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Multivesicular liposomes containing bleomycin for subcutaneous administration*

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Summary. Optimal cancer treatment with cell-cycle-specific agents requires maintenance of a cytotoxic drug level for a prolonged period. We explored the use of multivesicular liposomes as a slow-release depot of bleomycin for systemic administration via the s.c. route. The average volume-adjusted liposome size was 19.1 μm, the half-life of leakage in human plasma was 32.1 h, and the half-life of s.c. liposomal bleomycin was 31.8 h. When tested against the s.c. B-16 melanoma model in BDF_1 mice, the therapeutic index of single-dose bleomycin given s.c. was significantly improved when the drug was encapsulated in multivesicular liposomes. The efficacy was improved as assessed by both inhibition of tumor growth and increased life span, and the toxicity appeared to be decreased.

Introduction

Bleomycin is an important antineoplastic drug used in several curative chemotherapeutic regimens for widespread cancer. It is a phase-specific drug [1, 2] that kills cancer cells most efficiently when they are in mitosis or in G2 phase. Therefore, prolonged exposure to the drug at a therapeutic concentration is required for optimal cell kill. There is also evidence that prolonged low-dose administration is less toxic than intermittent high-dose bolus injections in mice [14] and in man [3]. An attempt to produce a slow-release preparation of bleomycin by suspending the drug in sesame oil was unsuccessful [15]. At present, the only way of maintaining drug levels over a prolonged period is through very frequent dosing or continuous administration, both of which are cumbersome, inconvenient, and costly. Therefore, an acceptable slow-release depot preparation of bleomycin is needed.

We have studied a new type of liposome (multivesicular liposomes) for the encapsulation of bleomycin [6–8, 10, 11]. Multivesicular liposomes differ from the traditional multilamellar and unilamellar liposomes by virtue of their containing numerous, nonconcentric chambers, each of which is separated from the adjacent chambers by a single bilayer membrane [8]. Multivesicular liposomes are uniquely suitable for use in drug delivery because of their nontoxic membrane components, the highly efficient drug incorporation, the slow drug-release rate, and their stability during storage [7].

Materials and methods

Materials. Bleomycin sulfate (Blenoxane) was a gift from Bristol Laboratories (Syracuse, N. Y.). Dioleoyl lecithin, dipalmitoyl phosphatidylglycerol, and cholesterol were purchased from Avanti Polar-Lipids (Birmingham, Ala.); triolein, triethylamine, and L-lysine were procured from Sigma (St. Louis, Mo.); and nanograde chloroform was obtained from Mallinckrodt (Paris, Ky.). All were used without further purification. BDF_1 and C57BL/6 mice were obtained from Simonsen Laboratories (Gilroy Calif.); the vortex mixer was supplied by American Scientific Products (McGaw Park, Ill.). All HPLC-grade solvents were obtained from Fisher Scientific, New Jersey, and heptane sulfonic acid was supplied by Eastman Kodak (Rochester, N. Y.).

Liposome preparation. For each batch of liposomes prepared, 1 ml bleomycin solution (30 mg/ml) in 4% glucose (w/v) containing 0.065 *N* hydrochloric acid was added to a 1-dram vial containing 9.3 μmol dioleoyl lecithin, 2.1 μmol dipalmitoyl phosphatidylglycerol, 15 μmol cholesterol, 1.8 μmol triolein, and 1 ml chloroform. The vial was attached to the head of the vortex mixer and shaken at the maximal speed for 6 min. Each half of the resulting water-in-oil emulsion was individually squirted rapidly through a narrow-tip Pasteur pipette into a 1-dram vial containing water (2.5 ml), glucose (3.2 g/100 ml), and lysine (40 mM), which were then shaken on the vortex mixer for 3 s at the maximal setting to obtain chloroform spherules. The chloroform-spherule suspensions in the two vials were transferred into the bottom of a 250-ml Erlenmeyer flask containing water (5 ml), glucose (3.2 g/100 ml), and lysine (40 mM). To evaporate the chloroform, a stream of nitrogen gas was flushed through the flask at a flow rate of

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7 l/min for 10–15 min at 37° C. The liposomes were then isolated by centrifugation at 600 *g* for 5 min and washed with 0.9% NaCl solution. They were used within 24 h. The liposomes were characterized according to previously published methods [9].

In vitro studies. For estimation of the rate of release of bleomycin from multivesicular liposomes in vitro, blood-bank human plasma was incubated with washed liposome pellets. The liposome suspension was placed in a syringe, 0.01% sodium azide was added to inhibit the growth of microorganisms, and air was excluded from the syringe, which was then incubated at 37° C. At appropriate points, aliquots were removed. The liposomes were separated from the plasma by the addition of 5 vol. 0.9% NaCl solution to the aliquot, followed by centrifugation for 2 min in an Eppendorf microfuge. The amount of bleomycin in the liposome pellets was compared with that determined at time zero and expressed as the percentage of drug remaining in liposomes.

In vivo studies. A pharmacokinetic study was done on male BDF_1 mice weighing 20–25 g. A group of mice were injected s. c. with 2 mg free or encapsulated drug in 0.2 ml 0.9% NaCl solution using a $^5/_8$-in., 28-gauge hypodermic needle in the middle of the abdominal skin. Care was taken to minimize back-leakage through the needle by tunneling through the skin before injecting the drug. At appropriate points, the animals were killed by cervical dislocation. The full thickness of the abdominal wall tissue, including the entire skin and the underlying peritoneal membrane, was excised. The specimens were minced and then homogenized on ice with distilled water using a glass Dounce tissue homogenizer. An aliquot of the homogenate was centrifuged in an Eppendorf microfuge for 5 min to remove tissue debris. The supernate was cleaned up by the addition of 2 vol. ice-cold acetonitrile, and the bottom fraction was analyzed using a Waters Associates (Milford, Mass.) high-performance liquid chromatograph (HPLC) according to a modification of previous method [12].

In brief, samples were isocratically eluted from a Beckman (San Ramon, Calif.) C-18 reverse-phase Ultrasphere ODS 5-μm steel column measuring 4.6 mm × 25 cm using a mobile phase consisting of methanol : acetonitrile : water : acetic acid (225 : 68 : 714 : 2, by vol.) containing 0.2 mM heptane sulfonic acid and 29 mM triethylamine (final pH = 5.4) at a flow rate of 1 ml/min. Bleomycins were detected at 254 nm using a Waters model 440 fixed-wavelength UV detector. The commercially available bleomycin is a mixture of two main components, bleomycins A2 and B2, and their retention times were 7 and 14 min, respectively. Data for the two components were summed to obtain total bleomycin values. The efficiency of bleomycin recovery from the skin samples after processing and extraction was 48% ± 5%. There were no interfering peaks and the standard curve was linear. The detection limit was 3 μg/ml for total bleomycin.

Efficacy study. B-16 (FO) melanoma cells initially obtained from American Type Culture Collection (Rockville, Md.) were maintained as s. c. tumors in male C57BL/6 mice. The tumors were dissected using a sterile technique, minced thoroughly in serum-free Dulbecco's modified Eagle's medium (DMEM), and made into a fine suspension by several passages through a 21-gauge needle. The suspension was centrifuged for 3 min at 600 *g* and the supernate was removed. A 0.2-ml sample of the pellet was inoculated into the caudal abdominal skin of male BDF_1 mice. At 24 h after inoculation (on day 1), the mice were treated with a single s. c. dose of free or encapsulated bleomycin at the nape of neck, distant from the tumor site. The ranges of injection volumes and lipid doses were 0.11–0.44 ml and 1.1–4.4 mg lipid/mouse, respectively, depending on the dose. The tumor sizes were measured with a caliper and the volumes were calculated. The tumor volume was converted into tumor weight, assuming a specific gravity of 1. Both the free and the liposomal bleomycin given to the mice were assayed by HPLC prior to drug administration. The increase in life span (ILS) was calculated by the following equation:

$$\text{ILS} = \frac{T - C}{C} \times 100\%$$

where T represents the median survival (in days) of the treated group and C represents that of untreated controls.

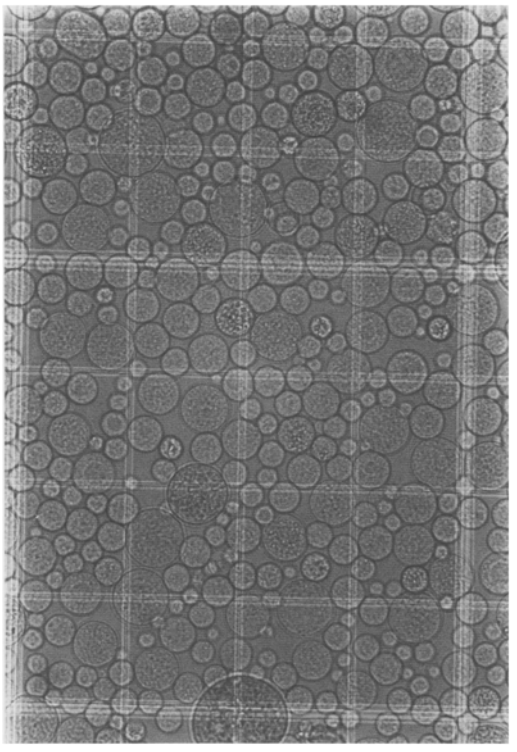

Fig. 1. Photomicrograph of multivesicular liposomes containing bleomycin. The size of each square is 50 × 50 μm

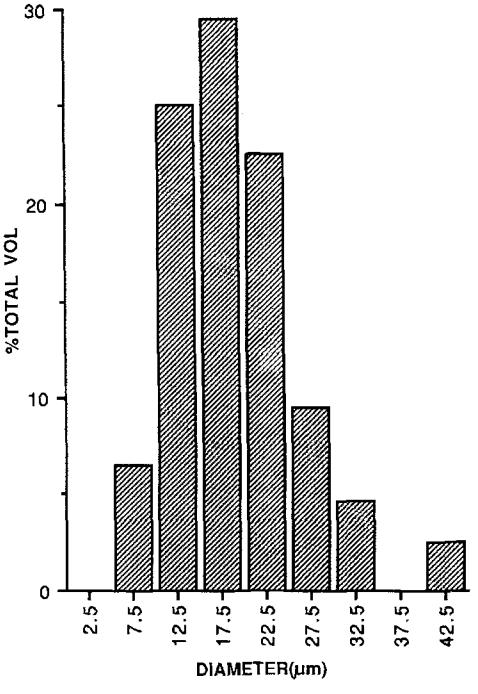

Fig. 2. Volume-adjusted size distribution of multivesicular liposomes containing bleomycin

Results

Characterization of bleomycin liposomes

Figure 1 shows the photomicrograph of multivesicular liposomes containing bleomycin sulfate. Figure 2 shows

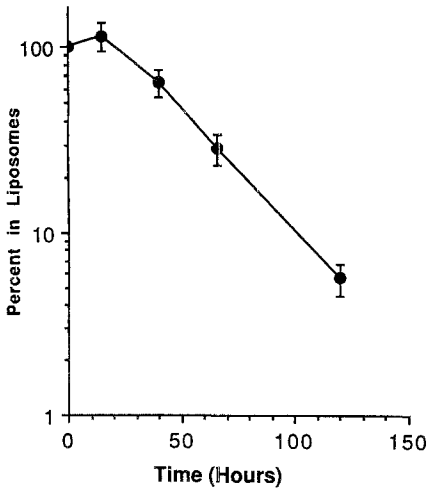

Fig. 3. Release of bleomycin from multivesicular liposomes suspended in 100% human plasma. Each data point represents the mean from 3 experiments; bars represent the SEM

the liposome size distribution. The average volume-adjusted diameter of the liposomes was 19.1 μm; 68% of the liposomes had diameters of between 13.5 and 26.5 μm. The percentage of capture (± standard deviation) was 72.3%±7.5% (n = 3) and the captured volume was 44.2±4.6 μl/mg lipids used. The ratio of bleomycin/lipids was 1.83±0.19 mg bleomycin/mg lipids.

In vitro drug-release studies

The liposome suspended in human plasma at 37° C demonstrated first-order release kinetics, showing a half-life of 32.1 h as shown in Fig. 3.

Subcutaneous pharmacokinetics

Following s.c. injection of free bleomycin, the amount of the drug under the skin decreased exponentially, demonstrating a half-life of 8 min. The drug encapsulated in multivesicular liposomes escaped from the injection site at a half-life of 31.8 h (Fig. 4). Analysis of blood from mice that had been injected with encapsulated drug did not reveal detectable levels of bleomycin (limit of detection = 3 μg/ml).

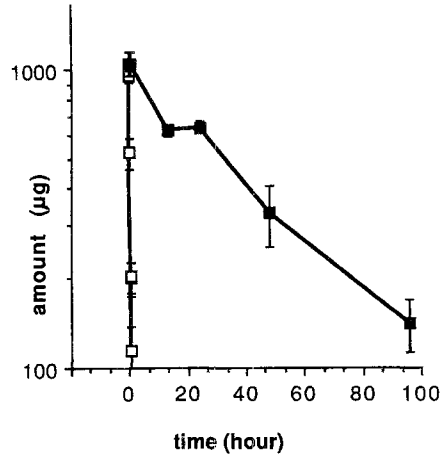

Fig. 4. Pharmacokinetics of bleomycin remaining at the injection site after s.c. injection of 2 mg unencapsulated free bleomycin (□) or liposome-encapsulated bleomycin (■). Each data point represents the mean from 4 animals, except for the 96-h point, for which 3 animals were used; bars represent the SEM

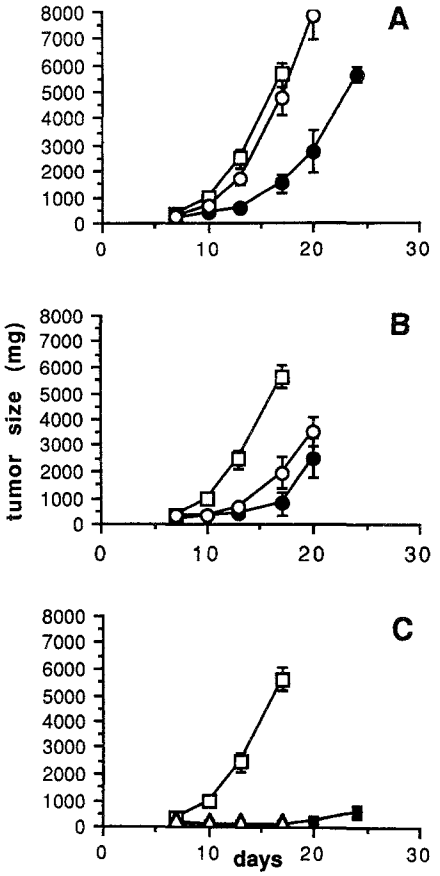

Fig. 5 A–C. Therapeutic effect of bleomycin in multivesicular liposomes as measured by tumor growth inhibition. Each data point represents the mean from 5 animals given **A** 106 or **B** 203 mg/kg as a single s.c. dose. (○), Free bleomycin; (●), liposomal bleomycin; (□), control. **C** Liposomal bleomycin given at 310 (■) and 412 mg/kg (△) as a single s.c. dose. At these doses, free bleomycin was lethal by day 7. Bars represent the SEM

Efficacy studies

Figure 5 shows the effect of liposome-encapsulated bleomycin on tumor growth. At doses of 106 (Fig. 5A) and

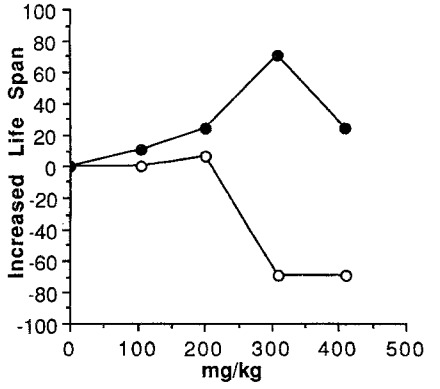

Fig. 6. The percentage of increase in life span (ILS) observed in animals given liposomal (●) and free drug (○) as compared with control values

203 mg/kg (Fig. 5B), greater inhibition of tumor growth was observed for liposomal bleomycin than for equivalent doses of free bleomycin ($P < 0.05$, Wilcoxon's signed-rank test). At higher doses, no direct comparisons with free drug could be made because all animals that had received free bleomycin at doses of 310 and 412 mg/kg died of toxicity early in the study. Nevertheless, animals receiving liposomal drug at 310 and 412 mg/kg demonstrated dose-responsive inhibition of tumor growth. As shown in Fig. 6, a 76% increase in life span was achieved in animals given a single optimal dose of 310 mg/kg liposomal bleomycin, whereas the free drug had minimal activity in increasing life span at an optimal dose of 203 mg/kg. Animals that had received the drug at levels above the optimal doses died of drug toxicities early in the study.

Discussion

The results of this study indicate that the therapeutic index of a single s.c. injection of bleomycin is improved when the drug is encapsulated in multivesicular liposomes; in other words, the efficacy appears to be improved and the toxicity is decreased. This holds true regardless of whether the inhibition of tumor growth (Fig. 5) or the increase in life span (Fig. 6) is used as the measure of efficacy.

Other investigators have previously encapsulated bleomycin into unilamellar and multilamellar liposomes and given these preparations via intracerebral [4, 5], i.p. [16], and i.v. [13] routes. To our knowledge, systemic therapy consisting of s.c. injection of slow-releasing liposomal bleomycin has not previously been reported. The intracerebral study in rats [4] showed that liposomal bleomycin released much lower concentrations and total amounts of drug into the systemic circulation and into the urine. The initial and terminal intracerebral half-lives of liposomal bleomycin appeared to be 9 and 26 h, respectively. The former value is much shorter than that obtained in the present study, although the terminal half-life is similar to our finding. The efficacy of intracerebral bleomycin entrapped in liposomes in two human patients with glioma was inconclusive [5]. The i.p. study showed that liposomal encapsulation of this drug results in increased efficacy against Ehrlich ascites carcinoma [16]; however, no pharmacokinetic data were reported. The i.v. study in humans used liposomal bleomycin labelled with indium 111 [13]; again, detailed pharmacokinetic data were not reported, but most of the liposomes were found to accumulate in the liver and spleen.

Although we did not study the levels of intratumoral bleomycin, we believe that all of the actions of liposomes are attributable to the slow-release effect and that no targeting is involved. For targeting to occur, the liposomes must first enter the capillaries, pass through the pulmonary capillary bed, then leave the capillary bed of the distant tumor and accumulate in the tumor. The liposomes we used are too large (average size, 19.1 μm) to pass through capillary lumina, let alone through endothelial barriers. Thus, the liposomes would not be expected to accumulate preferentially at the distant s.c. tumor site.

The results of this study may have an immediate implication in clinical oncology. The results appear to support the contention that administration of bleomycin by continuous infusion is better than the standard bolus injection [3, 14]. The practicality of using s.c. injection of multivesicular liposomes to approximate the effects of continuous infusion may make it easier to carry out randomized clinical trials.

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