Liposome-Mediated Therapy of Intracranial Brain Tumors in a Rat Model

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Purpose. Malignant brain tumors represent a serious therapeutic challenge, and survival often is low. We investigated the delivery of doxorubicin (DXR) to rat brain tumors *in situ via* liposomes, to test the hypothesis that intact liposomes undergo deposition in intracranial tumor through a compromised blood-tumor vasculature. Both therapeutic effect and intra-tumor drug carrier distribution were evaluated to identify variables in carrier-mediated delivery having impact on therapy.

Methods. The rat 9L gliosarcoma tumor was implanted orthotopically in Fischer 344 rats in the caudate-putamen region. The tumor-bearing rats were treated with DXR, either free or encapsulated in long-circulating, sterically-stabilized liposomes. Anti-tumor efficacy was assessed by survival time. In parallel, liposomes labeled with a fluorescent phospholipid analog were injected into tumor-bearing rats. At predetermined intervals, the brains were perfused with fixative, sectioned, and imaged with laser scanning confocal microscope (LSCM) to investigate the integrity of the tumor vascular bed and the intratumor deposition of liposomes.

Results. Free DXR given in 3 weekly iv injections was ineffective in increasing the life span of tumor-bearing rats at cumulative doses $\leq$ 17 mg/kg, and at the highest dose (17 mg/kg) decreased survival slightly, compared to saline-treated controls. In contrast, DXR encapsulated in long-circulating liposomes mediated significant increases in life span at 17 mg/kg. Rats showed a 29% percent increase in median survival, respectively, compared to saline-control animals. The delay of treatment after tumor implantation was a major determinant of therapeutic effect. Fluorescent liposomes were deposited preferentially in tumor rather than normal brain, and were distributed non-uniformly, in close proximity to tumor blood vessels.

Conclusions. Liposomes can be used to enhance delivery of drugs to brain tumors and increase therapeutic effect. The therapeutic effect may arise from release of drug from liposomes extravasated in discrete regions of the tumor vasculature and the extravascular space.

KEY WORDS: brain tumor; liposomes; drug delivery; doxorubicin; confocal fluorescence microscopy.

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ABBREVIATIONS: BBB, blood-brain barrier; Chol, cholesterol; DXR, doxorubicin; DSPC, distearoyl phosphatidylcholine; FBS, fetal bovine serum; IC_{50}, concentration for 50% growth inhibition; PEG-DSPE, polyethylene glycol (1900) conjugated to distearoyl phosphatidylethanolamine; Rh-DPPE, N-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt.

INTRODUCTION

Malignant brain tumors often represent a difficult therapeutic challenge. Among children, the incidence of brain tumors is second only to acute lymphoblastic leukemia (1), and they are more likely to be fatal. Forty to 60% of all primary brain tumors are gliomas (2). *Glioblastoma multiforme* is one of the most common forms of glioma, and is highly lethal; median survival after diagnosis averages approximately 12 months (3). Conventional treatment includes surgery, radiotherapy and chemotherapy. Because surgery of most malignant brain tumors serves primarily for debulking the tumor, and often is not curative, radiation therapy and chemotherapy are employed for removal of residual microscopic tumor (4). For children, chemotherapy is recommended as the first line of therapy, since radiation therapy can cause developmental delays. However, chemotherapy is of limited effectiveness because many drugs do not reach therapeutic concentrations in the brain tumor, either due to limited lipid solubility and/or active drug efflux through the blood-brain barrier (BBB) mediated by P-glycoprotein (5). Increased delivery of chemotherapeutic agents to tumors could improve survival of patients with malignant brain tumors.

Liposomes are versatile drug delivery vehicles that have proven to be useful in reducing toxicity and enhancing the activity of a variety of pharmacologically active agents, including antineoplastic drugs. Liposomes having extended blood circulation time have been devised, and these are composed of high phase-transition (T_m) lipids, high cholesterol content, and a component such as phosphatidyl inositol, monosialoganglioside (GM_1), or synthetic phospholipid bearing a polyethylene glycol (PEG) headgroup, which provides a steric barrier against plasma protein access to the liposome surface. Such liposomes have a slower clearance from the plasma compartment after intravenous injection and a longer circulation half-life, and show increased deposition in tumor tissue (6–9). Small ($\leq$0.08 μm diameter), long-circulating liposomes have been shown to extravasate in various models of solid tumors, apparently through leaky tumor vasculature (10,11).

Some intracranial tumors such as *Glioblastoma multiforme* may be highly vascularized, with leaky vasculature, and therefore may be accessible to blood-borne contrast agents or radioactive tracers (12–16). Such tumors may be amenable to liposome-enhanced drug delivery, provided liposomes can breach the compromised vascular barrier. In the present work, our objective was to test the hypothesis that the integrity of the blood:brain tumor barrier may be compromised in an orthotopic model for an invasive brain tumor, and that small (<100 nm), long-circulating liposomes may accumulate into such tumors, thereby leading to enhanced drug deposition and therapeutic effect. We employed the 9L model, an invasive rat brain tumor that has been used extensively as a model of malignant *Glioblastoma multiforme*. The 9L tumor has many characteristics of *Glioblastoma* (15), and also expresses P-glycoprotein (17), which is implicated as a mechanism by which tumor cells reduce the intracellular concentration of agents such as DXR. 9L has been used previously in developing chemo- and radiotherapeutic treatments (15,18,19). Under the conditions used here, we have observed previously the uptake of impermeant blood-borne tracers into 9L tumors (16).

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Laser scanning confocal microscopy (LSCM) was used to seek morphological evidence of extravasation of fluorescent liposomes into rat brain tumors *in situ*, and anti-tumor experiments were undertaken to examine the therapeutic effect of DXR encapsulated in long-circulating liposomes. DXR was used as a model drug, since it is not known to accumulate in brain, perhaps due to active efflux by the MDR1 P-glycoprotein present in the BBB (5,20). We have also compared the dependence of therapeutic effect on tumor bulk and development time.

MATERIALS AND METHODS

Materials

Doxorubicin HCl was obtained from Cetus (Emeryville, CA), and phospholipids, including polyethylene glycol conjugated to dipalmitoylphosphatidylethanolamine (PEG-DPPE) or to distearoylphosphatidylethanolamine (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent phospholipid analog N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh-DPPE) was from Molecular Probes (Eugene, OR). Cholesterol was purchased from Sigma and recrystallized three times from methanol. The 9L gliosarcoma cell line, designated 9L-72, was obtained from D. Deen, University of California at San Francisco, and was maintained in vitro in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum.

Preparation of Fluorescent Liposomes

Liposomes were composed of phosphatidylcholine:cholesterol: PEG-DPPE (PC: Chol:PEG-DPPE 9:5:1 molar ratio). Rhodamine-DPPE was included at 1 mole%. Lipids were initially mixed in chloroform, and a thin film of lipid was produced by evaporation of solvent. The lipids were hydrated in a buffer consisting of NaCl (145 mM), Tris[Hydroxymethyl]-2-aminoethane-sulfonic acid (TES: 10 mM), and ethylenediamine tetraacetate (EDTA: 0.1 mM) buffer, pH 7.2. The liposomes were extruded several times through 0.08 μm polycarbonate filters.

Preparation of Doxorubicin-containing Liposomes

DXR liposomes composed of distearoylphosphatidylcholine (DSPC):Chol:PEG-DSPE (9:5:1 mole ratio) were prepared using a "remote loading" (21) method adapted from (22). Remote loading results in the encapsulation of high concentrations of DXR within the liposome aqueous core. Briefly, a thin film of lipids was hydrated in ammonium sulfate (250 mM, pH 5.5). The lipid suspension was extruded through 0.08 μm polycarbonate filters at 60°C and dialyzed overnight against isotonic sucrose to remove free ammonium sulfate. Doxorubicin was hydrated in 10% (w/v) sucrose and incubated with the preformed liposomes for 1 hour at 65°C. The preparation was dialyzed against isotonic sucrose to remove the minor residual fraction of unencapsulated DXR. This method yielded encapsulation efficiencies of ≥90% of the initial DXR. For a typical preparation, 4.4 μmoles of DXR were encapsulated in 20 μmoles of phospholipid, yielding a drug:lipid ratio of 0.22 (mole:mole).

Cytostatic Activity of Free and Liposomal DXR *In Vitro*

9L cells were plated at a density of 2×10^4 cells in 24-well plates (Costar) and allowed to adhere overnight. Wells in triplicate were treated with different concentrations of free- or liposomal DXR diluted in isotonic saline or sucrose, respectively. Control wells were treated in triplicate with drug-free vehicle. In some control experiments liposomes containing no drug were tested at concentrations of 0.2 mM phospholipid. The cells were allowed to grow at 37°C in an atmosphere containing 5% CO_2. After 72 hours, cells were trypsinized and counted using a Coulter Counter (Hialeah, FL), and the drug concentration inhibiting cell growth by 50% (IC_{50}) was calculated graphically.

Uptake of Fluorescent Liposomes by 9L Cells *In Vitro*

9L cells were plated on sterile glass coverslips in complete growth medium, and allowed to adhere overnight. Prior to the addition of drug or liposomes, the growth medium was replaced with serum-free medium, and 50–100 nmoles of fluorescent liposomes were added. After 24 hr, cells were washed free of unbound liposomes with ice-cold phosphate-buffered saline and imaged using an Olympus laser scanning confocal microscope (23).

Orthotopic Implantation of Brain Tumors

Male Fischer 344 rats weighing 130–160 gm (7–8 week old, Harlan Sprague-Dawley, Indianapolis IN) were anesthetized by intramuscular injection with 66.7 mg/kg Ketamine and 6.7 mg/kg Xylazine. The incision site was shaved and the head secured in a stereotaxic frame (Kopf, Tajunga, CA). The scalp was opened and a burr hole was drilled 1.5 mm anterior and 2.4 mm lateral to Bregma. Four $\times 10^4$ 9L cells in 4 μL RPMI 1640 media (buffered with 20 mM HEPES (N(2)hydroxyethyl-piperazine-N′-[2 ethanesulfonic acid])) were injected slowly into the caudate/putamen through a sharp 26-gauge Hamilton syringe at a depth of 4.5 mm below the surface of the brain. After injection, the burr hole was covered with GelFoam absorbable gelatin foam (Upjohn, MI), and the scalp closed. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and were approved in advance. The research adhered to the "Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985)".

Biodistribution of Liposomes in Brain Tumor

Fluorescent liposomes were administered to rats *via* tail vein injection 14 or 21 days after tumor implantation. The total lipid dose administered was 16 μmole. At 4, 24 and 48 h post-injection, rats were anesthetized with Ketamine/Xylazine and perfused with 250–300 mL normal saline containing 5 U/mL heparin, and then with 250–300 mL zinc-containing isotonic aldehyde fixative (Z-fix, Anatech). Brain, liver, spleen and any extracranial tumor were harvested. After flash freezing in liquid propane, the tissues were sectioned on a cryostat (Reichert Jung) to 10–60 μm thickness, depending on the experimental objectives, and the sections were examined by laser scanning confocal microscopy. Some sections were stained subsequently with hematoxylin and eosin to observe landmarks.

Therapy of 9L Tumors with DXR-Containing Liposomes

Rats bearing 9L brain tumors were treated by tail vein injection of free- or liposome-encapsulated DXR. The highest single dose of liposomal phospholipid administered in any therapeutic study was approximately 12 μmoles (to achieve 17 mg/kg), which is not toxic in the absence of encapsulated drug. Free DXR was administered in saline, and a control group was administered saline alone. Animals were observed for signs of increased intracranial pressure due to growth of tumor (eg. seizures, paralysis in contralateral limbs, pigmentation around eyes, mouth and nose), and were weighed every 2 days to detect toxicity of treatment. Moribund animals were sacrificed, and the death was recorded as if it occurred on the following day. The percent change in median survival of the treated groups was compared to that of the control group using the Cox-Mantel test, as implemented in the program 'Solid Tumor' (24).

RESULTS

Cytostatic Activity of Free and Liposomal DXR

The cytostatic activity of free and liposome-encapsulated DXR was evaluated against 9L tumor cells in order to compare potency in a simple *in vitro* system. DXR in PEG-containing liposomes was at least 100 fold less potent than free DXR (Figure 1); the $IC_{50} \pm SD$ (the concentration required for inhibition of cell growth by 50%) of L-DXR was 700 ± 100 ng/mL, while the $IC_{50} \pm SD$ of free DXR was 5 ± 1 ng/mL. The highest concentration of liposome phospholipid added to cells was 0.1 mM. Control liposomes lacking drug, which were prepared in the same manner as DXR-containing liposomes, were not cytostatic at a concentration of 0.2 mM phospholipid (data not shown).

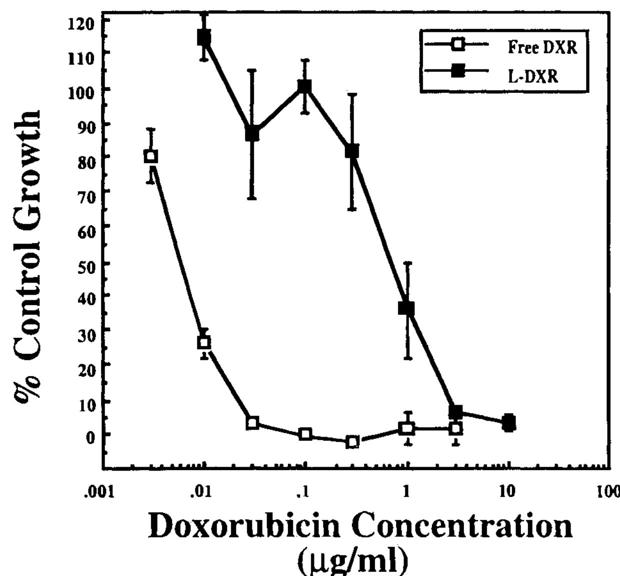

Fig. 1. Growth inhibition of 9L gliosarcoma cells by free and encapsulated doxorubicin. Cells in culture were incubated in the presence of varying concentrations of doxorubicin (DXR); open squares: free DXR; filled squares: DXR encapsulated in liposomes of distearoylphosphatidylcholine: cholesterol:polyethylene glycol-distearoylphosphatidylethanolamine (DSPC:Chol:PEG-DSPE, 9:5:1 mole ratio).

Uptake of Fluorescent Liposomes by 9L Cells *In Vitro*

The large differences in the potency of free and liposomal DXR *in vitro* may arise from inefficient interaction of liposomes with 9L cells. Therefore, we investigated directly the cellular uptake of liposomes using fluorescence microscopy. PEG-PE-containing liposomes labeled with the fluorescent phospholipid Rhodamine-DPPE were incubated with 9L cells for 24 hr, and imaged after washing to remove unbound liposomes. Fluorescent liposomes of DSPC:Chol (1:1) were investigated for comparison, because of previous mechanistic studies on the interaction of similar liposomes with cells (25,26). Figure 2a shows that relatively little fluorescence was associated with 9L cells when PEG-PE-containing liposomes were used, and suggests that uptake by 9L cells is as low as that observed for other tumor cell types (7,8,27). Confocal microscopy, with the ability to provide 3-dimensional reconstructions of objects *via* serial optical sections, suggested that the majority of cell-associated liposomes were intracellular (analysis not shown).

Figure 2b shows that uptake of liposomes by 9L cells was greater in the absence of the PEG polymer surface coating.

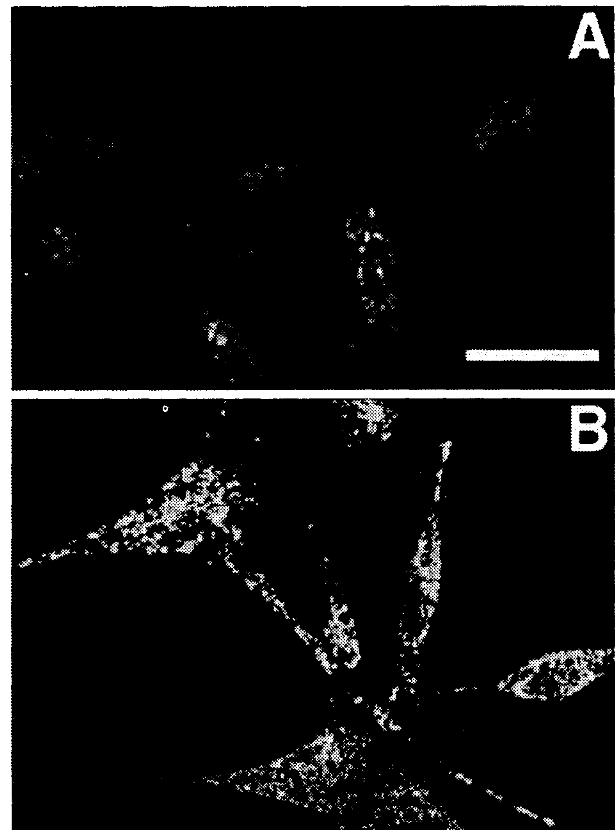

Fig. 2. Uptake of fluorescent liposomes by 9L gliosarcoma cells *in vitro*. Cells in culture were incubated overnight with 50-100 nmoles of liposomes that contained 1 mole% of the fluorescent phospholipid analog Rhodamine-DPPE (N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethamolamine), washed free of unbound liposomes, and imaged by laser scanning confocal microscopy, using wavelengths appropriate for the fluorescent dyes. (A) Cells incubated with DSPC:Chol:PEG-DSPE (9:5:1 mole ratio) liposomes; (B) Cells incubated with DSPC:Chol (1:1 mole ratio) liposomes. Bar: 50 μm.

Although DSPC:Chol (1:1) liposomes have comparatively low electrostatic charge and are taken up less efficiently than charged liposomes (25,26,28), DSPC:Chol liposomes were taken up by 9L cells more readily and in higher amounts than were PEG-PE-containing liposomes. Figure 2b shows extensive uptake of liposomes into the endocytic apparatus of 9L cells.

Delivery of Fluorescent Liposomes to 9L Tumors and Normal Brain

In order to investigate the deposition of liposome-associated materials in normal brain and in the 9L tumor, liposomes were trace-labeled with fluorescent phospholipids (Rh-DPPE), injected intravenously in tumor-bearing rats, and frozen sections from brain and other organs were examined by laser scanning confocal microscopy. Figure 3a shows non-uniform, focal deposition of fluorescence distributed sporadically around vessels within the tumor 24 h after injection. Regions of normal brain in close proximity to the tumor were virtually devoid of fluorescence. Confocal imaging allowed optical sectioning of tissue to a depth of 20 μm. Stereo projections of 3-D image reconstructions showed clear examples of intense fluorescence accretions lining tumor capillaries or blood vessels (Figure 3b).

Fluorescent label deposition in tumor was higher at 24 hr after administration than it was at 4 or 48 hr after injection (data not shown). At 48 hr, fluorescence was distributed relatively uniformly throughout some regions of tumor, indicating the probable breakdown of liposomes and the metabolism of the fluorescent label (data not shown).

Antitumor Activity of Liposomal DXR

The therapeutic effect of liposomal DXR (L-DXR) on advanced 9L tumors was investigated by initiating treatment 11 days after tumor implantation. Animals received three weekly injections of saline (control), or free and liposomal DXR at a cumulative dose of 15 mg/kg.

The median survival for the saline-treated control group was 25.5 days (Table I). The median day of death for the group receiving 15 mg/kg drug in the form of L-DXR was day 27, slightly later (~6%) than that of saline-treated controls. Free DXR at a cumulative dose of 15 mg/kg was toxic to animals, and was not effective in controlling tumor growth. The median day of death for the free drug group was day 24 (Table I), and was 97% of control survival, suggesting that drug toxicity may have hastened death.

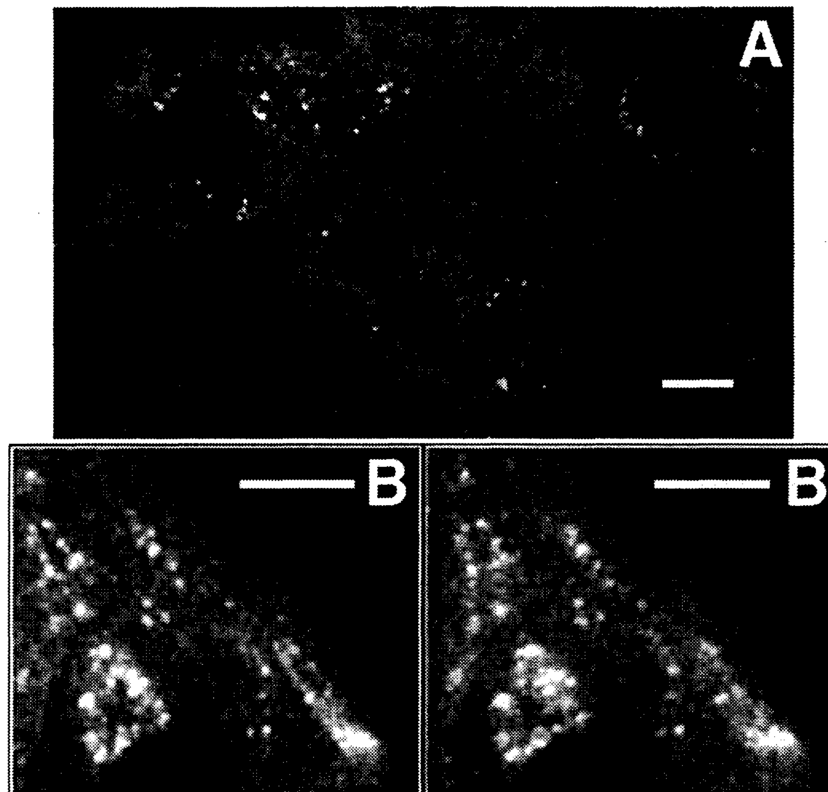

Fig. 3. *In vivo* deposition of fluorescent liposomes in 9L brain tumors. 9L tumor cells were implanted stereotaxically in rat brains as described in *Methods*. After 14 days, rats were injected intravenously with liposomes of DSPC:Chol:PEG-DSPE (9:5:1 mole ratio) that contained 1 mole% of the fluorescent phospholipid analog Rhodamine-DPPE and sacrificed 24 hours later. Frozen sections (5 μm thickness) were prepared, and optical sections were acquired at 0.5 μm intervals along the Z-axis using a laser scanning confocal microscope. (A) Representative optical slice through tumor. Bar: 20 μm. (B) Stereo projection prepared from 13 sequential optical sections. Note the accretions of punctate fluorescence near and around blood vessels. Bar: 20 μm.

Table I. Survival of Rats with Late-Stage Tumor (Day 11)

Treatment groups	Dose[a] (mg/kg)	Rats per group	Median days survival (Range)	% IMLS[b]	p value[c]
L-DXR	15	8	27.0(21–30)	6	0.544
F-DXR	15	8	24.0(18–31)	−6	0.851
Control (Saline)	0	6	25.5(18–31)	—	

[a] Cumulative dose (mg/kg) given as 3 weekly iv injections.
[b] Percent Increase in Median Life Span relative to control.
[c] From statistical analysis of each group *vs.* control using Cox-Mantel analysis (no adjustment for multiple comparisons).

In order to test the significance of differences in lifespan among various groups, the survival data was subjected to statistical analysis by the Cox-Mantel test, as described in *Methods*. Unlike median day of death (MDD) comparisons, Cox-Mantel analysis compares the data from individual animals in order to calculate the statistical significance of differences among treatment groups. Cox-Mantel analysis showed that the small increase in lifespan mediated by L-DXR was not significant (p = 0.544). Similarly, free drug did not extend survival (p = 0.851) compared to saline-treated controls.

To investigate the effect of treatment on tumors of lower mass, animals were treated 7 days after implantation of 9L tumors. The 7-day 9L tumors were well-established and vascularized, as indicated by histological examination (data not shown). Free or liposome-encapsulated DXR was given at a cumulative dose of 17 mg/kg, administered i.v. as three weekly injections. When given by this treatment schedule, 17 mg/kg DXR was approximately the maximum survivable dose (data not shown). A considerable increase in survival time was observed for the group receiving L-DXR (Fig. 4), which showed a 29% increase in median survival compared to saline-treated controls (Table II). In contrast, free DXR appeared to be toxic, and the median survival was only 96% of that for the saline-treated control group (Table II). In groups treated with free or encapsulated DXR, weight loss was observed immediately following drug administration (data not shown). However, animals receiving L-DXR resumed weight increase 2–3 days post-injection. In contrast, a progressive and greater loss of weight was observed in animals receiving free DXR (data not shown).

Cox-Mantel comparison of the control- and L-DXR-treated animals showed that the increase in lifespan mediated by L-DXR at 17 mg/kg was significant at p = 0.003. In contrast, free DXR did not change survival relative to controls (p = 0.452).

The therapeutic effects of free and liposome-encapsulated DXR were also investigated at a dose approximately 30% below the maximum survived dose of 17 mg/kg, to determine whether a reduction in drug-mediated toxicity would further enhance survival. Free and encapsulated DXR were given at a cumulative dose of 12 mg/kg in 3 weekly injections. Animals treated with L-DXR showed a median survival that was 18% greater than saline-treated controls, while animals treated with free DXR showed a 2% increase in median survival compared to controls (Table II). Cox-Mantel analysis showed that the increase in lifespan mediated by L-DXR at 12 mg/kg was significant at p = 0.014. In contrast, free DXR did not extend survival relative to controls (p =0.307).

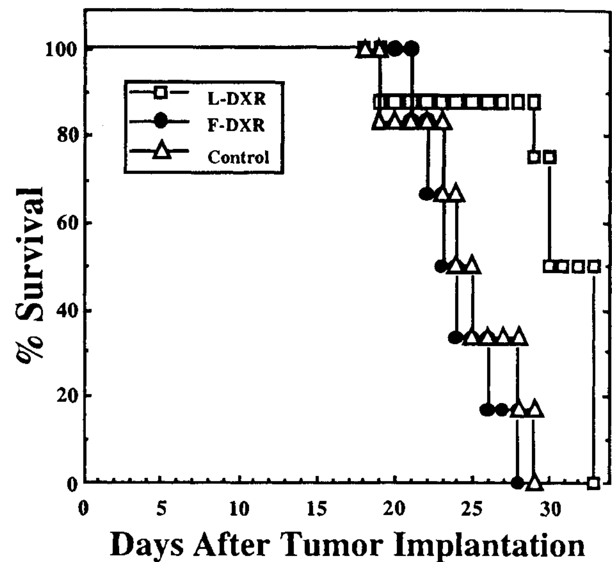

Fig. 4. Antitumor effect of free and liposome-encapsulated doxorubicin. Rats (230 gm body weight) were implanted with 9L brain tumors by stereotaxic inoculation of 4×10^4 9L cells. On days 7, 14, and 21, animals were treated by intravenous injection of free DXR (F-DXR) or liposome-encapsulated doxorubicin (L-DXR) at a dose of 17 mg/kg body weight; average weight at the initiation of treatment was 260 gm. Control animals received equivalent volumes of saline. Animals were observed daily, and the day of death was recorded. Moribund animals were sacrificed, and the death recorded as if occurring on the following day.

DISCUSSION

In both clinical cases and animal models (12–16), the blood-brain barrier in brain tumors may be disrupted, or may be more permeable than the normal vessels in brain; horseradish peroxidase, protein-bound dyes such as Evans Blue, serum proteins, and various radioactive probes have been shown to extravasate in such tumors. In spite of this increased permeabil-

Table II. Survival of Rats with Established Tumor (Day 7)

Treatment[a] groups	Dose[b] (mg/kg)	Rats per group	Median days survival (Range)	% IMLS[c]	p value[d]
L-DXR	17	8	31.5(19–33)	29	0.003
F-DXR	17	6	23.5(21–28)	−4	0.452
Control (Saline)	0	6	24.5(19–29)	—	
L-DXR	12	6	33.0(29–35)	18	0.014
F-DXR	12	7	28.5(21–35)	2	0.307
Control (Saline)	0	5	28.0(21–32)	—	

[a] The two dose levels were investigated in separate experiments.
[b] Cumulative dose (mg/kg) given as 3 weekly iv injections.
[c] Percent Increase in Median Life Span relative to control.
[d] From statistical analysis of each group *vs.* control using Cox-Mantel analysis (no adjustment for multiple comparisons).

ity, the BBB still represents a formidable barrier to drugs, and the action of drug-export proteins such as MDR1 P-glycoprotein (20) may further provide 'sanctuary' to neoplasms.

Overall, the chemotherapy of brain tumors is not very effective. Intraarterial (intracarotid) infusions, osmotic modification of the blood brain barrier, intrathecal drug administration, hyperthermia, and high-dose chemotherapy have been attempted, and are not curative. Drug delivery approaches offer considerable promise, with large increases in survival observed in the 9L tumor model, mediated by the implantation of drug-impregnated polymers (18,29). Although the overall improvement in clinical survival is limited, results appear promising, with a doubling in 6-month survival in one trial (30). Liposomes offer an alternative approach for targeted delivery of drug to brain tumors *via* the systemic circulation, and for reduced systemic drug toxicity. Several liposomal drugs are in Phase I/II clinical trials, and a liposome formulation of DXR (Doxil®) has been approved in the U.S. for use against Kaposi's Sarcoma in AIDS patients. Thus the pharmaceutical development of liposome carriers has progressed to the point that widespread clinical application of this carrier system can be envisaged, provided the appropriate rationale for treatment can be established.

A number of studies in laboratory animals have shown that liposomes do not cross the normal BBB (31,32). However, disruption of the BBB in brain tumors may permit extravasation of liposomes into the tumor interstitial space. In the present work, we have shown greater antitumor activity of liposome-encapsulated DXR, a drug which does not cross the BBB appreciably, compared to equal doses of unencapsulated drug. In parallel, we have observed deposition of liposome-borne labels in brain tumor tissue, but not in normal brain. These results are consistent with those obtained (9) with a malignant fibrous histiocytoma (T-749) brain tumor model, in which similar liposomes mediated peak DXR concentrations 15-fold greater than achieved with free DXR, along with sustained high intratumor drug concentrations. The chemical fate of the intra-tumor label is not known here; it has been shown that phospholipid labels similar to Rh-DPPE are intact (10) in the first hour after injection, and deposition of the rhodamine liposome label within tumor has been observed for up to one week (11). However, it is possible that significant processing of liposomes and fluorescent label occurs in the time frame of the present experiments (24–48 hours), just as encapsulated DXR has been shown to be metabolized (9) in this time frame. Nonetheless, the rhodamine lipid label indicates regions of deposition of liposome-borne materials, if not the fate of intact liposomes themselves.

These results suggest that liposomes can be used for relatively selective, enhanced tumor delivery of a variety of existing drugs that otherwise do not accumulate in brain tumors. We observed that most liposome-delivered fluorescent label was distributed non-uniformly within tumors, and resided at highest concentration around blood vessels. Due to the particulate nature of liposomes and the lack of lymphatic drainage in tumors (33), the liposomes would be expected to remain in close proximity to blood vessels, without significant movement across the extravascular space.

Paradoxically, *in vitro* cytostatic studies showed that the DXR-containing liposomes were 100-fold less potent than the free drug. The high phase-transition (T_m) phospholipids and cholesterol components increase the stability of the liposomes, and thereby reduce the rate at which DXR is released from the liposome aqueous core. Horowitz et al (27) have shown that longer incubation times can reverse the depressed cytostatic activity of liposomal DXR compared to free drug. Additionally, the hydrophilic PEG coating reduces *in vitro* interaction of liposomes with 9L cells, thereby decreasing both the endocytic uptake of the liposomes and the magnitude of cell-mediated leakage. These same properties enhance the deposition of liposomes in tumors (8,9) compared to free drug. In the absence of significant endocytosis of liposomes, the cytostatic effect is mediated predominantly by the slow release of drug sequestered in liposomes (27).

The overall antitumor effect observed is the aggregate of several phenomena. The improvement in circulating half-life of liposomal DXR, mediated by liposome composition, results in greater drug uptake into tumor tissue (9,34). Unlike free drug, however, liposome-entrapped drug appears not to be distributed uniformly within tumors, nor appears to access regions of tumor that are distant to the vasculature (6). Furthermore, those liposome compositions showing the longest circulating half-life are the least likely to interact with the cell surface, undergo endocytosis, and delivery their contents intracellularly. Likely the enhanced deposition of liposomes through the compromised vasculature, followed by slow release of drug, are responsible for the enhanced antitumor activity observed.

Further enhancement of the therapeutic effect may be possible though optimization of the drug carrier's properties. Drug release rates may be sub-optimal, and triggered release following liposome deposition may be required to supply cytotoxic concentrations of drug within the tumor.

Tumor biology may also impact the efficacy of liposome-based therapy. Intracranial tumors are lethal due to rapid growth and increased intracranial pressure; similar growth in tumors residing elsewhere in the body may not have the same degree of lethality. Early, aggressive treatment of tumors, with drug doses near the maximum tolerated dose, may be necessary to reduce the size of intracranial lesions or to retard their growth. We compared the efficacy of treatment initiated at different stages in tumor development, and found that an earlier start of treatment was found to be more effective in controlling the tumor growth. In both cases, tumors were well-established, and had developed extensive vascularity. Tumor bulk, and its effect on the surrounding tissue, were significant differences between early- and late-treated tumors.

Siegal et al (9) tested the efficacy of DXR liposomes of similar composition against the intracerebral T-749 histiocytoma, and observed increases in life-span (relative to untreated control animals) of 89% with liposomal DXR and 23% with free DXR. Using the 9L gliosarcoma model, we observed somewhat lower efficacy of liposome-encapsulated DXR, and essentially no antitumor effect of free DXR. The difference in efficacy may arise from intrinsic differences in the properties of the tumors, such as sensitivity to DXR.

In summary, we have shown that liposomes can enhance the efficacy of antineoplastic drug therapy against an invasive brain tumor *in situ*. Liposomes undergo extravasation and deposition non-uniformly within the tumor, and appear to reside at greatest concentration in close proximity to blood vessels. Both in vitro and in vivo studies suggest that the liposomes themselves do not interact at high frequency with the majority of tumor cells; thus sustained efflux of drug deposited outside

the tumor vasculature may be responsible for the enhanced antitumor effects observed. Further increases in antitumor activity may result from optimization of the release rates of drug from the liposomes.

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