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Fluorescence Imaging Studies for the Disposition of Daunorubicin Liposomes (DaunoXome) within Tumor Tissue[1]

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ABSTRACT

Unilamellar liposomes that retain their contents in the systemic circulation can alter the pharmacokinetics of anticancer agents in favorable ways. It has long been recognized that certain liposome compositions will extravasate at sites of growing tumors and may increase the local drug concentration substantially above that achievable with a free drug. We report here that liposomes can alter the *in vivo* disposition of an entrapped drug not only on a macroscopic but also on a microscopic scale. We show through *in vitro* studies that intact liposomes composed of distearoylphosphatidylcholine and cholesterol and containing daunorubicin (DaunoXome) are taken up into P1798 tumor cells. These liposomes produce an enhanced cytotoxicity relative to the free drug for incubation times longer than about 8 h. For *in vivo* studies, we developed and used a noninvasive fluorescence imaging technique to follow the accumulation of liposomal daunorubicin within murine tumors. With this method, we show that the maximum concentration of the available liposomal drug in tumors exceeds that of the free drug, and additionally, liposomal daunorubicin persists at high levels for several days. Total liposome-delivered drug fluorescence from whole tumor extracts peaks at about 8 h. In comparison, the fluorescence intensity of daunorubicin released from vesicles seen with the *in vivo* imaging experiment peaks at 28–32 h. This apparent delay is due to a sustained release of the drug from liposomes in the tumor. Fluorescence microscopy of thin sections of tumors from animals injected i.v. with liposomal daunorubicin demonstrate persistent high levels of daunorubicin fluorescence within cells and throughout the tumor masses. Free daunorubicin, in contrast, transiently achieves modest levels of fluorescence and rapidly drops to background within a few h. These results indicate distinct mechanisms for the localization of free and liposomal daunorubicin, suggesting that liposomal daunorubicin can provide sustained intracellular levels of the drug within the tumor.

INTRODUCTION

Liposome formulations have evolved over the past 2 decades from preparations comprising a limited selection of available materials to those now consisting of highly purified lipids, used in precisely defined compositions. Early preparations, frequently called "conventional liposomes," were often used as research tools to investigate cell biology and membrane physical chemistry. Although intended to improve the efficacy profile of therapeutic agents when encapsulated, precursory liposome formulations often performed poorly due to inadequate stability in physiological environments. As the understanding of membrane physical chemistry and the availability of high-purity lipids has progressed, liposome compositions with improved stability have been formulated. Recently, two leading strategies for the clinical application of liposome-formulated antineoplastic agents have been pursued. One has been to fabricate liposomes with prolonged half-lives (1), whereas the other has been to design formulations with enhanced delivery to solid tumors *in vivo* (2).

DaunoXome is a liposome formulation of daunorubicin entrapped within the inner aqueous spaces of small unilamellar vesicles and is designed to target solid tumors (3). We had demonstrated previously that similar unilamellar liposomes, ranging 60–80 nm in diameter and composed of distearoylphosphatidylcholine plus cholesterol, could deliver high concentrations of imaging agents to animal (4–6) and human (7–9) tumors. This work led to preparation of chemotherapeutic liposomes based on the same carrier. Investigations in animal models showed that delivery of daunorubicin to tumor tissue was 10-fold greater (peak values and AUCs[3]) for the liposome preparation compared with the free drug (3). The levels achieved (up to 100 μg/g tumor tissue) are among the highest reported for liposomal delivery of anthracyclines. A balance between producing sustained high levels of the drug in blood and permitting the carrier to escape from the circulation into the target tissues seems to be pivotal. Thus, although the circulation lifetime is a factor, the rate of egress from the blood into target sites and the efficient delivery of liposome contents into cells at these sites may be more important. Liposomal daunorubicin has been developed into the commercial product DaunoXome. It has demonstrated improved activity against solid tumors in animal models (3, 10) and against Kaposi's sarcoma in AIDS patients (11–14). Clinical trials are ongoing in other solid tumors (15).

In the studies reported here, we have used the fluorescent properties of daunorubicin to compare dispositions of free and liposome-entrapped daunorubicin in tumor cells *in vitro* and *in vivo*. Fluorescence micrographs of P1798 lymphosarcoma cells in culture and of tissue sections from the same cell line grown as solid tumors in mice show the spatial distribution of the drug released from liposomal daunorubicin. These data are complemented by noninvasive *in vivo* fluorescence imaging of daunorubicin near the surface of intact tumors in live animals.

MATERIALS AND METHODS

Liposome Preparation. Liposomes composed of distearoylphosphatidylcholine and cholesterol in a 2:1 molar ratio and containing daunorubicin were prepared as described previously (3). A dry mixture of the lipids was hydrated in an aqueous solution containing 50 mM citric acid and homogenized to produce a suspension of small, unilamellar vesicles. Daunorubicin was entrapped as a citrate salt within the vesicles.

Cell Line and Tumor Preparation. P1798 lymphosarcoma cells, originally derived from BALB/c mice, were obtained from the Tumor Repository of the Department of Cancer Treatment of the National Cancer Institute. For *in vitro* studies, the cells were incubated in Iscove's medium supplemented with 10% FCS and 1% penicillin and streptomycin (complete Iscove's medium). For *in vivo* investigations, P1798 cells were passaged i.p. in BALB/c mice and grown intradermally as solid tumors from implants in the right flanks of BALB/c × DBA/2F$_1$ (hereafter called CD2F$_1$) mice. Areas of normal skin and

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[3] The abbreviations used are: AUC, area under the curve; IC_{50}, 50% inhibitory concentration; FACS, fluorescence-activated cell sorting.

the tumor implantation site that were to be illuminated for the *in situ* laser-induced fluorescence imaging studies were cleared of fur (which causes unacceptable light scattering) by shaving or application of a depilatory. Levels of daunorubicin and fluorescent metabolites in tumors and normal skin were determined by measuring the total fluorescence of normal butyl alcohol extracts, following previously published methods (3, 16, 17).

***In Vitro* Cytotoxicity Assays.** P1798 lymphosarcoma cells were added in 100-μl aliquots to a 96-well plate to produce a final concentration of 1×10^4 cells/well. Free and liposomal daunorubicin were diluted in complete Iscove's medium, and 100 μl of each dilution were added to each well. The range of final drug concentrations was 0.1–1000 ng/ml. A single IC_{50} determination was made for free daunorubicin HCl (Rhone-Poulenc). For liposomal daunorubicin, four separate preparations were run, and the IC_{50} values at each time point were averaged. [^{3}H]Thymidine (0.5 μCi in 20 μl) was added 4 h prior to harvesting the well contents onto filter discs (Skatron) using a Skatron cell harvester (model 11022) with two aqueous washes. The filter discs were placed in 10 ml Biofluor (DuPont) in scintillation vials and counted for tritium in an LKB-Wallac scintillation counter (model 1219).

***In Vitro* Fluorescence Microscopy.** Daunorubicin, as either the free or liposomal drug, was added to P1798 cell suspensions at 10^6 cells/ml for *in vitro* fluorescence microscopic studies. Daunorubicin concentrations, both free and liposome entrapped, were 7.4 μg/ml (daunorubicin base, 14.0 μM). Cells were incubated with the daunorubicin preparations at 37°C for up to 20 h. Cell samples were removed at 1.5, 3, 7, 9, 14, and 20 h and centrifuged to concentrate the cells and to remove excess liposomes or free drug. The cells were then resuspended in drug-free medium and examined by fluorescence microscopy.

FACS. P1798 lymphosarcoma cells were incubated at a concentration of 10^6 cells/ml in complete Iscove's medium. Free or liposomal daunorubicin was added at a concentration of 7.4 μg/ml. At various times, cells were removed, washed to remove unbound liposomes, fixed with 1% formaldehyde, and stored on ice until FACS determinations were made (on the same day). The intensity of daunorubicin-associated fluorescence in P1798 tumor cells was estimated by FACS using a Coulter Epics Elite instrument equipped with an air-cooled, argon laser, 488-nm light source and fitted with a 550–575-nm bandpass filter.

Fluorescence Microscopy of Tumor Sections. Bright-field color images of H&E-stained sections were obtained and stored digitally using a Sony charged coupled device color video camera (model DXC-151) and a VIDAS image analysis system (Roche Image Analysis Systems). Using the same system with a low-light (very-high-sensitivity) video camera (Hamamatsu model C2400), very-low-intensity fluorescence from unstained sections was imaged through red and green filters. The gray scale black-and-white camera images were converted to red and green scale images and added digitally using the VIDAS system to yield a "true color" image of the unstained section. (There is no blue light component to these images, because fluorescence excitation by mercury arc lamp is at short wavelengths, and a blue filter is used in the microscope to prevent "pass through" of the excitation wavelength). For the *in vivo* microscopic study, female $CD2F_1$ mice bearing P1798 lymphosarcoma solid tumors were treated by injection in the tail vein with 20 mg/kg free or liposomal daunorubicin. At 2, 8, 24, and 48 h after injection, groups of three mice each were sacrificed. Tumors were excised, frozen at −70°C, and sectioned serially by cryostat. Sections cut through tumor centers were used in this study. Alternate 5-μm-thick sections were stained with H&E for visualization of cell morphology.

***In Situ* Laser-induced Fluorescence.** Female $CD2F_1$ mice bearing P1798 lymphosarcoma solid tumors received i.v. daunorubicin, as liposomal daunorubicin or the free drug, at a dose of 18.7 mg/kg (daunorubicin base, equivalent to 20 mg/kg as the HCl salt). Treatment was administered when tumors had grown to about 50–100 mm^3 (approximately day 6 following tumor implantation). Tumors *in situ* were illuminated with the 488-nm line of an argon ion laser (Innova 90–5; Coherent Corp., Palo Alto, CA) to induce drug-associated fluorescence. Both tumors and normal skin were illuminated with 25 mW/cm^2 light for 0.1 s. Fluorescence emission images were isolated spectrally with a 600-nm bandpass interference filter (50-nm bandwidth; Corion Corp., Holliston, MA). Back-scattered light was recorded using a 450-nm bandpass interference filter (50-nm bandwidth; Corion Corp).

All images were recorded digitally using a slow-scan, thermoelectrically cooled charged coupled device camera (model TE576/ST135; Princeton Instruments, Quakerbridge, NJ) with a 16-bit/pixel dynamic range. Image acquisition, camera control, and signal processing were performed using a MacIntosh computer with IP lab software (Signal Analytics Corp., Vienna, VA). All fluorescence images were normalized by the following algorithm to correct for animal position variations and nonuniform illumination:

$$NFI = \frac{\textit{600-nm Red Image}\ (\text{fluorescence} - \text{dark noise})}{\textit{450-nm Blue Image}\ (\text{fluorescence} - \text{dark noise})}$$

where *NFI* is the normalized fluorescence image. The blue image is composed primarily of 488-nm light that is not rejected by the blue (450-nm) filter. The resulting red/blue image provides an accurate representation of drug distribution, because it accounts for spatial variations in light intensity that can influence fluorescence levels. Dark noise images, corresponding to the electronic background noise, were acquired under conditions identical to fluorescence, without laser excitation. Background fluorescence images were recorded for each mouse before drug injection and were subtracted from the subsequent postinjection images. Fluorescent imaging data for liposomal daunorubicin were acquired from seven tumor-bearing mice through 88 h after injection. For free daunorubicin, four mice were used, with data gathered through 72 h after injection. Levels of daunorubicin-associated fluorescence were determined by averaging the fluorescence of the computer-stored ratio images across regions of tumor tissue or corresponding normal skin.

Curve fitting was performed using the R-Strip (MicroMath, Salt Lake City, UT) exponential regression program. Fits were made to biexponential functions with one increasing and one decreasing component, similar to a simple absorption model (18) of the form:

$$RTF = A \times (e^{-k_{el}} - e^{-k_a})$$

where *RTF* is the relative tissue fluorescence; k_{el} and k_a are the rate constants for elimination and accumulation, respectively, of daunorubicin-associated fluorescence by skin or tumor tissues *in situ*; and *A* is a weighting factor proportional to net accumulation. No time advance or delay was factored into the equations; *i.e.*, tissue fluorescence at time 0 was set to a relative fluorescence of zero. More complex functions of greater than two exponential terms could not be justified based on the data spread.

Separately, calibration studies were conducted to correlate *in situ* fluorescence intensities with tissue drug levels. Free daunorubicin was administered i.v. at doses of 10, 20, and 40 mg/kg, and fluorescence images were acquired 3 h after injection, the time when tumor drug levels were at or near maximum. Liposomal daunorubicin was administered at 10, 20, and 30 mg/kg, and images were acquired 24 h after injection, when levels of the liposome-released drug were near maximum. Following acquisition of the *in situ* fluorescent images, the animals were sacrificed, and areas of normal skin and tumor that had been imaged were excised and assayed for daunorubicin-associated fluorescence by normal butyl alcohol extraction.

RESULTS

Cytotoxicity of Free and Liposomal Daunorubicin. Liposomal daunorubicin was compared with the free drug for cytotoxicity in cultures of P1798 lymphosarcoma cells. The IC_{50}s were determined at several intervals from 2 through 48 h following drug addition (Fig. 1). Free daunorubicin has a lower IC_{50} than liposomal daunorubicin (0.29 *versus* >1 μg/ml) at 2 h of drug exposure. (An IC_{50} was not achieved over the concentration range tested for liposomal daunorubicin at 2 h). However, liposomal daunorubicin treatment is more potent than treatment with the free drug for incubation times greater than about 8 h. At 48 h, the potency of liposomal daunorubicin (IC_{50}, 0.003 μg/ml) is more than 7-fold greater than that of the free drug (IC_{50}, 0.022 μg/ml).

Intracellular Dispositions of Free and Liposomal Daunorubicin. Fluorescence microscopy of P1798 tumor cells *in vitro* indicates significantly different patterns of daunorubicin-associated fluorescence for free and liposomal drugs. The free drug accumulates rapidly within the cells (Fig. 2*A* and *B*). By 1.5 h, strong fluorescence can be noted throughout the cytoplasm, with significant fluorescence within the nucleus. At this time, there are localized areas of more intense

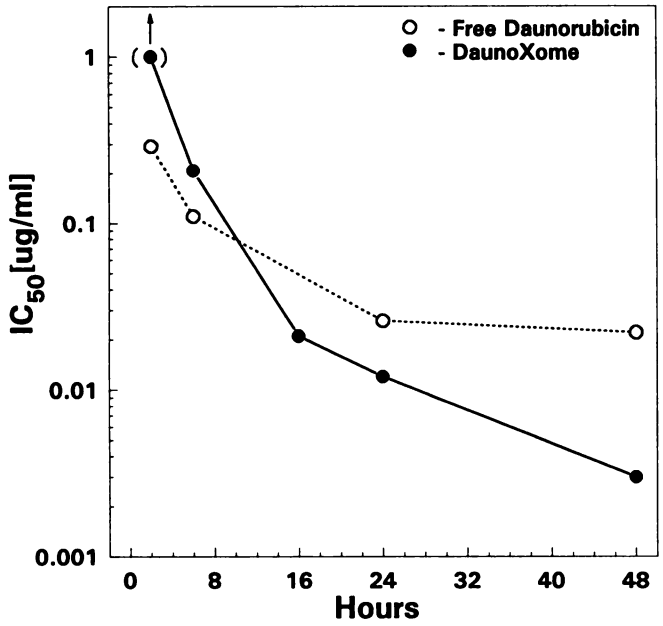

Fig. 1. Free and liposomal daunorubicin concentrations for IC_{50} as a function of incubation time. P1798 lymphosarcoma cells were incubated at a concentration of 10^4 cells/ml in Iscove's medium supplemented with 10% FCS and 1% penicillin and streptomycin. The IC_{50} values at each time point were determined by inhibition of [^{3}H]thymidine incorporation. The 2-h IC_{50} value for liposomal daunorubicin is in excess of the highest concentration tested (1 μg/ml) and, therefore, is shown in *parentheses*. For incubation times longer than 8 h, liposomal daunorubicin is more cytotoxic than the free drug.

fluorescence in the cytoplasm, possibly corresponding to preferential accumulation within cell organelles such as endosomes and/or mitochondria. By 3 h and at all later times, the fluorescence is dispersed more uniformly throughout each cell, although there continue to be local areas of more intense fluorescence.

Cells treated with liposomal daunorubicin demonstrate an overall less intense fluorescence, relative to the free drug, at 1.5 h (Fig. 3*A*). The fluorescence pattern within the cytoplasm is punctate, appearing less diffuse throughout the cytoplasm than that of the free drug. At this time, there also seems to be much less fluorescence within the nucleus compared with the free drug. The 3-h pattern for liposomal daunorubicin treatment is relatively unchanged from that at 1.5 h, although an increased cytoplasmic fluorescence seems evident, along with the appearance of a stronger fluorescent signal within the nucleus (Fig. 3*B*). From 7 through 14 h, there is a continuous but gradual increase in nuclear fluorescence with a corresponding loss in the punctate appearance of the cytoplasm (Fig. 3, *C* and *D*). At 14 and 20 h (not shown), the nuclear areas seem to be more fluorescent than the cytoplasm, and the distribution of fluorescence throughout each cell seems similar to that of free drug.

FACS results are in accord with the microscopic observations noted above. Free daunorubicin rapidly produces high levels of cell-associated fluorescence (78% of maximum fluorescence intensity, arbitrary units) within 1.5 h, plateauing at 7 h (100% of maximum). For liposomal daunorubicin-treated cells, daunorubicin-associated fluorescence increases more gradually (37 and 64% of maximum at 1.5 and 7 h, respectively) through 14 h (100% of maximum), plateauing at 20 h. Between 14 and 20 h, the fluorescence intensities for free and liposomal drugs are not significantly different.

***In Vivo* Disposition of Free and Liposomal Daunorubicin.** *In situ* laser-induced fluorescence studies demonstrate peak levels of daunorubicin-associated fluorescence in tumor tissue at about 3 h for the free drug, decreasing thereafter (Fig. 4*A*). A representative fluorescent image near the 3-h time of the peak tumor drug level (as determined in Ref. 3) for free daunorubicin is shown in Fig. 5*A*. In this image, the observed fluorescence intensity of the tumor is comparable to that of the surrounding normal skin. One region of the tumor that seems to be of relatively lower intensity is an area in which the tumor has ulcerated and has become partially scabbed over. Localized bright spots, away from the tumor region, are areas of skin that were nicked during shaving and likely are due to porphyrins.

In contrast to the free drug, liposomal daunorubicin produces a steady increase in tumor fluorescence through 24 h, peaking at approximately 30 h (Fig. 4*B*). The drug is distributed throughout the tumor, with the most intense signal visible near the tumor center (Fig. 5*B*). Furthermore, the tumor is substantially brighter than the surrounding normal tissues, indicating the selective localization brought about by the liposome carrier.

Normal skin demonstrates a uniformly high signal from daunoru-

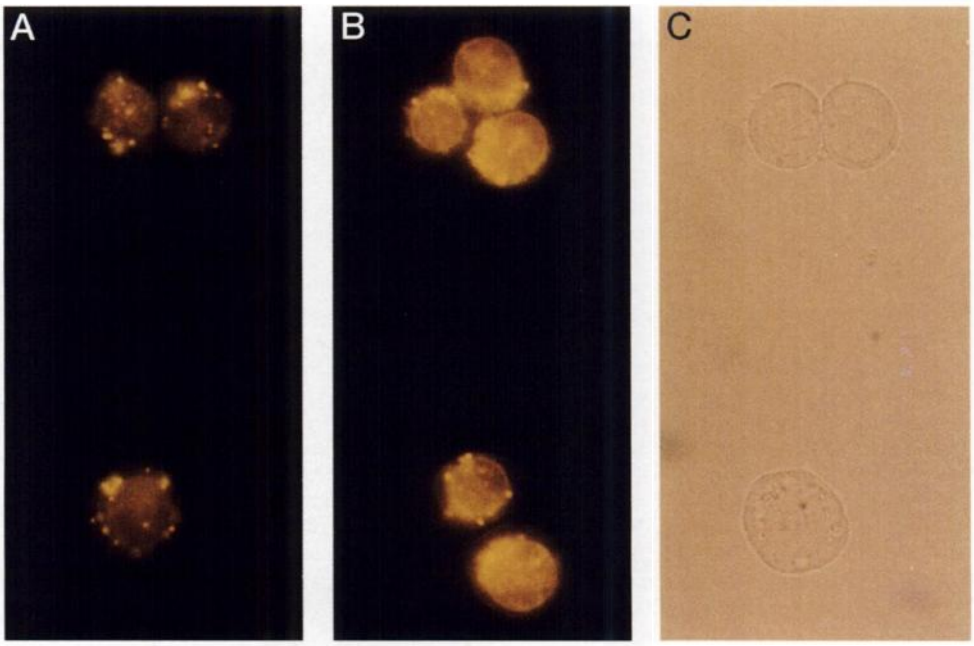

Fig. 2. Fluorescence microscopy of daunorubicin-treated P1798 lymphosarcoma cells *in vitro*. The cells were incubated with free daunorubicin, 7.4 μg/ml, at 37°C. Cell samples were removed, centrifuged to remove excess drug, resuspended in drug-free medium, and examined by fluorescence microscopy. *A*, cells at 1.5 h; *B*, 3 h; *C*, bright-field image of the P1798 cells at 1.5 h, as shown in *A*, for reference.

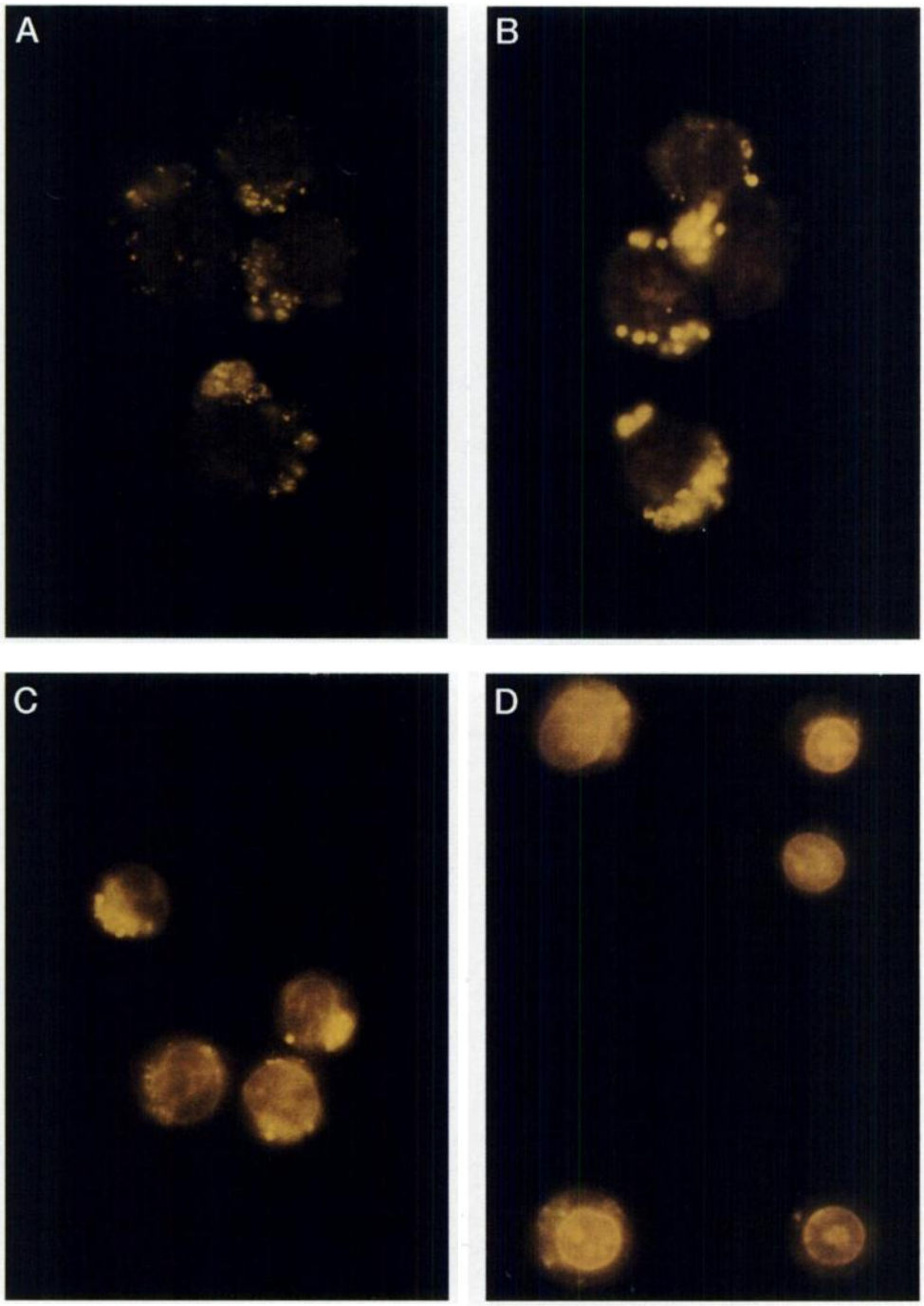

Fig. 3. Fluorescence microscopy of P1798 lymphosarcoma cells treated *in vitro* with liposomal daunorubicin. The cells were incubated with liposomal daunorubicin at a drug concentration of 7.4 μg/ml. *A*, cells at 1.5 h; *B*, 3 h; *C*, 7 h; *D*, 14 h.

bicin-associated fluorescence throughout the imaged areas in mice treated with the free drug. Over the 72-h period of the study, daunorubicin levels in normal skin closely parallel those of the tumor (Fig. 4*A*). In comparison, liposomal daunorubicin produces much less fluorescence in areas of normal skin relative to areas with underlying tumor tissue (Fig. 4*B*). Also, the fluorescence intensity of normal skin is observed to peak at an earlier time (between 16 and 24 h) when compared with the tumor (between 28 and 40 h).

The relative exposures of tumor and normal skin to free and liposomal daunorubicin may be estimated by comparing data for the relative tissue fluorescence over time (Table 1). This comparison indicates that although free daunorubicin demonstrates nearly comparable levels in normal and tumor tissues, liposomal daunorubicin exhibits about a 5-fold greater preference for tumor accumulation.

Comparison of Image Intensity with Extractable Fluorescence Levels. Skin and tumor levels of daunorubicin are proportional to the daunorubicin dose over the ranges of 10–40 (free drug) and 10–30 (liposomal daunorubicin) mg/kg, as determined by fluorescence intensity measurements of excised extracted tissue (data not shown). The values of daunorubicin concentration were compared with image intensity for the corresponding tissue before excision to generate calibration plots (Fig. 6, *A* and *B*). Image intensity correlated with tissue level for free daunorubicin in skin ($r^2 = 0.938$) and tumor ($r^2 = 0.848$) and for liposomal daunorubicin in tumor tissue ($r^2 = 0.858$). However, liposomal daunorubicin fluorescence intensity is not well correlated with drug levels in skin tissue ($r^2 = 0.296$). For given levels of extractable drug in the tissues, the relative fluorescence image intensity values were greater for free

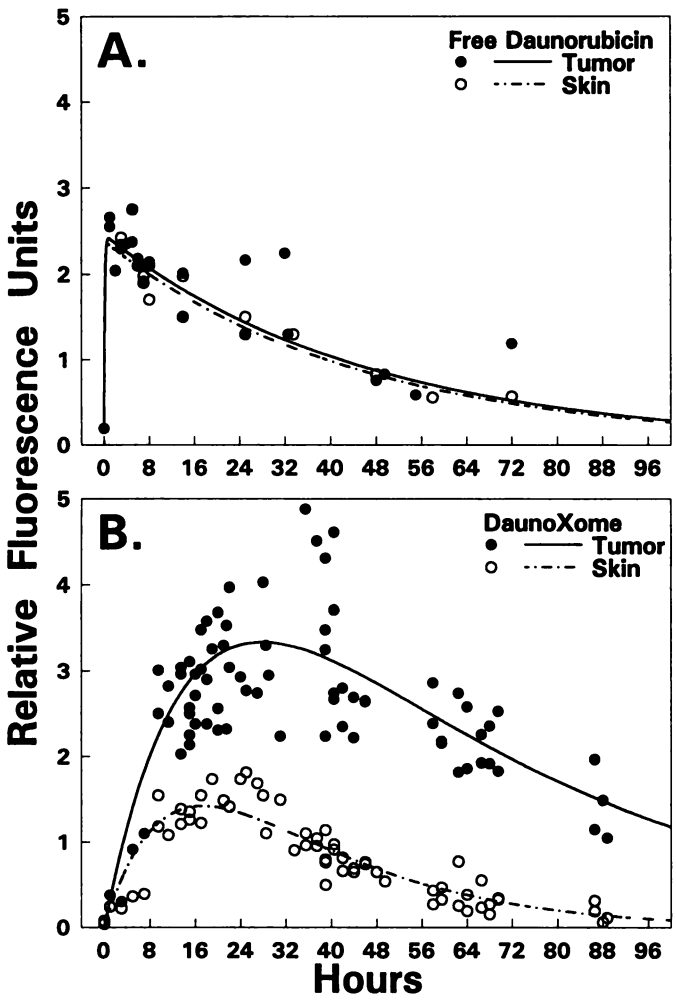

Fig. 4. *In situ* fluorescence intensity of tumor and normal skin. Normalized fluorescence intensities of digitized fluorescence images were determined noninvasively for areas of tumor and normal skin. Data *points* are individual determinations at the times indicated for four free drug-treated and seven liposomal daunorubicin-treated mice.

daunorubicin than for liposomal daunorubicin. This is most likely due to self-quenching of the drug still contained within liposomes during image acquisition, but that is released later during the extraction process.

Fluorescence Microscopy of Tumor Sections. Free daunorubicin produces a relatively low level of drug-associated fluorescence (orange) in most tumor cells 2 h after i.v. administration (Fig. 7*A*). The fluorescence pattern is diffuse throughout the tissue cross-section, with an apparently higher concentration near the tumor margins. This intensity diminishes with time thereafter, and by 24 h after injection, the tumor fluorescence (Fig. 7*B*) is similar to the background autofluorescence, as observed in tumor sections from untreated control animals (not shown).

Liposomal daunorubicin produces a noticeably more intense orange fluorescence throughout most of the tumor section, relative to the free drug, at 2–24 h after administration (Fig. 8, *A* and *B*). Because the encapsulated drug self-quenches, the fluorescence is principally from released daunorubicin. There seems to be little extracellular fluorescence. Areas of enhanced fluorescence intensity may be associated with capillaries (Fig. 8*B*), suggesting that sites of increased permeability within the tumor vasculature allow liposomes to move into the surrounding tumor tissue. For sections removed 24 h after administration of liposomal daunorubicin, the fluorescence pattern is essentially similar to that of free daunorubicin at 2 h, although the overall intensity is increased substantially (Fig. 8*B*). At 2 days following injection of liposomal daunorubicin, bright daunorubicin-associated fluorescence is still present in most of the tumor cells (not shown).

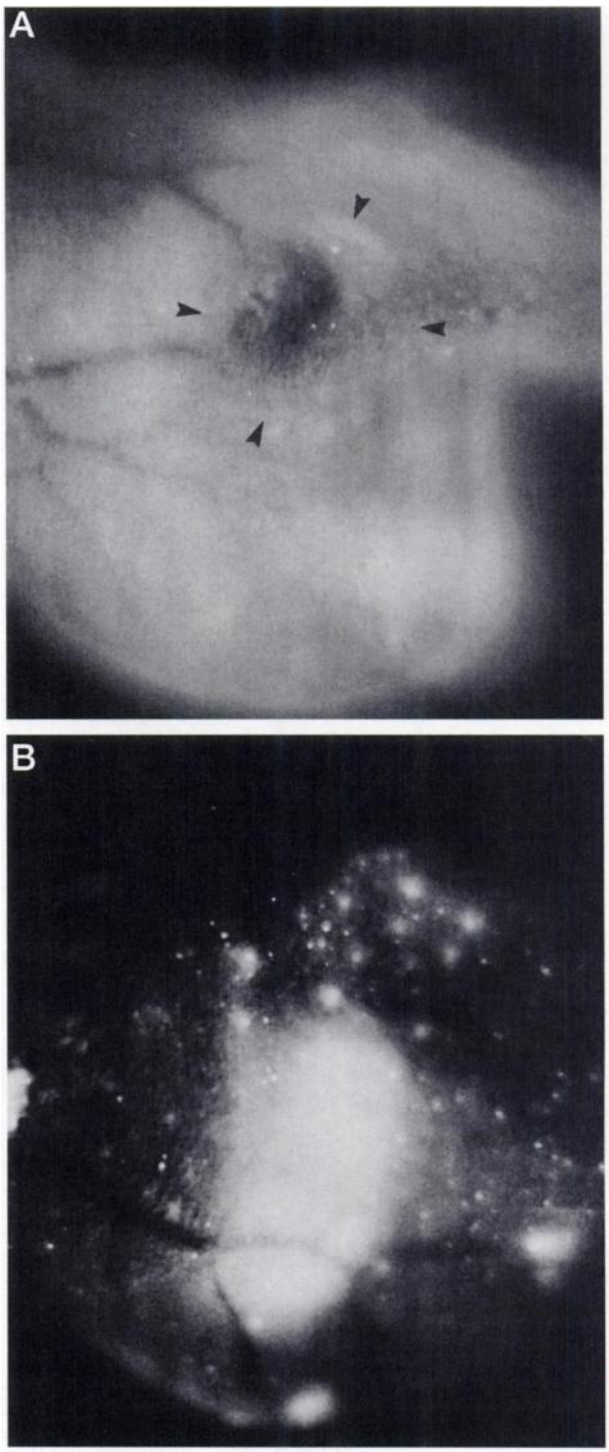

Fig. 5. *In situ* fluorescence image of P1798 tumor and surrounding normal skin. *A*, image at 3 h following administration of free daunorubicin, corresponding to peak tumor drug levels. The tumor, situated in the *upper center*, is delineated by *arrows*. *B*, image at 24 h following administration of liposomal daunorubicin. The tumor, clearly located at the *center* of the image, is intensely fluorescent, particularly in comparison to the surrounding normal skin. Brighter spots in normal skin correspond to porphyrins in nicks that occurred during shaving of the mice to remove hair, which interferes with the fluorescent signal. Image intensities for *A* and *B* are scaled similarly. The tumors in each image are approximately 1 cm across.

Table 1 *Relative exposures of tumor and normal skin to free and liposomal daunorubicin*

AUC values are time-integrated ($t = 0 \rightarrow \infty$) relative fluorescence units (Fig. 4, *A* and *B*). Mice were treated with 20 mg/kg daunorubicin base.

Tissue	AUC (relative fluorescence units × h)	
	Free daunorubicin	Liposomal daunorubicin
Tumor	113.4	254.4
Normal skin	107.2	50.6
Ratio	1.06	5.03

DISCUSSION

Previous work has shown that liposomal daunorubicin is substantially more efficacious than conventional (free) daunorubicin against solid tumors in animal models (3, 19). This liposomal formulation increases peak tumor drug concentrations and AUCs by about 10-fold relative to the free drug at similar doses (3). Increased tumor concentration undoubtedly contributes to increased efficacy, but other factors, such as distribution of the drug within the tumor mass, uptake of liposomes into cells, rates of drug release from liposomes, and intracellular trafficking, may play significant roles. We begin to address these factors with the studies presented here. In its free drug form, daunorubicin is strongly fluorescent at dilute concentrations in aqueous solutions, with an absorption maximum at 476 nm and an emission maximum at 580 nm. When entrapped in liposomes, however, the fluorescence emission of daunorubicin is quenched efficiently, due to both reabsorption (inner filter effect) and energy transfer among the highly concentrated daunorubicin molecules entrapped within the liposomes. Fluorescence intensity increases by 50–100-fold as the drug is released from the liposomes on detergent-induced vesicle disruption. Thus, although the drug that is still entrapped within intact liposomes does produce a small fluorescent signal, the observed fluorescence in treated cells and tissues arises primarily from the released drug.

The altered subcellular disposition of daunorubicin brought about by liposome delivery is reflected in the change of relative IC_{50} values over time. For shorter incubation periods with P1798 cells *in vitro*, free daunorubicin is more cytotoxic than the liposomal drug. At 2 h, for example, free daunorubicin has more than three times the activity of liposomal daunorubicin. Although both the free and liposome-entrapped drug demonstrate progressively decreasing IC_{50} values as the incubation periods are extended, liposomal daunorubicin becomes increasingly more cytotoxic than the free drug over time. Thus, by 48 h, liposomal daunorubicin is nearly seven times more potent than the free drug.

The fluorescence distribution pattern for liposomal daunorubicin is noticeably different from that of the free drug and is consistent with an uptake mechanism predominated by endocytosis, followed by intracellular drug release from the liposomes. Extracellular release of daunorubicin from liposomes followed by uptake of the released drug is not supported by these data. Indeed, liposomal daunorubicin in buffer or 50% calf serum at 37°C is stable to drug leakage, losing less than 5% of the entrapped drug over 48 h (3). Confocal fluorescence microscopy of cells treated with liposomal daunorubicin (data not shown) shows punctate intracellular fluorescence, which may be associated with internal vacuoles. These observations indicate that liposomal daunorubicin does not remain at the cell surface, but that, over time, it is internalized and releases its contents into the cytoplasm. The greater cytotoxicity of free daunorubicin for shorter incubation periods is not surprising, considering its rapid cellular uptake. The delay in full potency of liposomal daunorubicin and its ultimately greater cytotoxicity may be accounted for, in part, by a slower but sustained accumulation of liposomal daunorubicin by the tumor cells, occurring over several h. This seems to be followed by a more gradual intracellular release of the drug from the liposomes. It is interesting to note that the cytotoxicity roughly follows the time course of total fluorescence measured by FACS, except that at later times, when liposomal daunorubicin is more potent, the fluorescence intensities are similar. This difference is not unexpected, because FACS measures total cell-associated fluorescence, which parallels, but is not identical to, drug concentration. These combined data suggest that liposome encapsulation of daunorubicin may alter the traffick-

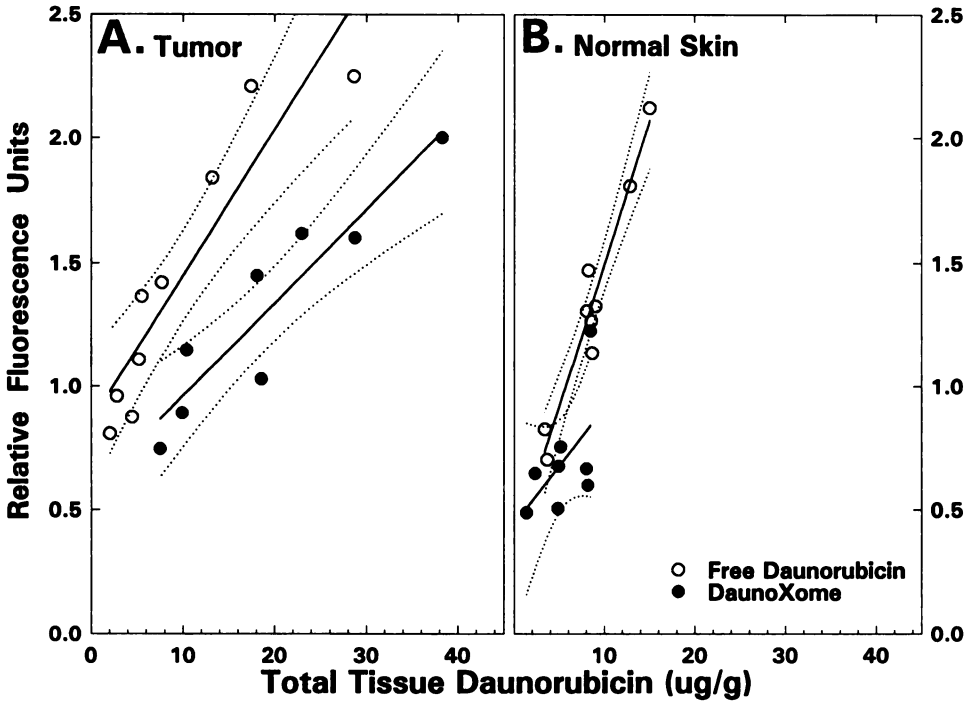

Fig. 6. Relative fluorescence as a function of tissue concentrations for daunorubicin and associated metabolites. *A*, P1798 lymphosarcoma tumor and overlying skin (r^2 = 0.858 and 0.848 for liposomal and free daunorubicin, respectively). *B*, normal skin away from tumor site (r^2 = 0.296 and 0.938 for liposomal and free daunorubicin, respectively). *Dotted lines*, 95% confidence intervals of the regression estimates.

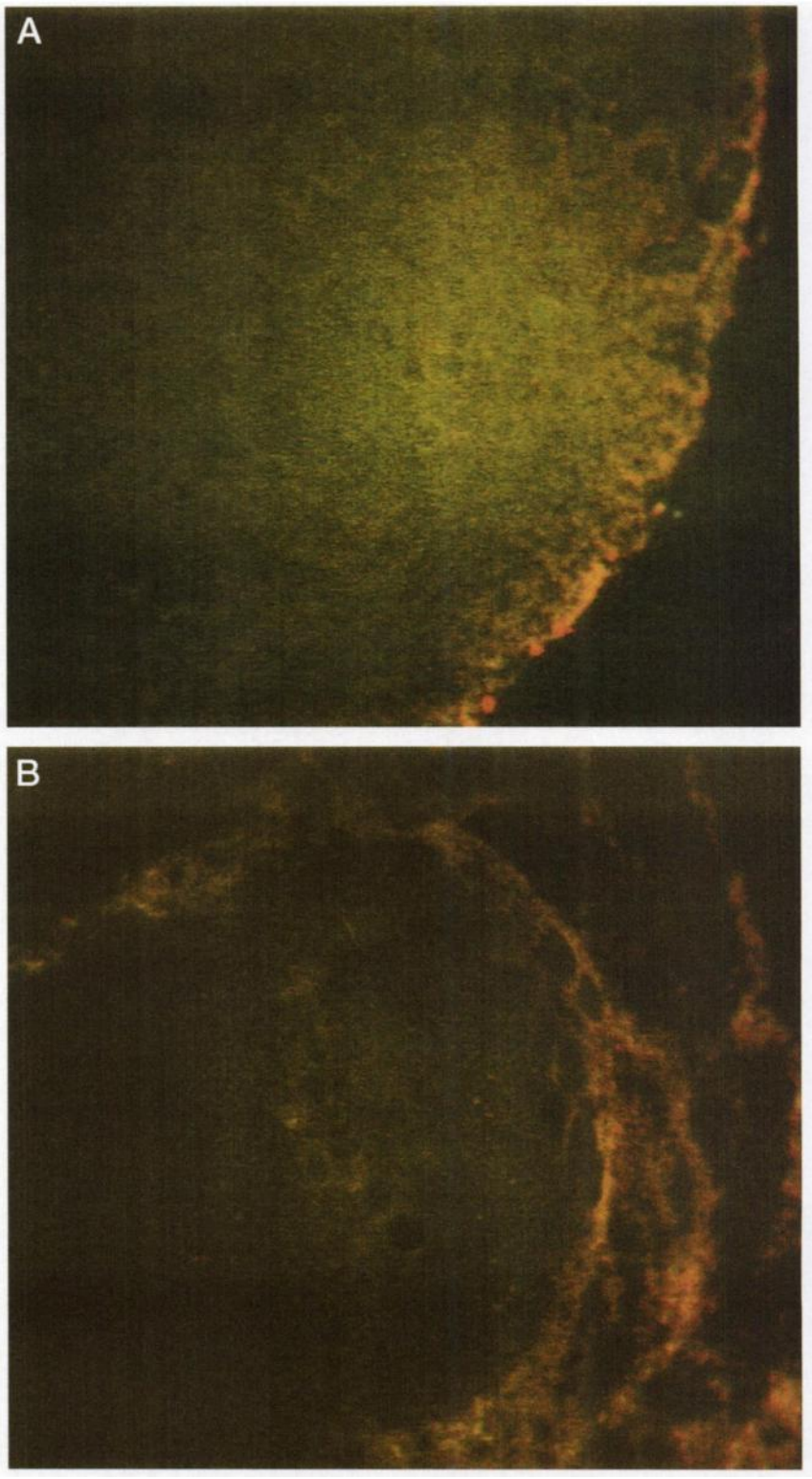

Fig. 7. Fluorescence microscopy of 5-μm cryostat sections of P1798 lymphosarcoma solid tumor from $CD2F_1$ mice treated with free daunorubicin at 20 mg/kg 2 (*A*) and 24 (*B*) h after injection.

ing of daunorubicin within tumor cells, potentially resulting in an increased cytotoxic effect.

The FACS data parallel the *in vitro* fluorescence microscopic results, demonstrating rapid cell association of the free drug. Although the fluorescence of liposome-delivered daunorubicin initially is less than that of the free drug, its accumulation is continuous over a prolonged period, attaining equivalent levels by 14 h. The fluorescence microscopic and FACS data are consistent with a process whereby liposomal daunorubicin accumulates within the tumor cell cytoplasm by a slower endocytotic process compared with the free drug.

In addition to the altered trafficking noted previously, an alternative or additional reason for enhanced liposomal daunorubicin cytotoxicity may be a protective effect provided by liposome encapsulation, preventing metabolic and/or chemical conversion of the drug to inactive forms. As demonstrated in earlier studies, liposomal daunorubicin is able to retain the entrapped drug for prolonged periods under physiological conditions.

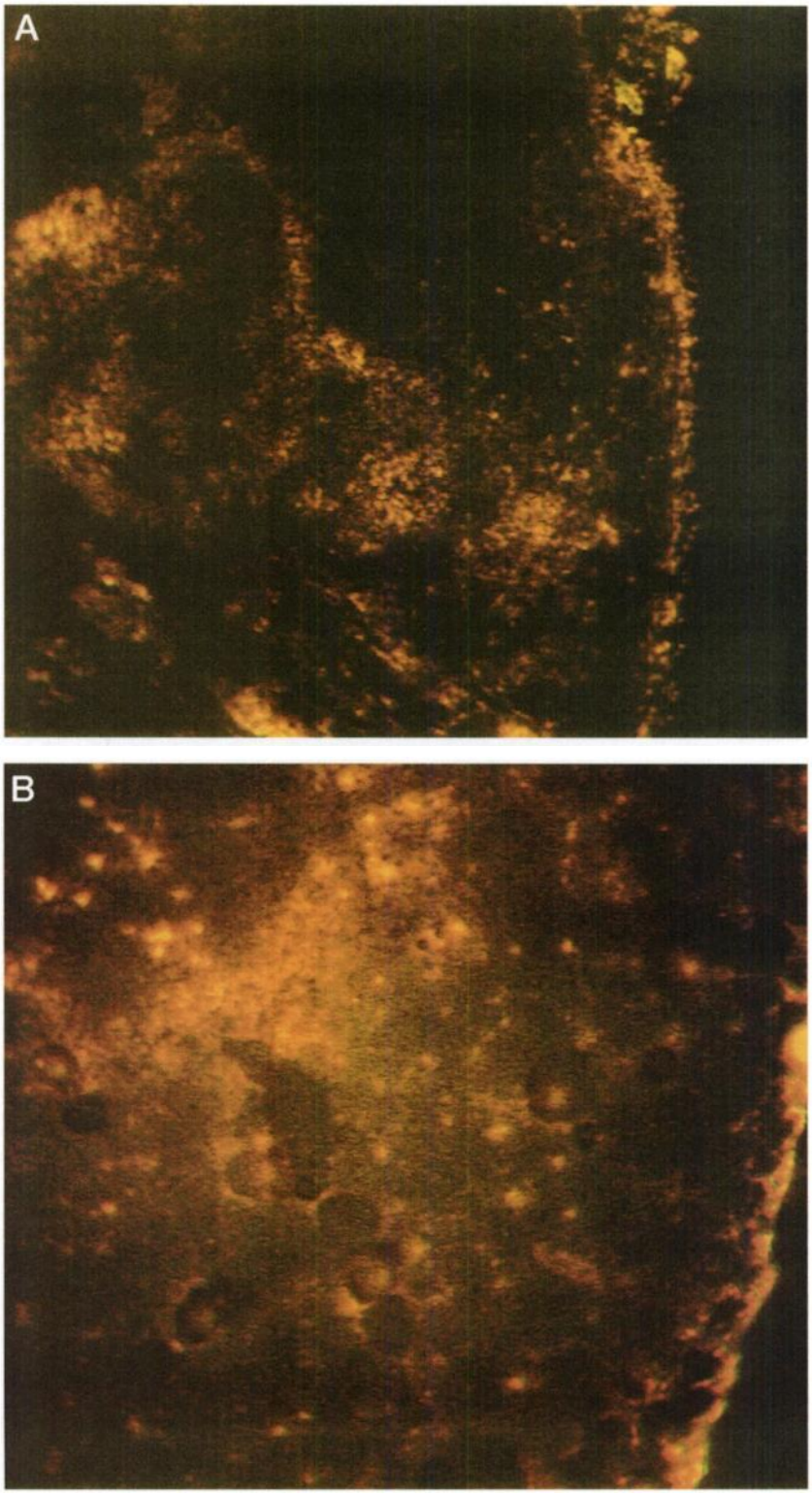

Fig. 8. Fluorescence microscopy of 5-μm cryostat sections of P1798 lymphosarcoma solid tumor from CD2F$_1$ mice treated with liposomal daunorubicin at 20 mg/kg 2 (*A*) and 24 (*B*) h after injection.

The technique of fluorescence imaging used in this investigation demonstrates that daunorubicin can be visualized, and its concentration can be estimated noninvasively within solid tumors *in situ*. This is accomplished by fluorescence excitation of daunorubicin and its associated metabolites within tissues using direct laser illumination. The resulting emission is then detected using a sensitive, low-level-light camera. Several physical factors must be recognized when interpreting these *in situ* data. First, the excitation light intensity is limited by the shallow blue light tissue penetration; therefore, the measured fluorescence is derived from only the first few mm in the tissue. Second, the fluorescence of liposome-entrapped daunorubicin is quenched significantly, due to its high local concentration within the vesicles, whereas the free and released drugs can be nearly fully fluorescent, depending on their specific environments. This has been shown in separate studies, in which preparations of liposomal daunorubicin approaching 100% encapsulation were found to have only about 1 or 2% of the fluorescence intensity of equivalent concentrations of the unencapsulated drug. These

images thus are weighted toward the relative amounts of unencapsulated daunorubicin in the tissues.

A comparison of calibration curves (Fig. 6) for *in situ* fluorescence signal as a function of tissue drug levels provides a qualitative sense for the differences in the disposition of daunorubicin within the tissues as a function of dosage form. The comparable slopes and correlation coefficients observed for liposomal and free daunorubicin in the tumor mass (r^2 = 0.858 and 0.848, respectively) are consistent with comparable dispositions for these dosage forms. This does not necessarily indicate that the dispositions of the drug throughout the tumor are similar for the two dosage forms. However, significantly different values for these coefficients would have indicated that the distributions of the drug within the tissue are different. The normal skin of free drug-treated mice shows the greatest slope for *in situ* fluorescence intensity as a function of drug levels in excised tissues. This is due to the free drug being less quenched and to the drug in the skin being closer to the surface where the signal is acquired, thus minimizing signal attenuation due to light scatter. This premise is supported by the high correlation coefficient (r^2 = 0.938) observed for this relationship. In contrast, normal skin tissue from mice treated with liposomal daunorubicin displays a limited *in situ* fluorescent response as a function of tissue drug levels (r^2 = 0.296). This is in part due to the very low levels of liposome-formulated drug that are delivered to the normal skin. Another factor is the likelihood that much of the observed *in situ* fluorescent signal is from intact liposomes in the circulation. The analysis of these calibration curves indicates that the ultimate dispositions of free and liposomal daunorubicin are much different in normal skin but similar within the tumor mass. This supports a model in which liposomal daunorubicin remains intact while in the circulation and normal tissues but breaks down within the tumor to release daunorubicin.

Comparison of the time courses for the relative fluorescence intensities *in situ* (Fig. 5, *A* and *B*) with previously published data (3) for drug levels assayed by extraction methods for the same tumor model is particularly informative. The course of *in situ* fluorescence for the free drug in the tumor closely follows the data for tissue extracts. For both observation methods, peak tumor levels occur within 3 h of administration. In addition, the calculated elimination rate constants are equivalent, at 0.031 and 0.029 h^{-1} for the extraction and *in situ* imaging methods, respectively. This equivalency is anticipated because, as noted above, the free drug is nearly fully fluorescent, and the *in situ* signal intensity is directly proportional to the tumor drug concentration. Liposomal daunorubicin, however, exhibits substantially different pharmacokinetic profiles for extractable drug when compared with *in situ* fluorescence. *In vivo*, liposome-delivered daunorubicin accumulates within tumors more slowly than the free drug, not reaching peak levels of total extractable material until between 8 and 9 h following administration. However, peak levels of *in situ* fluorescence are reached even later, 24–32 h after injection. Additionally, the calculated elimination rate constant for *in situ* fluorescence, 0.019 h^{-1}, is about one-half that determined for extractable fluorescence, 0.038 h^{-1}. These observations of delayed times to peak levels and a slower elimination rate constant support a model of liposomal daunorubicin being accumulated within the tumor intact, followed by their breakdown and a slow release of fully fluorescent drug. Thus, although the total accumulation of liposomal daunorubicin in the tumor may peak at 8 h, much of the drug remains entrapped at this time. As the liposome-delivered drug is slowly cleared from the tumor, the loss of its fluorescence is balanced by the release of additional drug from the liposomes within the tumor. The continuous release of daunorubicin from the liposomes provides sustained high levels of available drug within the tumor.

The increased exposure of tumor tissue relative to normal skin provided by liposomal daunorubicin (as indicated by comparing AUC values of *in situ* fluorescence) is consistent with previous findings of *in vivo* targeting by daunorubicin liposomes (3, 11). The use of AUC values rather than single time points is a more reliable basis for comparison of free and encapsulated drugs, because it more accurately reflects the total tissue exposure to an agent independent of pharmacokinetic differences. However, tumor-associated liposomal daunorubicin remains mostly intact at early time points, significantly quenching drug fluorescence. This results in a diminished AUC value for liposomal daunorubicin as determined by *in situ* fluorescence, underestimating the amount of daunorubicin present. Thus, the ratio of the AUC in the tumor to that in skin is about 5 from the present *in situ* study (Table 1), whereas the same ratio for total extractable daunorubicin is approximately 10 (3).

Due to the optical properties of biological tissues, *in situ* imaging measures daunorubicin-associated fluorescence principally from the first 1–2 mm of the skin surface. A concern thus arises over differences in the relative distributions of free and entrapped daunorubicin within the tumor. For example, if liposome encapsulation causes daunorubicin to localize at the outer tumor margins, the *in situ* images may not be representative of the tumor as a whole. Findings from the thin-section fluorescence microscopic studies demonstrate that both free and liposomal daunorubicin penetrate deeply into the tumor mass. At 2 h after i.v. injection, liposomal daunorubicin produces intense fluorescence throughout the implanted P1798 tumor sections. The major portion of the fluorescent signal seems to come from within the tumor cells. Some particularly bright areas may be associated with discontinuities in the vascular endothelium, which could be sites where liposomal daunorubicin selectively leaks from the circulation into the tumor. Microscopic visualization of free drug fluorescence in tumor sections indicates a transient presence of the drug in the tumor, consistent with the *in situ* fluorescence imaging.

Fluorescence imaging with the liposomal and free drugs provides data complementary to conventional pharmacokinetic methods of sacrificing animals at various times and measuring tissue drug levels. Tissue extraction assays typically use solubilization methods that break down both the tissue and liposomes; therefore, the total drug is measured. The studies reported here provide data that begin to elucidate the *in vivo* disposition of liposomal daunorubicin, indicating the mode of delivery and release of the active drug into the tumor cells. Future studies are planned to investigate differences in subcellular localization of daunorubicin that are brought about by liposome delivery. Investigations that examine the nature of the *in situ* tumor tissue fluorescence, such as fluorescence lifetimes, polarization, and photon migration studies, may add additional information concerning differences in the local tissue and cellular environments into which daunorubicin distributes following uptake of the free or liposomal drug.

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