Cancer Chemother Pharmacol (1995) 35: 447–456

ORIGINAL ARTICLE

Atsuhide Mori · Stephen J. Kennel
Marjan van Borssum Waalkes · Gerrit L. Scherphof
Leaf Huang

Characterization of organ-specific immunoliposomes for delivery of 3′,5′-O-dipalmitoyl-5-fluoro-2′-deoxyuridine in a mouse lung-metastasis model

Received: 13 May 1994 / Accepted: 22 September 1994

Abstract A previous study has shown that lipophilic prodrugs can be delivered efficiently to normal lung endothelium by incorporation into liposomes covalently conjugated to monoclonal antibody (mAb) 34A against the lung endothelial anticoagulant protein thrombomodulin. In the present study, the potential use of these lung-targeted immunoliposomes (34A-liposomes) for delivery of a lipophilic prodrug, 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine (dpFUdR), to the tumor-bearing lung was examined using BALB/c mice bearing experimental lung metastasis induced by i.v. injection of EMT-6 mouse mammary tumor cells. Immunohistochemical examination of the tumor-bearing lung showed specificity of mAb 34A to lung endothelium. Tumor cells appeared to localize just outside of the normal blood vessels and were within a small diffusion distance from the mAb 34A-binding sites. ^{111}In-labeled 34A-liposomes containing monosialoganglioside (G_{M1}) were prepared that included [^{3}H]-dpFUdR at 3.0 mol% in the lipid mixture. In vitro cell binding studies further demonstrated that 34A-liposomes bound specifically to normal mouse lung cells that expressed thrombomodulin but not to EMT-6 cells. Biodistribution study showed efficient and immunospecific accumulation of [^{3}H]-dpFUdR incorporated into 34A-liposomes in the lung at a level parallel with that of ^{111}In-labeled 34A-liposomes, indicating that the drug is delivered to the target organ in intact liposomes. Liposomal dpFUdR appeared to be metabolized in the lung to the parent drug FUdR at a rate slower than in the liver and spleen. Furthermore, treatment of lung-metastasis-bearing mice with dpFUdR incorporated into 34A-liposomes on days 1 and 3 after tumor cell injection resulted in a significant increase in the median survival time of treated mice as compared with control mice (%T/C value, 165%). dpFUdR either dispersed in emulsion or incorporated into antibody-free liposomes was ineffective in prolonging the survival of mice. These results indicate the potential effectiveness of organ-specific immunoliposomes containing a lipophilic prodrug for the targeted therapy of metastatic tumors.

Key words Immunoliposome
Lipophilic prodrug · Lung targeting

Abbreviations *Chol* Cholesterol · *dpFUdR* 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine · *DTPA-SA* diethylenetriamine pentaacetic acid distearylamide complex G_{M1} monosialoganglioside · IC_{50} 50% inhibitory concentration · *mAb* monoclonal antibody · *MST* mean survival time · *NGPE N*-glutaryl phosphatidylethanolamine · *NMLC* normal mouse lung cells · *OG* octyl-β-D-glucopyranoside *PBS* phosphate-buffered saline · *PC* egg phosphatidylcholine · *%T/C* (median survival time of treated mice divided by the median survival time of control mice) ×100 *RES* reticuloendothelial system

A. Mori · L. Huang (✉)
Department of Pharmacology, 13th Floor, Biomedical Science Tower, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

S.J. Kennel
Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

M. van Borssum Waalkes · G.L. Scherphof
Groningen Institute for Drug Studies, Laboratory of Physiological Chemistry, Groningen University, Groningen, The Netherlands

Introduction

The use of monoclonal antibodies (mAbs) for targeting anticancer drugs to tumors has been a major area of investigation in cancer research [1–3]. Most anticancer drugs are nonspecific in their actions. Low therapeutic indices together with high toxicities of the drugs, arising

from nonspecific and unfavorable localization of the drug, often limit their effectiveness. The ultimate goal of antibody-directed drug delivery is to improve the therapeutic index of a drug by delivering it specifically to tumor cells and minimizing accumulation in sensitive normal cells. However, several limiting factors make such direct tumor targeting inefficient. Even with tumor-selective mAbs, efficient targeting to tumor cells in vivo is often hindered by the inaccessibility of the target antigen due to poor vascularization of the tumor [4]. Tumor cell heterogeneity for antigen expression is also a problem in such a direct immunotargeting approach.

We have been examining the potential of organ-specific antibody-directed targeting of liposomes as an alternative approach. Organ-specific immunotargeting takes advantage of a relatively abundant antigen in the target organ. The use of organ-specific immunotargeting may avoid some of the problems found in direct immunotargeting to tumor cells. MAb 34A has previously been shown to bind specifically to a surface glycoprotein (P112), now identified as the endothelial anticoagulant protein thrombomodulin, which is expressed at high concentrations on the lumenal surface of capillary endothelial cells of mouse lung [5–7]. Systemically delivered mAb 34A accumulates selectively in the mouse lung due to a highly accessible localization of the target antigen [8].

Antibody-directed liposomes, or immunoliposomes, have been studied extensively and show good promise for liposome targeting [9]. One favorable characteristic of liposomes as a drug-carrier system is their high degree of drug-carrying capacity as compared with mAb directly conjugated to a drug molecule [10]. In addition, both hydrophobic and hydrophilic drugs can be loaded into liposomes, irrespective of the chemical structure of the drug. Thus, use of immunoliposomes potentially offers both target specificity and versatility for anticancer drugs.

Initial studies with liposomes bearing mAb 34A (34A-liposomes) showed efficient and immunospecific accumulation of 34A-liposomes in the mouse lung [11–14]. Several variables in immunoliposome construction have been optimized for efficient target binding (for a review, see [15]). Under optimal conditions, approximately 70% of the total i.v. injected dose of 34A-liposomes accumulated in the lung [16]. An important finding has been that when immunoliposomes were designed to exhibit a reduced affinity to the reticuloendothelial cells in the liver and spleen by including monosialoganglioside (G_{M1}) or polyethylene glycol conjugated to phosphatidylethanolamine (PEG-PE) in the lipid composition, the resulting immunoliposomes showed more efficient target binding and prolonged retention in the target organ [16–18]. Furthermore, our recent studies have demonstrated that lipophilic prodrugs can be efficiently delivered to lung endothelium by 34A-liposomes [19].

The present study was intended to examine further the concept of organ-specific immunotargeting of liposomes for lipophilic prodrug delivery. Using a mouse lung-metastasis model, we examined the potential use of 34A-liposomes for delivery of 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine (dpFUdR), a lipophilic prodrug of 5-fluoro-2′-deoxyuridine (FUdR), to the tumor-bearing lung. The results indicate the effectiveness of organ-specific immunoliposomes for the targeted therapy of metastatic tumors.

Materials and methods

Reagents

Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids Inc. (Birmingham, Ala.). Cholesterol (Chol) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Bovine monosialoganglioside (G_{M1}) was obtained from Matreya Inc. (Mount Pleasant, Pa.). ^{111}In and ^{125}I were supplied by New England Nuclear (Boston, Mass.). All other chemicals were of reagent grade. Syntheses of diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) [20], dpFUdR [21], and *N*-glutarylphosphatidylethanolamine (NGPE) [18] have been described previously. Radiolabeling of DTPA-SA with ^{111}In was performed as described elsewhere [22]. [^{3}H]-dpFUdR was synthesized at a specific activity of ~35 μCi/mg as previously described [23].

Antibodies

Rat mAb 34A (IgG_{2a}) recognizes thrombomodulin expressed in high concentrations in the BALB/c lung endothelium [8]. Rat mAb 14 (IgG_{2a}), which has no known binding specificity [24], was used as a negative control. The mAbs were purified from nu/nu mouse ascites fluid by ammonium sulfate precipitation followed by ion-exchange chromatography on DE52 cellulose as described previously [25]. MAbs were radiolabeled with ^{125}I using the Iodo-Gen method (Pierce, Rockford, Ill.) to a specific activity of ~4×10^5 cpm/μg protein. ^{125}I-labeled antibody was purified using a BioGel P-4 (Bio-Rad, Richmond, Calif.) spin column.

Cell cultures

EMT-6 tumor, originally derived from a spontaneous breast tumor of a female BALB/c mouse [26, 27], was obtained from the DCT Tumor Repository, Frederick Cancer Research Facility, National Cancer Institute (Frederick, Md.). The tumor was maintained in vivo by s.c. implantation of tumor tissues into the left hind legs of female BALB/c mice. A single-cell suspension was prepared from tumor tissue dispersed by collagenase and was grown as monolayer cultures in Waymouth's medium supplemented with 15% (v/v) fetal calf serum (FCS), penicillin (200 units/ml), and streptomycin (100 μg/ml) [27]. Normal mouse lung cells (NMLC) were prepared from a mixed population of dissociated NMLC and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS [9]. These cells were nearly 100% positive when stained with fluorescent mAb 34A (data not shown), indicating that they express thrombomodulin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were detached from the culture flask plate by incubation in 0.05% trypsin/0.53 m*M* ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution (HBSS) at 37 °C.

Animals and metastasis model

Female BALB/c mice, 6–10 weeks old, were purchased from Charles River Laboratories (Wilmington, Mass.). Experimental mouse lung metastases were induced as follows [27]. Trypsinized EMT-6 tumor cells were washed three times with serum-containing medium and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS at a concentration of 5×10^4 cells/ml. Cell viability was ascertained by trypan blue exclusion. Cells were injected i.v. into mice (5×10^3 cells in 100 μl per mouse) using a tuberculin syringe with a 27-gauge 0.5-in. needle.

MAb distribution

For autoradiography experiments, BALB/c mice with EMT-6 tumor cells growing in the lung from i.v. injection were injected with ^{125}I-labeled mAb 34A in phosphate-buffered saline (PBS; 1370 m*M* NaCl, 27 m*M* KCl, 15 m*M* KH_2PO_4, 1 m*M* Na_2HPO_4; pH 7.4) containing bovine serum albumin (5 mg/ml) at a dose of 8 μg protein in 100 μl per mouse. At appropriate intervals, mice were killed by lethal injection of Nembutal, and their lungs were inflated with 10% buffered formalin through a tracheal catheter. The lungs were excised and fixed for 24 h in the same fixative. Sections (~5 μm) were cut from paraffin blocks, deparaffinized, and dipped in Kodak NTB-2 emulsion before being developed after 3 days of exposure. Slides were then stained with hematoxylin and eosin, dehydrated, and coverslipped in Permount.

MAb derivatization

Conjugation of mAb 34A and mAb 14 with NGPE was performed as described elsewhere [12, 28]. Briefly, NGPE dissolved in $CHCl_3$ was dried with N_2 gas and vacuum-desiccated. The dried NGPE was then solubilized with 0.15 *M* octyl-β-D-glucopyranoside (OG) at an NGPE/OG ratio of 0.06:1 (mol/mol) in 2-[*N*-morpholino] ethanesulfonic acid (MES) buffer (10 m*M* MES/150 m*M* NaCl, pH 5.0). Then 0.25 *M* 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in H_2O and 0.10 *M* *N*-hydroxysulfosuccinimide (Sulfo-NHS) in H_2O were added to the above NGPE reaction mixture to an NGPE/EDC/Sulfo-NHS molar ratio of 1:50:20 and then incubated for 10 min at room temperature. The mixture was neutralized with 100 m*M* HEPES buffer (pH 7.5) and 1 *N* NaOH to pH 7.5. Antibodies containing trace amounts of ^{125}I label were then added at an antibody/NGPE ratio of 1:20 (mol/mol) and incubated for 8 h at 4 °C with gentle stirring. Antibody-NGPE conjugates were stored at 4 °C until use.

Liposome preparation

Antibody-free (bare) liposomes and immunoliposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1) with or without dpFUdR at 3.0 mol% of the lipid mixture were prepared by the detergent-dialysis method as described previously [12, 28]. In some experiments, [^{3}H]-dpFUdR was included in the lipid mixture as a label. Briefly, the solvent-free lipid mixture containing [^{111}In]-DTPA-SA as a nonexchangeable and nonmetabolizable lipid marker [20] at 1.0 mol% of the lipid mixture was solubilized with OG in PBS (pH 7.4) at a lipid/OG ratio of 1: 5 (mol/mol). The resultant solution was mixed vigorously with mAb-NGPE conjugates at an antibody/lipid ratio of 1:4 ~ 1:8 (w/w), and detergent was removed by dialysis against PBS (pH 7.4) for 36 h at 4 °C. Bare liposomes were prepared without addition of mAb-NGPE conjugates before dialysis. The resulting liposomes were extruded at room temperature eight to ten times through stacked Nucleopore membranes (0.4- and 0.2-μm pore size; Costar, Cambridge, Mass.) using a syringe-type filter holder (Whatman, OL) to generate liposomes measuring ~200 nm in diameter with homogeneous size distribution. Liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, Fla.) and was expressed as the average diameter with SD. The extruded immunoliposome preparation was chromatographed on a BioGel A1.5M column (Bio-Rad Laboratories, N.Y.) to remove unincorporated antibody. Normally, ~40% of the amount of antibody added was incorporated into liposomes. The liposome fractions were pooled and diluted appropriately with PBS. The antibody/lipid weight ratio of immunoliposomes was determined by calculations using the specific activities of [^{111}In]-DTPA-SA and [^{125}I]-antibody.

In vitro cell binding of liposomes

[^{111}In]-labeled liposomes were prepared to include [^{3}H]-dpFUdR at 3.0 mol% of the lipid mixture as described above. The EMT-6 cells and NMLC were prepared as described above and plated onto 6-well plates at 5×10^5 cells in 2 ml of serum-containing medium in each well. After incubation for 36 h at 37 °C, cells were washed with fresh cold medium and kept in 0.9 ml of the same media for 1 h at 4 °C before the addition of liposomes. Next, 100 μl of the liposome suspension (2 μmol total lipid/ml) was added to each well to a final lipid concentration of 200 nmol/ml. The cells were further incubated for 1 h at 4 °C with liposomes. Cells were washed three times with cold HBSS, detached from the plates by trypsinization with 1 ml trypsin-EDTA solution, transferred to a 50- × 10-mm tube, and assayed for ^{111}In radioactivity using a Beckman gamma-counter. For analysis of cell-associated [^{3}H]-dpFUdR, the cells were subsequently solubilized with 0.5 ml Solvable (NEN) for 4 h at 50 °C and decolored with hydrogen peroxide, and the radioactivity was determined in scintillation fluid (Ultima Gold; Packard Instrument Co., Downers Grove, Ill.) using a Beckman LS-1801 counter. Data were expressed as amounts of lipid (in nanomoles) and dpFUdR (in picomoles) bound to 10^6 cells as determined by calculations using the specific activities of [^{111}In]-DTPA-SA and [^{3}H]-dpFUdR, respectively.

In vitro cytotoxicity assay

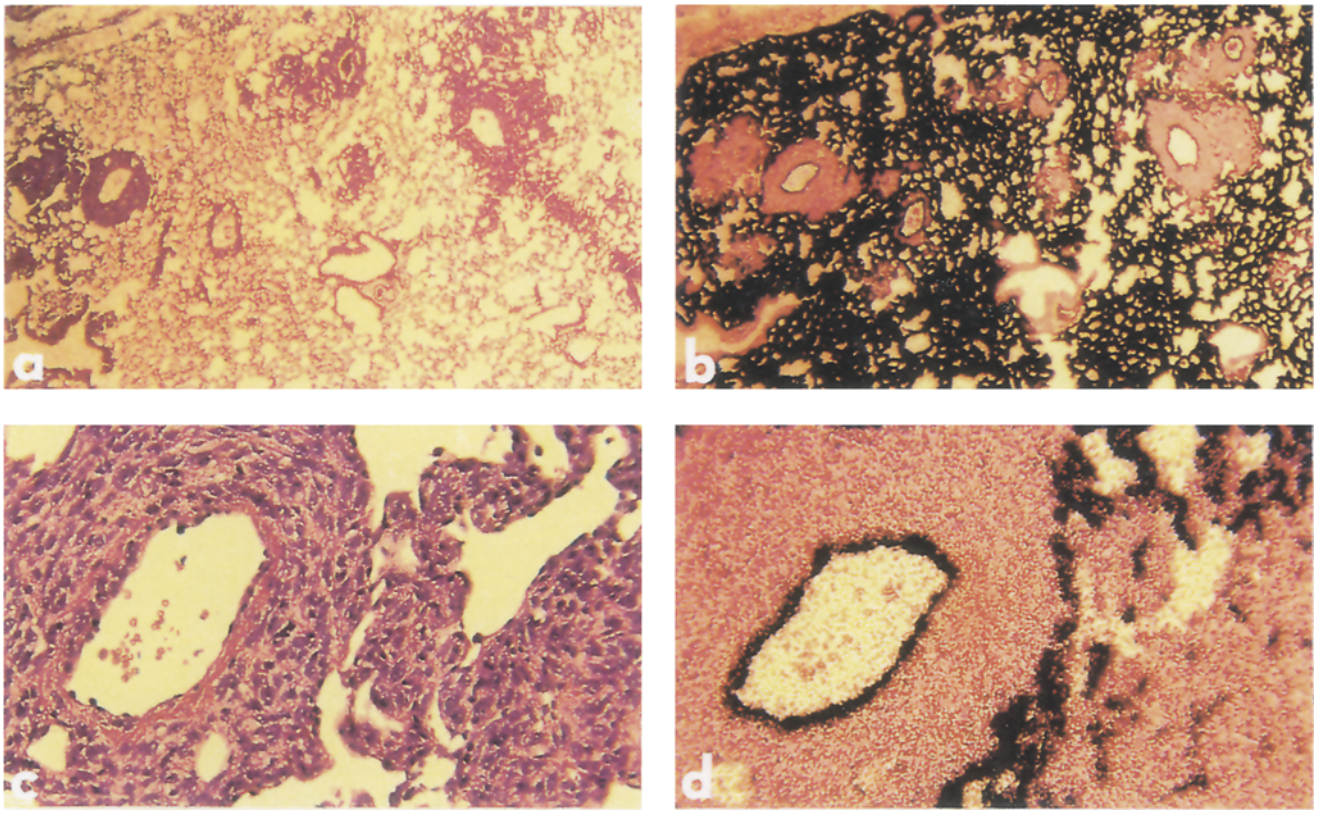
Liposomes containing dpFUdR were prepared as described above. NMLC and EMT-6 tumor cells were prepared as described above, plated onto 24-well plates at 1×10^4 cells in 1 ml serum-containing medium per well, and incubated for 24 h at 37 °C. After washing of the cells, 1 ml of the medium containing varying concentrations (0.1–5000 n*M*) of free FUdR (Sigma Chemical Co.) or dpFUdR incorporated into liposomes was added. After incubation for 72 h at 37 °C, the cells were washed three times with HBSS, trypsinized, resuspended in sterile isotonic saline solution (Curtis Matheson Sci. Inc., Marietta, Ga.), and enumerated using a model ZM Coulter Counter. Data were expressed as the percentage of viable cells with respect to the control cultures incubated in the medium without drugs.

Liposome biodistribution

[^{111}In-labeled liposomes with or without [^{3}H]-dpFUdR at 3.0 mol% of the lipid mixture were prepared as described above and diluted to 1.0–1.6 mg lipid/ml PBS, then injected i.v. into mice at a dose of 0.2–0.3 mg lipid per mouse in 0.2 ml PBS. Administration of liposomes in this dose range does not cause saturation of liposome uptake by the reticuloendothelial system (RES), and the biodistribution of liposomes is independent of both the amount and the number of injected liposomes (data not shown). At specified intervals, mice were lightly anesthetized, bled by retroorbital puncture, and then killed by cervical dislocation. Major organs, including the lung, spleen, liver, heart, and kidney, were collected and weighed. Biodistribution of liposomes was determined by analysis of the ^{111}In radioactivity in each organ using a Beckman gamma-counter. For analysis of [^{3}H]-dpFUdR, weighed samples of blood and solid tissues were transfered to glass scintillation vials, solubilized with Solvable at 50 °C according to the manufacture's instructions, decolored with hydrogen peroxide, and analyzed for ^{3}H radioactivity in scintillation fluid using a Beckman counter. The radioactivity in each sample was corrected for quenching using the internal standard method as described elsewhere [29]. Data were expressed as percentages of the total injected dose of ^{111}In-labeled liposomes and [^{3}H]-dpFUdR in each organ. Levels of liposomes and dpFUdR in the blood were determined by assuming that the blood volume of a mouse is 7.3% of the total body weight [30]. The amounts in organs were corrected for blood contamination as previously described [18].

Survival studies

Lung metastases were induced by i.v. injection of 5×10^3 EMT-6 tumor cells into BALB/c mice as described above. Liposomes containing dpFUdR were prepared as described above and diluted to 8.0 mg lipid/ml or 0.25 mg dpFUdR/ml in PBS. dpFUdR in emulsion was prepared using vegetable oil/Tween 20 (2:1, v/v) and diluted with PBS



to 0.25 mg dpFUdR/ml. The effect of dpFUdR incorporated into liposomes on the overall survival of mice bearing EMT-6 lung metastases was studied by the administration of 2 mg/kg dpFUdR per treatment. Four different treatment groups of mice received PBS, dpFUdR in emulsion, dpFUdR incorporated into bare liposomes, or dpFUdR incorporated into 34A-liposomes. Treatments were given on days 1 and 3 after the injection of tumor cells. Survival was examined daily over a 60-day period. Tissues from autopsied animals were examined by histology. The cause of death was usually lung hemorrhage due to tumor burden.

Fig. 1 a–d Autoradiographic analyses of mouse lungs from animals injected 7 days earlier with 1×10^4 EMT-6 cells. At 1 h before the animals were killed, 8 μg ^{125}I-labeled mAb 34A was injected i.v. (~10,000 cpm/μg). Lungs were fixed in buffered formalin, paraffin embedded, and sectioned for staining or autoradiography. **a**, **c** Section stained with hematoxylin and eosin (magnification, ca. 100 ×). **b**, **d** Autoradiography with ^{125}I-labeled mAb 34A (magnification, ca. 640 ×)

Results

Distribution of mAb 34A in the mouse lung with growing EMT-6 tumor

When EMT-6 tumor cells were injected i.v. into the BALB/c mice, the mice developed tumor nodules in the lung with virtually no growth of the tumor in any other organs [27]. To examine locations of the target antigen of mAb 34A and EMT-6 tumor cells in the lung, we injected ^{125}I-labeled mAb 34A into mice bearing lung metastases and performed an immunohistochemical analysis of the lung (Fig. 1). Sections stained with hematoxylin and eosin (Fig. 1 a, c) showed that metastatic EMT-6 tumor cells were growing around preexisting normal blood vessels in the lung. The tumor mass was densely packed without well-developed neovascularization. Subsequent autoradiography of serial sections (Fig. 1 b, d) showed that the normal blood vessels, but not the tumor-cell mass, were labeled with mAb 34A to lung endothelium. It was also clear at a higher magnification (Fig. 1 c, d) that the EMT-6 tumor at this stage of growth was within six to ten cell layers of the lung endothelium.

In vitro cell binding of liposomes

The intense labeling of the normal lung capillaries observed in the immunohistochemical analysis is due to a high accessibility of mAb 34A to the target antigen [10]. EMT-6 tumor cells growing in the lung may not be readily accessible to mAb 34A and, thus, may not be stained with ^{125}I-labeled mAb 34A. To rule out this possibility and demonstrate further the specificity of mAb 34A to lung endothelium, we examined in vitro cell binding of 34A-liposomes using normal mouse lung cells (NMLC) and EMT-6 tumor cells. In this study, ^{111}In-labeled, bare, and mAb 34A-/mAb 14-conjugated (34A-/14-) liposomes containing [^{3}H]-dpFUdR were prepared by the detergent-dialysis method. Cells were incubated with liposomes for 1 h at 4 °C.

As shown in Fig. 2, nonspecific binding of bare liposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1) to NMLC as determined by analysis of cell-bound ^{111}In

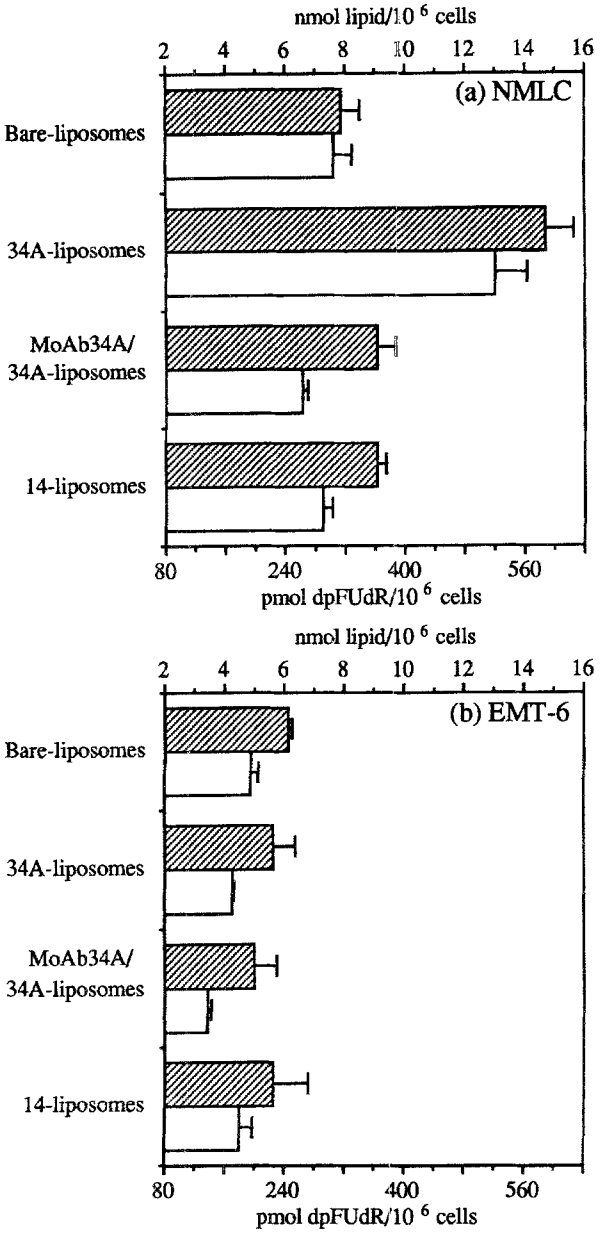


Fig. 2 a, b Binding of 34A-liposomes to **a** NMLC and **b** EMT-6 tumor cells. NMLC and EMT-6 tumor cells were incubated at 4 °C with ^{111}In-labeled liposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1), additionally containing [^{3}H]-dpFUdR at 3.0 mol% of the lipid mixture, at a lipid concentration of 200 nmol/ml. Amounts of cell-bound liposomes and dpFUdR were determined after 1 h of incubation and were plotted as amounts of lipid (nmol, ▨ 1) and dpFUdR (pmol, □) bound to 10^6 cells. In a competition experiment with free mAb 34A, cells were preincubated for 1 h with a 10-fold excess amount of mAb 34A before addition of 34A-liposomes. Bar represent SD (n = 3). Average diameters of bare, 34A-, and 14-liposomes were 142(56), 148(54), and 141(66) nm, respectively. Antibody/lipid weight ratios of 34A- and 14-liposomes were 1:8.6 and 1:7.1, respectively. Amounts of 34A-liposomes containing dpFUdR bound to NMLC differed significantly from those of bare liposomes ($P < 0.003$ for ^{111}In; $P < 0.008$ for ^{3}H), 34A-liposomes with pretreatment with mAb 34A ($P < 0.001$ for ^{111}In; $P < 0.001$ for ^{3}H), or 14-liposomes ($P < 0.001$ for ^{111}In; $P < 0.001$ for ^{3}H).

radioactivity was 7.9 nmol lipid/10^6 cells (Fig. 2a). Incorporation of mAb 34A-NGPE conjugates into liposomes at an antibody/lipid weight ratio of 1:8.6 resulted in an approximately 2-fold increase (14.7 nmol) in liposome binding to NMLC. This enhanced binding of 34A-liposomes over bare liposomes was almost completely blocked when cells were preincubated with an excess amount of free mAb 34A. Furthermore, 14-liposomes, control immunoliposomes, with a similar antibody/lipid weight ratio (1:7.1) bound to NMLC at the same level as the bare liposomes. Similarly, an increased amount of cell-associated dpFUdR as determined by analysis of ^{3}H radioactivity was observed only when the drug was incorporated into 34A-liposomes. These data clearly show immunospecific interactions of 34A-liposomes with NMLC. It was also noted that cell-bound lipid/dpFUdR molar ratios were close to the initial molar ratio of 97:3 in the liposome preparation, indicating that intact liposomes bound to cells. Binding of liposomes to EMT-6 cells was not significantly different for the 34A-liposomes and the control 14-liposomes, with the amounts of cell-bound lipid ranging from 5.0 to 6.1 nmol lipid/10^6 cells (Fig. 2b), indicating little, if any, immunospecific interaction of 34A-liposomes with the EMT-6 tumor cells. Binding of [^{3}H]-dpFUdR to EMT-6 cells parallel to the binding of ^{111}In label (Fig. 2b), again showing no immunospecific binding of the drug incorporated into 34A-liposomes.

In vitro cytotoxicity of dpFUdR incorporated into liposomes on NMLC and EMT-6 tumor cells

One potential problem in organ-specific immunotargeting is the toxicity of the drug concentrated to the target organ. Figure 3 shows the growth-inhibition curves generated for NMLC and EMT-6 tumor cells continuously exposed to dpFUdR incorporated into liposomes. Incubation of cells with dpFUdR incorporated into 34A-liposomes resulted in similar growth-inhibition curves for NMLC and EMT-6 tumor cells, with IC_{50} values being approximately 9 and 7 ng/ml, respectively. These data indicate that both cells show a similar sensitivity to dpFUdR when it is delivered by liposomes.

Biodistribution of [^{3}H]-dpFUdR incorporated into 34A-liposomes

We have previously shown that incorporation of dpFUdR at 3.0 mol% of the lipid mixture does not alter 34A-liposomes homing to the lung as analyzed with a lipid marker, [^{111}In]-DTPA-SA [19]. Consequently, dpFUdR is expected to be delivered efficiently to the lung by incorporation into 34A-liposomes. For direct detection of a drug molecule in the lung target, ^{111}In-labeled liposomes containing [^{3}H]-dpFUdR were prepared and injected into mice. The biodistribution of liposomes and liposomal dpFUdR was examined at 2 h after injection by the analysis of ^{111}In and ^{3}H radioactivity in tissues, respectively. This interval was

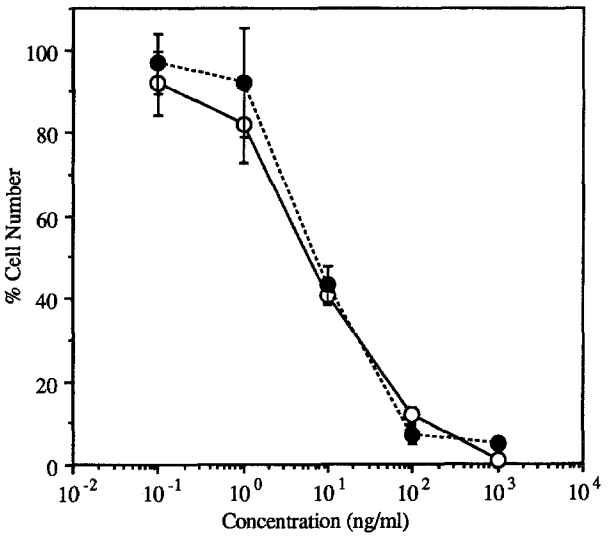


Fig. 3 Growth inhibition of NMLC and EMT-6 tumor cells induced by liposomal dpFUdR. NMLC (●) and EMT-6 tumor cells (○) were incubated with the indicated concentrations of dpFUdR incorporated into 34A-liposomes with the average diameter of 183(56) nm and an antibody/lipid weight ratio of 1:10. Cells were enumerated after incubation for 72 h. Cell numbers in cultures incubated in different concentrations of drugs are expressed as percentages of the control culture incubated in media without drugs. Bars represent SD (n = 5).

chosen since the maximal target binding of 34A-liposomes is normally obtained at 15 min to 2 h after injection [15, 18]. Data were expressed as percentages of the injected dose of liposomes and dpFUdR in the lung, blood, spleen and liver (Table 1).

At 2 h after injection, the majority (51%) of the bare liposomes persisted in the blood, whereas only a small fraction (1.2%) accumulated in the lung as determined by analysis of ^{111}In radioactivity. Analysis of [^{3}H]-dpFUdR showed that similar percentages of the total injected dose of dpFUdR were detected in the blood (52%) and the lung (1.3%), indicating that dpFUdR did not dissociate from the liposomes and that the disposition of drug molecules paralleled that of liposomal lipid at this interval. When dpFUdR was incorporated into 34A-liposomes with an antibody/lipid weight ratio of 1:8.6, the level of dpFUdR accumulation in the lung was 56% of the total injected dose, in accordance with the level of accumulation of 34A-liposomes (52%) in this organ. Thus, with the total injected dose of dpFUdR (10 μg/mouse), 5.6 μg dpFUdR accumulated in the lung at 2 h after injection. Lung binding of 34A-liposomes was completely blocked by preinjection of an excess of free mAb 34A, indicating that the observed accumulation of 34A-liposomes was due to immunospecific interactions with the lung. Furthermore, dpFUdR incorporated into 14-liposomes with a similar antibody/lipid weight ratio (1:7.1) did not show any significant accumulation in the lung. Thus, the biodistribution of dpFUdR depends on the liposomes to be used for incorporation, and dpFUdR can be efficiently delivered to the lung by its incorporation into 34A-liposomes. Both 34A-liposomes, with preinjection of free mAb 34A, and 14-liposomes showed increased accumulation in the liver (56% and 36%, respectively) as compared with bare liposomes (19%), presumably as a result of Fc receptor-mediated uptake of these immunoliposomes by this organ [31, 32].

Biodistribution data obtained on [^{3}H]-dpFUdR-containing bare and 34A-liposomes at 5 h after injection showed substantially lower levels of [^{3}H]-dpFUdR in the spleen and liver as compared with the levels of ^{111}In-labeled liposome accumulation in these organs. For example, 24% of the total injected dose of 34A-liposomes accumulated in the liver, whereas only 13% of the total injected dose of dpFUdR was detected in this organ at 5 h after injection. The total recovery in major organs at 5 h after the injection of [^{3}H]-dpFUdR incorporated into bare and 34A-liposomes was 51% and 64%, respectively, whereas that of ^{111}In-labeled

Table 1 Biodistribution of ^{111}In-labeled liposomes containing [^{3}H]-dpFUdR[a]

Liposomes[b]	Time (h)	% Injected dose[c]							
		Lung		Blood		Spleen		Liver	
		^{111}In	^{3}H	^{111}In	^{3}H	^{111}In	^{3}H	^{111}In	^{3}H
Bare liposomes	2	1.2 (0.3)	1.3 (0.1)	50.7 (6.4)	51.9 (4.9)	14.6 (0.6)	5.5 (0.1)	18.7 (1.0)	17.8 (1.4)
	5	0.9 (0.2)	0.8 (0.0)	27.2 (1.4)	30.9 (3.1)	20.5 (0.5)	4.3 (0.3)	31.2 (1.5)	12.9 (1.1)
34A-liposomes	2	52.1 (0.5)	56.4 (1.2)	4.1 (0.7)	6.8 (0.6)	2.8 (0.3)	2.1 (0.2)	17.6 (1.7)	16.5 (0.6)
	5	47.9 (3.8)	41.0 (3.8)	3.4 (0.9)	5.9 (0.6)	4.8 (0.9)	2.2 (0.3)	24.2 (2.0)	12.7 (0.5)
mAb 34A/ 34A-liposomes[d]	2	1.3 (0.5)	1.3 (0.3)	31.6 (4.3)	33.2 (6.2)	5.0 (0.4)	4.7 (1.9)	55.9 (6.4)	46.8 (4.7)
14-liposomes	2	2.3 (0.4)	2.9 (0.5)	45.8 (3.1)	40.0 (3.3)	2.7 (0.1)	1.9 (0.1)	36.3 (1.9)	29.8 (1.9)

[a] ^{111}In-labeled liposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1) additionally containing [^{3}H]-dpFUdR at 3 mol% in the lipid mixture were injected i.v. into mice at 0.32 mg lipid and 10 μg dpFUdR per mouse. The biodistribution of [^{111}In]-liposomes and [^{3}H]-dpFUdR was examined at the indicated intervals and expressed as the percentage of the injected dose detected in the lung, blood, spleen, and liver.

[b] Average diameters of bare, 34A-, and 14-liposomes were 225(76), 191(60), and 141(66) nm, respectively. Antibody/lipid weight ratios of 34A- and 14-liposomes were 1:8.4 and 1:7.1, respectively.

[c] Data are expressed as mean values (SD), n = 3.

[d] Free mAb 34A was injected i.v. into mice at 500 μg per mouse at 1 h prior to the injection of 34A-liposomes.

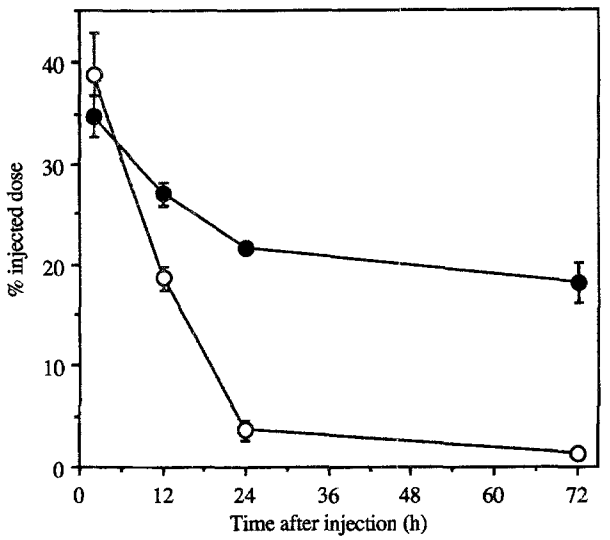


Fig. 4 Retention of [^{3}H]-dpFUdR incorporated into 34A-liposomes in the lung. ^{111}In-labeled 34A-liposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1) additionally containing [^{3}H]-dpFUdR at 3.0 mol% in the lipid mixture were prepared by the detergent-dialysis method with an average diameter of 174(81) nm and an antibody/lipid weight ratio of 1:17. Liposomes were injected i.v. into mice at 0.32 mg lipid and 10 μg dpFUdR per mouse. Levels of (●) ^{111}In-labeled liposomes and (○) ^{3}H-labeled drug in the lung were examined at the indicated intervals and expressed as the percentage of the injected dose. Bars represent SD (n = 3).

Table 2 Effect of injection frequency on the target binding of 34A-liposomes[a]

Number of injection	% Injected dose[b]			
	Lung	Blood	Spleen	Liver
1	43.7 (3.0)	23.2 (2.2)	4.2 (0.6)	20.1 (2.2)
2	41.0 (0.9)	29.9 (3.0)	6.1 (0.4)	20.7 (2.1)
3	29.0 (2.8)	24.9 (3.0)	8.2 (0.2)	40.4 (5.3)
4	21.6 (1.8)	21.8 (0.6)	6.4 (0.2)	49.9 (4.9)
5	2.5 (0.3)	12.2 (1.9)	6.2 (5.5)	81.4 (3.2)
6	1.6 (0.3)	9.2 (0.4)	5.5 (0.5)	82.9 (0.3)

[a] ^{111}In-labeled 34A-liposomes composed of PC/Chol/G_{M1} (molar ratio, 10:5:1) with an average diameter of 199(55) nm and an antibody/lipid weight ratio of 1:20 were repeatedly injected i.v. into mice every other day at 0.2 mg lipid per mouse. The biodistribution of liposomes was examined at 15 min after injection and was expressed as the percentage of the injected dose detected in the lung, blood, spleen, and liver.

[b] Data are expressed as mean values (SD), n = 3.

liposomes was 81% and 85%, respectively. The difference in the total recovery of ^{111}In versus ^{3}H label was accounted for mainly by the lower levels of ^{3}H label detected in the spleen and liver. These data indicate rapid intracellular metabolism of liposomal [^{3}H]-dpFUdR in these organs. On the other hand, in the case of 34A-liposomes, only a slightly lower level (41%) of ^{3}H label than of ^{111}In label (48%) remained in the lung at 5 h after injection, suggesting that once bound to the endothelial cells, liposomal dpFUdR is metabolized more slowly there than in the spleen and liver, where it is mainly taken up by macrophages.

To examine the residence time in the lung of dpFUdR delivered by incorporation into 34A-liposomes, the level of ^{111}In-labeled 34A-liposomes containing [^{3}H]-dpFUdR was examined at different intervals after injection (Fig. 4). The level of 34A-liposomes in the lung decreased slowly with a half-life of approximately 72 h. The drug level in the lung decreased relatively rapidly with a half-life of approximately 12 h. The observed decrease in the drug level in the lung was presumably due to intracellular metabolism of the drug. Thin-layer chromatographic analyses of the drug in $CHCl_3$ extract showed that approximately 55% and 20% of the ^{3}H-labeled drug detected in the lung was the 3′/5′-monopalmitoyl form and the fully hydrolyzed form, respectively (data not shown), suggesting that the prodrug was in fact activated in the lung.

Effect of injection frequency on target binding of 34A-liposomes

For an effective tumor therapy protocol, multiple treatments with drug-containing immunoliposomes would be desirable. We thus studied the effect of injection frequency on the level of lung accumulation of 34A-liposomes. ^{111}In-labeled 34A-liposomes with an antibody/lipid weight ratio of 1:20 were injected into mice every other day, and the biodistribution was determined at 15 min after each injection. As shown in Table 2, the first two injections of 34A-liposomes showed high levels of lung accumulation (44% and 41% of the total injected dose of liposomes, respectively). However, subsequent (the third through the sixth) injections showed a progressive decrease in lung accumulation of 34A-liposomes with a concomitant increase in liver accumulation. At the sixth injection, only a small fraction (1.6%) of 34A-liposomes accumulated in the lung, whereas the majority (83%) were found in the liver. It should be noted that 34A-liposomes used for each injection showed high levels of lung accumulation in mice that had received no previous injection with 34A-liposomes (data not shown). The level of 34A-liposome accumulation in the spleen was relatively constant upon repeated injections.

Effect of dpFUdR incorporated into 34A-liposomes on the survival time of mice bearing lung colonies

To evaluate the therapeutic potential of dpFUdR incorporated into 34A-liposomes, an experimental mouse lung-metastasis model was used. Upon i.v. injection of EMT-6 tumor cells, the lung eventually (15–20 days) becomes nearly a solid tumor, depending on the cell dose. The mice die mainly of tumor burden or thoracic hemorrhage within a reasonably short period (20–30 days), thus providing a useful model to assess the effectiveness of lung tumor therapy. A group of mice received i.v. injections of dpFUdR dispersed in emulsion or dpFUdR incorporated into bare or 34A-liposomes at a dose of 2 mg/kg dpFUdR. This dose was selected on the basis of our previous finding that dpFUdR is toxic in doses far lower than those used for the parent drug

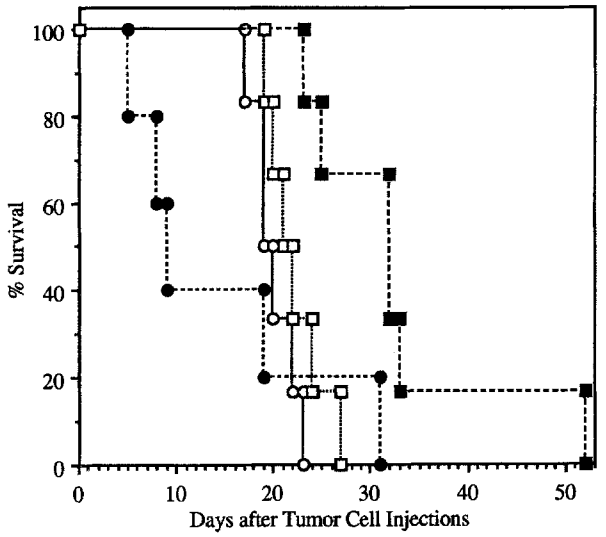


Fig. 5 Survival curves generated for BALB/c mice bearing EMT-6 lung metastases and treated with dpFUdR incorporated in liposomes. Lung metastases were induced by i.v. injection of 5×10^3 EMT-6 tumor cells. Mice were randomized into the following treatment groups (5–6 mice/group): ○, PBS (6 mice); ●, dpFUdR in emulsion (5 mice); □, dpFUdR incorporated into bare liposomes with an average diameter of 191 nm (6 mice); and ■, dpFUdR incorporated into 34A-liposomes with an average diameter of 203 nm and an antibody/lipid weight ratio of 1:17 (6 mice). All dpFUdR formulations were given at a dose of 2 mg/kg dpFUdR and 64 mg/kg lipid for bare and 34A-liposomes, respectively. All mice received treatments twice on days 1 and 3 after the injection of tumor cells. A significant increase in MST was observed in the group of mice treated with dpFUdR incorporated into 34A-liposomes (MST, 33 days) as compared with the groups of mice treated with PBS (MST, 20 days; $P < 0.016$), dpFUdR in emulsion (MST, 14 days; $P < 0.015$), or dpFUdR incorporated into bare liposomes (MST, 22 days; $P < 0.020$).

(~7 mg/kg dpFUdR versus 150 mg/kg FUdR) [33]. In addition, on the basis of the data shown in Table 2, mice were treated with different dpFUdR formulations twice on days 1 and 3 after tumor cell inoculation. Biodistribution studies confirmed the lung targetability of 34A-liposomes containing dpFUdR used in this study, with the level of lung accumulation being 49% at 2 h after injection, whereas the level of bare liposomes in the lung was only 2.7% as determined by analysis of a lipid marker, [^{111}In]-DTPA-SA. Figure 5 shows the results of a single experiment.

The median survival time (MST) of the control mice treated with PBS was 20 days. A significant increase ($P < 0.016$) in MST (33 days) was observed when mice were treated with dpFUdR incorporated into 34A-liposomes, with the T/C value being 165%. On the other hand, treatments with dpFUdR either dispersed in emulsion or incorporated into bare liposomes did not show any significant therapeutic effect (MST, 14 and 22 days, respectively). In a separate set of experiments, treatments with dpFUdR incorporated into 14-liposomes or equimolar amounts of free FUdR did not show any significant therapeutic effect (data not shown).

Discussion

We have previously reported the characterization of lipophilic prodrug-containing targeted immunoliposomes with a reduced affinity for the reticuloendothelial system (RES) [19]. These studies showed that lipophilic prodrugs can be incorporated into 34A-liposomes without affecting their ability to accumulate in the lung. The potential use of 34A-liposomes for delivery of a lipophilic prodrug to tumor-bearing lung was examined in this study using an experimental mouse lung-metastasis model. Lung metastases of EMT-6 tumor cells have been the model used by a number of investigators [27]. Upon i.v. injections of EMT-6 tumor cells, a large fraction of cells lodge in the lung. After 5–7 days, tumor nodules are well established. The tumors do not appear to form isolated surface colonies but instead seem to grow surrounding medium-sized vessels. This metastasis model thus provides a model to examine the concept of organ-specific immunotargeting of liposomes containing antitumor drugs.

One important observation shown in Fig. 1 is that the tumors utilize preexisting normal blood vessels of the lung for their site of growth. The tumor mass itself is poorly vascularized but is separated from the intravascular compartment only by the endothelium. Targeting of immunoliposomes to sites localized outside the vascular compartment, however, is limited due to the inability of immunoliposomes to cross the continuous endothelial-cell lining of the vascular wall. Therefore, direct targeting of liposomes to EMT-6 tumor masses in the lung with a tumor-specific mAb, even if available, would be difficult. Also shown in Fig. 1 is that normal capillaries of the lung are fully and uniformly labeled with radiolabeled mAb 34A. This clearly illustrates the feasibility of organ-specific immunotargeting to the sites where the site of growing tumor is within small diffusion distances.

We have examined several lipophilic antitumor prodrugs for their suitability in the organ-specific delivery system [19]. An advantage of using lipophilic drugs for liposomal formulations is their high degree of incorporation and retention into liposomes due to their hydrophobic properties [34]. Furthermore, organ-specific immunoliposomes are not designed to deliver the drugs to the tumor cells directly; rather, the drugs are required to diffuse from bound liposomes to the tumor cells. In this regard, lipophilic drugs are likely to diffuse more efficiently across the cell membrane.

In the present study, dpFUdR, a lipophilic prodrug of FUdR, was chosen to be delivered to the lung by 34A-liposomes. dpFUdR was originally developed by Nishizawa et al. [34]. Liposomal formulations of dpFUdR were introduced by Schwendener and co-workers [21, 35]. They examined the antitumor activities of dpFUdR incorporated into small unilamellar liposomes using several mouse tumor models. These studies show that although the antitumor activity of dpFUdR incorporated into liposomes is higher than that of the parent drug, a decreased therapeutic index of the drug results from the increased toxicity to the host [21, 36]. More recently, we reported that modifications of lipid

compositions, drug/lipid ratios, and particle sizes of liposomes failed to improve the therapeutic index of dpFUdR incorporated into liposomes [37, 38]. The increased toxicity of dpFUdR incorporated into liposomes was believed to be due to the predominant localization of the prodrug in the liver macrophages and the subsequent release from those cells of the parent drug FUdR into the blood as a result of intracellular hydrolysis of dpFUdR [37, 38]. In these studies, however, we used conventional liposomes, i.e., antibody-free liposomes, as a carrier of dpFUdR and, thus, the majority of the delivered liposomal dpFUdR accumulated in the liver due to the high affinity of conventional liposomes for this organ.

Since 34A-liposomes with a reduced affinity to the RES are shown to deliver drugs to the lung and to reduce drug accumulation in the liver macrophages, the use of 34A-liposomes as carriers for dpFUdR is expected to lead to an improved therapeutic index of the drug together with reduced toxicity in the therapy of lung tumors. The toxicity of the delivered drug to the target organ may be a matter of concern in organ-specific drug targeting. In vitro cytotoxicity assay showed a comparable cytotoxic effect of liposomal dpFUdR on NMLC as compared with EMT-6 tumor cells (Fig. 3). However, these NMLC are rapidly growing cells in vitro and, thus, the observed toxic effect of liposomal dpFUdR on NMLC may not directly reflect the actual toxicity to the lung in vivo. More experiments are needed to assess the toxicity of immunotargeted liposomal dpFUdR to the lung.

Efficient delivery of dpFUdR incorporated into 34A-liposomes to the lung was demonstrated by direct analysis of [^{3}H]-dpFUdR (Table 1). Under the conditions used in the present study, approximately 50% of the total injected dose of liposomal dpFUdR accumulated in the lung at 2 h after injection, which paralleled the level of liposome accumulation (Table 1). Previous studies have shown that the half-life of residence in the lung is ~5 h for 34A-liposomes composed of PC/Chol [13]. Inclusion of G_{M1} in the lipid composition was later shown to increase the retention time of 34A-liposomes in the lung, presumably due to a lower affinity of G_{M1}-containing liposomes to the circulating macrophages [16]. Thus, tumor cells in the lung are expected to be exposed to a relatively high prodrug concentration for a prolonged period when the drug is delivered by G_{M1}-containing 34A-liposomes.

Upon binding of immunoliposomes to the target organ, it is expected that lipophilic prodrugs are released and/or transfered from bound liposomes to the cell membranes, from which they diffuse across the endothelial barrier to the nearby tumor cells, where the prodrug is metabolized to form the active drug. We have previously observed that dpFUdR incorporated into liposomes undergoes rapid intracellular metabolism in the spleen and liver [38]. These findings are consistent with the data listed in Table 1, showing substantially lower levels of ^{3}H label in the spleen and liver than of ^{111}In label for bare liposomes at 5 h after injection. Since the lipid marker used in this study, [^{111}In]-DTPA-SA, is not readily metabolized [20], lower levels of [^{3}H]-dpFUdR in the spleen and liver probably result from intracellular metabolism of the prodrug and release of label in these organs, in line with earlier observations [33]. In contrast, an appreciable amount of [^{3}H]-dpFUdR incorporated into 34A-liposomes remains in the lung at 5 h after injection (Table 1), with the residence half-life being approximately 12 h (Fig. 4), compatible with the notion that these liposomes are mainly extracellularly bound. Thus, the release and/or transfer of the drug from bound immunoliposomes to cell membranes could be the rate-limiting step for intracellular metabolism of the prodrug in this model.

Data in Table 2 show that repeated injections of 34A-liposomes resulted in less efficient target binding. One potential mechanism of reduced target binding of subsequently injected immunoliposomes is that an immune response induced by repeated injections might prevent immunoliposomes from reaching the target. This mechanism is consistent with the observation that increasing amounts of 34A-liposomes were found in the liver upon repeated injections (Table 2), presumably due to Fc receptor-mediated uptake of 34A-liposomes by this organ [32]. The use of immunosuppressive agents may be effective in reducing such an immune response to ensure efficient target binding of repeatedly injected immunoliposomes.

Survival studies showed that dpFUdR was effective in prolonging the survival of mice only when it was incorporated into 34A-liposomes (Fig. 5). Therapeutic effects of dpFUdR incorporated into 34A-liposomes were obtained after only two injections with a relatively low dose of the drug (2 mg/kg). It should be noted that the experiment utilized a suboptimal treatment protocol with a limited number of animals. Due to the limited availability of the antibody reagent, we could not perform experiments with a larger number of mice and with larger doses of the drug. Previously, we found the maximal tolerated dose of dpFUdR incorporated into conventional liposomes to be ~10 μmol (7 mg)/kg [38]. Because of the high specificity of 34A-liposomes to the target organ, one would expect that treatments with increasing doses of the drug would show more pronounced therapeutic effects without inducing any severe toxicity in the host. This mAb has also been used in therapy studies as a direct ^{131}I conjugate with limited success [39].

Ahmad and co-workers [40, 41] have recently reported that doxorubicin-containing liposomes conjugated to tumor-specific mAbs are effective in prolonging the survival of mice bearing spontaneous lung metastases with a squamous-cell carcinoma. Although the liposomes used in these studies were designed to be tumor-specific, our present strategy is to target liposomes to the lung itself. A potential advantage of using organ-specific immunoliposomes for tumor therapy is their versatility. Since organ-specific immunoliposomes are designed to deliver the drug to normal cells, not to tumor cells, this drug-delivery system should be theoretically effective in treating many different types of tumor cells, thus providing a general delivery system for any tumor in the target organ. The major advantage remains the high dose of drug delivered near the tumor site. Therapy in humans will require increased targeting efficiency over what is

presently achievable; thus, organ-specific, efficient drug delivery to endothelial sites may have advantages in patients.

Acknowledgement This work was supported by NIH grant CA 24553 (L.H.).

References

1. Brodsky FM (1988) Monoclonal antibodies as magic bullets. Pharm Res 5: 1
2. Rowlinson-Busza G, Epenetos AA (1992) Targeted delivery of biologic and other antineoplastic agents. Curr Opin Oncol 4: 1142
3. Kosmas C, Linardou H, Epenetos AA (1993) Review: advances in monoclonal antibody tumour targeting. J Drug Targeting 1: 81
4. Jain RK (1990) Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metastasis Rev 9: 253
5. Rorvik MC, Allison DP, Hotchkiss JA, Witschi HP, Kennel SJ (1988) Antibodies to mouse lung capillary endothelium. J Histochem Cytochem 36: 741
6. Ford VA, Stringer C, Kennel SJ (1992) Thrombomodulin is preferentially expressed in BALB/c lung microvessels. J Biol Chem 267: 5446
7. Kennel SJ, Lankford T, Hughes B, Hotchkiss JA (1988) Quantitation of a murine lung endothelial cell protein, P112, with a double monoclonal antibody assay. Lab Invest 59: 692
8. Kennel SJ, Lee R, Bultman S, Kabalka G (1990) Rat monoclonal antibody distribution in mice: an epitope inside the lung vascular space mediated very efficient localization. Nucl Med Biol 17: 193
9. Wright S, Huang L (1989) Antibody-directed liposomes as drug-delivery vehicles. Adv Drug Deliv Rev 3: 343
10. Ostro MJ (ed) (1987) Liposomes: from biophysics to therapeutics. Marcel Dekker, New York
11. Hughes BJ, Kennel S, Lee R, Huang L (1989) Monoclonal antibody targeting of liposomes to mouse lung in vivo. Cancer Res 49: 6214
12. Holmberg E, Maruyama K, Litzinger DC, Wright S, Davis M, Kabalka GW, Kennel SJ, Huang L (1989) Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. Biochem Biophys Res Commun 165: 1272
13. Maruyama K, Holmberg E, Kennel SJ, Klibanov A, Torchilin VP, Huang L (1990) Characterization of in vivo immunoliposome targeting to pulmonary endothelium. J Pharm Sci 79: 978
14. Holmberg E, Maruyama K, Kennel S, Klibanov A, Torchilin V, Ryan U, Huang L (1990) Target-specific binding of immunoliposomes in vivo. J Liposome Res 1: 393
15. Mori A, Liu D, Huang L (1992) Novel immunoliposome targeting system avoiding reticuloendothelial cell. In: Junginger HE (ed) Drug targeting and delivery: concepts in dosage form design. Ellis Horwood, New York, p 26
16. Maruyama K, Kennel SJ, Huang L (1990) Lipid composition is important for highly efficient target binding and retention of immunoliposomes. Proc Natl Acad Sci USA 87: 5744
17. Mori A, Klibanov AL, Torchilin VP, Huang L (1991) Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside G_{M1} on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. FEBS Lett 284: 263
18. Litzinger DC, Huang L (1992) Biodistribution and immunotargetability of ganglioside-stabilized dioleoylphosphatidylethanolamine liposomes. Biochim Biophys Acta 1104: 179
19. Mori A, Kennel SJ, Huang L (1993) Immunotargeting of liposomes containing lipophilic antitumor prodrugs. Pharm Res 10: 507
20. Kabalka G, Buonocore E, Hubner K, Moss T, Norley N, Huang L (1987) Gadolinium-labeled liposomes: targeted MR contrast agents for the liver and spleen. Radiology 163: 255
21. Schwendener RA, Supersaxo A, Rubas W, Weder HG, Hartmann HR, Schott H, Ziegler A, Hengartner H (1985) 5′-*O*-Palmitoyl- and 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine–novel lipophilic analogues of 5-fluoro-2′-deoxyuridine: synthesis, incorporation into liposomes and preliminary biological results. Biochem Biophys Res Commun 126: 660
22. Klibanov AL, Maruyama K, Torchilin VP, Huang L (1990) Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 268: 235
23. Borssum Waalkes M van, Galen M van, Morselt H, Sternberg B, Scherphof GL (1993) In vitro stability and cytostatic activity of liposomal formulations of 5-fluoro-2′-deoxyuridine and its diacylated derivatives. Biochim Biophys Acta 1148: 161
24. Kennel SJ, Foote LJ, Lankford PK (1981) Analysis of surface proteins of mouse lung carcinomas using monoclonal antibodies. Cancer Res 41: 3465
25. Kennel SJ, Foote LJ, Flynn KM (1986) Tumor antigen on benign adenomas and on murine lung carcinomas quantitated by a two-site monoclonal antibody assay. Cancer Res 46: 707
26. Rockwell SC, Kallman RF, Fajardo LF (1972) Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. J. Natl Cancer Inst 49: 735
27. Rockwell S (1977) In vivo-in vitro tumor systems: new models for studying the response of tumor to therapy. Lab Animal Sci 27: 831
28. Mori A, Huang L (1992) Immunoliposome targeting in a mouse model: optimization and therapeutic application. In: Gregoriadis G (ed) Liposome technology, 2nd edn, vol. III. CRC Press, Boca Raton, p 153
29. Osterman LA (ed) (1984) The use of radioisotopes. In: Methods of protein and nucleic acid research. Springer, New York Berlin Heidelberg, p 67
30. Wu MS, Robbins JC, Bugianesi RL, Ponpipom MM, Shen TY (1981) Modified in vivo behavior of liposomes containing synthetic glycolipids. Biochim Biophys Acta 674: 19
31. Derksen JTP, Morselt HWM, Kalicharan D, Hulstaert CE, Scherphof GL (1987) Interaction of immunoglobulin-coated liposomes with rat liver macrophages in vitro. Exp Cell Res 168: 105
32. Aragnol D, Leserman L (1986) Immune clearance of liposomes inhibited by an anti-Fc receptor antibody in vivo. Proc Natl Acad Sci USA 83: 2699
33. Borssum Waalkes M van (1992) Lipophilic derivatives of 5-fluoro-2′-deoxyuridine as liposomal anticancer agents. Thesis, Groningen University, Groningen, The Netherlands
34. Schwendener RA (1986) The preparation of large volumes of homogeneous, sterile liposomes containing various lipophilic cytotoxic drugs by the use of a capillary dialyzer. Cancer Drug Deliv 3: 123
35. Nishizawa Y, Casida JE, Anderson SW, Heidelberger C (1965) 3′,5′-Diesters of 5-fluoro-2′-deoxyuridine: synthesis and biological activity. Biochem Pharmacol 14: 1605
36. Supersaxo A, Rubas W, Hartmann H, Schott H, Hengartner H, Schwendener RA (1988) The antitumor effect of lipophilic derivatives of 5-fluoro-2′-deoxyuridine incorporated into liposomes. J Microencapsulation 5: 1
37. Borssum Waalkes M van, Scherphof GL (1990) Liposome-incorporated 3′,5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine as a slow-release anti-tumor drug depot in rat liver macrophages. Select Cancer Ther 6: 15
38. Borssum Waalkes M van, Fichtner I, Dontje BHJ, Lemm M, Becker M, Arndt D, Scherphof GL (1992) In vivo distribution and antitumor activity of liposomal 3′, 5′-*O*-dipalmitoyl-5-fluoro-2′-deoxyuridine. J Microencapsulation 9: 335
39. Blumenthal RD, Sharkey RM, Haywood L, Natale AM, Wong GY, Siegel JA, Kennel SJ, Goldenberg DM (1992) Targeted therapy of athymic mice bearing GW-39 human colonic cancer micrometastases with ^{131}I-labeled monoclonal antibody. Cancer Res 52: 6036
40. Ahmad I, Allen TM (1992) Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. Cancer Res 52: 4817
41. Ahmad I, Longenecker M, Samuel J, Allen TM (1993) Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. Cancer Res 53: 1484