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Membrane Interaction of an Antitumor Antibiotic, Mithramycin, with Anionic Phospholipid Vesicles

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ABSTRACT. Small unilamellar vesicles (SUV) composed of zwitterionic phosphatidylcholine and two different anionic phospholipids, phosphatidic acid and phosphatidylserine, in different compositions, were employed to study the membrane interaction of an antitumor antibiotic, mithramycin (MTR). Binding of MTR to dimyristoylphosphatidylcholine (DMPC) liposomes containing the anionic phospholipid dimyristoylphosphatidic acid (DMPA) was estimated by measuring the increase in intensity of the intrinsic fluorescence of MTR with increasing concentrations of phospholipids. Membrane perturbations were observed in acidic SUV of DMPC/DMPA and DMPC/bovine brain phosphatidylserine by MTR and its magnesium complex as studied by monitoring the leakage of the entrapped fluorescent marker carboxyfluorescein and by electron microscopic measurements of the size of the liposomes. These results indicated a possible role of anionic phospholipids in mediating binding of MTR and its magnesium complex to the cell surface membranes before reaching the target DNA. BIOCHEM PHARMACOL **57**;9:981–987, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. mithramycin; anionic phospholipids; fluorescence; carboxyfluorescein leakage

MTR† is a potent antitumor antibiotic produced by *Streptomyces plicatus* [1]. It is employed for the treatment of testicular carcinoma and Paget's disease under the name of plicamycin [2, 3]. This antibiotic, along with the structurally related antibiotics chromomycin A_3 and olivomycin, belongs to the aureolic acid group [4]. MTR inhibits DNA-dependent RNA synthesis via reversible interaction with DNA only in the presence of divalent metal ions such as Mg^{2+} at and above neutral pH [5, 6]. It has also been shown that, in the absence of DNA, Mg^{2+} binds to the antibiotic; two types of MTR:Mg^{2+} complexes are formed depending on the concentration of the divalent metal ion, and both of them have been shown to bind differently to calf thymus DNA [7, 8].

The interactions of the aureolic acid group of antibiotics, chromomycin A_3, and MTR with polynucleotides have been studied in great detail, and it has been recognized for a long time that such an interaction requires a divalent cation such as Mg^{2+} [8–13]. On the other hand, very little is known about the interaction of these antibiotics with other cellular components, e.g. membranes and proteins. The antitumor activity is, most often, directly attributed to their interaction with DNA. However, many other anticancer antibiotics have been shown to interact with molecular and cellular components other than nucleic acids. For example, doxorubicin has been shown to interact with DNA topoisomerase II [14] and to bind to various cellular membranes [15], thereby affecting vital cellular processes such as signal transduction [16]. We have shown previously that the cytoskeletal protein spectrin can bind MTR with high affinity [17]. Very few attempts have been made thus far to study the interaction of MTR with phospholipid membranes [18]. The study of the membrane interactions of MTR is essential to understand the role of membrane lipids during the passage of the anticancer antibiotic through the lipid bilayer. Anionic phospholipids are implicated in membrane interactions of the anthracycline antibiotic doxorubicin, which indicates a specific interaction of the antibiotic with anionic phospholipids [19]. The anticancer drug cisplatin also has been shown to bind membranes composed of anionic phospholipids [20]. Such selective interactions with anionic phospholipids are often linked with its physiological effects. DNA replication in *Escherichia coli* is associated with cell membranes containing anionic phospholipids; these lipids have been shown to reactivate inactivated DnaA protein, essential for initiation of DNA replication [21]. In this work, interactions of MTR and its dimeric magnesium complex [abbreviated as $(MTR)_2Mg^{2+}$] have been studied with SUV of phosphatidylcholine containing two different anionic phospholipids, e.g. phosphatidic acid and phosphatidylserine, using fluorescence spectroscopy and electron microscopy. We have worked with the magnesium complex of MTR that is formed at the physiological millimolar concentration of Mg^{2+} [7, 22].

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† *Abbreviations*: MTR, mithramycin; SUV, small unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DMPA, dimyristoylphosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; PS, bovine brain phosphatidylserine; CF, carboxyfluorescein; and F_{540} (A.U.), fluorescence at 540 nm (arbitrary units).

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In this study, we have investigated the binding of MTR to the phospholipid vesicles at physiological pH from a phospholipid concentration-dependent change in the intrinsic fluorescence intensity of the antibiotic and estimated the binding constant of MTR to the membranes. Leakage of the entrapped fluorescent marker CF, upon interaction with MTR, from phospholipid vesicles composed of different proportions of anionic and zwitterionic phospholipids was measured. The sizes of a large number of individual liposomes before and after incubation with the antibiotic and its Mg^{2+} complex were estimated by electron microscopy. Results from these experiments indicated a possible involvement of anionic lipids in membrane interaction of MTR and its Mg^{2+} complex, leading to bilayer membrane instability in lipid vesicles containing acidic phospholipids.

MATERIALS AND METHODS

MTR, $MgCl_2$ solution (4.9 mol/L), DMPC, PS, CF, Sephadex G-50, Triton X-100, and Tris were purchased from the Sigma Chemical Co. DMPA was from Fluka. Solvents used were of spectroscopic grade. Deionized water from a Milli Q source (Millipore Corp.) was used for preparation of the buffer and all other solutions. $(MTR)_2Mg^{2+}$ complex was prepared by incubating 10–20 μM MTR with 10 mM Mg^{2+} for 1 hr.

Fluorescence measurements were performed in a Shimadzu RF-540 spectrofluorometer using both 2- and 10-mm pathlength quartz cuvettes. Excitation wavelength was at 470 nm, the red edge of the excitation spectrum (EX: 400 nm), in order to avoid photodegradation of MTR [12]. Excitation and emission slits with bandpass of 5 and 10 nm, respectively, were used in all experiments. For all experiments, the absorbance of MTR at 470 nm did not exceed 0.02, which is well below the limit of correction for the inner filter effects [23]. Absorbance measurements were carried out in 10-mm pathlength cuvettes using a Hitachi U-2000 spectrophotometer. The concentration of MTR was estimated using a molar absorptivity of 10,000 M^{-1} cm^{-1} at 400 nm as described earlier [8].

Unilamellar liposomes of phospholipids containing different mole percentages of DMPA and PS were prepared by sonication [24]. Thin films of phospholipids with different compositions were deposited on the walls of glass tubes from a solution in chloroform:methanol (2:1, v/v) by evaporation under dry nitrogen. The lipid films (5–10 μmol each) were dried in a desiccator for more than 48 hr and then hydrated in the buffer. The phospholipids were dispersed by vortexing and then sonicated with a B. Braun LabSonic 1510 sonicator for 10–12 min using 30-sec cycles until no further optical clearing of the suspension was observed. SUV were centrifuged to remove titanium particles before use. The vesicles were annealed for 2 hr before adding MTR for binding studies. Small aliquots of increasing concentrations of SUV were added from a stock solution to 15 μM MTR and $(MTR)_2Mg^{2+}$ complex in the buffer containing 10 mM Tris–HCl, 50 mM NaCl, pH 7.5 (henceforth, abbreviated as Tris–saline buffer), and mixed well. Samples were incubated for 3 hr before measuring the fluorescence. At least 2 hr of incubation of MTR with the phospholipid SUV was necessary to measure binding. Background intensities of liposomes without MTR were subtracted from all experimental data points to avoid scatter artifacts. All studies were done at a concentration of 10–15 μM to avoid aggregation of MTR in liposomes. The phospholipid concentration was measured after digestion with perchloric acid following a published protocol [25].

CF (30 mM was used as the self-quenching concentration) was entrapped in liposomes by sonication, and the untrapped CF was removed by gel filtration on Sephadex G-50. Fluorescence intensity corresponding to 100% leakage was obtained by treating with 0.5% Triton X-100. Percentage leakage was determined from a previously published procedure [26]. CF concentrations were determined spectrophotometrically assuming a molar extinction coefficient of 72,000 M^{-1} cm^{-1} at 492 nm. Liposomes of different lipid compositions were incubated with various concentrations of MTR and the $(MTR)_2Mg^{2+}$ complex at room temperature for 3 hr before leakage measurements. For each measurement, liposomes without MTR in the absence and presence of 10 mM Mg^{2+} were taken as controls. Fluorescence of CF was measured at 518 nm upon excitation at 488 nm.

Results from spectrofluorimetric titrations of MTR and $(MTR)_2Mg^{2+}$ complex with increasing concentrations of phospholipids (DMPC containing 10% DMPA) were analyzed according to the following equation [27]:

$$1/\Delta F = 1/\Delta F_{max} + 1/(K_{app} \cdot \Delta F_{max} \cdot [\text{Lipid}]) \quad (1)$$

where ΔF is the change in fluorescence intensity at 540 nm and ΔF_{max} is the same at the saturating molar concentration of lipid phosphate, [Lipid]. ΔF_{max} is obtained from a plot of $1/\Delta F$ against 1/[Lipid]; the resulting straight line is extrapolated to the y-axis and the intercept gives the value of $1/\Delta F_{max}$. The ratio of intercept to slope gives the apparent binding constant, K_{app}.

A Hitachi H-600 transmission electron microscope, operating at 75 kV, was used to measure the size of the phospholipid vesicles. Mean diameter was obtained by measuring a large number of individual liposomes from different samples. Liposomes were treated with either 10 mM Mg^{2+}, free MTR (10–20 μM), or $(MTR)_2Mg^{2+}$ complex containing 10–20 μM MTR and 10 mM Mg^{2+} for 2 hr before electron microscopic measurements. Samples were placed on grids precoated with mixed films of formvar and carbon. Staining was done with 2% phosphotungstic acid.

RESULTS

Fluorescence emission spectra of MTR both in the aqueous buffer and in two different concentrations of DMPC/

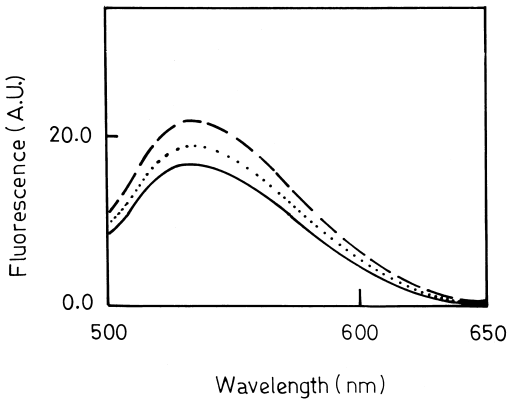

FIG. 1. Fluorescence emission spectra (EX: 470 nm) of MTR (15 μM) in Tris–saline buffer (_____) and in DMPC/10% DMPA liposomes at L/D = 15 (. . . . .) and L/D = 25 (— —). L/D stands for the molar ratio of phospholipid to MTR.

DMPA SUV are shown in Fig. 1. Maximum emission of MTR was at 536 nm in aqueous buffer; it was blue-shifted by 2 nm in phospholipid vesicles, emitting maximally at 534 nm. Enhancement of the intensity of MTR fluorescence was observed in the presence of increasing concentrations of the phospholipid vesicles. Similar enhancement of fluorescence intensity also was observed when phospholipid vesicles were added to the $(MTR)_2Mg^{2+}$ complex (not shown). The results from fluorescence titrations of MTR and $(MTR)_2Mg^{2+}$ complex at 27° in DMPC liposomes containing 10% DMPA are shown in Fig. 2. The

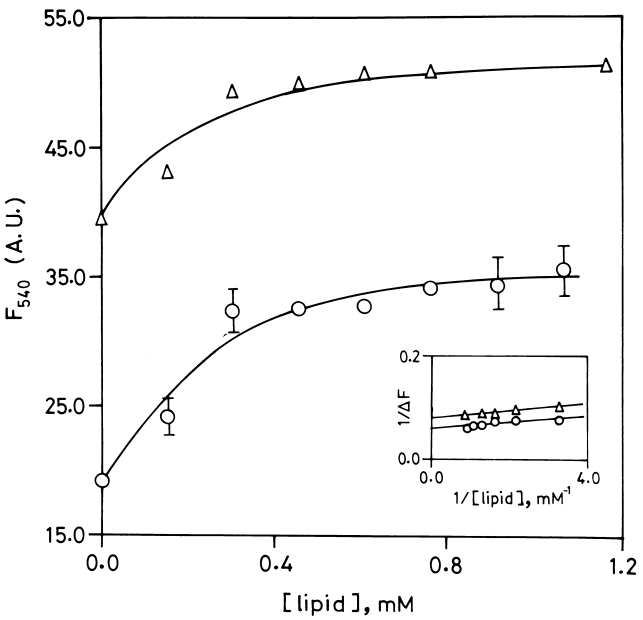

FIG. 2. Binding isotherms for the association of MTR (15 μM, Δ) and $(MTR)_2Mg^{2+}$ complex ([MTR] = 15 μM, $[Mg^{2+}]$ = 10 mM, ○) with DMPC/10% DMPA liposomes in Tris–saline buffer at 27°. The inset shows representative linear plots of 1/ΔF against 1/[Lipid], from which K_{app} values were determined by equation 1. The same symbols are used as in binding isotherms. The error bars are SEM for five independent determinations.

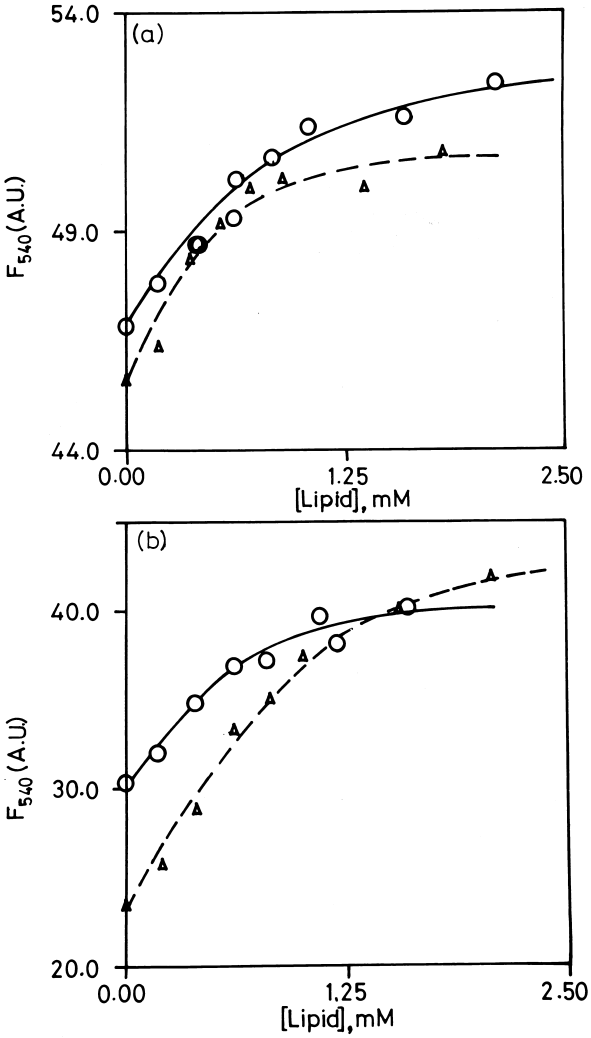

FIG. 3. (a) Binding isotherms for the association of MTR (15 μM) with DMPC/10% DMPA liposomes in Tris–saline buffer at 15° (○) and 35° (△). (b) Binding isotherms for the association of $(MTR)_2Mg^{2+}$ complex ([MTR] = 15 μM, $[Mg^{2+}]$ = 10 mM) with DMPC/10% DMPA liposomes in Tris–saline buffer at 15° (○) and 35° (△). Data points shown are means of duplicate measurements.

fluorescence intensity of MTR and $(MTR)_2Mg^{2+}$ complex increased up to about 30% of the initial, at 540 nm, when 15 μM MTR was titrated with DMPC/DMPA liposomes at 27°. The increase in fluorescence intensity indicated binding of MTR to the phospholipid bilayer. Since the extent of change in fluorescence was not large, typical error bars are shown as SEM for five independent determinations, indicating the reproducibility of the data (Fig. 2). The inset shows linear representative plots of 1/ΔF against 1/[Lipid], which was used to evaluate the K_{app}. Figure 3 shows similar titration profiles for both MTR (Fig. 3a) and $(MTR)_2Mg^{2+}$ complex (Fig. 3b) in DMPC/10% DMPA liposomes at two different temperatures, 15° and 35°, below and above the phase transition temperature of DMPC, which is ~24°. The apparent binding constants for the free MTR and the $(MTR)_2Mg^{2+}$ complex are summarized in Table 1.

TABLE 1. Apparent binding constants for the interaction between MTR and $(MTR)_2Mg^{2+}$ complex with phospholipid vesicles

Membrane system	Temperature (°)	K_{app} (10^4 M^{-1})	
		MTR	$(MTR)_2Mg^{2+}$
DMPC/10% DMPA	15	0.14	0.1
DMPC/10% DMPA	27	1.10	1.0
DMPC/10% DMPA	35	0.24	0.08

Five independent experiments were done at 27°, and duplicate experiments were done at 15° and 35°.

Figure 4 represents the data on the leakage of CF expressed as leakage percentage [26] at different micromolar concentrations of the antibiotic after a 3-hr incubation of liposomal membranes having different phospholipid compositions with MTR and $(MTR)_2Mg^{2+}$ complex, at 27°. These data on CF leakage at a fixed molar ratio of phospholipid to MTR (L/D = 25) are summarized in Table 2 for different negatively charged phospholipid vesicles composed of DMPC/DMPA and DMPC/PS liposomal membranes.

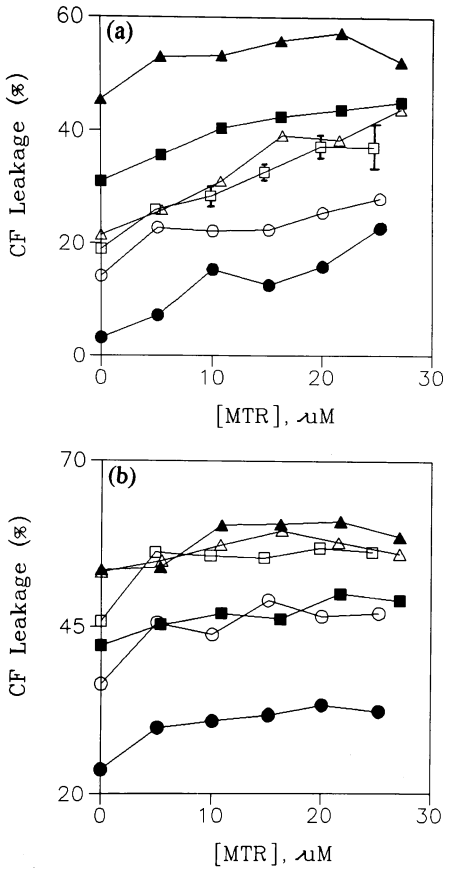

FIG. 4. (a) Change in leakage percentage of entrapped CF (30 mM) as a function of increasing concentration of MTR for the following membranes: pure DMPC (●); DMPC/2% DMPA (○); DMPC/5% DMPA (□); DMPC/10% DMPA (△); DMPC/5% PS (■); DMPC/10% PS (▲). Incubation (3 hr) and fluorescence measurements in all CF leakage experiments were done at 27°. Error bars are SEM from three independent experiments. (b) Change in leakage percentage of entrapped CF as a function of increasing concentration of $(MTR)_2Mg^{2+}$ complex. The symbols and other experimental conditions are the same as in Fig. 4a.

TABLE 2. Percent leakage of entrapped CF from liposomal membranes of different lipid composition upon treatment with MTR and its Mg^{2+} complex at 27°

Membrane composition	CF leakage (%)	
	In the presence of MTR	In the presence of $(MTR)_2Mg^{2+}$
DMPC	15.6 (3.2)*	33.3 (23.6)
DMPC/2% DMPA	22.4 (14.2)	49.0 (36.4)
DMPC/5% DMPA	34.0 ± 2.6† (19.0)	55.6 ± 2.0† (45.8)
DMPC/10% DMPA	39.3 (21.5)	59.5 (53.2)
DMPC/5% PS	42.6 (31.0)	46.2 (43.2)
DMPC/10% PS	57.4 (45.5)	60.9 (53.6)

The molar ratio of phospholipid to MTR (L/D) is 25 in each case.

*Values represent the means from two experiments, each conducted in duplicate.

†Values are means ± SD of four individual experiments, each conducted in duplicate. These representative standard deviations are within 4–8% of the mean values. Values in parentheses are those of the controls in the absence of MTR.

Both zwitterionic (DMPC) and acidic (DMPC/DMPA) liposomes were used for electron microscopic measurements of the diameter of the lipid vesicles. Figure 5 shows the histogram representation of the sizes of the zwitterionic DMPC liposomes and of the same in the presence of 5% DMPA after treatment with 10 mM Mg^{2+}, 15 μM MTR, and $(MTR)_2Mg^{2+}$ complex, respectively, for 2 hr.

DISCUSSION

The antitumor activities of antibiotics such as actinomycin, chromomycin, and the anthracycline group of antibiotics are often attributed directly to their interaction with DNA [28, 29]. However, studies with other antitumor antibiotics, such as doxorubicin, indicate that the drug can be cytotoxic

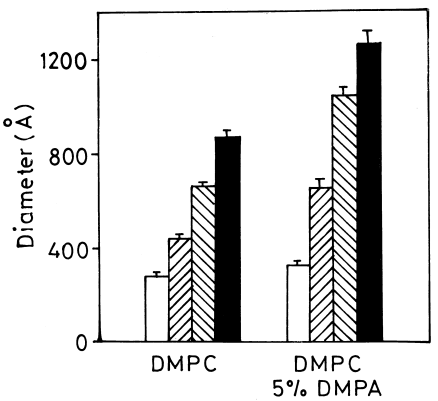

FIG. 5. Histograms of the average diameter of SUV of pure DMPC and DMPC in the presence of 5% DMPA. Diameter values are shown as means ± SEM (N = 50). Key: liposomes only, □; in the presence of 10 mM Mg^{2+}, ▨; upon treatment with MTR (15 μM), ▧; and upon treatment with $(MTR)_2Mg^{2+}$ complex ([MTR] = 15 μM, [Mg^{2+}] = 10 mM), ■. MTR incubations with the membranes were done at 27°.

even without entering the cells [30, 31]. A growing body of literature indicates that the cytotoxicity could be related directly to drug–membrane and in particular to drug–lipid interactions [31]. Studies on membrane interactions of the anthracycline antibiotic doxorubicin have indicated specific interaction of the antibiotic with anionic phospholipids such as phosphatidic acid (PA) and PS, inducing disordering of the acyl chains [19, 31, 32]. Cisplatin, another anticancer drug, also has been shown to bind erythrocytes and membranes containing anionic phospholipids such as PS [20, 33].

The membrane interaction of MTR with DPPC/cholesterol membranes was studied earlier using circular dichroism spectroscopy [18]. Binding of MTR to DPPC liposomes containing cholesterol has been shown to take place in the presence of the divalent cations Zn^{2+} and Mg^{2+}, where two molecules of MTR are complexed with one molecule of Zn^{2+} or Mg^{2+} in a left-handed conformation of the drug. It also has been indicated that MTR interacts with DPPC/cholesterol only in the gel phase, and no binding was observed above the phase transition temperature, i.e. in the liquid crystalline state [18]. Results shown in the present study clearly indicated binding of MTR and the DNA-binding ligand, $(MTR)_2Mg^{2+}$ complex, both above and below the phase transition temperature of the phospholipid membranes as detected by the change in the intrinsic fluorescence of the antibiotic (Table 1). These results are consistent with the earlier reports of fluorescence anisotropic studies on binding of anthracycline antibiotics with phospholipid membranes [34]. In the negatively charged DMPC membranes containing 10% DMPA, binding of both MTR and $(MTR)_2Mg^{2+}$ complex was detected at 15°, the pretransition temperature of DMPC, at 27°, just above the T_m, where the phospholipids are partially in gel and partially in the liquid-crystalline state, and at 35°, well above the phase transition temperature of the bulk DMPC. Previous studies have shown that 10 mol% of DMPA or PS, the maximum amount that is used in the present study, does not alter the phase transition temperature of DMPC [35–37]. Binding affinities of MTR and $(MTR)_2Mg^{2+}$ complex are comparable with the earlier fluorescence studies on carminomycin ($K_{app} = 2.2 \times 10^4\ M^{-1}$) with DPPC membranes and Adriamycin ($K_{app} = 6 \times 10^3\ M^{-1}$) with DMPC membranes containing 1% cardiolipin [38, 39]. Although the CF leakage and the electron microscopic data indicate that there are some effects of MTR and the $(MTR)_2Mg^{2+}$ complex on zwitterionic membranes, binding of MTR was not measurable in the pure DMPC membranes by similar fluorescence measurements. The affinities of binding of MTR and $(MTR)_2Mg^{2+}$ complex were an order of magnitude higher at 27°, very close to the phase transition temperature of DMPC, with a K_{app} of $1 \times 10^4\ M^{-1}$ (Table 1). This suggests that the interaction could be enhanced by the exposure of hydrophobic defects during the highly fluctuating state at or near phase transition of the bilayer.

The present study has aimed to investigate whether membrane interaction of MTR and its interaction with other macromolecules also contribute to the antitumor activity of MTR in addition to its DNA-binding capacity. In an earlier report, we demonstrated for the first time that MTR can bind to the membrane cytoskeletal protein spectrin with an affinity higher than that observed in calf thymus DNA [17]. Keeping in view the implication of anionic phospholipids in membrane interactions of anthracycline antibiotics, we have studied the effect of anionic phospholipids such as PA and PS with MTR in zwitterionic phospholipid membranes. The membrane-perturbing effect of MTR was measured with the water-soluble fluorescent marker CF. Its fluorescence is self-quenched during containment in liposomes, and leakage into the surrounding medium could be monitored as an increase in fluorescence due to the dilution of the marker [26]. The CF leakage data, summarized in Table 2, clearly indicated that liposomal membranes of different compositions showed appreciable differences in the extent of leakage of the fluorescent marker at a fixed ratio of phospholipid to the MTR (L/D = 25). In the membranes composed of DMPC with different amounts of the anionic phospholipids DMPA and PS, the CF leakage data also showed dependence on the density of the negatively charged phospholipids DMPA (2, 5, and 10%) and bovine PS (5 and 10%) (Fig. 4). Even in membranes of pure DMPC, there was appreciable leakage of CF in the presence of MTR and $(MTR)_2Mg^{2+}$ complex (Fig. 4). In the presence of 10 mM Mg^{2+}, the control values of CF leakage in the acidic membranes were significantly higher, but still we could detect the $(MTR)_2Mg^{2+}$ complex-induced changes well above the control values. The control values for membranes containing anionic phospholipids were higher due to a 3-hr stay at room temperature in the absence of MTR (Fig. 4).

This work has provided evidence of membrane interaction of MTR mediated by anionic phospholipids. The data on leakage of entrapped CF from acidic membranes, measured at 27°, indicated disruption of the local membrane structure by the antibiotic and its Mg^{2+} complex in which binding affinity of MTR is the strongest (Table 1). To correlate the increase in vesicle permeability with the reorganization of bilayer membrane, we have further examined the morphology and size of the phospholipid vesicles by electron microscopy. Size analysis of the electron microscopic data, summarized in the form of histograms (Fig. 5), indicated a significant increase in the size of the phospholipid vesicles after treatment with MTR and its Mg^{2+} complex. As can be seen from Fig. 5, there are definite increases in the sizes of the SUV after treatment with MTR and its Mg^{2+} complex in the presence of 5% DMPA versus in the absence of DMPA. The electron microscopic data point toward either membrane fusion or induction of multilamellar structures by MTR, with slower kinetics, as one probable reason for the leakage of CF into the bulk aqueous medium.

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