCD20)] was significantly better than the combination of nontargeted liposomal VCR and free antibodies ($P < 0.01$).

Previous studies have reported that *Vinca* alkaloid-containing immunoconjugates do not require internalization for therapeutic activity (36, 37). The authors proposed that cytotoxicity of these immunoconjugates might be due to the release of free drug from the conjugate at tumor cell periphery, followed by intracellular transport. On the other hand, studies have shown that Adriamycin conjugates linked to internalizing antibodies have significantly improved therapeutics over Adriamycin conjugates linked to noninternalizing antibodies (αCD20; 38), which is in accordance with our previous observations for DXR-containing immunoliposomes (1). In another study, antibody-drug conjugates composed of αCD20 (Rituxan) and the drug monomethyl auristatin demonstrated significantly improved therapeutic outcomes over conjugates made from DXR and Rituxan (39). Monomethyl auristatin is a derivative of

synthetic analog of dolastatin 10, which belongs to the antimitotic class of chemotherapeutics. Clearly, important differences exist between antimitotic drugs and schedule-independent drugs such as DXR in their therapeutic effects in ligand-targeted systems. Anti-mitotic agents such as the *Vinca* alkaloids, the taxanes, and the dolastatins act by interfering with the polymerization and depolymerization of microtubules, an intracellular transport apparatus which facilitates the endocytosis of macromolecules (40). Agents like nocodazole, which promote the disassembly of microtubules, have been reported to increase the endocytosis of transferrin in CV-1 cells; by contrast, paclitaxel, which promotes the assembly of microtubules, had the opposite effect (40). It is possible that the initial entry of the microtubule disassembly agent VCR into the target cells in the free form (after its partial release from liposomes) may subsequently increase the rate of intracellular trafficking of CD20 and promote an increased uptake of liposomal VCR into the cells.

This study demonstrates for the first time that immunoliposomal anticancer drugs used in combination can result in improved therapeutics, when the appropriate antigens, drugs, and drug release rates are chosen. Additional studies, using combinations of immunoliposomal drugs, are, hence, warranted.

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