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Comparative immunotoxicity of free doxorubicin and doxorubicin encapsulated in cardiolipin liposomes

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Summary. The immunologic and pharmacologic effects of free doxorubicin and of doxorubicin entrapped in liposomes were compared in mice at a dose of 20 mg/kg. Liposomes for encapsulation of doxorubicin were prepared by using 39.35 μ*mol* drug, 19.65 μ*mol* cardiolipin, 100 μ*mol* phosphatidylcholine, 68.4 μ*mol* cholesterol, and 38.9 μ*mol* stearylamine. Pharmacologic disposition studies after a dose of 20 mg/kg demonstrated 7- to 10-fold higher drug concentrations in the spleen at all time points following administration of doxorubicin entrapped in cardiolipin lipsosomes than after the free drug. The levels in liver were 4- to 5-fold higher with liposomal drug, whereas the cardiac uptake with liposomal doxorubicin was significantly lower than with free drug. Mice were sacrificed on days 1, 8, 15, and 22 after drug administration and spleen cells were isolated for studies of sensitization to alloantigens for cell-mediated cytolysis and of proliferation in response to mitogens. Mice treated with free doxorubicin demonstrated a decrease of more than 50 fold (compared with saline control) in allospecific cytotoxic activity on day 15; normal levels were recovered by day 22. The animals treated with doxorubicin encapsulated in liposomes showed a similar but not more pronounced fall to low levels. The total lytic activity per spleen after free drug or drug encapsulated in liposomes was markedly reduced at day 8, but this activity was fully recovered by day 15 in animals receiving liposomal doxorubicin; in those receiving free drug it had not recovered fully even at day 22. The proliferative response to concanavalin A was affected by the two forms of doxorubicin in a pattern very similar to the cytotoxic response. The proliferative response to lipopolysaccharide was markedly depressed by doxorubicin delivered in either form, and the kinetics were not altered by the mode of administration. The concentration of doxorubicin in spleen was markedly increased with liposomal delivery, but did not result in greater toxicity than that of free durg according to the immunologic parameters evaluated.

Introduction

Doxorubicin is an important antitumor antibiotic of the anthracycline group. It produces clinically useful responses in a wide range of human cancers, including lymphomas [2, 3], leukemias [13, 25], and solid tumors [23, 38]. The drug also produces acute adverse side effects, such as bone marrow depression, alopecia, and mucosal ulceration, but the treatment-limiting toxicity is a cumulative dose-dependent cardiac toxicity taking the form of refractory congestive heart failure [4, 34]. Doxorubicin is concentrated in the myocardiun, and histological studies have shown degeneration of myofibrils, mitochondrial distortion, and a decrease in the number of myocytes [34]. Juliano's, group [16–18] have demonstrated that liposomes can serve as effective carriers of anticancer drugs by altering the pharmacokinetic of these agents in vivo. We have recently shown that encapsulation of doxorubicin in cardiolipin liposomes imparts a highly selective decrease in cardiac concentration of the drug [32] and provides protection against drug-induced cardiotoxicity in beagle dogs [14]. This protection is correlated with decreased mortality after the administration of high doses of liposomal doxorubicin [36].

Doxorubicin and its congeners are known to affect antibody production and various aspects of cellular immunity [28, 35]. We previously demonstrated higher concentrations of doxorubicin in spleen after the administration of liposonal than after the administration of free doxorubicin. In this study we have explored certain immunologic consequences of this relative splenic concentration following a single dose of liposome-encapsulated doxorubicin. We report that liposomal doxorubicin suppresses antigen specific cellular cytotoxicity and proliferative responses to the mitogens concanavalin A and lipopolysaccharide; the suppression is altered in timing but not in magnitude, and it is of shorter duration than after free doxorubicin according to the cellular cytotoxicity and proliferative response to concanavalin A.

Materials and methods

Mice. Male mice of the (BALB/c × DBA/2) F1 hybrid strain CDF 1 and weighing 20–25 g were obtained commercially from Charles River Co. (Boston, Mass). C57BL/-6J males aged 6–8 weeks were obtained from Jackson Laboratories (Bar Harbor, Me). Mice were maintained according to accredited procedures in our facility and enjoyed uniformly good health at the initiation of the studies.

Cell lines. The murine cell lines EL-4 (H-2^b) and P-815 (H-2^d) were obtained originally from Dr John Wunderlich.

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Both have been tested for mycoplasma, and mycoplasma-free aliquots have been frozen. Cells maintained in culture or freshly thawed were used as targets.

Preparation of liposomes. Doxorubicin was kindly supplied by Farmitalia Carlo Erba, Milan, Italy. Phosphatidyl choline, cardiolipin, cholesterol, and stearylamine were purchased from Sigma Chemicals Co. (St. Louis, Mo). The lipids were tested for purity by thin-layer chromatography on silica gel with the solvent system chloroform/methanol/water 70:30:5 (volume), and phosphatidyl choline, cardiolipin, cholesterol, and stearylamine were found to be 99%, 98%, 99%, and 90% pure, respectively. Doxorubicin was incorporated in to liposomes by using 39.35 μmol drug in methanol with 19.65 μmol cardiolipin. The mixture was evaporated to dryness under N_2. To this dried mixture were then added 100 μmol phosphatidyl choline, 68.4 μmol cholesterol, and 38.9 μmol stearylamine. The mixture was stirred gently to achieve a homogenous solution and evaporated to dryness under N_2. The dried mixture was resuspended in 10 ml 0.01 *M* phosphate buffer with 0.85% NaCl, pH 7.4 (PBS). After a swelling time of 30 min the liposomes were stirred for 15 min, followed by sonication (Heat System, W-220F) under N_2 in a fixed-temperature bath at 37 °C for 90 min. The nonentrapped doxorubicin was separated from liposomal-encapsulated drug by extensive dialysis against 0.001 *M* phosphate buffer with 0.85% NaCl, pH 7.4, at 4 °C over a period of 20 h with at least two changes of buffer solution. The percentage entrapment of doxorubicin in cardiolipin liposomes was determined by fluorescence [1] after the completion of dialysis and was found to be 55% of the total input doese [12, 29]. Thus for each milligram of doxorubicin administered in liposomes 13 mg lipid needs to be administered. The size of the lipsomes as determined by electron microscopy ranged from 900 to 1100 Å. Liposomes were prepared fresh each day they were studied, and were diluted with 0.01 *M* phosphate buffer with 0.85% NaCl to permit the administration of total doses of doxorubicin equivalent to those of free drug. These liposomes were used to perform comparative immunotoxicity and pharmacology studies.

Sequence of studies. Doxorubicin, either as free drug or entrapped in liposomes, was administered to CDF1 mice via a lateral tail vein at a dose of 20 mg/kg and at a volume of 0.02 ml/g body weight. Controls received saline or empty liposomes. The dose of lipids injected to mice was 280 mg/kg. At days 1, 8, 15, and 22 after drug administration mice were killed by cervical dislocation, and the spleens were removed quickly under aseptic conditions. Three mice in each treatment group were killed at each time point. The spleen of each mouse was processed for immunologic evaluation as described below.

In vitro sensitization. Spleen cells obtained from mice sacrificed by cervical dislocation were used as responders and stimulators for in vitro generation of cytotoxicity. The isolated spleens were disrupted mechanically and erythrocytes were removed with lysing buffer. Responder cells were suspended at 10^6/ml in Eagle's Minimal Essential Medium (MEM) with added nutrients, antibiotics, and 10% preselected fetal calf serum (FCS). Stimulator cells were irradiated at 2000 r and added at 2×10^6/ml. Cultures were incubated at 37 °C in humidified 5% CO_2 in air for 5 days.

Assay of cell-mediated cytolysis. Targets were labeled for 1 h in 1 ml MEM containing antibiotics and 5% FCS with 0.1 mCi $Na_251Cr04$ (Amersham, Arlington Heights, Ill). Targets were washed three times and plated at 5000/well in 200 μl round-bottom microwells in 96-well plates. Cells to be assayed for cytotoxicity were added in appropriate concentrations to achieve attacker-to-target ratios of 40:1, 20:1, 10:1, and 5:1. All conditions were tested in triplicate. Releasable isotope (MAX) was determined by adding 0.1 *N* HCl to targets. Spontaneous release (SR) was determined from medium added to targets. Wells were spun at 50 *g* for 2 min at 23 °C and incubated at 37 °C in 5% CO_2 in air for 4 h. Plates were spun at 775 *g* for 5 min at 4 °C, harvested by means of the Titertek Supernatant Collection System (Flow Laboratories, Rockville, Md), and counted (Beckman Gamma 4000, Fullerton, Calif). The corrected percentage lysis is (experimental SR) ÷ (MAX − BG), where BG is machine background. SR was less than 10% in most cases.

Calculation of lytic units and lytic activity. Lytic units were calculated with a computer program generated by Ms E. Phillips as perviously described [24]. In brief, straight lines were fitted to the titration curve for percentage lysis versus the logarithm of the attacker-to-target ratio. Normal animals or saline-treated controls were assumed to have a standard lytic activity concentration of 1.0. The distance between the two lines of the experimental values and the standard or control values is a measure of the relative concentration of lytic activities. The value for total lytic activity per spleen is an arbitrary number obtained by multiplying the concentration of lytic activity by the number of nucleated cells in the spleen tested.

Proliferation assays. Spleen cells obtained as above were plated in 200-μl round-bottom wells in 96-well plates at a concentration of 5×10^5/ml in quadruplicates. The mitogens concanavalin A and lipopolysaccharide were added to appropriate wells at concentrations of 5 μg/ml and 10 μg/ml, respectively. Controls with no mitogen were also plated. Plates were incubated for 36–44 h, then pulsed with 0.5 μCi ^{3}H-thymidine for 4 h, and harvested with a MASH II (Microbiological Associates, Rockville, Md) onto absorbent strips. These were dried, placed in scintillation fluid (Beta Fluor, National Diagnostics, Somerville, NJ), and counted on a Beckman LS7000.

Pharmacologic disposition studies. To correlate the immunologic studies, pharmacologic disposition studies were carried out in CDF_1 mice at a dose of 20 mg/kg doxorubicin. Doxorubicin, either as free drug or entrapped in liposomes, was administered to mice via a lateral tail vein in a volume of 0.02 ml/g body wt. At 1.5, 2.5, 6.0, 8.0, 24.0 and 48.0 h after drug treatment, four mice in each treatment group were bled from the orbital sinus in to heparinized tubes and were killed by cervical dislocation. The liver, spleen, kidney, lungs, and heart were rapidly exised, rinsed in ice-cold 0.9% NaCl solution, and stored at −20 °C.

Plasma and tissues were analyzed for doxorubicin equivalents by our modification of the method of Formelli et al. [7]. Plasma (0.25 ml) was diluted to 1 ml with distilled water, followed by the addition of 0.2 ml $AgNO_3$ (33% wt/vol). Tissues were homogenized in 1 ml/water in a Polytron homogenizer. To each tube was then added 0.2 ml Ag No_3. The tubes were vortexed vigorously, followed by the addition of 3 ml *n*-butanol saturated with water. Each tube was vortexed for 1 min and then was centrifuged at 5000 rpm for 10 min. The organic layer was removed, followed by further extraction of the residue with 2 ml *n*-butanol. The tubes were vortexed for 30 s and then centrifuged again for 10 min at 5000 rpm. The second organic layer was removed and pooled with the first extract. The butanol extract was read in a spectrofluorometer at 470 nm excitation and 585 nm emission. Control plasma and tissues obtained from mice treated with blank liposomes were treated in the same way and read in the fluorometer to correct for any endogenous flourescence. Fresh doxorubicin samples were prepared in *n*-butanol each day to calculate the concentration of doxorubicin in the plasma and tissue samples.

Results

Pilot immunologic studies were performed on groups of mice receiving IV injections of saline, free doxorubicin 6 mg/kg, free doxorubicin 20 mg/kg, blank liposomes, liposome-encapsulated doxorubicin 6 mg/kg, or encapsulated doxorubicin 20 mg/kg. The most striking effects occurred with free doxorubicin 20 mg/kg, which ablated the capacity to generate a cell-mediated cytotoxic response 14 days later. Mitogenic reponses were descreased during the first 2 weeks after free or encapsulated doxorubicin at the higher dose. No clear alterations in the activity of natural killers was detected. These initial observations were confirmed by the detailed observations below.

Survival of animals

The mice treated with free doxorubicin at a dose of 20 mg/kg demonstrated dose-related toxicity by day 3. Loss of general body weight, lethargy, and loss of the hairy coat were evident. Over the period of the experiment four mice died (4/18): all four had received free doxorubicin 20 mg/kg. However, no mortality occurred in the group of mice treated with the same dose of doxorubicin entrapped in cardiolipin liposomes, nor was any sign of toxicity noticed in that group of mice.

Generation of allospecific cytotoxicity

Spleen cells from sacrificed mice were sensitized to allogeneic ($H\text{-}2^b$) transplantation antigens by sensitization in vitro. The kinetics of this activity are shown in Fig. 1. The saline control is used as the standard for generation of the lytic unit calculation for each of the other three groups. The activity of the saline control is arbitrarily assumed to be 1.0 units. The animals injected with blank liposomes showed a modest but very definite increase in activity, which persisted until day 22, the fourth observation point. By contrast, the animals treated with free doxorubicin showed a marked (greater than 50-fold) decrease in the capacity to mount an allospecific cytotoxic response on day 15; this defect had been fully repaired by day 22. The animals treated with encapsulated doxorubicin showed a similar fall to low levels, but the decrease was observed earlier and was almost fully repaired by day 15. The cytotoxicity observed was allospecific, as seen from the fact that syngeneic and irrelevant allogeneic targets were not killed by any of the cytotoxic populations. Natural killing was evaluated in companion experiments (data not shown). Relatively low levels of killing against the NK-susceptible target YAC-1 were detected in these animals, and no clear changes could be detected among any of the groups.

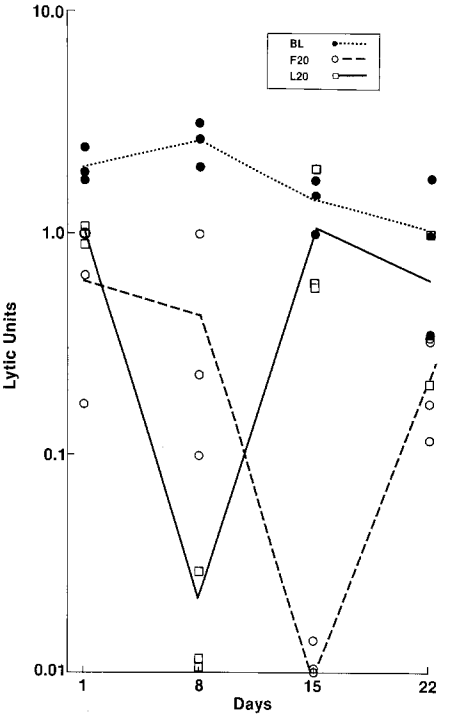

Fig. 1. T-cell mediated cytotoxicity against $H\text{-}2^b$ alloantigens. Saline-treated controls are used to normalize the response as lytic units. Animals received empty liposomes *(BL)*, doxorubicin 20 mg/kg *(F20)*, or doxorubicin encapsulated in liposomes *(L20)*. Individual *symbols* represent single animals and the lines represent the arithmetic mean response

Despite the relative preservation of concentration of lytic activity at 8 days, the animals treated with free drug showed a marked decrease in spleen size and in the number of nucleated spleen cells. Thus, when total lytic activity per spleen was calculated these animals, like those treated with liposome-encapsulated doxorubicin, showed a marked decrease at 8 days (Fig. 2). The total activity per spleen of the latter animals was replaced by 15 days, but the animals treated with free drug had still not returned to normal even by day 22. The discordance between concentration and total activity per spleen in the animals treated with free drug implies that other cell populations are more profoundly affected by the free drug than the cell types that are important in the generation of an allospecific cytotoxic response.

Proliferative responses. The spleens of the same animals were tested for their capacity to mount a proliferative response. Figure 3 shows the mean response of each group to the mitogen concanavalin A. The absolute number of

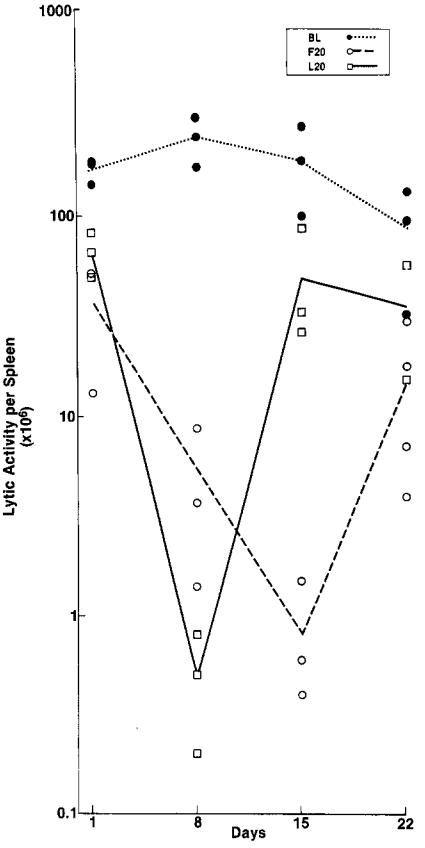

Fig. 2. T-cell mediated cytotoxicity against H-2^b alloantigens on a per spleen basis. See legend to Fig. 1

counts taken up by the stimulated cells of the saline control varies considerably from assay to assay, in these and other experiments; the test animals in each group are normalized to a response index by diving the counts by those of the saline controls. It should be noted that the counts in the animals receiving blank liposomes were lower at day 1, but were similar to those in the saline control by day 8. The drug-treated animals had depressed responses at day 1 and at day 8, with recovery by day 15. The animals treated with liposome-encapsulated doxorubicin had more markedly depressed responses at day 1 than animals treated with free drug; most of this effect appears to be due to the liposome itself, rather than to the doxorubicin, since the response in the liposome-treated controls was also depressed. The response to the B cell mitogen was even more profoundly depressed than that to concanavalin A from day 1 onward in animals receiving either free or encapsulated doxorubicin. This effect persisted until day 22, and may suggest that cells in the antibody-forming series are more profoundly affected by doxorubicin than are cells in the T cell lineage.

Table 1. Pharmacologic disposition of free and liposomal encapsulated doxorubicin in mice at a dose of 20 mg/kg IV

	Spleen	
Time (h)	Free doxorubicin (μg/g)	Liposomal doxorubicin (μg/g)
1.5	32.16 ± 4.3 (2.18)[a]	218.2 ± 31.1 (15.6)
2.5	25.23 ± 1.73 (2.18)	125.1 ± 11.4 (11.2)
6.0	27.92 ± 5.06 (2.16)	160.9 ± 5.9 (12.6)
8.0	26.34 ± 2.69 (2.06)	123.5 ± 7.1 (9.4)
24.0	25.42 ± 4.97 (1.31)	188.1 ± 7.2 (12.4)
48.0	33.96 ± 4.03 (1.33)	190.8 ± 15.8 (12.5)

[a] Values in parenthesis represent average drug concentration (μg) measured per spleen

Pharmacologic studies of doxorubicin

Table 1 shows the comparative disposition of doxorubicin as free drug or entrapped in cardiolipin liposomes in spleen when administered at a dose of 20 mg/kg. The higher drug concentration in spleen persisted very effectively throughout the period of observation when administered as free drug or entrapped in lipsomes. The concentration of doxorubicin in spleen following free drug administration was 32 μg/g tissue at 1.5 h and about 34 μg/g tissue at 48 h. There was apparently no change in the drug concentration throughout the observation period. The same pattern of drug disposition in the spleen was observed when doxorubicin entrapped in cardiolipin liposomes was administered. Nevertheless, the concentration

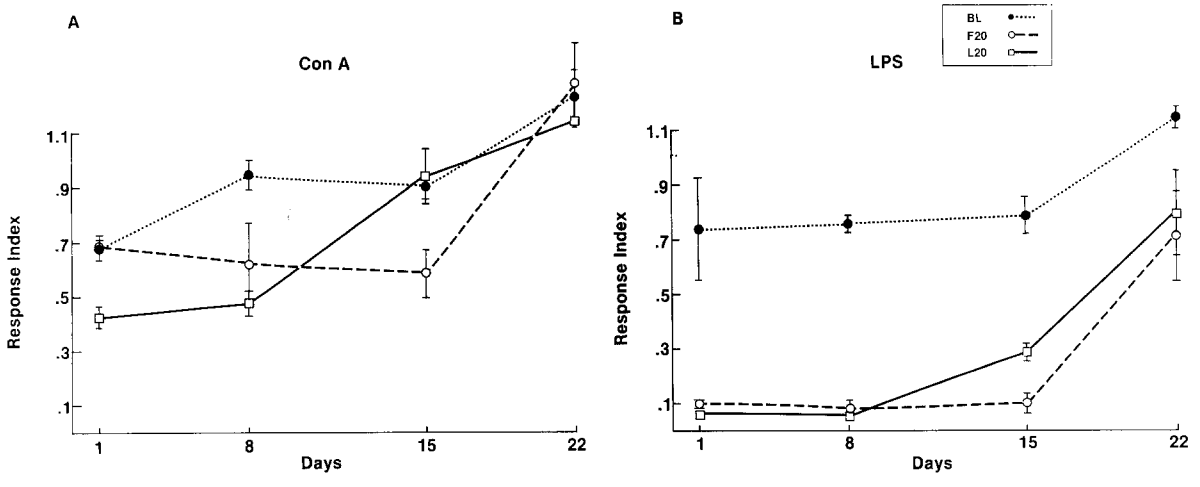

Fig. 3. *A, B.* Proliferative responses of spleen cells to the mitogens concanavalin A *(A)* and lipopolysaccharide *(B)*. The response index is obtained by dividing experimental counts per minute by the saline control value. *Error bars* indicate SEM. Animals received empty liposomes *(BL)*, doxorubicin 20 mg/kg IV *(F20)*, or doxorubicin in liposomes *(L20)*

Table 2. Pharmacologic disposition of free and liposomal-encapsulated doxorubicin in mice at a dose of 20 mg/kg IV

Time (h)	Liver		Kidney		Lungs	
	Free[a]	Liposomal	Free	Liposomal	Free	Liposomal
1.5	59.9	61.2	48.73	26.84	36.55	30.83
2.5	60.7	60.8	36.83	16.13	33.64	22.64
6.0	24.77	50.6	19.26	–	36.47	26.41
8.0	15.6	30.3	17.26	9.62	27.09	16.19
24.0	7.8	30.2	7.84	7.96	15.37	9.11
48.0	6.3	29.4	6.35	4.44	10.89	9.65

[a] Free, free doxorubicin (concentration in μg/g); Liposomal, encapsulated doxorubicin (concentration in μg/g)

of drug in spleen was 7- to 10-fold higher following liposomal doxorubicin administration than after free drug. Table 1 shows the values recorded in individual spleens in parentheses. The same relationship was observed in drug concentration when the per spleen values for free drug and liposomal drug were compared. A 6- to 10-fold higher drug concentration was obtained in spleen with liposomal doxorubicin than with free doxorubicin.

Table 2 shows the disposition of free drug and liposome-entrapped drug in liver. At 1.5 and 2.5 h the drug equivalents were similar in liver following administration of free drug or liposomal drug. However, from 6 h to 48 h after administration the concentration in liver was 3- to 5-fold higher with liposomal doxorubicin than with free drug. The levels fell off rapidly in liver with both forms of administration, in contrast to those in spleen. The drug levels in other tissues demonstrated the same temporal relationship as was observed earlier [32].

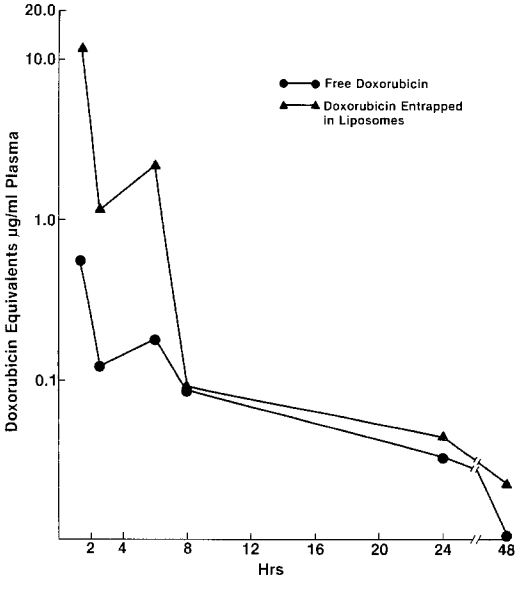

Fig. 4. Plasma doxorubicin levels. Plasma doxorubicin levels were determined by fluorescence up to 48 h after animals received either free (●———●) or liposome-encapsulated (▲———▲) doxorubicin. Each *point* represents the mean plasma level (μg doxorubicin/ml plasma) of four animals

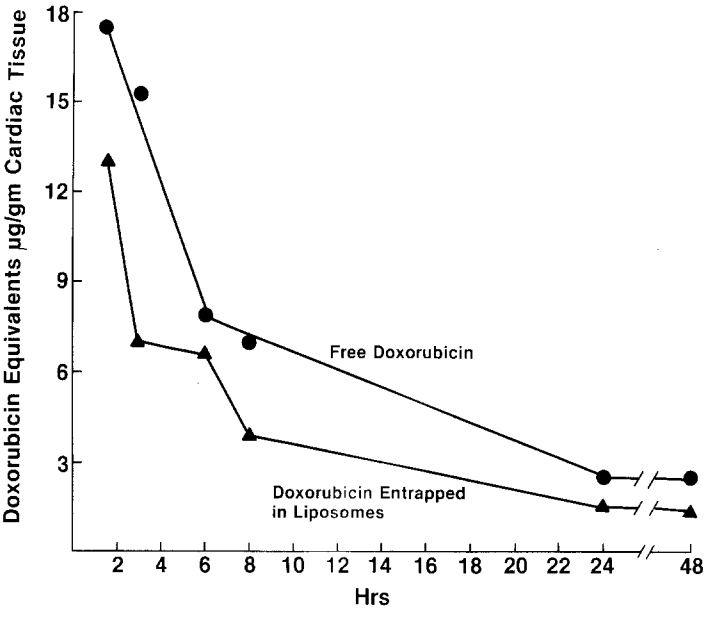

Fig. 5. Cardiac doxorubicin levels. Doxorubicin levels in cardiac tissue were determined up to 48 h by fluorescence after animals received either free doxorubicin (●———●) or doxorubicin entrapped in liposomes (▲———▲). Each *point* represents the mean cardiac levels (μg/gm tissue) of four animals

Figure 4 shows that following equivalent doses (20 mg/kg) of doxorubicin the plasma clearance of drug was markedly delayed in animals receiving drug encapsulated in liposomes. Initially, there was a drug level at least 10- to 15-fold higher in plasma following liposomal drug administration; this result is consistent with previous observations [16, 26, 31]. The enhanced drug levels in plasma after liposomal doxorubicin indicate the different rate of clearance occurring during the first pass through circulation and also a decrease in the volume of distribution compared with free drug. However, by 8 h after drug administration the plasma levels of doxorubicin were similar in both groups.

Figure 5 shows that following free doxorubicin administration, the maximum cardiac concentration, 17.6 μg/g, was seen at 1.5 h. The corresponding value with liposomal drug was 13.3 μg/g. Both concentrations decayed at similar rates during the observation period of 48 h.

Discussion

During the last decade liposomes have proven to be useful and effective tools as drug delivery systems to alter the pharmacokinetics, tissue distribution, and metabolism of drugs [8–10, 12, 16, 27]. Our studies have demonstrated a reduced uptake of doxorubicin in cardiac tissue of mice when the drug is injected entrapped in positive liposomes, with a concomitant decrease in acute and chronic cardiotoxicity [30]. Studies in our laboratories have also demonstrated that the intrinsic properties of liposome components can be exploited to retard the uptake of anthracycline drugs in cardiac tissue effectively [32]. Recently, Goormaghtigh et al. [11] have demonstrated a high affinity of cardiolipin for doxorubicin in a membrane-model system. This affinity is thought to be stabilized by two essential in-

teractions, an electrostatic interaction between the protonated amino groups of the sugar residues and the ionized phosphate residues, and an interaction between the adjacent anthraquinones and chromophores. We have utilized these properties in preparing the liposomes of doxorubicin and cardiolipin with a net positive charge for effective control of the drug-induced toxicity in mice [36, 37], with a concomitant increase in antitumor activity in P388 ascitic leukemia [31, 36]. We have further demonstrated that encapsulation of doxorubicin in cardiolipin liposomes provides complete protection against drug-induced chronic cardiotoxicity in beagle dogs [14]. Free doxorubicin given every 3 weeks at a dose of 1.75 mg/kg over a period of 21 weeks caused extensive myocardial alterations in beagles. The prominent features of the cardiomyopathy included cytoplasmic vacuolization and loss of myofibrils. In contrast, a marked reduction in cardiotoxicity was noted in dogs treated chronically with equivalent doses of doxorubicin entrapped in cardiolipin liposomes. The hearts of all dogs given liposomal doxorubicin remained essentially normal despite the administration of a cumulative dose of the agent of 245 mg/m^2 [14].

The present study was undertaken to evaluate the role of free and liposome-entrapped doxorubicin on the suppression of immunogenicity, since liposomal drug is preferentially concentrated in spleen and liver [18, 30, 31, 33]. Previous studies demonstrated that doxorubicin administered IV depressed the synthesis of circulating antibodies, hemagglutinin, and hemolysin, and that the immunodepressive action of doxorubicin was dose-dependent [15, 20]. Since the participating host defense mechanisms may influence the antitumor activity of cancer chemotherapeutic agents, the exaggerated concentration of doxorubicin in spleen when administered in cardiolipin liposomes may have an adverse effect on the chemotherapeutic potential of the drug.

Mice were evaluated for generation of allospecific cytotoxicity when treated with free doxorubicin or doxorubicin entrapped in cardiolipin liposomes at 20 mg/kg, a supralethal dose of free drug. The animals treated with free doxorubicin demonstrated a significant (greater than 50-fold) decrease in the capacity to mount an allospecific cytotoxic response on day 15. Mice treated with doxorubicin entrapped in cardiolipin liposomes showed a similar, but not more profound, decrease in cytotoxic capacity (Fig. 1). Moreover, the kinetics of cytotoxic depression were altered; the decrease occured earlier and was of shorter duration with liposomal doxorubicin than with free drug. This difference in kinetics could lead to the hypothesis that chronic administration of free doxorubicin would be more likely to cause cumulative immunotoxicity than chronic administration of liposomal drug. The blank liposomes did not seem to cause any toxicity; on the contrary, a stimulating effect was observed on the generation of cytotoxicity with blank liposomes. However, blank liposomes did appear to depress the proliferative responses of spleens on day 1 compared to saline control, but this effect was rather transient since complete recovery was observed on day 8 (Fig. 2). In this parameter of evaluation, spleens obtaiend from mice treated with doxorubicin liposomes demonstrated more pronounced depression on day 1 than those from free-drug-treated animals, which may have been partly due to the synergistic effect of liposomes. Animals treated with either free drug or drug entrapped in liposomes demonstrated more profound depression of the response to the B cell mitogen than to concanavalin A (Fig. 3).

Comparative pharmacologic disposition studies at a dose of 20 mg/kg free doxorubicin and doxorubicin entrapped in cardiolipin liposomes demonstrated 7- to 10-fold higher drug concentration in spleen of mice treated with liposomal drug (Table 1). Nevertheless, this pronounced concentration of doxorubicin in spleens with liposomal delivery failed to demonstrate any higher toxicity than was seen after free drug. Though the mechanisms of protection from this toxicity are not fully understood, one of the possible explanations for these observations may be that encapsulation in cardiolipin liposomes alters the site of action, subcellular distribution, and/or metabolism of doxorubicin. On the other hand, doxorubicin may remain associated with the cardiolipin liposomes and be unable to cause tissue damage in the same way as free doxorubicin.

Liposomal encapsulation offers a potentially useful means of directing immunomodulators to lymphoid tissue including spleen. The discordance between concentration of lytic activity and total lytic activity in spleen of animals treated with free doxorubicin suggests a differential access of free drug to subpopulations of splenic immunocytes. The distribution of liposomal drug appears to differ with regard to these subpopulations. Such differences may have important implications both for immunotoxicity and for the treatment of lymphomas. Other studies of immune modulators with a liposomal carrier system [5, 6, 19, 21, 22, 29] underscore the need for additional investigations of the localization of liposomes to particular populations of immunocytes.

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References

1. Bachur NR, Moore AL, Bernstein JG, Lio A (1970) A tissue distribution and disposition of daunomycin in mice. Fluorometric and isotopic methods. Cancer Chemother Rep 54: 89–94
2. Bonadonna G, Monfardini S, DeLena MD, Fossati-Bellani F, Beretter G (1970) Phase 1 and preliminary phase II evaluation of Adriamycin (NSC-123127) Cancer Res 30: 2572–2582
3. Bonadonna G, Delenar MD, Monfardini S, Milani F (1975) Combination chemotherapy with Adriamycin in malignant lymphoma. In: Adriamycin review. European Press Medicon, Ghent, pp 200–215
4. Chabner BA, Myers CE (1982) Clinical pharmacology of cancer chemotherapy. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Cancer: Principles and practice od oncology. J. B. Lippincott Company, Philadelphia, p 182
5. Fidler IJ, Hart IR, Raz A, Fogler W, Kirsh R, Poste G (1980) Activation of tumorcidal properties in macrophages by liposome-encapsulated lymphokines: In vivo studies. In: Tom BH, Six HR (eds) Liposomes and immunobiology. Elsevier/North-Holland, New York, pp 109–118
6. Fidler IJ, Sone S, Fogler WE, Barnes ZL (1981) Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing muramyl dipeptide. Proc Natl Acad Sci USA 78: 1680–1684

7. Formelli F, Pollini C, Casazza AM, diMarco A, Mariani A (1981) Fluorescence assays and pharmacokinetic studies of 4'-deoxydoxorubicin and doxorubicin in organs of mice bearing solid tumors. Cancer Chemother Pharmacol 5: 139–144
8. Forssen EA, Tokes ZA (1979) In vitro and in vivo studies with Adriamycin liposomes. Biochem Biophys Res Commun 91: 1295–1307
9. Gabizon A, Dagan A, Goren D, Barenholz Y, Fuks Z (1982) Liposomes as in vivo carriers of adriamycin: reduced cardiac uptake and preserved antitumor activity in mice. Cancer Res 42: 4737–4739
10. Goldin A, Rahman A, Harris M, Fumegalli A, Schein P (1983) Antitumor and pharmacologic disposition studies of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. Proc Am Assoc Cancer 24: 260
11. Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM (1980). Evidence of a specific complex between adriamycin and negatively charged phospholipids. Biochem Biophys Acta 597: 1–14
12. Gregoriadis G (1977) Targeting of drugs. Nature 265: 407–411
13. Haanen C, Hillen G (1975) Combination chemotherapy with doxorubicin in 'bad risk' leukemia patients. In: Adriamycin review. European Press Medicon, Ghent, pp 193–199
14. Herman E, Rahman A, Ferrans V, Vick J, Schein P (1983) Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation. Cancer Res 43: 5427–5432
15. Isetta AM, Intini C, Soldati M (1971) On the immunodepressive action of Adriamycin. Experientia 27: 202–204
16. Juliano RL, Stamp D (1978) Pharmacokinetic of liposome-encapsulated antitumor drugs. Biochem Pharmacol 27: 21–27
17. Juliano RL, Stamp D, McCullough N (1978) Pharmacokinetics of liposomes encapsulated antitumor drug and implications for therapy. In: Papahadjopolous D (ed) Lipsosomes and their uses in biology and medicine. Ann NY Acad Sci 308: 411–423
18. Juliano RL, Lopez-Berestein G, Mehta R, Hopfer R, Mehta K, Kasi L (1983) Pharmacokinetic and therapeutic consequences of liposomal drug delivery: Fluorodeoxyuridine and amphotericin B as examples. Biol Cell 47: 39–46
19. Lopez-Berestein G, Milas K, Hunter N, Mehta K, Hersh EM, Kurahara CG, Vanderpas M, Eppstein AD (1984) Prophylaxis and treatment of experimental lung metastases in mice after treatment with liposome-encapsulated 6-0-stearoyl-*N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine. Clin Exp Metast 2: 127–137
20. Mantovani A, Polentaruttl N, Lulul W, Perl G, Spreafico F (1979) Role of host defense mechanisms in the antitumor activity of Adriamycin and daunomycin in mice. JNCI 63: 61–66
21. Mehta K, Lopez-Berestein C, Hersh EM, Juliano RL (1982) Uptake of liposomes and liposome-encapsulated muramyl dipeptide by human peripheral blood monocytes. J Reticuloendothel Soc 32: 155–164
22. Mehta K, Juliano RL, Lopez-Berestein G (1984) Stimulation of macrophage protease secretion via liposomal delivery of muramyl dipeptide derivatives to intracellular sites. Immunology 51: 517–527
23. Middleman E, Luce J, Frei E (1971) Clinical trials with Adriamycin. Cancer 28: 844–850
24. Neefe JR, Sullivan JE, Silgals RE (1983) Preliminary observations of immunomodulatory activity of lymphoblastoid interferon-alpha administered every other day or weekly. J Biol Resp Modifiers 2: 441–449
25. Oldham RK, Pomeroy TC (1972) Treatment of Ewing's sarcoma with Adriamycin (NSC-123127). Cancer Chemother Rep 56: 635–639
26. Olson F, Mayhew E, Marlow D, Rustrum Y, Szoka F (1982) Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. Eur J Cancer Clin Oncol 18: 167–176
27. Parker RJ, Hartman KD, Sieber SM (1981) Lymphatic absorption and tissue disposition of liposome-entrapped (14C) Adriamycin following intraperitoneal administration to rats. Cancer Res 41: 1311–1317
28. Pfeiffer RW, Bosmann HB (1982) Modulation of antitumoral antibody-dependent cellular cytotoxicity and natural killer activity by Adriamycin and daunorubicin. Agents Actions 12: 635–644
29. Poste G, Kirsh R, Fogler WE, Fidler IJ (1979) Activation of tumoricidal properties in mouse macrophages by lymphokines encapsulated in liposomes. Cancer Res 39: 881–892
30. Rahman A, More N, Schein PS (1982) Doxorubicin-induced cardiotoxicity and its protection by liposomal administration. Cancer Res 42: 1817–1825
31. Rahman A, Fumagalli A, Goodman A, Schein PS (1984) Potential of liposomes to ameliorate anthracycline-induced cardiotoxicity. Semin Oncol 2: 45–56
32. Rahman A, White G, More N, Schein PS (1985) The pharmacologic, toxicologic and therapeutic evaluation of doxorubicin entrapped in cardiolipin liposomes. Cancer Res 45: 796–803
33. Rahman YE, Kilieski WE, Buess EM, Cerny EA (1975) Liposomes containing ^{3}H-actinomycin D: differential tissue distribution by varying the mode of drug incorporation. Eur J Cancer 11: 883–889
34. Rinehart JJ, Louis RP, Baleerzak SP (1974) Adriamycin cardiotoxicity in man. Ann Intern Med 81: 475–478
35. Santoni A, Riccardi C, Socei V, Herberman RB (1980) Effects of Adriamycin on the activity of mouse natural killer cells. J Immunol 124: 2329–2335
36. Smith F, Rahman A, Jenson A, Kline I, Schein PS (1983) Toxicity evaluation of doxorubicin entrapped in cardiolipin liposomes in mice. Proc Am Assoc Cancer Res 24: 260
37. Van Hennik M, Carmichael D, Nester B, Sarngadharan MG, Schein PS, Rahman A (1983) Pharmacologic disposition of doxorubicin (Dx) as influenced by liposome charge. Proc Am Assoc Cancer Res 24: 254
38. Wang JJ, Cortes E, Sinks LF, Holland JF (1971) Therapeutic effect and toxicity of Adriamycin in patients with neoplastic disease. Cancer 28: 837–843

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