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# Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin

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**Summary.** Numerous studies have demonstrated that liposomal encapsulation decreases the life-threatening chronic and acute toxicities of doxorubicin in the face of unaltered or improved antitumor activity. Minimal attention has been paid to the encapsulation effect on the lesser toxicities of the drug, specifically the vesicant properties. In this report we assess the effect of the encapsulation of doxorubicin in an egg-yolk phosphatidylcholine (EPC) cholesterol liposome on the drug's topical toxicity. In addition, to ensure acceptable activity and reduction in toxicity comparable with those of previously assessed formulations, the cardiac and acute toxicities and antitumor activity of the liposomal doxorubicin complex were also investigated. Antitumor efficacy was assessed using the metastatic murine P815 mastocytoma model. Equivalent doses of free and encapsulated doxorubicin possessed the same antitumor activity in the prolongation of animal survival in 14-day survival studies conducted to assess the effect of liposomal encapsulation on the acute toxicity of this drug. The $LD_{50}$ of liposomal doxorubicin was found to be 40 mg/kg, 53% higher than that of free doxorubicin (26 mg/kg). Histologic examination of cardiac sections taken from DBA/2J mice 7 days after a single i.v. injection of free or liposomal doxorubicin (25 mg/kg) revealed that the liposomal preparation was much less cardiotoxic. In animals receiving the free drug, edema, monocytic infiltration, and cell necrosis were evident. In contrast, those receiving the liposomal preparation demonstrated slight cellular edema but showed no evidence of cellular necrosis. To assess vesicant properties, DBA/2J mice were given a single s.c. injection (0.2 ml) of free or liposomal doxorubicin (2 mg/ml). Those receiving the free drug immediately developed erythema and edema at the injection site, which progressed to ulceration. Those receiving the liposomal complex developed slight erythema and edema but did not ulcerate at any time. All signs of irritation in this group had subsided 3 weeks postinjection. In summary, the liposomal complex used eliminated the vesicant properties of doxorubicin as well as significantly decreasing its cardiac and acute toxicities in the face of unaltered antitumor activity.

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## Introduction

Although one of the most efficacious antineoplastic agents available, doxorubicin is also one of the most toxic. Myelosuppression, affecting both platelets and white blood cells, constitutes the major acute, dose-limiting side effect [4]. Doxorubicin stimulates dose-dependent nausea and vomiting and induces alopecia in 100% of patients on full-dose therapy [5]. Unique to doxorubicin are its cardiotoxic properties; chronic administration can result in a fatal cardiomyopathy if the total lifetime dose exceeds 450–550 mg/m$^2$ (depending on patient age and previous radiotherapy) [5]. The frequency of fulminant cardiac failure is, fortunately, low (2.2%) when total dose limits are observed but rises sharply to 30% if these limits are exceeded [5].

Numerous reports indicate that both chronic and acute toxicities of doxorubicin can be greatly diminished in the face of unaltered or improved antitumor activity by entrapment of the drug in liposomes [9, 16, 18–20, 22]. Although liposomal specifics (including lipid composition, size, and charge) can influence the drug's performance from a therapeutic and toxic point of view [1, 3, 11, 21], liposomal encapsulation of doxorubicin generally reduces significantly the chronic cardiotoxic effects of the drug as well as decreasing weight loss and alopecia. However, minimal attention [7] has been focused on the ability of liposomal encapsulation to reduce the topical toxicity of doxorubicin, i.e., its vesicant properties. Accidental perivenous infiltration of the drug results in painful necrotic lesions. In severe cases, treatment of such ulcers can only be achieved by surgical debridement and skin grafting. No "rescue therapy" available (i.e., saline flushing, "antidote" injections such as sodium bicarbonate) significantly alters the outcome of extravasation [5, 12]. It is possible that a liposomal carrier of doxorubicin may preclude the pain and ulceration typically noted on extravasation of the drug.

In this report we assess the effect of liposomal encapsulation on the vesicant properties of doxorubicin. In addition, in an attempt to characterize the behavior of the particular liposomal preparation used and to ensure performance comparable with that of previously reported formulations, its antitumor activity, cardiotoxic potential, and acute toxicity were assessed.

## Materials and methods

*Liposomes.* Egg-yolk phosphatidylcholine (EPC) was obtained from The Liposome Company (Princeton, NJ); it was >99% pure. Cholesterol and all salts were obtained from Sigma Chemical Co. (St. Louis, Mo). Doxorubicin was obtained from Adria Laboratories (Mississauga, Ontario). Vesicles were prepared by hydrating an EPC/cholesterol (55:45, mol:mol) film (vacuum-dried from $CHCl_3$ for 12 h) in 150 m*M* citric acid (pH 4.0), freezing and thawing the multilamellar vesicles (MLVs) five times as previously described [15] and extruding them five times [10] through polycarbonate filters (pore size, 200 nm). Doxorubicin was encapsulated using ΔpH-driven entrapment procedures as previously described by Mayer et al. [13]. Briefly, vesicles whose external medium had been brought to pH 7.8 with sodium hydroxide were added to powdered doxorubicin to achieve a drug-to-lipid ratio of 0.25:1 (wt/wt). This sample was heated at 60°C for 5 min, at which time doxorubicin-trapping efficiencies >98% were obtained. Solutions for injection were further diluted to appropriate concentrations with sterile normal saline (0.9% NaCl).

*Antitumor efficacy (murine P815 mastocytoma).* Female DBA/2J mice (Jackson Laboratories, Bar Harbor, Mo), 6–8 weeks old and weighing approximately 20 g each, were inoculated with the murine P815 mastocytoma (1500 cells/animal; EG & G Mason Research Institute, Worcester, Mass) by tail-vein injection. When injected i.v., this tumor seeds throughout the animal and becomes established in the lungs, liver, colon, and pancreas. Following tumor inoculation, the mice (in groups of 20) received free or liposomal doxorubicin at a dose of 1, 4, or 8 mg/kg via tail-vein injection. The doxorubicin dose was given over 2 days, with the first injection given immediately after tumor inoculation and the second 24 h later. Control mice received tail-vein injections of normal saline or blank liposomes (at a dose equivalent to that given with liposomal doxorubicin at a dose of 8 mg/kg). Animal survival was recorded in days and the experiment was terminated 90 days after tumor inoculation. The data were analyzed for statistical significance using the Mann-Whitney test.

*Acute toxicity.* A 14-day survival study was conducted to assess the effect of liposomal encapsulation on the acute toxicity of doxorubicin. DBA/2J mice received free or liposomal doxorubicin by tail-vein injection on day 0. Free doxorubicin was injected at doses of 10, 20, 25, 30, and 40 mg/kg; liposomal doxorubicin was injected at doses of 10, 30, 40, 50, and 70 mg/kg. Five animals were treated per group and animal survival was recorded in days.

*Cardiac toxicity.* Female DBA/2J mice received free or liposomal doxorubicin at doses of 15 and 25 mg/kg by tail-vein injection on day 0. Control mice were given an injection of normal saline. Animals were sacrificied by cervical dislocation on day 3 or day 7. The hearts were excised and fixed in 10% buffered formalin, processed for routine histology, and stained with hematoxylin and eosin (H&E).

*Vesicant properties.* Female DBA/2J mice were injected s.c. with 0.2 ml free or liposomal doxorubicin (2 mg/ml), empty liposomes (at a concentration equal to that of the liposomal doxorubicin dose), or normal saline. The drug or control was injected into the clean, shaven skin on the lateral aspect of the abdomen. The area of extravasation was observed daily for 30 days.

## Results

*Antitumor efficacy (murine P815 mastocytoma)*

Figure 1 depicts the results of the efficacy study. As expected, the survival of mice treated with free doxorubicin was dose-dependent, with 1, 4, and 8 mg/kg doxorubicin producing 10%, 30%, and 60% survival, respectively, at day 90. On a mg/mg basis, there was no significant difference ($P > 0.05$) between the survival of animals treated with free and that of those treated with liposomal doxorubicin, nor was there a significant difference in survival between saline-treated controls and those receiving empty liposomes. Thus, liposomal encapsulation of doxorubicin neither enhanced nor detracted from the drug's activity in this tumor model.

*Acute toxicity*

As shown in Figs. 2 and 3, the 14-day survival curves exhibit dramatic dependence on the doxorubicin dose in both the free and liposomal forms. The $LD_{50}$ for the free form of

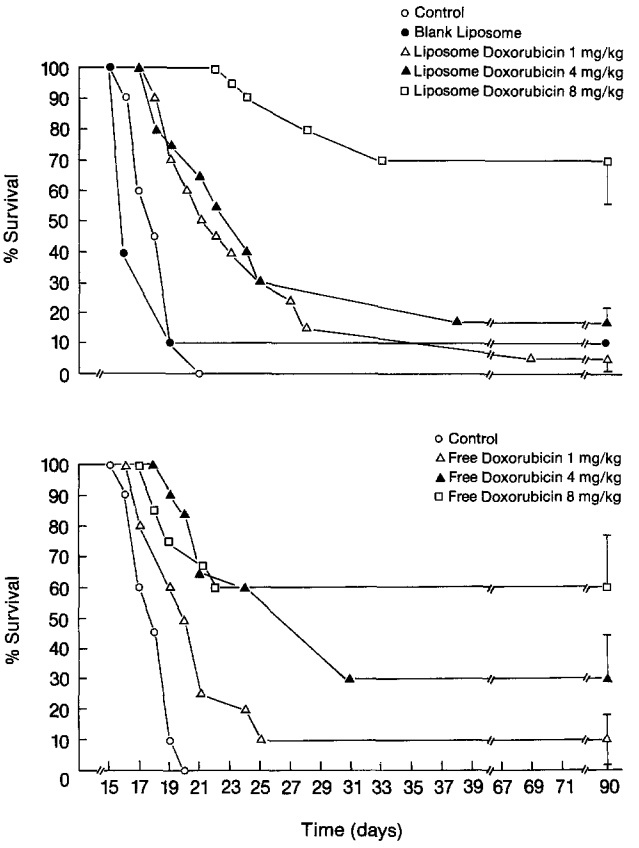


**Fig. 1.** Antitumor activities of free and liposomal doxorubicin against the P815 mastocytoma. DBA/2J mice were inoculated with 1500 P815 cells and treated with i.v. injections of free or liposomal doxorubicin at doses of 1, 4, and 8 mg/kg. Injections of normal saline or blank liposomes were given as controls. On a mg/mg basis, there was no significant difference in survival ($P > 0.05$) between animals treated with free and those treated with liposomal doxorubicin

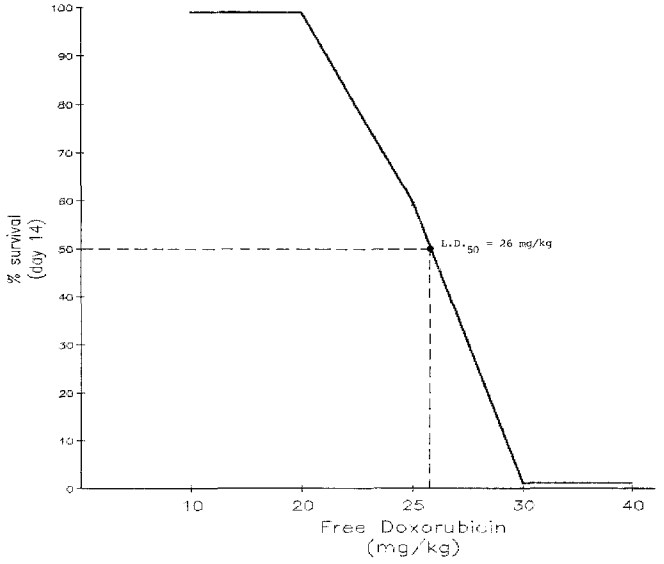


**Fig. 2.** The 14-day survival curve for DBA/2J mice given a single i.v. injection of free doxorubicin. The $LD_{50}$ was 26 mg/kg

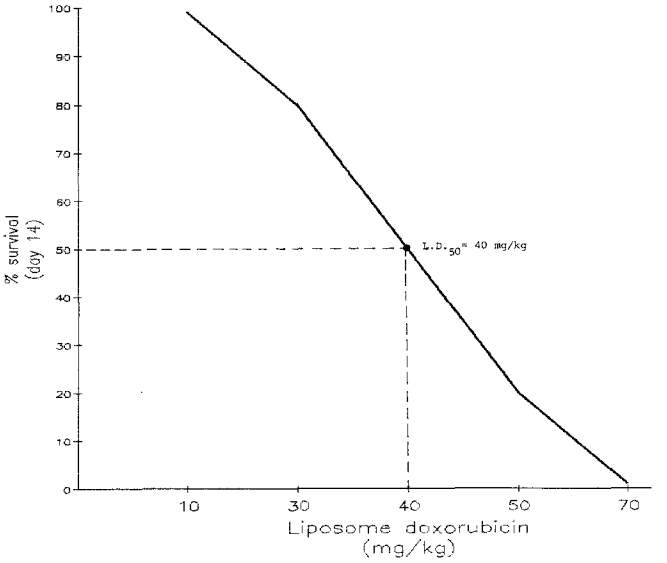


**Fig. 3.** The 14-day survival curve of DBA/2J mice given a single i.v. injection of liposomal doxorubicin. The $LD_{50}$ was 40 mg/kg

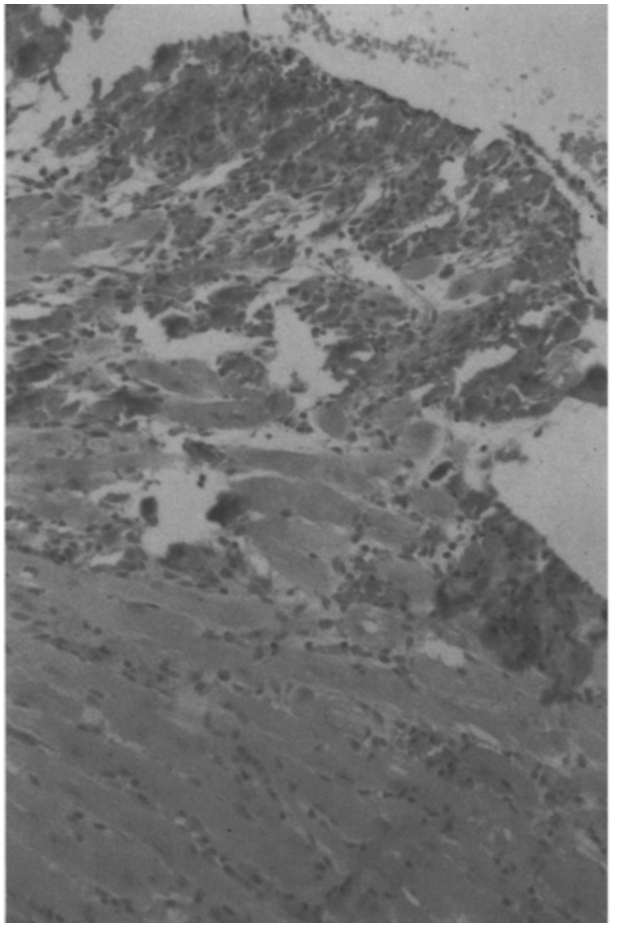

**Fig. 4.** Cardiac section from a female DBA/2J mouse 7 days after a single i.v. injection of free doxorubicin (25 mg/kg). Cellular edema, monocytic infiltration, and cell necrosis were evident

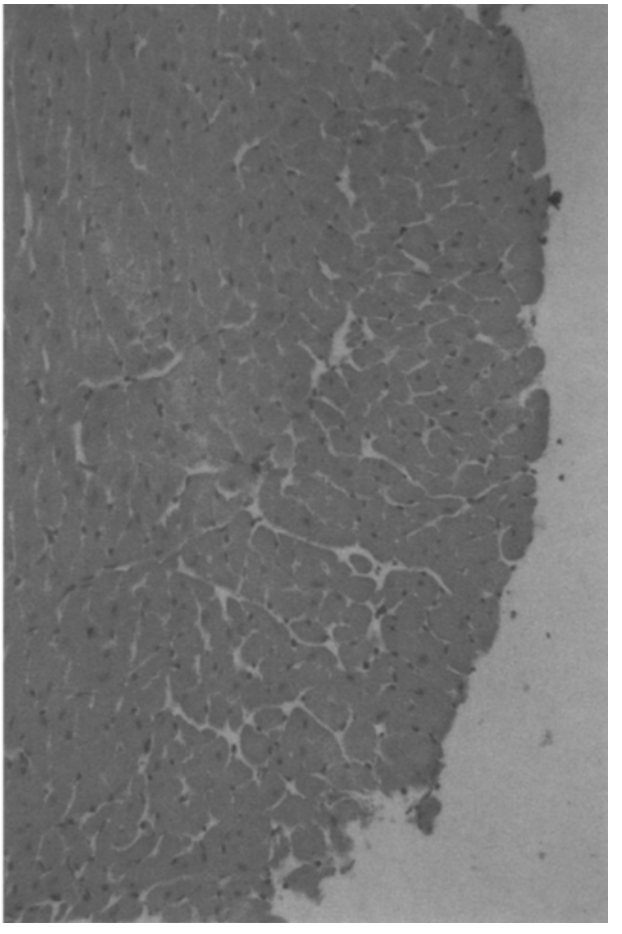

**Fig. 5.** Cardiac section from a female DBA/2J mouse 7 days after a single i.v. injection of liposomal doxorubicin (25 mg/kg). In contrast to Fig. 4, note the lack of cell necrosis and only slight edema

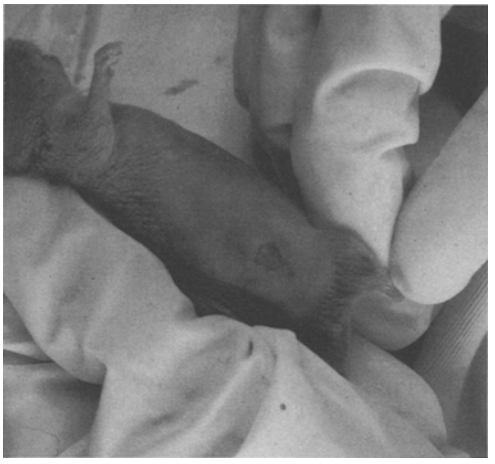

**Fig. 6.** Female DBA/2J mouse given a single s.c. injection (0.2 ml) of free doxorubicin (2 mg/ml). Erythema and edema developed at the site immediately after injection. The inflammatory response progressed and ulcerated into an open lesion by day 4. No spontaneous healing was evident by day 6 and the animal was sacrificed

**Fig. 7.** Female DBA/2J mouse given a single s.c. injection (0.2 ml) of liposomal doxorubicin (2 mg/ml). Slight erythema and edema were evident at day 4. In contrast to Fig. 6, no ulceration was evident at any time following the injection. By day 20, all signs of irritation had subsided and fur regrowth commenced

doxorubicin (Fig. 2) is 26 mg/kg and that for liposomal doxorubicin (Fig. 3) is 40 mg/kg, representing a 53% increase over the former. It should be noted that no additional deaths were observed in any group after the extension of the time course to 30 days (data not shown).

*Cardiac toxicity*

Light microscopic examination of cardiac sections taken from mice receiving 15 mg/kg free or liposomal doxorubicin did not reveal any abnormalities. However, free doxorubicin at a dose of 25 mg/kg produced noticeable histologic changes on day 3, and by day 7 (Fig. 4) the cardiac damage observed was severe. Cellular edema, monocytic infiltration, and cell necrosis were evident in both the pericardium and internal aspects of the heart. In contrast, the analysis of sections taken from animals receiving 25 mg/kg liposomal doxorubicin demonstrated only slight edema and no cell necrosis by day 7 (Fig. 5).

*Vesicant properties*

Figures 6 and 7 are photographs of mice given s.c. injections of free and liposomal doxorubicin, respectively, on Day 4. Those treated with free doxorubicin developed erythema and edema within minutes at the injection site. This inflammatory response progressed and necrotized into an open lesion (Fig. 6) by day 4. The original intention was to maintain the animals for the assessment of spontaneous healing of the area; however, by day 6 the progressing severity of the lesion necessitated sacrificing the animals. Those treated with liposomal doxorubicin also developed an inflammatory reaction (erythema, edema) at the site of injection, but at no time did this response progress to necrosis and ulceration. These animals were maintained for 30 days; by day 20, fur regrowth had commenced and all signs of irritation had subsided. Control animals given empty liposomes or saline demonstrated only a slight inflammatory response.

## Discussion

The ability of liposomal encapsulation to reduce doxorubicin's toxicities and redirect its tissue distribution is now well documented [9, 16, 18–20, 22]. Liposomes preferentially distribute into tissues with sinusoidal capillary systems and those enriched with phagocytic reticuloendothelial cells, such as the liver and spleen [13]. They are not generally sequestered in the extravascular compartment of tissues with continuous capillaries, such as the nervous system, or in skeletal and cardiac muscle [13]. For these reasons, the cardiotoxicity classically associated with long-term and/or high-dose doxorubicin therapy has been reported to be decreased by its administration in a liposomal form. Indicators of acute toxicity, such as weight loss, can also be reduced or eliminated [9, 18].

Discrepancies between reports concerning quantitative and qualitative decreases in the drug's toxicity brought about by liposomal encapsulation may be due to differences in the specific type of liposome used. In particular, the toxicity and efficacy spectrum of liposomal doxorubicin is likely to be affected by any factor altering the clearance of the liposome from the blood and its final tissue distribution. Therefore, the size, lipid content, and surface charge of a particular liposome could affect the therapeutic characteristics of the liposomal complex [1, 3, 11, 21]. The liposomal preparation used in this series of investigations demonstrates acceptable activity and reduction in cardiac and acute toxicities comparable with those reported for previously tested formulations. In addition, the $\Delta$pH-dependent encapsulation procedure used in the present study provides improvements in trapping efficiency and stability over those of previously used, passive trapping procedures [14].

In the present study, doxorubicin produced comparable long-term survival in DBA/2J mice carrying the murine P815 mastocytoma, whether the drug was given in the free or liposomally encapsulated form. The majority of studies assessing the in vivo activity of liposomal doxorubicin in tumor models have also found liposomal and free doxorubicin to be equally effective [6, 8, 18, 19, 22]. Those reporting enhanced activity for liposomal doxorubicin often use tumors in tissues and/or organs possessing a sinu-

soidal capillary system or enriched with phagocytic endothelial cells.

We assessed doxorubicin's cardiotoxicity with the qualitative histologic examination of cardiac sections taken from DBA/2J mice given equal doses of free and liposomal doxorubicin and demonstrated lower cardiotoxic potential for the liposomally encapsulated form. By day 7, animals receiving 25 mg/kg free doxorubicin experienced severe cardiac damage compared with controls; cellular edema, monocytic infiltration, and cell necrosis were evident. In contrast, those receiving 25 mg/kg liposomal doxorubicin developed little cardiac edema and no cell necrosis by day 7. Such dramatic differences in cardiotoxic potential have been attributed to the decreased cardiac uptake of doxorubicin in its liposomal form [18, 19]. The present findings are comparable with those previously reported. For instance, Rahman and co-workers [19] have found the cardiac level of liposomal doxorubicin in male DBA/2J mice to be approximately half that attained with the free drug. Qualitative analysis revealed that the myocytic and myofibrillar structure of the cardiac muscle was markedly well preserved when liposomal doxorubicin was given, compared with the free drug.

The acute toxicity of liposomal doxorubicin was assessed by examination of the effect of the dose on animal survival. Previous work has demonstrated that the primary nature of acute toxicity involves both the degenerative action of anthracycline on the heart tissue and myelosuppressive effects [5, 17]. The $LD_{50}$ observed in the present study at 14 days for free doxorubicin was 26 mg/kg, comparable with that reported in the literature [19]. The $LD_{50}$ similarily calculated for liposomal doxorubicin was approximately 50% higher, at 40 mg/kg.

The final phase of this study investigated the effect of liposomal encapsulation on the vesicant properties of doxorubicin. Whereas the efficacy of encapsulation in reducing the cardiotoxic potential of the drug has been well studied, as is evident from the above discussion, its effect on the topical toxicity of doxorubicin has received only minor attention [7]. This is due in part to the fact that the vesicant properties of doxorubicin are neither dose-limiting nor fatal [5, 12]. Nevertheless, its topical toxicity is cause for concern, as the extravasation of doxorubicin can seriously and adversely affect the patient's quality of life.

The inadvertent perivenous infiltration of doxorubicin can lead to severe, protracted ulceration and necrosis [5]. The mechanism of this anthracycline-induced skin necrosis is poorly understood. Doxorubicin has a high affinity for cellular DNA, to which it rapidly binds and intercalates between nucleic base pairs, resulting in the inhibition of DNA synthesis and cell death. With respect to skin injury, there have been speculations that doxorubicin released from dying cells in the dermis induces damage and death in neighbouring cells, only to be released again, thus forming a repetitive cycle. This would account for the indolent and progressive nature of the tissue damage [2]. Alternatively, it has been suggested that doxorubicin undergoes enzymatic alterations to form a primary or secondary free-radical species that causes cell death/skin necrosis by rapidly binding DNA and/or membrane lipids [2]. Regardless of the mechanism, the various rescue therapies that have been recommended (i.e., intradermal injections of steroids, chemical neutralizing agents) have not significantly improved the outcome of the extravasation [4, 5].

Forssen et al. [7] have demonstrated the ability of liposomal encapsulation to reduce the vesicant properties of doxorubicin. Using ΔpH-dependent encapsulation to provide improved trapping efficiency and stability, we demonstrated that liposomal encapsulation dramatically prevented doxorubicin from inducing necrosis at the site of infiltration at doses fourfold higher than those tested by Forssen. Animals receiving s.c. injections of liposomal doxorubicin experienced a slight inflammatory response and some discomfort, but at no time did the site of injection ulcerate. Hair regrowth occurred and the area of insult appeared normal by day 20. In contrast, those receiving free doxorubicin experienced a pronounced inflammatory response that progressed to severe necrosis. This lack of topical toxicity due to liposomal encapsulation is likely due to the fact that free doxorubicin, or enzymatic degradation products, will rapidly bind cellular DNA/membrane lipids upon perivenous administration, whereas the encapsulated drug is not available to the tissue, being washed away by the lymphatics prior to its release from the liposomes. This hypothesis is supported by the finding that in an attempted rescue, the injection of empty liposomes exhibiting a transmembrane pH gradient (inside acidic) into the site of doxorubicin infiltration did not alter the outcome of the extravasation (data not shown). This indicates that the cellular binding of doxorubicin must occur very rapidly, as the empty liposomes displaying a ΔpH (which efficiently incorporate free doxorubicin) could not encapsulate any of the extravasated drug.

In summary, it is evident that liposomal encapsulation of doxorubicin greatly reduces the drug's toxicities without impeding its antitumor efficacy. In the clinical setting, this translates into an enhanced effect in those malignancies in which doxorubicin is an established part of the therapeutic regimen, potential efficacy in doxorubicin-resistant systems, and an improved quality of life for the cancer patient.

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