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Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes

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Abstract A problem associated with the intravenous delivery of vincristine concerns drug extravasation at the site of injection or infusion. This can result in extensive local soft-tissue damage. A new formulation of vincristine has recently been developed based on encapsulation of the drug in liposomes. The liposomal drug is somewhat less toxic and substantially more efficacious than free drug. The studies described here assessed, using a murine model of drug extravasation, whether vincristine encapsulation in liposomes influences drug-induced dermal toxicity. It was shown that subcutaneous injection of vincristine in liposomes does not result in the gross skin necrosis and ulceration observed following injection of free drug. Histological analysis of the dermal tissue surrounding the injection site suggests that free drug induces a pronounced inflammatory reaction as judged by the presence of infiltrating leukocytes. In contrast, the liposomal formulation of vincristine engenders a mild prolonged inflammatory condition. These toxicological studies were correlated with an evaluation of drug retention at the site of administration. It was shown using radiolabelled vincristine as a drug marker, that free vincristine is rapidly eliminated from the injection site. In contrast, the level of drug at the site of injection was far greater when the drug was given in liposomal form.

Key words Vincristine · Liposomes · Dermal toxicity

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Introduction

Vincristine is a widely used antineoplastic agent that displays effectiveness against a wide variety of neoplasms including Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukemia, embryonal rhabdomyosarcoma, neuroblastoma, breast carcinoma, and Wilm's tumor [1, 2]. A dose-limiting neurotoxicity is associated with vincristine use, manifested mainly as peripheral neuropathy. Vincristine is also known for its ability to produce soft-tissue necrosis and ulceration if accidently extravasated during intravenous (i.v.) administration or an inadvertent intramuscular (i.m.) injection [3, 4]. Although several studies have focused on developing and assessing procedures to halt or reverse the necrosis that occurs following an extravasation event [5, 7], there is at present no effective procedure for controlling the outcome after accidental exposure. Further, given the potential hazards associated with vincristine extravasation, the development of treatment protocols based on long-term i.v. infusions have not been developed. This is despite reports strongly suggesting that the therapeutic activity of this cell-cycle-specific drug would be improved significantly if given by long-term infusion [8, 9].

This laboratory has developed a liposomal formulation of vincristine to take advantage of the ability of liposomes to exhibit long residence times in the plasma compartment following i.v. administration [10]. Drug released from circulating liposomes could provide a convenient "microinfusion" system for this drug. In addition, studies from our laboratory and others have demonstrated that after i.v. administration liposomes containing anticancer agents can accumulate preferentially in regions of disease, such as tumors [11, 12]. Preclinical studies assessing the antitumor activity of this formulation have shown that vincristine is significantly more active when given in liposomal form [10, 13].

The studies described in this report assessed the effect of liposomal encapsulation on vincristine-induced dermal toxicity. It was anticipated, based on previous studies demonstrating reduced dermal toxicity of doxorubicin [14, 15],

when given in liposomal form, that liposomal vincristine would not cause ulceration or other damage when given subcutaneously (s.c.) to mice. The studies presented here also determined drug levels within the injection site over time and the results suggest that long-term drug exposure can be achieved safely within defined regions when the drug is given s.c. in liposomal form.

Materials and methods

Distearoyl phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids and was >99% pure. Cholesterol, HEPES, and citric acid were obtained from Sigma (St. Louis, Mo.). Vincristine sulfate was purchased from Lynphomed (Markham, Ont.). [^{14}C]Cholesteryl hexadecyl ether was produced by special order from New England Nuclear (Ontario, Canada) and was >95% pure. It was chosen as a lipid marker due to its stability in vivo [16]. [^{3}H]Vincristine was obtained from Amersham (Oakville, Ontario, Canada). Female BALB/c mice (retired breeders) were purchased from Charles River Laboratories.

DSPC/Chol (55:45; mol/mol) liposomes were prepared by first dissolving the lipid mixture in 95% ethanol at 60 °C for 30 min (100 mg lipid/ml). Multilamellar vesicles (MLVs) were formed by adding 300 m*M* citrate, pH 4.0, and vortex mixing vigorously (25 mg lipid/ml final mixture). The resulting MLVs were then incubated at 60 °C for an additional 30 min to ensure equilibration of citrate buffer across the lipid bilayers. Following incubation, large unilamellar vesicles (LUVs) were produced by extruding the MLVs through an extruder containing two Nucleopore polycarbonate filters with 100 nm pore size. The extrusion device was obtained from Lipex Biomembranes (Vancouver, British Columbia, Canada) and was equilibrated at 60 °C. Following extrusion, the liposomes were dialyzed against two changes of 100 volumes of citric acid buffer (pH 4.0) over a 24-h period. Spectra/Por 2 dialysis tubing was used (cutoff 12–14 kDa). The resulting liposomes displayed a mean diameter of 110 nm as demonstrated by quasielastic light scattering.

Vincristine was loaded into the liposomes as follows. The vesicles were passed down a G25 Sephadex column equilibrated with Hepes buffered saline, pH 7.4, to exchange the external buffer. Vincristine sulfate was then added to the liposomes to achieve a drug-to-lipid ratio of 0.1:1. The resulting drug/lipid mixture was then incubated at 60 °C for 10 min. This procedure ensures a >95% trapping efficiency of the drug [10].

The procedure used for assessing skin toxicity has been previously described [14]. Briefly, an approximately 3-cm^2 area of hair above the hindleg of adult BALB/c mice was removed by vigorous rubbing with Neet topical depilatory lotion (Whitehall Laboratories, New York, N.Y.). This procedure, which causes no adverse skin effects in itself [14], was followed after 24 h by s.c. injection of 10 µg of either free or liposomal vincristine (diluted to 50 µl in normal saline) using a 25-gauge needle (bevel up). Animals were monitored twice daily for any sign of skin irritation or damage. If ulceration was observed the animals were terminated immediately. A more refined assessment of dermal toxicity was based on histologic evaluation of the injection site. Briefly, at selected time points, mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (160 mg/kg) and xylazine (20 mg/kg) prior to cervical dislocation. Skin around the injection site was removed and placed in 10% formalin. The samples were left in formalin for at least 24 h prior to processing and paraffin embedding. The tissue blocks were cut into 8-µm sections and deparaffinized prior to staining with hematoxylin and eosin.

The level of liposomal lipid and/or vincristine within the injection site was quantified using radiolabelled tracers. Liposomal lipid was measured by incorporating [^{14}C]cholesteryl hexadecyl ether, a non-exchangeable and non-metabolizable lipid marker. Vincristine was measured through use of a [^{3}H]vincristine label. For these studies, skin around the injection site was removed and subsequently homogenized in saline (0.9%) using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ont.). The skin homogenates (total volume) were then digested with 500 µl of "Solvable" (DuPont Canada, Mississauga, Ont.) for 3 h at 50 °C. Subsequently, the samples were cooled to room temperature before decolorizing with 200 µl of 30% hydrogen peroxide. Samples were then analyzed using dual label liquid scintillation counting.

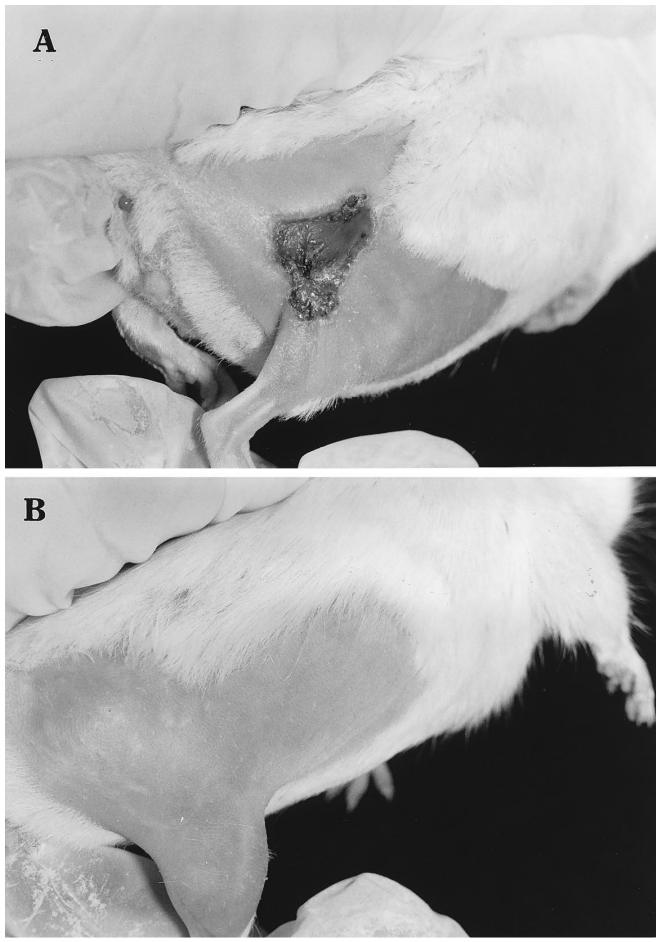

Fig. 1 A, B Female BALB/c mouse given s.c. vincristine either as free drug (**A**) or in liposomal form (**B**). Each mouse was given a single s.c. injection (50 µl) of free or liposomal vincristine (10 µg)

Results

Gross ulceration studies

Figure 1A and B are photographs of mice 9 days after being given s.c. injections of free and liposomal vincristine, respectively. Neither treatment group displayed any evidence of erythema or edema at the site of injection over the first 7 days following injection. On day 7, five of the ten mice given free vincristine showed ulcerations with diameters of 2–3 mm. The frequency of ulcerations in mice given free drug was ten/ten with a total of nine mice showing evidence of ulceration by day 10 and all mice ulcerated by day 11. In contrast, mice injected with liposomal vincristine showed no evidence of skin necrosis or ulceration throughout the time course of the experiment.

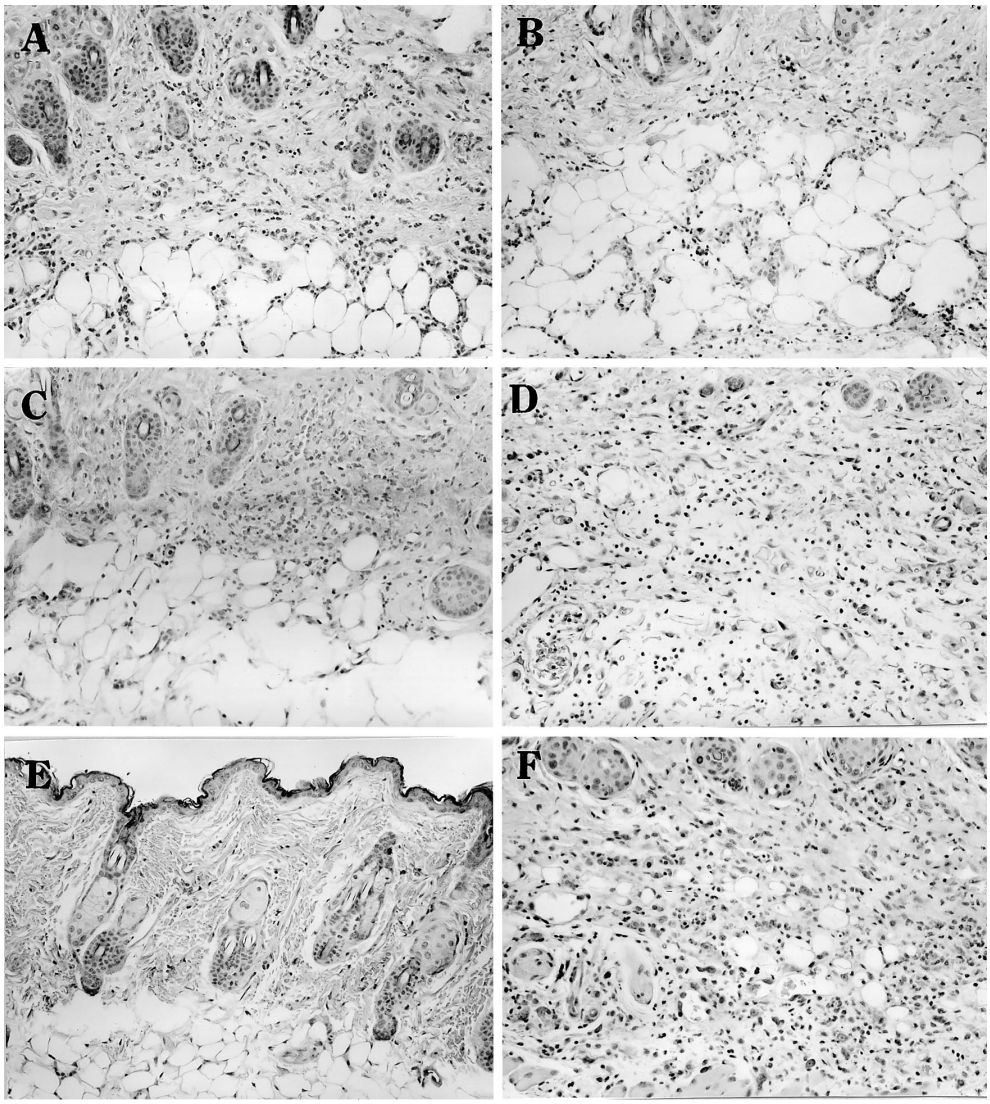

Fig. 2 A–F Histologic skin sections from BALB/c mice given free or liposomal vincristine. Each mouse was given a single s.c. injection (50 µl) of free or liposomal vincristine (10 µg). All sections are shown at × 100 magnification and stained with hematoxylin and eosin. **E** Control mice; **A, C** free drug at 1 and 3 days following injection, respectively; **B, D, F** liposomal drug at 1, 3, and 7 days following injection, respectively

Control animals given saline or empty liposomes showed no evidence of an inflammatory response.

Histological studies

In an attempt to correlate ulcer formation with more subtle changes in tissue histology, skin sections were examined. Skin was isolated 1, 3, 5, and 7 days after s.c. administration of either free vincristine or liposomal vincristine. The photomicrographs presented here are from representative skin sections. The H&E-stained sections of dermal tissue showed that for animals given free drug there were numerous inflammatory cells in the s.c. injection site within 1 day (Fig. 2A). There were significantly fewer of these cells within sections derived from mice 3 days after drug administration (Fig. 2C). These sections appeared similar to those derived from control animals (Fig. 2E). In contrast, sections obtained from animals given s.c. injections of liposomal vincristine showed a much less intense inflammatory response 1 day after drug administration (Fig. 2B). However, the presence of inflammatory cells in the dermal area was prolonged, lasting throughout the 7-day study (Fig. 2D,F). In an attempt to quantify the observed changes in inflammatory cells, the number of basophilic cells (blue cells) within selected fields of each section was estimated. Fields were selected on the basis of regions that appeared enriched in inflammatory cells. The results of this analysis are summarized in Table 1. It should be noted that the number of inflammatory cells within the dermal area were relatively constant following s.c. administration of free or liposomal drug. Further, the number of leukocytes within this area was consistant with that observed for control sections. The number of inflammatory cells in the subcutaneous area derived from treated animals confirms the acute and prolonged inflammatory conditions observed following injection of free and liposomal vincristine, respectively. For animals given s.c. injections of liposomal drug there were almost tenfold more inflammatory cells observed on day 7 compared with untreated animals and animals given free drug. As indicated above, no gross lesions were observed in animals given liposomal drug.

Table 1 Inflammatory response following subcutaneous injection of free or liposomal vincristine

Injection	Dermal inflammatory cells (cells/mm²)	Subcutaneous inflammatory cells (cells/mm²)
Controls	8.2×10^4	9.8×10^3
Free vincristine (10 μg)		
Day 1	1.9×10^5	1.3×10^6
Day 3	1.8×10^5	6.8×10^4
Day 5	1.5×10^5	9.0×10^4
Day 7	1.1×10^5	9.0×10^4
Liposomal vincristine (10 μg)		
Day 1	4.3×10^5	6.9×10^5
Day 3	2.0×10^5	5.4×10^5
Day 5	4.6×10^5	6.6×10^5
Day 7	7.2×10^5	9.0×10^5

These animals were observed for periods in excess of 30 days.

Analysis of drug levels within the injection site

In an attempt to establish a pharmacodynamic correlate for drug-induced skin ulceration, the level of drug and/or liposomal lipid was measured within the injection site 1, 3, 5, and 7 days after administration of free or liposomal vincristine. The results, shown in Fig. 3A, indicate that free drug was cleared rapidly from the site of injection. Less than 0.4% of the drug remained at the injection site 1 day after administration. This residual amount of drug, easily detectable by the assay system employed, remained essentially constant over the 7-day time course. In contrast, almost 6% of the drug remained at the injection site 1 day after animals were given liposomal vincristine. This level of drug was more than ten times the level observed after injection of free drug. The level of drug within the injection site gradually declined over the time course of the experiment, with less than 0.3% remaining after 7 days. In addition to monitoring drug levels following s.c. injection of liposomal vincristine, the level of liposomal lipid was determined. It is interesting to note that approximately 45% of the injected lipid dose remained at the injection site 1 day after administration. This level of liposomal lipid was essentially constant throughout the time course. If it is assumed that all the vincristine within the injection site was associated with the liposomal carrier, it can be estimated that the drug-to-lipid ratio decreased approximately 90% within 1 day after administration. This value is comparable to that observed following i.v. administration of the drug, where the drug-to-lipid ratio of liposomes retained in the plasma changes from 0.1 to 0.01 within 24 h [10].

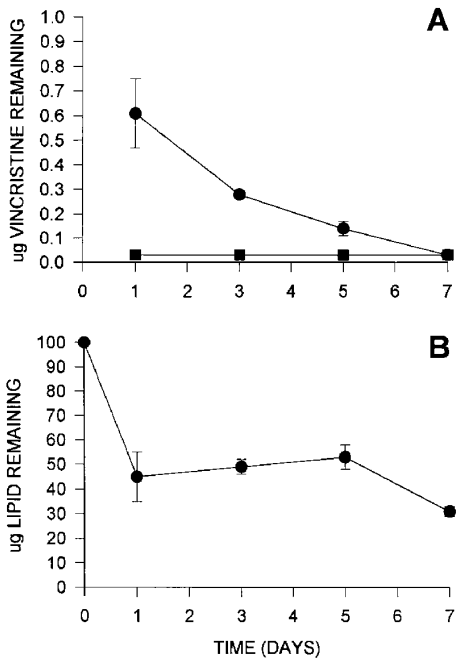

Fig. 3 A, B Cutaneous retention of liposomal vincristine. Cutaneous retention of vincristine (**A**) and lipid (**B**) following s.c. injection of 10 μg of free (■) or liposomal (●) vincristine. The drug-to-lipid ratio of liposomal vincristine was 0.1:1 (w/w). *Error bars* are the standard deviations of the results from four mice

Discussion

This study investigated the effect of liposomal encapsulation on the vesicant properties of vincristine. It has previously been shown that encapsulation of the drug reduces vincristine-induced acute toxicity in mice as evidenced by a decrease in weight loss over time [10, 13]. The soft tissue toxicity of vincristine, however, has received minimal attention until now. In this study we focused on the vesicant properites of the drug since extravasation can seriously affect the patient's quality of life, particularly since vincristine is widely used in pediatric patients for the treatment of acute lymphoblastic leukemia. Extravasation injuries have been shown to be highest in the pediatric and geriatric age groups [17] and has forced the use of more invasive central lines in these patients to administer drug. Extravasation following standard i.v. administration is a relatively common occurrence, occurring in as many as 1–2% of chemotherapy infusions [18]. In patients following the extravasation of the antineoplastic drug doxorubicin, blistering and skin loss become apparent in a few days, followed by progressive tissue necrosis that can continue for as long as 3 months [19]. Full-thickness skin necrosis can eventually ensue exposing underlying tendons and neurovascular structures [18].

The mechanism by which soft-tissue necrosis occurs is widely assumed to be due to a directly cytotoxic effect of the drug. Histologic analysis has been reported on two patients following inadvertent extravasation of cytotoxic

drugs [20, 21]. These studies revealed a nonspecific chronic inflammation with a patent microvasculature. In addition to the gross ulceration observed following s.c. injection of free vincristine using the murine model described here (see Fig. 1A), there were also observed short-term histopathologic changes in the tissue consistent with an inflammatory response (Fig. 2B and Table 1). For these reasons we believe that the murine model used here is representative of the dermal toxicity observed in humans following vincristine extravasation during drug administration. Regardless of the mechanism and severity of vincristine-induced necrosis, various antidote therapies have had questionable efficacy [22, 23].

Since it has been shown previously that liposomal encapsulation of doxorubicin can dramatically reduce the vesicant properties of the drug [15, 24], the use of liposomes to abrogate the vesicant properties of vincristine was assessed. As indicated in Fig. 1B, liposomal encapsulation of vincristine dramatically reduced soft-tissue damage by the drug. There was virtually no evidence of inflammatory response seen grossly when liposomal vincristine was administered s.c. In contrast, free drug produced gross ulceration in 100% of the treated animals within 11 days after injection. Histologic analysis of the injection site suggested that liposomal vincristine induced a mild, but prolonged inflammatory response that was distinct from the intense, short-lived inflammatory response observed after free drug administration. The prolonged inflammatory response observed following s.c. injection of liposomal vincristine may be due to the fact that vincristine levels within the injection site are higher than can be achieved with free drug and are maintained at these levels for periods in excess of 5 days (Fig. 3). The drug leaks slowly from the liposome interior, resulting in the tissue being exposed to a long-term, low dose of free drug. In comparison, when free drug is administered, there is a brief exposure of the tissue to the full drug dose before it is absorbed by the circulatory system. This peak level of exposure to free drug may be the causal factor leading to soft-tissue necrosis.

It is tempting to speculate on the basis of the results presented here that liposomal vincristine could be used safely when treatment consists of local administration. Although it has been suggested that the therapeutic activity of anticancer drugs could be improved through regional delivery [25], clinical studies have shown that unacceptable drug toxicities limit the utility of locally injected free anticancer drugs. It may be possible to avoid these toxicities when the drug is given in liposomal form. The results presented here clearly demonstrate that vincristine-induced dermal toxicity can be reduced when the drug is given in liposomal form. Previous studies from our research group demonstrate that the therapeutic activity of liposomal vincristine is greater than free drug [10, 13]. Therefore, in addition to providing a more potent formulation of the drug, liposomes reduce the potential of vincristine to cause tissue necrosis upon accidental extravasation. This could improve patient quality of life and allow the drug to be administered more safely via standard or alternative routes of administration.

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