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Tissue distribution and antitumor effect of liposome-entrapped doxorubicin (adriamycin) in Ehrlich solid tumor-bearing mouse.

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Abstract

The usefulness of liposomes (in neutral, positively and negatively charged forms) as a carrier for adriamycin (ADM) was studied by examining the distribution of ADM and related fluorescent compounds in Ehrlich solid tumor-bearing mice. The mice were given free or liposome-entrapped ADM intraperitoneally. The distribution of ADM and related fluorescent compounds between the administration of the free form and liposome-entrapped form was measured by high performance liquid chromatography : The distribution was dependent on the form of the liposomes. The amounts of ADM and its metabolites in the mouse serum 20 min after administration of neutral-liposome-entrapped ADM were 10 times those after the administration of free ADM, 6 times those after the administration of a negatively charged form, and 3.5 times those in the administration of positively charged form. There was no marked difference in the concentrations of these compounds 5 h after administration. The concentration of these compounds in the liver 60 min after administration of each liposome-entrapped form of ADM were in inverse correlation with the concentrations in the serum obtained at 20 min after administration. Total concentrations of ADM and its metabolites in the tumors 20 min after administration of each entrapped form of ADM were 4-5 times that in administration of free ADM after 20 min. There were no marked differences in the concentration of ADM for administration of the various liposome forms. Statistically significant decreases in mean tumor weight were seen in the groups given neutral, positively and negatively charged liposome-entrapped forms compared to corresponding control groups given with free ADM.

KEYWORDS: adriamycin, charged liposomes, tissue distribution, antitumor effect, high-performance liquid chromatography (HPLC).

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TISSUE DISTRIBUTION AND ANTITUMOR EFFECT OF LIPOSOME-ENTRAPPED DOXORUBICIN (ADRIAMYCIN) IN EHRLICH SOLID TUMOR-BEARING MOUSE

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Abstract. The usefulness of liposomes (in neutral, positively and negatively charged forms) as a carrier for adriamycin (ADM) was studied by examining the distribution of ADM and related fluorescent compounds in Ehrlich solid tumor-bearing mice. The mice were given free or liposome-entrapped ADM intraperitoneally. The distribution of ADM and related fluorescent compounds between the administration of the free form and liposome-entrapped form was measured by high-performance liquid chromatography : The distribution was dependent on the form of the liposomes. The amounts of ADM and its metabolites in the mouse serum 20 min after administration of neutral-liposome-entrapped ADM were 10 times those after the administration of free ADM, 6 times those after the administration of a negatively charged form, and 3.5 times those in the administration of positively charged form. There was no marked difference in the concentrations of these compounds 5 h after administration. The concentration of these compounds in the liver 60 min after administration of each liposome-entrapped form of ADM were in inverse correlation with the concentrations in the serum obtained at 20 min after administration. Total concentrations of ADM and its metabolites in the tumors 20 min after administration of each entrapped form of ADM were 4-5 times that in administration of free ADM after 20 min. There were no marked differences in the concentration of ADM for administration of the various liposome forms. Statistically significant decreases in mean tumor weight were seen in the groups given neutral, positively and negatively charged liposome-entrapped forms compared to corresponding control groups given with free ADM.

Key words : adriamycin, charged liposomes, tissue distribution, antitumor effect, high-performance liquid chromatography (HPLC).

Adriamycin (ADM) is useful in the treatment of acute leukemias and malignant tumors (1, 2), but its usefulness is complicated by toxic responses such as cardio-toxicity and marrow depression. The initial half-life time after intravenous injection is only a few minutes (3), and poses a problem for reaching tumor cells.

In recent years, liposomes have been used as a carrier for drugs in the field of cancer chemotherapy in order to increase the affinity of the drug to target cells and to decrease side effects (4, 5). Some estimation of the effectiveness of liposome-entrapment was obtained in our previous work : decreasing the side

effect of the drug (6), increasing the antitumor effect (7) and altering the drug distribution (8).

In the present series of experiments, we examined the usefulness of liposomes as a carrier for ADM.

MATERIALS AND METHODS

Reagents. Adriamycin hydrochloride, adriamycinone, adriamycinol and daunomycin were kindly donated by Farmitalia (Milan, Italy). Egg lecithin was purified from chicken egg york by the method previously reported (9). Cholesterol was purchased from Nakarai Chemical Co. (Kyoto, Japan) and recrystallized. Dicetyl phosphate was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and stearylamine from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Determination of ADM and related fluorescent compounds. ADM and related fluorescent compounds in the serum and tissues of Ehrlich tumor-bearing mice were determined by using high-performance liquid chromatography (HPLC) (10). In brief, HPLC was carried out by using Zorbax Sil as the stationary phase and 3.8% sodium acetate in isopropanol as the mobile phase. Measurements were made with a fluorescence detector at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. The extraction procedure and lower limit for detection of the compounds have been described elsewhere (10, 11). All operations with ADM and related compounds were carried out in the dark.

Preparation of liposomes. Fifteen mg egg lecithin and 2.2 mg cholesterol (neutral, molar ratio 7:2) or the same supplemented with 1.5 mg dicetyl phosphate (negatively charged) or 0.8 mg stearylamine (positively charged, molar ratio 7:2:1) were dissolved in 5 ml of chloroform in a round-bottom flask and evaporated until the formation of thin film which was then immediately dispersed under N_2 vapor in 2 ml of ADM solution (adriamycin hydrochloride 1 mg/ml in sterilized saline). The suspension (without glass beads) was sonicated in N_2 vapor for 30 min at 0°, using a Bransonic sonicator (bath type, 98V) then washed twice with sterilized saline at 35,000 g for 30 min. Determination of ADM in liposomes was carried out as previously reported using HPLC (11).

Animal experiments. Ehrlich ascites tumors (2×10^6 cells/mouse) were inoculated on the back of ICR male mice (25-30 g in body weight, 6 weeks old). Mice had free access to food and water. Seven days later, the mice were injected intraperitoneally with free ADM, a mixture of free ADM and "empty" liposomes (neutral, negatively and positively charged forms) and liposome-entrapped ADM (neutral, negatively and positively charged forms) at a dose of 2.85 mg/kg. The mice were sacrificed at 20 min, 60 min and 5 h after injection. There were 3 mice in each group. For the therapeutic experiments, mice were inoculated with 2×10^6 Ehrlich tumor cells on the back on day 0, and treated with three i.p. injections of either saline, free ADM, a mixture of free ADM and "empty" liposomes (neutral, negatively and positively charged forms) or liposome-entrapped ADM on days 7, 8 and 9. There were 10 mice in each group. All mice were sacrificed on day 11, and the solid tumors were weighed. The tumor weights were expressed as mean ± S.E. for each experimental group. The significance of the mean was determined with Student' unpaired test.

RESULTS

Determination of ADM in the biological samples by HPLC with a fluorescence detector at an excitation wavelength of 470 nm and an emission wavelength of 585 nm was found to minimize the effect of contaminating substances, and the separation of ADM, adriamycinone, adriamycinol and daunomycin was good (Fig. 1).

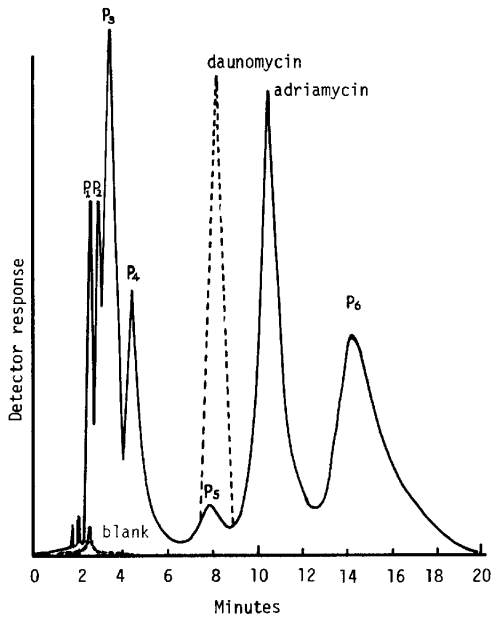

Fig. 1 High-performance liquid chromatogram of adriamycin and related fluorescent compounds. P3 peak=adriamycinone; P6 peak=adriamycinol; Blank=contaminating serum and tissue blank. Apparatus: Hitachi Model 635 A high-performance liquid chromatograph. Column, Zorbax Sil (150×4.6 mm I.D.) at room temperature; Mobile phase, 3.8 % sodium acetate in isopropanol; flow-rate, 1 ml/min. Detector; Hitachi Model 650-10S fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm); sensitivity, 10,; fine, 6; pen range, 5 mV.

Peak 1 through peak 6 (P1 metabolite-P6 metabolite) of ADM related compounds were detected in ADM-administered mouse serum: P3 had been identified as adriamycinone and P6 as adriamycinol in previous experiments (10). The contaminating substance in serum and the tissue blank was detected at the same site as P1 but in trace amounts. P5 was detected at the same site as daunomycin but in trace amounts. Therefore, the content of P5 was not included in the total concentration of ADM and related compounds. By this method, artificial production of ADM related compounds (mainly adriamycinone) was less than 0.1 % against standard ADM solution.

Entrapment of ADM in liposomes was 4.0 % in positively charged liposomes (liposome$^+$ (ADM)), 6.6 % in nuetral liposomes (neutral (ADM)) and 25.7 % in negatively charged liposomes (liposome$^-$ (ADM)) against dispersed ADM solution used preparation of liposome (Table 1).

Figs 2-4 show the total concentration of ADM and related compounds in plasma or tissues of Ehrlich tumor-bearing mice administered with free or liposome-entrapped ADM examined by HPLC (per cent to administered dose per ml or gram of tissues). The total concentration of ADM and related compounds in

the mice treated with free ADM only was about equivalent to that of mice treated with a mixture of ADM and "empty" liposomes in neutral, negatively or positively charged forms. Administration of ADM in the liposome-entrapped form not only increased the total amount of ADM accumulated in the serum and tissues, but also significantly altered the relative distribution of ADM among each tissues. The total concentration of ADM and related compounds in mouse serum 20 min after administration of neutral liposome-entrapped ADM was 10 times that after the administration of free ADM, 6 times that after the adminis-

TABLE I. EFFICIENCY OF THE ENTRAPMENT OF ADRIAMYCIN (ADM) BY LIPOSOMES AS DETERMINED BY HPLC

Composition of liposomes	Proportion incorporated (%)* Mean ± S.D.
Neutral (ADM) (EL, CHOL)	6.6 ± 2.0 (n = 15)
Liposome⁻ (ADM) (EL, CHOL, DICE)	25.7 ± 4.9 (n = 12)
Liposome⁺ (ADM) (EL, CHOL, STEAR)	4.0 ± 1.1 (n = 15)

A 2-ml volume of adriamycin hydrochloride solution (1 mg/ml) was entrapped in liposomes composed of 15 mg of egg lecithin (EL) supplemented with 2.2 mg of cholesterol (CHOL) and 0.8 mg of stearylamine (STEAR) or 1.5 mg of dicetyl phosphate (DICE). The molar ratio of each set of phospholipid, cholesterol and charged lipid was 7 : 2 : 1.

*Proportion of incorporated ADM to dispersed ADM solution used for preparation of liposomes

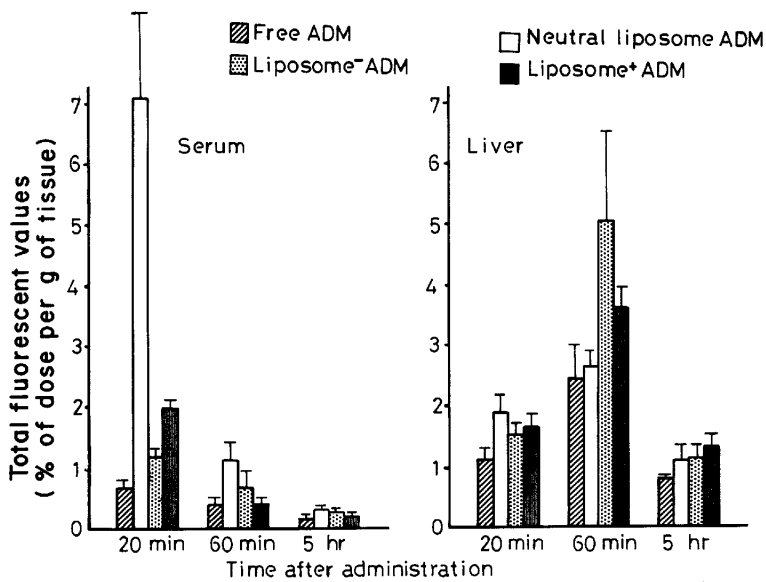

Fig. 2. Total concentration of ADM and related compounds in plasma and liver of Ehrlich tumor-bearing mice administered with free or liposome-entrapped ADM. Total concentrations of P1, P2, P3, P4 and P6 in Fig. 1 are expressed as per cent to administered dose per ml or gram of tissue (mean ± S.E. of duplicate determinations from three mice).

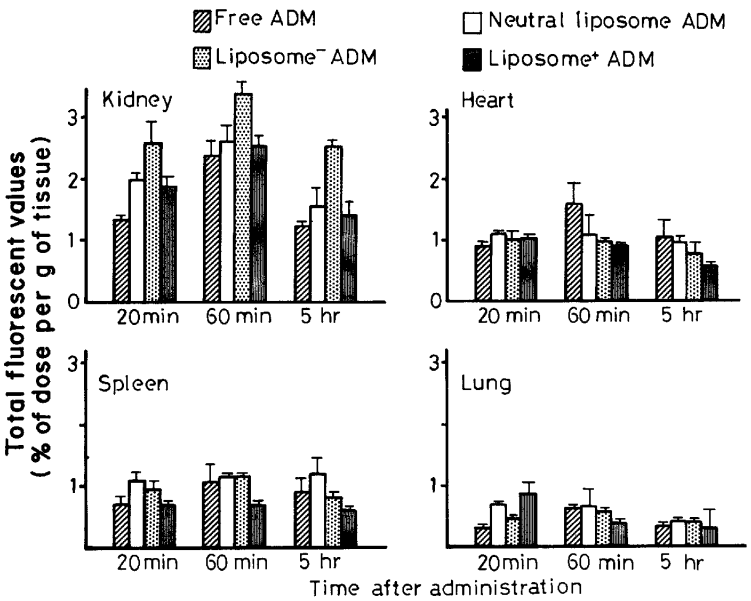

Fig. 3. Total concentration of ADM and related compounds in kidney, spleen, heart and lung of Ehrlich tumor-bearing mice administered with free or liposome-entrapped ADM. The concentrations are expressed as in Fig. 2.

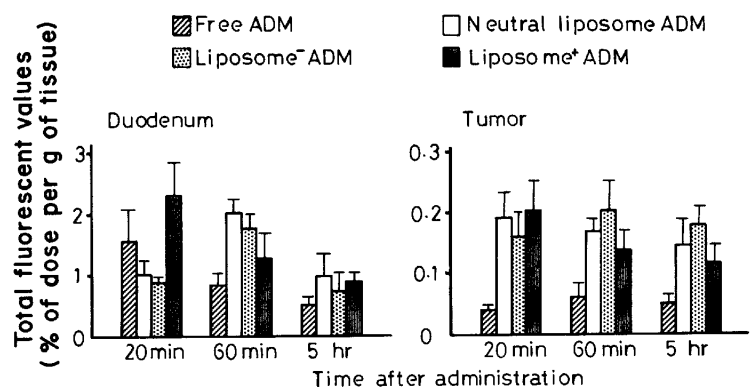

Fig. 4. Total concentration of ADM and related compounds in duodenum and the tumor of Ehrlich tumor-bearing mice administered with free or liposome-entrapped ADM. The concentrations are expressed as in Fig. 2.

tration of the negatively charged form, and 3.5 times that after the administration of the positively charged form. There was no marked difference in the concentrations 5 h after administration. The total concentration in the serum 20 min after administration of ADM in the positively charged form was higher than that for the negatively charged form, but at 60 min the concentration of

Table 2. Concentration of ADM and related fluorescent compounds (μG ADM equivalent per ml or gram of tissues) in the serum, liver, kidney and spleen in Ehrlich tumor bearing mice given free or liposome-entrapped ADM, examined by HPLC

ADM	Metabolites	Serum 20 min	Serum 60 min	Serum 5 h	Liver 20 min	Liver 60 min	Liver 5 h	Kidney 20 min	Kidney 60 min	Kidney 5 h	Spleen 20 min	Spleen 60 min	Spleen 5 h
Free ADM	AD-NE*	0.31	0.19	0.06	0.85	2.03	0.45	0.85	1.87	0.80	0.41	0.79	0.57
	P4 metabolite	0.08	0.07	0.03	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	ADM	0.24	0.07	0.03	0.21	0.28	0.27	0.23	0.25	0.25	0.21	0.19	0.30
	Adriamycinol	0.03	0.05	0.02	0.04	0.12	0.06	0.20	0.24	0.15	0.04	0.04	Tr.
Neutral liposome (ADM)[a]	AD-NE*	3.92	0.50	0.11	1.21	1.96	0.78	1.32	2.13	1.28	0.47	0.62	0.58
	P4 metabolite	0.80	0.15	0.08	0.08	Tr.	Tr.	0.14	Tr.	Tr.	0.13	Tr.	Tr.
	ADM	2.03	0.35	0.07	0.53	0.56	0.23	0.30	0.34	0.18	0.36	0.47	0.47
	Adriamycinol	0.33	0.12	0.03	0.07	0.15	0.02	0.19	0.24	0.05	0.07	0.05	0.02
Liposome (ADM)[b]	AD-NE*	0.51	0.39	0.13	0.97	4.49	0.79	1.87	2.81	2.04	0.48	0.75	0.42
	P4 metabolite	0.15	0.10	0.05	Tr.	Tr.	Tr.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	ADM	0.41	0.12	0.04	0.47	0.45	0.27	0.46	0.41	0.29	0.36	0.36	0.33
	Adriamycinol	0.09	0.05	0.03	0.07	0.15	0.06	0.21	0.21	0.15	0.03	Tr.	Tr.
Liposome$^+$ (ADM)[c]	AD-NE*	1.31	0.17	0.07	1.22	3.32	1.04	1.50	2.08	0.99	0.43	0.40	0.29
	P4 metabolite	0.22	0.07	0.04	Tr.	Tr.	Tr.	0.07	Tr.	Tr.	N.D.	N.D.	N.D.
	ADM	0.40	0.10	0.03	0.40	0.20	0.21	0.25	0.23	0.21	0.21	0.23	0.24
	Adriamycinol	0.04	0.04	0.02	0.03	0.13	0.08	0.06	0.21	0.19	0.02	Tr.	Tr.

The values are expressed as mean of duplicate determinations from three mice. *P2 metabolite + adriamycinone. Tr.= Trace(<0.01). N.D.= Not detected. *a* Neutral liposome-entrapped ADM *b* Negatively charged liposome-entrapped ADM *c* Positively charged liposome-entrapped ADM

TABLE 3. CONCENTRATION OF ADM AND RELATED FLUORESCENT COMPOUNDS (μG ADM EQUIVALENT PER ML OR GRAM OF TISSUES) IN THE HEART, LUNG, DUODENUM AND TUMOR IN EHRLICH TUMOR BEARING MICE GIVEN FREE OR LIPOSOME-ENTRAPPED ADM, EXAMINED BY HPLC

Dosage	Metabolites	Heart			Lung			Duodenum			Tumor		
		20 min	60 min	5 h	20 min	60 min	5 h	20 min	60 min	5 h	20 min	60 min	5 h
Free ADM	AD-NE*	0.48	1.03	0.73	0.13	0.36	0.12	1.22	0.47	0.22	0.03	0.05	0.04
	P4 metabolite	0.08	Tr.	Tr.	N.D.	N.D.	N.D.	0.12	Tr.	Tr.	0.01	Tr.	Tr.
	ADM	0.16	0.29	0.22	0.10	0.15	0.12	0.19	0.30	0.21	Tr.	0.01	0.01
	Adriamycinol	0.13	0.18	0.08	0.01	0.03	Tr.	0.05	0.06	0.07	Tr.	0.01	Tr.
Neutral liposome (ADM)[a]	AD-NE*	0.38	0.63	0.38	0.25	0.33	0.27	0.59	1.42	0.69	0.01	0.10	0.11
	P4 metabolite	0.22	Tr.	Tr.	0.07	Tr.	Tr.	0.13	Tr.	Tr.	0.04	Tr.	Tr.
	ADM	0.38	0.38	0.29	0.25	0.25	0.10	0.21	0.55	0.25	0.04	0.03	0.03
	Adriamycinol	0.11	0.13	0.17	0.05	0.03	Tr.	0.05	0.12	0.02	0.02	0.01	0.01
Liposome$^-$ (ADM)[b]	AD-NE*	0.43	0.66	0.50	0.19	0.33	0.19	0.63	1.26	0.51	0.09	0.19	0.15
	P4 metabolite	0.11	Tr.	Tr.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.02	Tr.	Tr.
	ADM	0.46	0.29	0.20	0.20	0.20	0.12	0.17	0.48	0.16	0.02	0.01	0.02
	Adriamycinol	0.06	Tr.	Tr.	0.01	Tr.	Tr.	0.05	0.05	0.05	0.01	Tr.	Tr.
Liposome$^+$ (ADM)[c]	AD-NE*	0.60	0.34	0.27	0.67	0.18	0.12	2.08	0.93	0.58	Tr.	0.11	0.08
	P4 metabolite	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.12	Tr.	Tr.	0.17	Tr.	Tr.
	ADM	0.36	0.28	0.21	0.13	0.15	0.12	0.14	0.28	0.18	0.03	0.02	0.02
	Adriamycinol	0.05	0.07	0.10	Tr.	Tr.	Tr.	0.03	0.06	0.08	Tr.	0.01	0.01

The values are expressed as mean of duplicate determinations from three mice. *P2 metabolite+adriamycinone. Tr.=Trace(<0.01). N.D.=Not detected. *a* Neutral liposome-entrapped ADM *b* Negatively charged liposome-entrapped ADM *c* Positively charged liposome-entrapped ADM

ADM in positively charged liposomes was lower than that for negatively charged liposomes. The total concentration of these compounds in the liver 60 min after administration of each liposome-entrapped form of ADM was in inverse correlation with the concentrations in the serum obtained at 20 min after administration. This phenomenon is attributed to continuous uptake of liposomes by the liver from the serum (12). In the kidney of the mouse given ADM in the negatively charged form, the total concentration of ADM was higher than that for the administration of other forms of ADM at every time examined. The concentration after administration of ADM in entrapped forms was higher than for free forms. In the spleen and lung, the total concentration of ADM after administration of entrapped forms were similar to those after administration of free ADM. In the heart, no differences in the total concentration of ADM for each method were noted at 20 min, but the concentration after administration of the free form was higher than that of entrapped forms at 60 min and 5 h. In the tumors, the total concentration of ADM was low for all administration methods. There was a marked difference in the concentrations after administration of free and entrapped forms of ADM at every time examined, but not after administration of liposome entrapped ADM. The total concentration of ADM in entrapped forms was 4-5 times that of free forms at 20 min and 2-3 times at 60 min and 5 h.

Tables 2 and 3 show the concentration of ADM and related fluorescent compounds (μg ADM equivalent per ml or gram of tissues) in the serum and tissues in Ehrlich tumor-bearing mice given free ADM or liposome-entrapped ADM. The concentrations of ADM and related fluorescent compounds in the serum and tissues of mice given liposome-entrapped ADM significantly differed from those in mice given free ADM. In serum, the concentration of ADM (as unmetabolized material) given with liposomes in the neutral form was the highest for the various methods of administration. After administration of ADM in the neutral form of liposome at 60 min, the total concentration of ADM and related compound was 3 times that of the free forms, and the concentration of ADM was 5 times. After administration of ADM entrapped in neutral and negatively charged forms of liposomes, the concentration of ADM in the liver, spleen and lung was about twice the concentration 20 and 60 min after administration of free ADM. In the heart, the concentration of AD-NE (P2 metabolite + adriamycinone) after administration of free ADM was higher than that after the administration of entrapped forms. The concentration of ADM in the tumors after administration of the neutral liposomes was 3-4 times, and for the negatively or positively charged form 2-3 times that for the free form.

The mean tumor weight (g) on day 11, after treatment with free ADM alone showed a slight decrease, being 2.48 ± 0.37 compared to 2.86 ± 0.38 for the saline control group. After the administration of ADM entrapped in liposomes, the mean tumor weight decreased significantly compared to the control

free ADM groups (treated with mixtures of free ADM and "empty" liposomes). There were statistically significant differences in the mean tumor weight between the group given liposome-entrapped ADM and the control group given free ADM (Table 4).

TABLE 4. EFFECT OF FREE AND LIPOSOME-ENTRAPPED ADM ON THE GROWTH OF EHRLICH SOLID TUMOR

Dosage form	Dose of ADM (μg/kg)	Mean tumor weight (g, mean ± S.E.)
Saline control	0	2.86 ± 0.38
Free ADM	1250	2.48 ± 0.37
Free ADM + empty liposomes$^+$	1250	2.47 ± 0.18
Free ADM + empty liposomes$^-$	1250	2.51 ± 0.35
Free ADM + empty neutral liposomes	1250	2.53 ± 0.22
Liposomes$^+$ (ADM)[a]	1250	1.35 ± 0.28[b]
Liposomes$^-$ (ADM)[c]	1250	1.13 ± 0.16[d]
Neutral liposomes (ADM)[e]	1250	1.02 ± 0.17[f]

a Positively charged liposome-entrapped ADM *b* Significantly different ($P < 0.05$) from the value obtained with control free ADM group (mixture of free ADM and positively charged liposomes) *c* Negatively charged liposome-entrapped ADM *d* Significantly different ($P < 0.01$) from the value obtained with control free ADM group (mixture of free ADM and negatively charged liposomes) *e* Neutral liposome-entrapped ADM *f* Significantly different ($p < 0.01$) from the value obtained with control free ADM group (mixture of free ADM and neutral liposomes)

DISCUSSION

After the administration of liposome-entrapped ADM to Ehrlich solid tumor-bearing mice, significantly different distributions of ADM were seen between the liposome-entrapped and free forms, and between the neutral and charged forms of liposomes. The concentrations of ADM and related fluorescent compounds given as liposome-entrapped forms were higher than for free forms in the serum, liver, kidney and tumor at every time examined. On the other hand, the concentration of ADM and related fluorescent compounds was lower in the heart at 60 min and 5 h than that after the administration of free ADM. Enhancement of the antitumor effect was obtained by the entrapment of ADM in liposomes compared to free ADM. However, the effect of liposomes as a carrier for ADM was smaller than that seen in cytosine arabinoside (13, 14), methotrexate (15, 16), daunomycin (12), actinomycin-D (17) and prednisolone (8). ADM, an amphophilic compound, can destabilized phospholipid membranes resulting in the loss of entrapped material (18, 19), has an electrostatic interaction with negatively charged lipids, especially cardiolipin (20), and causes changes of liposomal phase transition temperature (21). Therefore, better results as a carrier might be obtained by entrapping ADM in a liposomal lipid phase.

The efficiency of the entrapment of ADM by negatively charged liposomes having electrostatic affinity (20) was highest among other forms of liposomes, and lowest in the positively charged liposomes.

The slowest clearance rate of entrapped drugs has been reported to be the positively charged form (13, 22), the neutral form (22), and the negatively charged form (23). In our experiments, it appeared that ADM entrapped in each of the three types of liposomes was cleared from the serum at more or less the same rate, but that ADM administered by entrapping into neutral liposomes reached higher initial serum concentrations than from charged liposomes, possibly reflecting more rapid absorption from the peritoneal cavity.

Forssen *et al.* (24) and Rahman *et al.* (25) examined the distribution of liposome-entrapped ADM using tritium labelled ADM and a spectrofluorometric method. In these cases, the ADM values do not always show ADM as unmetabolized materials or biologically active compounds; therefore, problems arise in determining the distribution of ADM.

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