Preparation and Characterization of Doxorubicin-Loaded Sterically Stabilized Immunoliposomes[1]

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Purpose. To compare the performance of sterically stabilized, doxorubicin-loaded liposomes with and without surface attached specific antibodies (D-SSIL and D-SSL, respectively).

Methods. Small (≤120 nm) unilamellar liposomes were prepared composed of hydrogenated soy phosphatidylcholine, hydrogenated phosphatidylethanolamine (HPE), cholesterol, and ^{2000}Da polyethylene glycol (^{2000}PEG) attached to the primary amino group of distearoyl phosphatidylethanolamine. Doxorubicin was remote-loaded into these liposomes by an ammonium sulfate gradient to form the D-SSL. Monoclonal IgG_3 NI32/2 antibodies directed against a polyoma virus tumor-associated antigen expressed on A9 ctc 102 murine fibrosarcoma cells were attached to the D-SSL HPE via a thioether bond to form the D-SSIL-32/2. A control of nonspecific D-SSIL was prepared by attaching nonspecific IgG_3-enriched immunoglobulins to D-SSL. All liposomes were physically and chemically characterized and then tested *in vitro* for tumor cell binding, specificity, and uptake by macrophages; and *in vivo* for the drug plasma pharmacokinetics after intravenous administration in mice.

Results. *(i)* The attachment of antibodies to D-SSL did not impair their chemical or physical stability and had a minimal effect on their size and level of loaded drug. *(ii)* The combination of specific antibodies and ^{2000}PEG grafted in the liposomes improved the specific binding to relevant target cells by reducing the level of unspecific binding to nonrelevant cells. *(iii)* D-SSIL retained the prolonged circulation and slow clearance typical of SSL lacking the antibodies.

Conclusions. Sterically stabilized immunoliposomes exhibited stability, ability to recognize target cells, and prolonged circulation time. This study also shows that it is feasible to prepare them in pharmaceutically acceptable dosage form. Thus, further investigation for tumor targeting and efficacy is warranted.

KEY WORDS: sterically stabilized immunoliposomes; targeting; doxorubicin; immunospecificity; tumor cells.

INTRODUCTION

Recent development of sterically stabilized liposomes (SSL) (reviewed in 1, 2) renewed interest in systemic application of immunoliposomes, mainly because they have a very low RES uptake and a long circulation time (1, 3–5). In humans, DOX-SL™, which is SSL loaded with doxorubicin (DOX), D-SSL ($t^1/_2$ ~45 h), was shown to reach tumors, where the drug was released and metabolized (4). When these liposomes are <120 nm they readily extravasate into extrahepatic tumors and inflamed sites. Their prolonged circulation time is related to size, combined with high flexibility of the PEG moiety which forms a steric barrier.

Targeted chemotherapy using sterically stabilized immunoliposomes (SSIL) loaded with DOX (D-SSIL) may further improve therapeutic efficacy relative to D-SSL, by increasing the level of active drug in the tumor without elevating its level in sensitive nontumor tissues. It may also increase the intracellular level of the drug in the tumor cells. However, the concept of SSIL seems to be contradictory to immunospecific recognition since vesicle stabilization using PEG, like protein pegylation, can reduce immunological recognition by molecules on the vesicle surface (reviewed in 6, 7). Indeed, the steric barrier of immunoliposomes having PEG of 5,000 Da attached to distearoyl PE (^{5000}PEG-DSPE) prevents an efficient immunological recognition (8). This aspect was recently studied by Blume *et al.* (9) for SSL which have as the targeting device plasminogen molecules attached to the ends of the long PEG chains of the PEG-PE. They demonstrated that such vesicles have both long *in vivo* circulation time and high *in vitro* target binding; however, *in vivo* targeting was not tested.

Successful SSIL should: (a) recognize and specifically bind to their target cells; (b) have long circulation time; (c) deliver active drug/agent into target cells, either through uptake of intact liposomes or by uptake of drug released near target cells; and (d) minimize delivery of drug/agent to nonrelevant tissues.

This paper describes preparation and characterization of SSIL loaded with DOX by means of an $(NH_4)_2SO_4$ gradient (10). *In vitro* tumor cell binding and specificity are also presented, as is *in vivo* plasma pharmacokinetics. Biodistribution of D-SSIL, DOX processing in various organs and in metastatic solid tumors in mice, as well as its antitumor effect, are being reported in a companion publication (11).

MATERIALS AND METHODS

Reagents for Liposome Preparation

^{2000}PEG-DSPE was a gift of SEQUUS, Menlo Park, CA. HPC and HPE, iodine values 3.0, were from Lipoid, Ludwigsha-

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ABBREVIATIONS: MAb, monoclonal antibodies; RES, reticuloendothelial system; SSL, sterically stabilized liposomes; SSIL, sterically stabilized immunoliposomes; D-SSL, doxorubicin-loaded SSL; D-SSIL, doxorubicin-loaded SSIL; DOX, doxorubicin; HPC, hydrogenated soy phosphatidylcholine; Lip, nonstabilized liposomes; D-Lip, doxorubicin-loaded Lip; PE, phosphatidylethanolamine; HPE, hydrogenated soy phosphatidylethanolamine; PEG, polyethylene glycol; PEG-DSPE, *N*-carbamyl-poly-(ethylene glycol methyl ether)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine triethyl ammonium salt; FITC-PE, fluorescein isothiocyanate-phosphatidylethanolamine; DTT, dithiothreitol; PAA, polyoma virus tumor-associated antigen; PL, phospholipid; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; SMPB, *N*-succinimidyl 4-(*p*-maleimidophenyl) butyrate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; MPB-, 4-(*p*-maleimidophenyl) butyryl; PDP-, 3-(2-pyridyldithio) propionyl; 32/2, NI32/2 MAb.

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fen, Germany; cholesterol (lipid purity by TLC > 97% (12), α-tocopherol, α-tocopherol succinate, bovine serum albumin, trinitrobenzene sulfonate, SMPB, SPDP, HEPES buffer, DTT, and Dowex 50WX-4, from Sigma; FITC-PE from Avanti Polar Lipids (Pelham, AL); DOX-HCl, from Farmitalia Carlo Erba (Milan); and Sephadex G-50 and Sepharose CL-6B from Pharmacia. All reagents and solvents were analytical grade or better.

Cell Lines

A9 ctc 102 tumor cells (hereafter, "A9 cells") were used as the specific target cells for binding IgG_3 mouse MAb NI32/2/4 (NI32/2) *in vitro* (13). The A9 murine fibrosarcoma cell line is derived from BALB/c 3T3 cells ($H\text{-}2^d$) transformed *in vitro* by polyoma virus. A9 cells express on their surface a high density of a polyoma virus tumor-associated antigen (PAA) (13). Nontransformed 3T3 cells lacking PAA were used as negative control. Cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES buffer, and antibiotics (Biological Industries, Beit-HaEmek, Israel).

Animals

Specific pathogen-free BALB/c and C57BL/6 female mice, aged 9–12 weeks, were obtained from Harlan Sprague Dawley (Indianapolis, IN).

Preparation of Antibodies

NI32/2 MAb (32/2) were isolated from ascitic fluid of BALB/c mice by precipitation with 50% $(NH_4)_2SO_2$, followed by purification using protein-A Sepharose CL-4B (Zymed, South San Francisco) affinity columns (14). An IgG_3-enriched fraction from serum of normal BALB/c mice, was used as a control of nonrelevant IgG antibodies. Both purified IgG preparations were brought to a concentration of 2 mg/ml. Binding of the two antibodies to A9 and 3T3 cells was quantified by flow cytometry.

Preparation of D-SSL, D-SSIL, and D-Lip

Preparation of D-SSIL (15) is described schematically in Fig. 1. D-SSIL, D-SSL, and D-Lip were characterized for DOX concentration, phospholipid concentration, drug and lipid degradation, and size distribution (12). Loading efficiency exceeded 90%. Vesicles retained >99.0% of the drug for at least 6 months at 4°C. Phospholipid hydrolysis and cholesterol oxidation were below assay detection limits (1% and 0.5%, respectively) (12).

(i) D-Lip

(DOX-loaded nonstabilized liposomes): Lip were prepared from HPC:HPE:cholesterol 55:5:40 (mole ratio), 0.1 mole % α-tocopherol, and 0.1 mole % α-tocopherol succinate. Preparation of Lip and DOX loading are identical to that described below for D-SSL.

(ii) D-SSIL

(a) *Preparation of D-SSL.* D-SSL were prepared from (HPC:HPE): ^{2000}PEG-DSPE:cholesterol at a mole ratio of 55 (HPC + HPE):5:40. Unless otherwise stated, the mole ratio of HPC:HPE was 50:5. Liposomes also contained 0.1 mole % α-tocopherol and 0.1 mole % α-tocopherol succinate. In some preparations, 0.1–0.2 mole % FITC-PE was also included.

Doxorubicin-loaded liposomes were prepared using an $(NH_4)_2SO_4$ gradient (10, 15, 16).

(b) *Preparation of IgG-PDP.* Thiol groups were attached to NI32/2 MAb or control IgG as PDP by 30 min incubation with SPDP (1:15 molar ratio protein to SPDP) (17), at pH 7.4. Unreacted reagent was removed using Sephadex G-50 columns. Antibodies were stored under N_2 at 4°C.

(c) *Preparation of D-SSL-MPB.* Binding of SMPB to HPE of the external surface of the vesicles was carried out immediately before attachment of thiolated antibodies. Generally, D-SSL containing 5 mole % of HPE were used. 64% of HPE was present in the outer surface of these vesicles, as determined by its exposure to trinitrobenzene sulfonate (18). SMPB in tetrahydrofuran was added to the liposomes in HEPES buffer, pH 7.8 (mole ratio SMPB:external HPE 20:1). After 2 h incubation at room temperature, unbound SMPB was removed by Sephadex G-50 gel exclusion chromatography in 0.1 M citrate buffer pH 6.5. No DOX release or lipid degradation occurred during SMPB binding. D-SSL-MPB were used immediately for conjugation with activated PDP-IgG.

(d) *Binding of PDP-IgG to D-SSL-MPB.* PDP-IgG pH was lowered to 5.5 with HCl; then DTT was added in 0.2 M sodium acetate buffer pH 5.5 to a final concentration of 25 mM. The "activated" PDP-antibodies (after removal of DTT) were incubated with the D-SSL-MPB in 0.1 M citrate buffer pH 6.5 not later than 3 h after the reaction of SMPB with D-SSL started. Routinely we used ~250 μg protein per μmole of vesicle PL. Incubation was carried out for 16–20 h with stirring under N_2 at room temperature. Unreacted IgG and other low molecular mass molecules

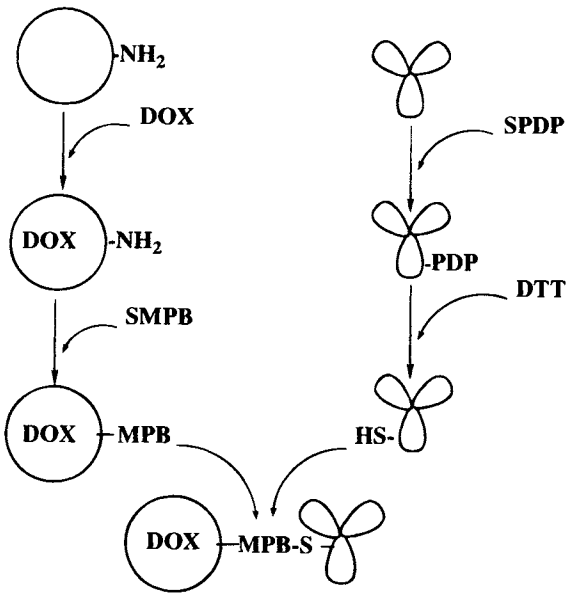

Fig. 1. Schematic flow chart describing the preparation of D-SSIL.

were removed by Sepharose CL-6B gel exclusion chromatography. The liposomal product was characterized as described above for D-SSL. To determine liposome-associated protein, two methanol precipitations were performed to remove the drug from the protein pellet, the pellet was suspended in 1% sodium dodecyl sulfate, and protein was determined by Minamide and Bamburg's method (19). The ratio protein:PL:DOX was identical after the first and second separations on Sepharose CL-6B, indicating negligible free IgG in the liposome fraction. No drug was released during the process (Table I).

Interaction of Fluorescent SSIL with Cells in Culture

Fluorescent SSIL having NI32/2 or irrelevant IgG on their surface were prepared by including in the liposomes 0.1–0.2 mole % FITC-PE. Cultured cells (A9 or 3T3) were detached with trypsin-EDTA, then washed with DMEM containing 5% FCS and 0.05% sodium azide. SSIL or other liposomes (0.25 μmole lipid) were added to 10^6 cells in a final volume of 0.1 ml. After a 40 min incubation on ice with gentle shaking, cells were washed twice in the same medium and analyzed using a Becton Dickinson flow cytometry analyzer.

Identical conditions were used for competition experiments between immunoliposomes and free NI32/2 MAb or control IgG anti H-2^d, except that cells were first incubated with varying amounts of free IgG for 20 min on ice in DMEM containing 5% FCS and 0.05% sodium azide before adding immunoliposomes.

Uptake of Liposomes by Peritoneal Macrophages in Culture

Peritoneal macrophages were collected from ascitic fluid of C57BL/6 mice 4 days after i.p. injection of 1.5 ml sterile 4% thioglycolate (DIFCO, Detroit). The macrophages were seeded for one day in 96-well, flat-bottomed microplates, in DMEM containing 10% FCS. After two washings, DOX-containing liposomes free of nonencapsulated drug (12) (0.1 μmol PL) in 5% FCS-DMEM were added to each well. Microplates were incubated 3 h at 37°C in 5% CO_2, and cell monolayers then washed 3× with phosphate-buffered saline at 37°C to remove unbound liposomes. To each well, 150 μl of acidic isopropanol (pH 2–3 using 0.75 N HCl) was added to extract DOX, and the well contents transferred to Eppendorf centrifuge tubes. This step was repeated. The tubes were centrifuged for 2 min at maximum speed of the Eppendorf centrifuge (model 5415 C), and DOX level determined from fluorescence intensity at 586 nm (excitation 472 nm) (12), measured with a Perkin-Elmer LS-5 spectrofluorometer (10, 12).

Table I. Physical and Chemical Characterization of D-SSL and D-SSIL

	Liposome Formulation		
	D-SSL	D-SSIL-IgG	D-SSIL-32/2
DOX/PL (μg/μmol) ± SD[a]	115 ± 3.5	92 ± 7.1	114 ± 12.9
Protein/PL (μg/μmol) ± SD	—	24.1 ± 1.3	22.3 ± 1.4
Mean diameter (nm) ± SD	79 ± 33	93 ± 41	89 ± 69
Shape of size distribution	Unimodal	Unimodal	Unimodal
Coupling efficiency (%)[b]		12.1 ± 0.65	9.7 ± 0.61
IgG/Liposome ± SD[c]		17 ± 1	15 ± 1

[a] The preparation of the liposomes was repeated 5 times with similar results.

[b] Percent coupling efficiency = amount of protein bound to liposomes/total protein used for binding × 100.

[c] Average number of antibody molecules per liposome. Calculations are based on the following assumptions: liposomes are unilamellar, average area per lipid molecule is 0.5 nm^2, and all liposome-associated protein is IgG.

In Vitro Release of DOX from Liposomes

Increase of fluorescence intensity at 586 nm (excitation 472 nm) was used to follow DOX release from liposomes in which the drug fluorescence is fully quenched (10).

Pharmacokinetic Studies

Mice were injected i.v. (tail vein) with 0.2 ml each of free DOX or preparations of the following liposomes containing 0.35 μmole PL: D-Lip, D-SSL, or D-SSIL-IgG (in sterile, pyrogen-free 10% sucrose in water), each having DOX concentration of 0.175 mg/ml (35 μg/~20-g BALB/c mouse).

At the desired time, 0.5 ml blood was collected from the retroorbital sinus, after ether anesthesia, into test tubes containing K_3-EDTA as anticoagulant, and the mouse was sacrificed. Plasma was separated by centrifugation. Hematocrit (~50%) was similar for all time points tested. Each time point (Fig. 5) = mean ± SD of 3 mice.

The DOX extraction procedure of Cummings and McArdle (20) with the following modification was used: To 0.2 ml plasma, 40 μl of 33% $AgNO_3$ (Sigma) was added to remove tightly bound DOX. Daunorubicin (10 μl of 0.05 mg/ml solution) was added as internal standard. After 10 min at 4°C, 1 ml of 2:1 (v/v) chloroform/isopropanol (both HPLC grade) was added. Samples were vigorously vortexed and then centrifuged at 1000 × g for 10 min. The chloroform-rich lower phase was collected, evaporated to dryness using N_2, and dissolved in 0.1 ml isopropanol immediately before injection into the HPLC system.

DOX was analyzed by HPLC (3, 4). RT of DOX was 4.46 min.

Extraction efficiency and reproducibility were tested by spiking plasma with known amounts of free DOX or various DOX-loaded liposomal preparations. Recoveries were always >80% and could be corrected by using daunorubicin as internal standard (RT, 8 min). Sensitivity limit of this HPLC determination is 0.5 ng per peak, equivalent to a concentration of 6.25 ng/ml plasma (~0.018% of total injected dose).

Mouse blood volume was assumed to be 77.8 ml/kg body weight (21).

Nonlinear least-squares analysis was performed on pharmacokinetic data using Rstrip software (Micromath, Salt Lake City) (4).

RESULTS

D-SSIL Characterization

Attaching IgG or NI32/2 had minimal or no effect on drug encapsulation (Table I); i.e., liposomes remained intact. It caused a slight increase in vesicle size but no immediate vesicle aggregation or fusion. Coupling efficiencies of antibodies to liposomes were similar (12.1 and 9.7% for IgG and NI32/2, respectively), as also expressed in similar levels of protein per μmole PL (24 and 22 μg, respectively) and average number of IgG molecules per liposome (17 and 15, respectively).

Effects of Antibody Attachment on SSIL Interaction with Serum Components

(a) *Liposome leakage.* DOX release kinetics in 50% FCS (inactivated by 30 min incubation at 56°C) in Hanks' balanced salt solution, pH 7.2, at 40°C was studied. Figure 2 shows that D-Lip (150 ± 60 nm) released their drug relatively fast, losing 76% DOX after 4 h, compared with <10% for both D-SSL and D-SSIL-IgG, indicating that covalent attachment of IgG to the D-SSL surface did not destabilize SSL.

The high rate of DOX leakage from D-Lip was temperature dependent and occurred only in presence of plasma. The reason it was higher than from DOX-SL™ (5,6) may be related to the presence of 5 mole % HPE, which was not present in DOX-SL.

(b) *Effect of complement.* D-Lip, D-SSL, D-SSL-IgG, and D-SSIL-32/2 were incubated with fresh guinea pig serum for 15 min at 25°C (conditions under which cells sensitized with the specific antibodies undergo complete complement-induced lysis). There was no leakage of DOX from any liposome formulation, suggesting that the complement system did not affect liposome integrity.

Characterization of the Binding of SSIL-32/2 to Their Tumor Target Cells

(a) *Binding and specificity.* The specific target cells, A9, having PAA on their surface, were compared with 3T3 cells which lack PAA. All liposomes, prepared without DOX, contained 5 mole % of ^{2000}PEG-DSPE in their bilayer and 0.1 mole % of the nontransferable fluorescent phospholipid, FITC-PE (16). Binding of fluorescent liposomes (SSL, SSIL-IgG and SSIL-32/2) to the two cells lines was determined by flow cytometry.

Of all SSL combinations tested, immunospecific binding occurred only when A9 tumor cells were reacted with SSIL-32/2 immunoliposomes. Figures 3A (percent positive cells) and 3B (fluorescence intensity) show that the average binding to A9 tumor cells of specific SSIL-32/2 is at least 22-fold that of nonspecific SSIL-IgG. Figure 3C (3T3, nonspecific cells)

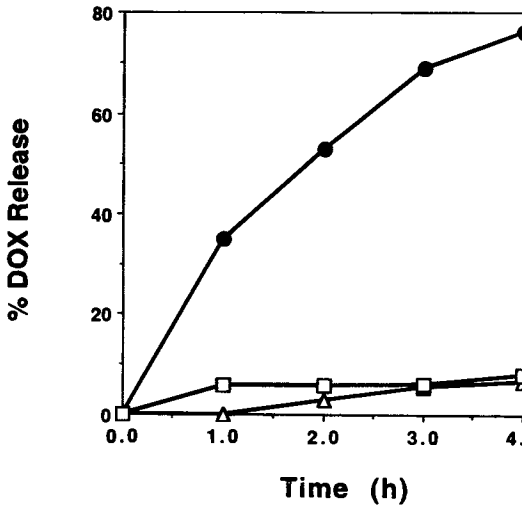

Fig. 2. Serum-induced DOX leakage from liposomes and immunoliposomes. D-Lip (●—●), D-SSL (△—△), and D-SSIL-IgG (□—□) in 10% sucrose were mixed with equal volumes of heat-inactivated FCS and incubated at 40°C. For more details see Methods.

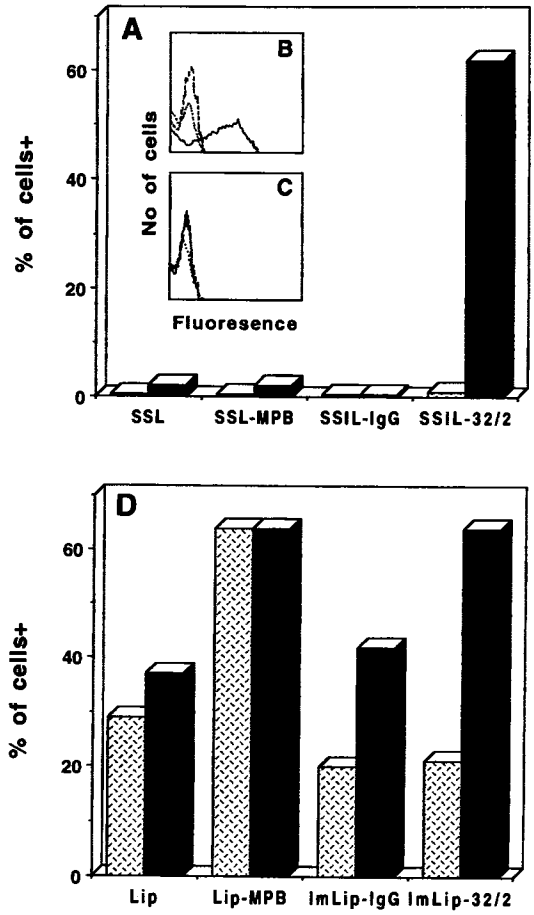

Fig. 3. Binding of unstabilized liposomes (Lip) and sterically stabilized liposomes (SSL) to the target cells, and the effect of ^{2000}PEG-DSPE on binding specificity. Liposomes before and after the binding of SMPB to HPE on their surface (Lip-MPB or SSL-MPB) and immunoliposomes with the control IgG or the NI32/2 MAb, all labeled with FITC-PE (and without doxorubicin), were incubated for 45 min in DMEM containing 5% FCS and 0.05% sodium azide with the nonrelevant 3T3 cells (▨) or the specific target A9 tumor cells (■). The unbound liposomes were washed and the fluorescence intensity of the cells was measured using a flow cytometer. Fig. 3A: SSL, SSL-MPB, SSIL-IgG, and SSIL 32/2. Figs. 3B and 3C (insets) show the fluorescence intensity of A9 tumor cells and 3T3 cells, respectively, for untreated cells (····), cells incubated with SSIL-IgG (----) and SSIL-32/2 (—). Fig. 3D: Lip, Lip-MPB or immunoliposomes (Lip-IgG, Lip-32/2). In both Fig. 3A and Fig. 3D the positive cells (cells +) are those which have a fluorescence intensity greater than the autofluorescence of the untreated cells. The experiment was repeated 3 times, with SD <8%.

indicates that there was no specific binding of SSIL-32/2 to 3T3 cells. Also, the liposome-related small increase in fluorescence of 3T3 cells was identical for SSL, SSIL-IgG, and SSIL-32/2 (Fig. 3A). Thus, only SSIL-32/2, but not SSIL-IgG or SSL, bind to A9 cells, while none binds appreciably to nonrelevant 3T3 cells (Figs. 3A, C), which suggests that SSIL-32/2 binding is immunospecific.

We also compared the binding to target cells of fluorescent liposomes at different stages of immunoliposome preparation containing or lacking ^{2000}PEG-DSPE. The following liposome (Lip or SSL) preparations were compared: (i) unmodified (containing 5 mole % HPE); (ii) after binding of SMPB; (iii) after binding of control IgG; (iv) after binding of NI32/2 MAb. Figure 3D describes the percent of fluorescent-stained cells, comparing binding to A9 cells and 3T3 cells; Both cell lines bind all types of fluorescent liposomes which lack PEG-DSPE (Lip). Noteworthy is the very high binding of Lip containing HPE-MPB (Lip-MPB) to both cell types. Although there is a 2.5-fold higher binding of Lip-32/2 to A9 cells than to 3T3 cells, specificity is much lower than that of SSIL-32/2 (Fig. 3A). Figure 3A also demonstrates that all of the high background of nonspecific binding obtained with Lip was eliminated when ^{2000}PEG-DSPE (5 mole %) was included in liposomes. Only SSIL-32/2 bound efficiently to specific A9 cells (>60% stained cells); SSL or SSIL-IgG, which lack specific MAb, have very low binding (<5% stained cells).

Figure 4 demonstrates that binding of fluorescent SSIL-32/2 to A9 cells was inhibited by soluble specific NI32/2 MAb, but almost not at all by nonrelevant anti-H-2^d antibodies, which also bind to A9 cells (H-2^d) with high affinity. Inhibition was dose-dependent, reaching saturation (90% inhibition) at 20 ng NI32/2 MAb. This indicates that SSIL-32/2 binding to A9 cells is indeed immunospecific.

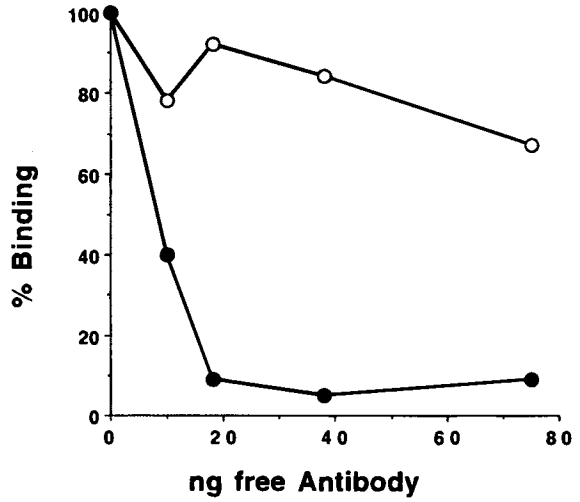

Fig. 4. Specific inhibition of SSIL-32/2 binding to A9 tumor cells by free NI32/2 MAb. A9 cells (1×10^6) were incubated at 4°C either alone (control) or with different amounts of free NI32/2 MAb (●—●), or anti-H-2^d Ab (○—○) in a final volume of 0.1 ml. Twenty minutes later the fluorescent FITC-labeled SSIL-32/2 (0.3 μmol PL in 0.1 ml) were added to every sample for an additional 45 min under the same conditions. The mean fluorescence intensity of the cells was analyzed using flow cytometry and percent binding relative to the control was calculated. Experiment was repeated 3 times. SD <8%.

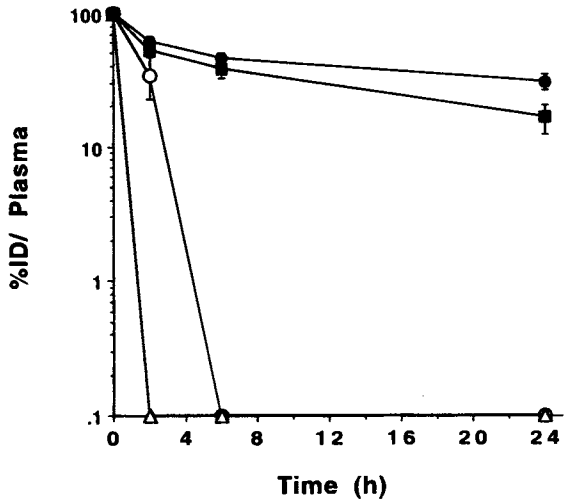

Fig. 5. Plasma pharmacokinetics of DOX encapsulated in liposomes and immunoliposomes in normal BALB/c mice: Free drug (△), D-Lip (○). D-SSL (●), or D-SSIL-IgG (■). Plasma DOX levels are given as percentage of injected dose (ID) corrected for total plasma volume (for more details see Materials and Methods).

(b) *Liposome interaction with macrophages.* Uptake of DOX by macrophages (see Methods) was very similar for cells incubated with D-SSL and D-SSIL and was approximately 40% that obtained with D-Lip.

(c) *Exposure of Fc domain in D-SSIL.* To study exposure of the Fc domain of IgG_3 NI32/2 MAb on the immunoliposome surface, aliquots of D-SSIL-32/2 and D-SSL (1 μg DOX) were passed through protein A Sepharose CL-4B columns (14). The columns were eluted using 1.5 M glycine buffer, pH 8.5, containing 3 M NaCl. Elution was followed by quantification of DOX fluorescence. Recovery of D-SSL was 50%, and all the liposomes were eluted in the void volume (0.8–1.2 ml), compared with only 15% recovery of D-SSIL-32/2, which was eluted at volumes larger than the void volume (1.8–2.4 ml). This suggests that D-SSIL interact with protein A and, therefore, Fc domains of at least part of the IgG_3 molecules on the SSIL surface are available for binding.

Plasma Pharmacokinetics

Figure 5 demonstrates superiority of D-SSL and D-SSIL to free DOX and D-Lip in normal mice plasma pharmacokinetics of the drug. Presence of antibodies on the surface of D-SSL has only a small effect on the long circulation time of the liposomes.

Table II shows the clearance, MRT, and AUC of DOX delivered via D-SSIL, D-SSL, and D-Lip in normal mice. AUC

Table II. Plasma Pharmacokinetics of DOX After I.V. Injection of DOX-loaded Liposomes and Immunoliposomes in Mice

Liposomes[a]	Clearance *(ml/h)*	MRT *(h)*	AUC^{24h} *(% of ID in total plasma, per h)*
D-SSL	0.08	7.9	1287
D-SSIL-IgG	0.17	5.9	593
D-Lip	0.59	1.5	76

[a] For doses injected, see Materials and Methods.

of DOX delivered via D-SSIL was about half that delivered by D-SSL. Both free drug (data not shown) and drug encapsulated in conventional liposomes (D-Lip) have much shorter MRT, and much smaller AUC, than the drug delivered by D-SSIL. Differences in pharmacokinetic parameters between DOX delivered by D-SSIL and D-SSL are much smaller than those between D-SSIL and D-LIP or free drug (Fig. 5, Table II). These observations indicate that steric stabilization was only slightly impaired by antibodies attached to the liposome surface. The faster than expected (compared with DOX-SL; 1, 3) clearance rate of D-Lip may be attributed to the presence of hydrogenated phosphatidylethanolamine (not present on DOX-SL) on the D-Lip surface.

DISCUSSION

General Aspects

Our objective was to assess the feasibility of preparing drug-containing targeted liposomes (D-SSIL) as a dosage form for treating tumors. The D-SSIL should: (a) be physically and chemically stable during preparation and storage; (b) carry enough drug to treat the tumor; (c) be small enough (<120 nm) to extravasate into the tumor; (d) be biocompatible regarding type of antibodies and mode of binding to the liposome; (e) be both immunospecific and sterically stabilized. Furthermore, the conjugated antibodies should not greatly reduce circulation time. This study responds to most of the above challenges.

Pharmaceutical Aspects

An ammonium sulfate gradient was used to obtain sufficient intraliposomal DOX accumulation, in the form of DOX sulfate gel (10, 15). This, together with the liposome phospholipids' high gel-to-liquid-crystalline phase-transition temperature, results in high stability (shelf life >6 months at 4°C) (10). It also explains the very slow release of drug from SSL in plasma *in vivo* (3, 4). In spite of the high stability of DOX loading, the drug is bioavailable, as demonstrated by its intracellular metabolism in human (4) and mouse (11) tumors. No leakage or degradation of drug or lipids occurred during the many steps needed for preparation and 4 months storage at 4°C of D-SSIL (Fig. 1, Table I), as proven by HPLC and TLC analyses (10, 12). We thus demonstrate formulation of a stable D-SSIL.

The method of attaching antibodies is very important, not only for immunotargeting, but also for biocompatibility and pharmaceutical acceptability, aspects not yet dealt with for SSIL and only to a small extent for conventional immunoliposomes (1, 2). Many methods are available for attaching proteins (including antibodies) to the liposome surface (reviewed in 16, 17). The importance of binding mode is illustrated by *in vivo* failure of attachment by disulfide bonds (22), which tend to undergo reduction in plasma, resulting in antibody loss. For a stable association, we selected the thioether bond between the HPE-MPB present only on the external face of the liposomes and the PDP-IgG antibodies (Fig. 1). This method is very reproducible and can be used successfully in a pharmaceutical setting (23). Furthermore, it does not involve a protein bridge (as does avidin, biotin, or protein A) which may induce an immune response against the SSIL (24).

The maleimide group of MPB degrades at a relatively fast rate. For example, at 30°C and pH 7.0, 35% and 87% degradation occurs after 1 h and 6 h, respectively (25). Therefore, the time available for efficient attachment of antibodies to MPB at the liposome surface is short. The advantage of the poor stability of the maleimide group is that the level of the active group remaining after attachment of the protein is low and should not impose serious compatibility problems.

The metabolic fate of antibodies conjugated to liposomal lipids by any of the available methods (17) has not yet been investigated.

The discrepancy between our results (stable DOX loading of D-SSIL) and those of Allen *et al.* (26) (unstable loading) may be related to (a) the difference in method of drug loading ($(NH_4)_2SO_4$ *vs.* pH loading, compare refs. 10 and 27), (b) the fact that in our SSL the HPE-MPB was present only on the external face of the liposomes, and (c) the sequence of steps leading to DOX-loaded SSIL (Fig. 1).

Bredehorst *et al.* (28) reported that attachment of Fab fragments to an MPB-PE anchor containing 5 mole % HPE-MPB induces liposome rupture. Our D-SSIL differ in containing ^{2000}PEG which stabilizes the liposomes so there is no drug leakage and reduces liposome-liposome interaction. At a lower level of PEG-DSPE (3 mole %) in the presence of the protein bound to the HPE-MPB at the liposome surface, liposome nonleaky aggregation occurred due to intervesicular protein-protein association (16).

A disadvantage of using SPDP for intact IgG is the randomness of the thiolation sites. As found here, the partial retention on the protein A Sepharose CL-4B column indeed indicates that the thiolation was not limited to the Fc fragment only. Exposure of the Fc domain did not, however, cause increased uptake of D-SSIL by macrophages, suggesting that the Fc domain exposed on the SSL is inaccessible to macrophages. Similar problems may exist with most other conventional methods used for attachment of intact IgG to the liposome surface (17). Still, use of a more specific association between antibodies and liposomes may be pharmaceutically advantageous (9, 16, 26).

Functional Aspects

The organization and topology of SSIL are schematically presented in Fig. 6, which depicts relative dimensions of ^{2000}PEG, ^{5000}PEG, a Fab fragment, intact IgG, and IgG attached to the far end of the PEG which is part of the PEG-DSPE molecule attached to the vesicle surface. Klibanov *et al.* (8) suggest that the ^{5000}PEG may be too large and thus interferes with the interaction of various recognition molecules attached to the vesicle surface with their targets. We have indications from other systems (unpublished) that ^{2000}PEG interferes with the specific binding to recognition molecules on the liposome surface of protein ligands having molecular mass ≤30,000. Thus, Fab fragments attached directly to a liposome surface containing ^{2000}PEG may be too short for immunotargeting of SSIL. This problem can be overcome by attaching the ligand at the far end of the PEG moiety (9). Like Allen *et al.* (26), we selected intact IgG which extends out from the ^{2000}PEG barrier of 5 nm.

Our *in vitro* results are in good agreement with those of Ahmad and Allen (29), who also demonstrated immunospecific

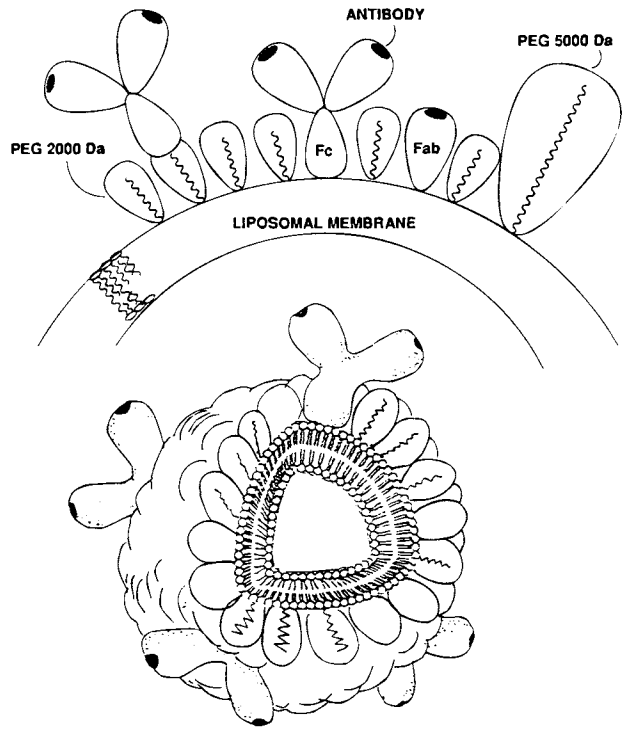

Fig. 6. Schematic diagram of SSIL (top) spatial relationships of the following ligands: PEG-5000, PEG-2000, intact IgG, and a Fab fragment; and (bottom) a 3-dimensional view of a small unilamellar immunoliposome with intact IgG and PEG-2000.

binding as well as DOX uptake by cells in culture despite the presence of ^{2000}PEG-DSPE on the liposome membrane. We demonstrate that ^{2000}PEG-DSPE actually improves immunospecific binding, probably by reducing nonspecific binding of liposomes and immunoliposomes to irrelevant cells (Figs. 3A, D).

Based on previous experience (3, 4) and on the plasma pharmacokinetic profile presented here, DOX measured in plasma after administration of D-SSL or D-SSIL is liposomal encapsulated. DOX delivered via D-SSIL has a somewhat shorter MRT, faster clearance, and smaller AUC than DOX delivered via D-SSL.

The identical and very high stability of D-SSL and D-SSIL *in vitro* in heat-inactivated serum even at 40°C, and their resistance to drug leakage by complement activation, speak against drug release from the liposomes. Furthermore, the similar clearance of FITC-PE-labeled SSIL (11) and of DOX delivered via D-SSIL indicates that DOX does not leak from the D-SSIL at a fast rate. These findings led us to assume that the faster clearance is of intact D-SSIL.

D-SSIL (and not D-SSL) may be modified in the circulation, for example by oligomerization induced by interaction of their surface protein with serum components. Thus, their size-dependent uptake by the spleen can be enhanced. This mechanism is supported by data showing that the extent of liposome accumulation in the spleen is correlated with their size (8). Phagocytosis by RES is not a good explanation for this behavior because presence of antibody did not accelerate uptake by macrophages *in vitro*. The mechanism of SSIL clearance has to be better understood in order to design optimally-effective SSIL formulations.

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