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ADRIAMYCIN-INDUCED CHANGES IN THE SURFACE MEMBRANE OF SARCOMA 180 ASCITES CELLS

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Adriamycin increases (a) the rate of agglutination of Sarcoma 180 cells by concanavalin A after brief exposure of 2–3 h and (b) membrane fluidity as measured by ESR within 30 min of exposure at concentrations of the anthracycline of 10^{-7}–10^{-5} M. The effect of adriamycin on agglutination is not due to an increase in the number of surface receptors for concanavalin A, since the extent of binding of the lectin is not altered by adriamycin and no change occurs in the rate of occupancy of the concanavalin A binding sites by the lectin in cells treated with the antibiotic. The order parameter, a measurement of membrane fluidity, decreases in cells exposed to adriamycin and is dose-related. The results indicate that adriamycin can induce changes in the surface membrane of Sarcoma 180 cells within a brief period of exposure to a low but cytotoxic level of this agent.

Introduction

The biochemical mechanism by which the anthracycline antibiotics exert their antineoplastic activity has generally been considered to be due to either intercalation with DNA and consequent inhibition of the synthesis of both DNA and RNA [1–4] or breakage of DNA by these drugs [5,6], perhaps as 'site-specific free radicals' after intracellular activation to a free radical state [7,8]. Although intercalative binding of agents of this class to DNA, blockade of DNA and RNA synthesis, and degradation of DNA may be important events for the expression of the cytotoxic action of these antibiotics, evidence is accumulating that these alterations may at best only account for part of the biochemical mechanism of action [9–12].

Since the plasma membrane is the first barrier encountered by the drug, it is important to consider the interaction of adriamycin with this cellular structure. In addition, examining this phenomenon may be important for understanding the cytotoxicity of agents of this class.

A variety of interactions and associations of adriamycin with the plasma membrane and membrane components have been reported [13–20]. For example, adriamycin binds in considerable amounts to erythrocytes and induces morphological changes in these cells without concomitant cell lysis [13]. Observations made on cultured heart cells suggest that ouabain antagonizes the toxic effects of adriamycin by interacting at the level of cellular membrane sites [14]. In addition, adriamycin binds to sulfated mucopolysaccharides [15], associates with membrane-lipid domains [16], increases the net transport of sodium ions across epithelium [17], and exhibits a high affinity for negatively charged phospholipids found in membranes [18], particularly cardiolipin [19,20].

Previous reports from this laboratory demonstrate that a correspondence exists between the concentration of adriamycin required for cytotoxicity to Sarcoma 180 cells in culture and effects on cell agglutination by concanavalin A [21]. Utilizing liposomal membranes as a model system, we also found that adriamycin interacts in a specific manner with cardiolipin-containing vesicles to alter fluidity and fusion characteristics [22,23].

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The present report is an expansion of our studies on the action of adriamycin on the plasma membrane of Sarcoma 180 cells. The results obtained indicate that the anthracycline rapidly produces (a) an alteration in the rate of cellular agglutination with no change in either the number of surface receptors for concanavalin A or their rate of occupancy by the plant lectin, and (b) an increase in the fluidity of the plasma membrane as measured by ESR spectroscopy.

Materials and Methods

Adriamycin was obtained from Dr. John Douros of the Division of Cancer Treatment, National Cancer Institute. Small aliquots of the drug were prepared periodically and stored below 0°C in the dark; high pressure liquid chromatography verified the purity of the compound [24]. The spin label, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl, referred to commonly as 5-doxyl stearic acid, was obtained from Syva (Palo Alto, CA) and stored in the dark under refrigeration. Stock solutions of 1 mg/ml in ethanol were prepared, and 10-μl aliquots were placed in individual tubes and dried to a thin film with nitrogen.

Concanavalin A (3-times recrystallized) was obtained from Miles Laboratories (Kankakee, IL). [^{3}H]Acetylated concanavalin A was obtained from New England Nuclear Corp. (Boston, MA). Fischer's medium, horse serum, and Hanks' balanced salt solution (pH 7.4), without calcium and magnesium, were purchased from Grand Island Biological Company (Grand Island, NY) Female CD-1 mice were obtained from the Charles River Breeding Laboratories (North Wilmington, MA).

The methodology employed for the measurement of concanavalin A-induced agglutination of Sarcoma 180 cells obtained either directly from the peritoneal cavities of CD-1 mice or from tissue culture has been described in detail [21,25]. In brief, Sarcoma 180 and Sarcoma 180/TG cells were collected from mice seven days after the intraperitoneal inoculation of $6 \cdot 10^6$ cells/animal. Cells were washed 2–3-times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.4, suspended in Fischer's medium plus 10% horse serum at a concentration of approx. 10^7 cells/ml with or without the addition of adriamycin, and incubated at 37°C with gentle shaking for variable periods of time as indicated. After incubation, the cells were washed twice with 10 ml Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and resuspended at approx. 10^7 cells/ml. The agglutination of cells by concanavalin A was measured as the initial rate of agglutination at 37°C defined by a change in optical absorbance at 546 nm and the lag time prior to the change in absorbance [25]. The binding of [^{3}H]acetylated concanavalin A (approx. 2 μCi/mg) to Sarcoma 180 cells was ascertained by exposure of the cells to adriamycin as indicated above [21,25]. This was accomplished by washing cells with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and incubating untreated and adriamycin-treated cells with a reaction mixture containing $1.6 \cdot 10^6$ cells and various concentrations of radioactive concanavalin A in a total volume of 0.25 ml. After 0–30 min at 23°C in a shaking water bath, the reactions were terminated by the addition of 5 ml ice-cold Ca^{2+}, Mg^{2+}-free phosphate-buffered saline. Cells were collected by centrifugation, washed two times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, dissolved in 0.3 ml 0.4 M NaOH, and radioactivity was determined with a Packard Scintillation Spectrometer using a toluene-liquifluor based scintillation fluid. The inclusion of 100 mM α-mannopyranoside in the incubation mixture was employed to determine the extent of nonspecific binding of [^{3}H]-concanavalin A to the cell surface in the treated and control samples.

For the analysis of membrane fluidity, Sarcoma 180 cells were grown in tissue culture to a cell density of $(1.25–1\ 6) \cdot 10^5$ cells/ml. 50 ml of cell suspension were centrifuged, and the cell pellet was washed with 20 ml Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution, pH 7.4, at 37°C. The cells were collected by centrifugation and resuspended in 5 ml Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution containing 2 μg/ml 5-doxyl stearic acid. After 15 min incubation at 37°C, the cells were collected, washed once, and resuspended in 50 μl of Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution. Exclusion of trypan blue was observed in 90–95% of the cells. The appropriate amount of buffer or adriamycin was added, the cell suspension was drawn into a 50-μl capillary pipette, and the tip was sealed. The capillary pipette was centrifuged for 1 min to gently sediment the cells and then placed in the 37°C temperature controlled cavity of a Brucker ER 200 D spectrometer. Spectra

were accumulated by computer for 10 min. Order parameters were obtained using a computer program to locate the positions of the hyperfine extrema. Measurements were made with a modulation amplitude of 2.0 G and a microwave power of 5 mW. Total time of exposure of the cells to adriamycin did not exceed 30 min. Both drug binding to the plasma membrane and transport into the cells reached equilibrium by this time [26]. The order parameters were calculated as defined by Hubbell and McConnell [27]. Both uncorrected and polarity-corrected order parameters were determined. Although the values for S (order parameter) were slightly different by the two methods, the trends within each experiment were not; for this reason, only uncorrected values have been reported.

Results

We reported that exposure of Sarcoma 180 cells to $1 \cdot 10^{-7}$ M adriamycin for 24 h in culture results in surface changes detected by measurement of changes in the rate of cellular agglutination by concanavalin A [21]. The present study indicates that long-term exposure to the anthracycline is not required for cell surface changes to occur as measured either by cell agglutination or by changes in membrane fluidity. An enhanced rate of agglutination by concanavalin A of Sarcoma 180 cells was produced after 3 h exposure to adriamycin and the increase in agglutination rate caused by the anthracycline was concentration-dependent (Table I). Similarly, a characteristic lag period prior to change in absorbance at 546 nm was altered by the treatment of the neoplastic cells with adriamycin. At the highest concentration of concanavalin A employed, which produced rapid agglutination, the lag time prior to an optical change at 546 nm was relatively short. However, differences were more distinct at a lower concentration of concanavalin A, where agglutination occurred more slowly and relatively long lag periods were observed. In other experiments in which Sarcoma 180 ascites cells were exposed to adriamycin at $7 \cdot 10^{-5}$ and $7 \cdot 10^{-6}$ M, an enhanced rate of cellular agglutination occurred as early as 2 h after incubation with the drug (data not shown).

To ascertain whether the effects of adriamycin were unique to Sarcoma 180 cells, the action of the drug on a closely related 6-thioguanine-resistant variant, Sarcoma 180/TG, was examined. Table II shows the comparative rates of agglutination and the lag time prior to the onset of cell clumping for untreated control and adriamycin-treated Sarcoma 180 and Sarcoma 180/TG cells. The resistant variant (Sarcoma 180/TG) did not agglutinate as readily as the parent Sarcoma 180 subline under identical conditions. These findings indicate that the spectrophotometric measurement of lectin-induced agglutination had sufficient specificity to detect surface differences between two related cell lines. Incubation of Sarcoma 180/TG cells with $7 \cdot 10^{-5}$ M adriamycin for 3 h produced an enhancement of the rate of agglutination similar to that observed for Sarcoma 180; this was characterized by a faster change in absorbancy at 546 nm and a decrease in the lag phase prior to cell agglutination.

Since the effect produced by adriamycin may be the result of an influence by the anthracycline on the interaction of concanavalin A with cell surface receptors, the effect of adriamycin treatment on the bind-

TABLE I

AGGLUTINATION OF SARCOMA 180 ASCITES CELLS BY CONCANAVALIN A AFTER INCUBATION WITH ADRIAMYCIN IN VITRO

Sarcoma 180 cells were suspended in Fischer's medium plus 10% horse serum at a concentration of approx. 10^7 cells/ml with or without adriamycin at the indicated levels for 3 h at 37°C. After incubation, the cells were collected and their rate of agglutination was measured as described in Materials and Methods. The data shown is a representative experiment. Although absolute values observed from one experiment to another varied depending upon the batch of concanavalin A (Con A), each experiment consistently demonstrated enhanced agglutination by adriamycin treatment.

Adriamycin (M)	Con A (μg/1.2 ml)	Initial rate of agglutination (ΔA_{546}/min)	Lag time (min)
0	50	0.026	6.7
$1 \cdot 10^{-6}$		0.044	4.4
$1 \cdot 10^{-5}$		0.091	3.4
$1 \cdot 10^{-4}$		0.106	3.7
0	250	0.104	1.3
$1 \cdot 10^{-6}$		0.114	1.2
$1 \cdot 10^{-5}$		0.176	1.2
$1 \cdot 10^{-4}$		0.296	1.1

TABLE II

COMPARATIVE EFFECTS OF ADRIAMYCIN ON THE AGGLUTINATION OF SARCOMA 180 AND SARCOMA 180/TG ASCITES CELLS BY CONCANAVALIN A

Cells were incubated and agglutination assays were performed as described in Table I, except that either Sarcoma 180 or Sarcoma 180/TG cells were employed. Con A, concanavalin A.

Neoplasm	Adriamycin ($7 \cdot 10^{-5}$ M)	Con A (μg/1.2 ml)	Initial rate of agglutination (ΔA_{546}/min)	Lag time (min)
Sarcoma 180	–	100	0.026	4.6
	+	100	0.084	2.7
	–	150	0.060	2.6
	+	150	0.112	2.5
	–	250	0.144	1.2
	+	250	0.288	1.1
Sarcoma 180/TG	–	100	0.014	6.5
	+	100	0.048	3.9
	–	150	0.018	5.7
	+	150	0.052	3.0
	–	250	0.026	3.4
	+	250	0.132	2.6

ing of concanavalin A to Sarcoma 180 cells was measured. The results and statistical fit are shown in Fig. 1. A biphasic interaction of [^{3}H]concanavalin A with

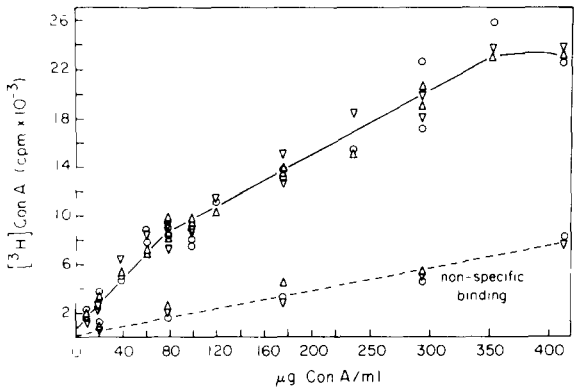

Fig. 1. Binding of [^{3}H]concanavalin A to Sarcoma 180 ascites cells after 3 h exposure to adriamycin. Sarcoma 180 cells were incubated in the presence and absence of adriamycin for 3 h, washed twice in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, resuspended in buffer, and incubated at 23°C in 0.25 ml of reaction mixture ($1.6 \cdot 10^6$ cells) containing increasing concentrations of [^{3}H]concanavalin A (2 μCi/mg) for 30 min. Binding was determined as described in Materials and Methods. The nonspecific binding represents the amount of [^{3}H]concanavalin A bound to cells in the presence of 100 mM α-D-mannopyranoside. (○) control; (▽) $7 \cdot 10^{-6}$ M adriamycin; (△) $7 \cdot 10^{-5}$ M adriamycin. Con A, concanavalin A.

Sarcoma 180 suggests the presence of at least two types of binding sites with different affinities for the plant lectin on the surface of Sarcoma 180 ascites cells. No statistically significant difference ($P > 0.5$) was seen in the amount of [^{3}H]concanavalin A bound to either untreated cells or to cells exposed to either $7 \cdot 10^{-6}$ or $7 \cdot 10^{-5}$ M adriamycin for 3 h. When 100 mM α-mannopyranoside was included in the incubation mixture to determine the extent of nonspecific binding of [^{3}H]concanavalin A to the cell surface, no statistically significant difference ($P > 0.5$) in the degree of nonspecific binding was observed between untreated and adriamycin-treated cells.

To determine if the rate of occupancy of cellular receptors by the lectin was altered by exposure of cells to adriamycin, the rate of binding of [^{3}H]concanavalin A was measured. Untreated cells and cells treated with either $7 \cdot 10^{-5}$ or $7 \cdot 10^{-6}$ M adriamycin for 3 h were exposed to 100 or 200 μg/ml [^{3}H]-concanavalin A for increasing intervals of time from 0 to 30 min at 23°C. The results obtained with 100 μg/ml [^{3}H]concanavalin A are shown in Fig. 2. No statistically significant rate difference ($P > 0.5$) in specific or nonspecific binding occurred between control and drug-treated cells. Similar results were observed for specific binding with 200 μg/ml [^{3}H]-concanavalin A.

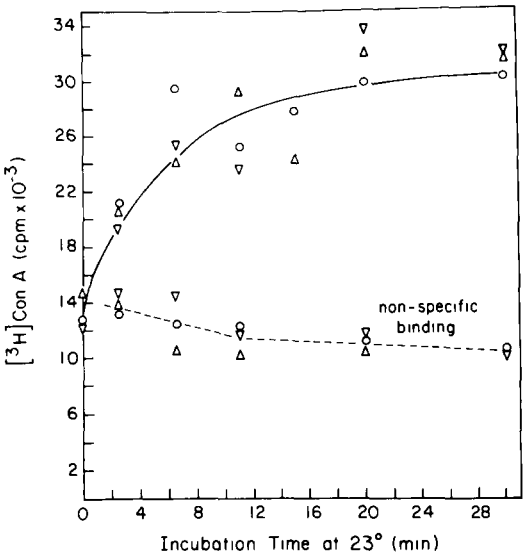

Fig. 2. Rate of binding of [^{3}H]concanavalin A to Sarcoma 180 ascites cells after 3 h exposure to adriamycin. Procedures used were the same as described in Fig. 1, except that the concentration of [^{3}H]concanavalin A was 100 μg/ml throughout, and the time of incubation was varied. (○) control; (▽) $7 \cdot 10^{-6}$ M adriamycin; (△) $7 \cdot 10^{-5}$ M adriamycin. Con A, concanavalin A.

The effect of various concentrations of adriamycin on the order parameter (S) determined from spin-labelled cells, is shown in Fig. 3. The data points were obtained from three independent experiments performed on different days and correspond to S values obtained by us and others [28,29] for Sarcoma 180 cells at 37°C utilizing 5-doxyl stearic acid as the spin label probe. Although the control values varied within a certain range depending upon the experiment, in each experiment shown, as well as in other experiments not reported here, the addition of adriamycin consistently decreased the order parameter at every concentration tested when compared to untreated control cells (Fig. 3). A detailed statistical analysis of variance supports these observations. There is not a significant interaction between drug concentrations and experiments, indicating consistent results within each experiment. The control values are significantly different (a) from values at the highest concentration of adriamycin in all three experiments ($P < 0.01$), and (b) from values at every concentration of adriamycin for two of the three experiments ($P < 0.01$).

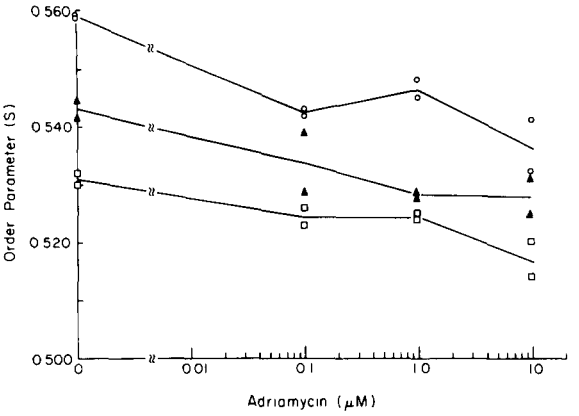

Fig. 3. The effect of adriamycin on the order parameter (S) of Sarcoma 180 cells. Three independent experiments are shown with duplicates taken at each concentration of adriamycin in each experiment. (○) Expt. I, (▲) Expt. II; (□) Expt. III. The lines connect the average values observed for S at each concentration of adriamycin.

To provide a model of the observed decrease in S, an analysis of covariance was performed utilizing the statistical software packages MINITAB and Statistical Analysis System [30,31]. The $\log_e$ concentration of adriamycin ($\ln C$) was defined as the independent variable and S as the dependent variable. For purposes of this analysis, the control values must be in terms of some concentration of adriamycin since $\ln 0$ is undefined. The value 10^{-9} M was chosen since this is well below the concentration of the drug that has a discernible effect on cell growth [21]. The adequacy of a common slope for all three experiments again bears out the overall consistency of the experiments. A 95% confidence interval for the true value of the common slope is given by -0.00179 ± 0.00045. It is important to recognize that while an equal-slopes model may be adequate to describe this particular set of data, it does not necessarily explain or describe the underlying process. Further delineation beyond this model would require more data, specifically, at intermediate concentrations in addition to the concentrations of the drug tested here and must be observed within the same experimental day preferably in duplicate.

Discussion

The finding of enhancement of agglutination of Sarcoma 180 cells by the plant lectin concanavalin

A after only relatively brief exposure to adriamycin, complements our previous study [21] in which exposure of neoplastic cells to the anthracycline antibiotic at low cytotoxic levels for a relatively long period of time (i.e., 24 h) also increased the rate of cellular agglutination. Together, these observations demonstrate that an agent whose principal target has generally been considered to be nuclear DNA, also elicits structural changes on the cell surface. The conclusion that the organization of the plasma membrane is indeed changed by the drug is enhanced by the demonstration that the affinity and number of lectin binding sites is not altered by the drug under conditions in which the agglutination rate is increased.

Endocytosis and subsequent internalization of concanavalin A has been reported to be dependent upon the clustering of receptors in mature erythrocytes of newborn humans [32] and to be independent of clustering in Chinese hamster ovary cells [33]. Internalization of the lectin may be occurring as part of the phenomenon reported in the present studies, however, the occurance of such an event does not negate the principal objectives of these experiments, namely to utilize concanavalin A as a probe to detect differences in the behavior of cell surface receptors for the lectin in the presence and absence of adriamycin. If the internalization rate of concanavalin A is altered by adriamycin and this in turn leads to an alteration in agglutination, then modulation of the cell surface by the antibiotic is occurring.

Cell agglutination involves not only static crosslinking of cells by multivalent lectin, but also dynamic mobility of lectin receptors [34]. This receptor mobility has important functional consequences. Wang and Edelman [35] have shown that there is a direct correlation between concanavalin A receptor mobility and the ability of the plant lectin to stimulate DNA synthesis in lymphocytes. Thus, components of the cell surface that control receptor mobility may be involved in the regulation of cell proliferation. Since adriamycin is a drug which influences cellular replication by exerting cytotoxicity, and promotes changes in the expression of lectin action, a mechanism of action for this agent could involve modulation of surface receptors involved in normal growth control.

The concept of receptor mobility envisions the membrane as a two-dimensional fluid, with the relative degree of 'fluidity' being one of the dominant elements in the control of motion in the plane of the membrane. The effect of adriamycin on membrane fluidity was assessed by ESR of a nitroxide-labeled fatty acid incorporated into Sarcoma 180 cell membranes. These spectra provide a measure (the order parameter, S) of the amplitude of motion about the average orientation of the fatty acid in the membrane. This motion is sensitive to the flexibility of the membrane lipids and is a measure of fluidity. Previous spin labeling work with Sarcoma 180 cells by Bales et al. [29] has shown that no change in membrane fluidity occurs upon interaction of the cells with concanavalin A and consequent agglutination. Thus, although lectin-induced cellular agglutination may require a certain degree of fluidity to support receptor mobility, a change in normal fluidity is not a necessary prerequisite for agglutination. Our work shows that a drug which increases agglutination also increases membrane fluidity, as observed with the paramagnetic probe. A direct functional linkage between these two events, however, cannot be implied.

The observed decrease in the order parameter (S) with increasing concentrations of adriamycin as reported by the fatty acid spin probe indicates that membrane fluidity is susceptible to perturbation in the presence of adriamycin. In addition, the observed increase in membrane fluidity occurred within 30 min of exposure to the drug, and an overall analysis of covariance showed a statistically significant dose response to the concentrations of adriamycin employed. The variations observed between experiments may be due to a variety of factors or cumulative effects of several factors, such as a variation in the rate of cell replication during the log phase when the cells were collected, a variation in the growth medium and availability or production of membrane lipids, or slight variations in the temperature control of the cavity of the spectrometer on different days. These variations, however, apply uniformly within each experiment, allowing a consistent pattern to evolve and be analyzed and described. Thus, the variations from day to day initiate and require a statistical model for analysis but do not preclude a comparative analysis of the experiments. Indeed, the model satisfies these variations and predicts the dimensions

and limitations of the experimental design.

The distribution of the spin label among various membranes in the cell is an important consideration in the interpretation of our results, and redistribution of the fatty acid probe from the plasma membrane (the site of initial labeling) to internal membranes probably occurs over some time scale. Klausner et al. [36] have shown that very little redistribution of free fatty acids occurs over a period of 30 min. Furthermore, work from Bales' laboratory [29,37] has provided evidence to suggest that in Sarcoma 180 ascites cells, over 80% of the fatty acid spin label resides in the plasma membrane. Since our experiments were performed within a maximum time period of 30 min, we assumed that the spin label reflects fluidity primarily in the plasma membrane.

The magnitude of the changes observed in the order parameter after exposure to adriamycin is biologically significant. After exposure to adriamycin at $1 \cdot 10^{-5}$ M for a relatively brief period of time (less than 30 min), the order parameter decreased 2.5–4.1%. A variety of experiments have shown that changes in the order parameter of this magnitude can result in major alterations in cell function [38,39]. Sinensky et al. [38] utilized a somatic cell mutant defective in the regulation of cholesterol biosynthesis to obtain cells in which plasma membranes displayed various cholesterol contents and acyl chain order parameters. With increasing amounts of cholesterol in the plasma membrane, the activity of the Na^+, K^+-stimulated adenosine triphosphatase (EC 3.6.1.3) present in these cells varied by a factor of 10, while the order parameter varied from 0.663 to 0.697, a change of 5.13%. Alterations in cholesterol content that did not change the order parameter, also did not alter enzyme activity. The authors concluded that the rate of catalysis by $(Na^+ + K^+)$-ATPase is determined by the order parameter and that variation of membrane lipid composition or factors which determine membrane lipid acyl chain ordering can result in variation in membrane enzyme activity. Likewise, comparable changes in the order parameter were shown to be coupled to the action of local anesthetics by Pang et al. [39]. General anesthetic concentrations were correlated with a 2–5% change in the order parameter and a 20–50% change in ion permeability. The functional change observed was about an order of magnitude larger than the structural change. Thus, small percentage changes in the order parameter can result in substantial modification of biological activity.

Furthermore, the magnitude of the fluidity change is consistent with the possibility that this change may be relevant to the cytotoxic action of this agent. The following considerations support this possibility: (a) the order parameter change exhibits a pharmacologic dose response to adriamycin; (b) the concentrations of adriamycin are biologically relevant and coupled biophysical responses should occur in this concentration range – the fluidity changes shown by adriamycin meet this criterion; and (c) modulation of membrane fluidity is the first response in time that can be measured in the biologically relevant concentration range. The increase in membrane fluidity occurs within 30 min of exposure to the antibiotic, whereas 1–2 h of drug treatment are required to affect the synthesis of DNA or the viability of these cells at low concentrations of antibiotic [26]. Thus, the modulation of membrane fluidity with associated effects on membrane functions may be a primary event in the initiation of cytotoxicity by adriamycin.

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