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PHOSPHOLIPID MEMBRANE STABILIZATION BY DIMETHYLSULFOXIDE AND OTHER INDUCERS OF FRIEND LEUKEMIC CELL DIFFERENTIATION

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SUMMARY

A large number of low molecular weight polar cryoprotective agents have recently been found to induce erythroid differentiation of Friend leukemic cells in vitro. The effect of these agents on membrane fluidity in phospholipid vesicles was studied by determining the solid-to-liquid crystalline phase transition using differential scanning calorimetry. Some of the inducing agents studies were found to raise the normal transition temperature (T_c) by a few degrees. All of these agents were found to produce a separate transition at a much higher temperature. Changes in the head group of the phospholipid, the pH, the presence of divalent cations, and the addition of other membrane-active compounds were found to significantly influence the inducing agent's effects on the T_c of phospholipid membranes.

The ability of the different agents to produce a new transition at a high temperature was found to correlate well with their ability to induce Friend leukemic cell differentiation. The possible mechanisms of action of the chemical inducers, and the significance of the observed membrane effects on differentiation and malignancy are discussed. It is concluded that inducing agents decrease the fluidity and stabilize phospholipid membranes, and that their effects in cell differentiation might be initiated by a similar change in the properties of cell membranes.

INTRODUCTION

Growth of Friend leukemic cells in media containing dimethylsulfoxide results in erythroid differentiation on the basis of morphological changes [1], the synthesis of hemoglobin and heme [2, 3], the appearance of globin mRNA [4], delta aminolevulenic acid synthetase [5] and red blood cell specific membrane antigens [6], the development of responsiveness to erythropoietin [7] and a loss of clonogenicity [8]. We have recently demonstrated that a large number of low molecular weight, basic, polar, cryoprotective compounds also induce cells to differentiate along erythroid lines [9, 10].

Although evidence has been presented that Dimethylsulfoxide must ultimately influence the control of differentiation at the transcription level possibly by inhibiting the binding of repressor molecules to DNA [11], the actual primary site of action of these agents remains unknown. The well recognized properties of dimethylsulfoxide as a penetrant-carrier and cryoprotectant along with considerable evidence indicating the occurrence of membrane alterations during both malignant transformation and differentiation [12], suggested to us that the initial and perhaps primary effects of dimethylsulfoxide might be on the cell membranes. This possibility has been strengthened by recently presented data from our laboratory [13] demonstrating a good correlation between the action of several inducers on cell differentiation and their effects on phospholipid membranes.

The studies reported here are based on differential scanning calorimetry of pure synthetic phospholipid membranes. The effect of the inducing agents was studied by following changes in the temperature (T_c) and enthalpy (ΔH) of the phospholipid phase transition from the solid to the liquid-crystalline state. The phase transition temperature is related to phospholipid membrane fluidity which may in turn regulate various biological functions [14–16]. The studies reported here indicate that the inducing agents tend to decrease membrane fluidity. Local anesthetics which have been shown to increase membrane fluidity [17, 18] were found to inhibit the action of the inducers on phospholipid membranes. On the other hand, divalent cations were found to enhance the effects of the inducing agents, as well as to reverse the effects of the local anesthetics on the phospholipid phase transition temperature. These findings suggest a direct interaction of dimethylsulfoxide and the other inducing agents with phospholipids resulting in alteration of membrane fluidity, an effect which may be related to their ability to induce cell differentiation in vitro.

MATERIALS AND METHODS

Chemicals

The inducing agents were used as provided or dissolved in phosphate buffered saline, 1 l of which contains: 900 ml 0.145 M NaCl; 80.40 ml 0.0667 M Na_2HPO_4; 19.60 ml 0.0667 M KH_2PO_4. They were obtained from the following companies: dimethylsulfoxide, pyridine-*N*-oxide, tetramethylurea, dimethylurea and butyric acid, all from Eastman Kodak, Rochester, N.Y.; dimethylformamide, urea and 6 H_2O · magnesium chloride, from Fischer Sci. Co., Fairlawn, N.J.; dibucaine hydrochloride from K and K Laboratories, Plainview, N.Y.; calcium chloride from Upjohn Co., Kalamazoo, Mich.; and procaine from McGaw Laboratories, Glendale, Calif.

Phospholipid vesicles

The phospholipids used in these studies were dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol and dimyristoylphosphatidic acid, synthesized as previously described [19]. The phospholipid was stored under nitrogen in sealed ampoules at −50 °C at a concentration of approximately 10 μmol per ml. Multilamellar vesicles were prepared by the method of Bangham et al. [20]. The phospholipid was dispersed in phosphate buffered saline prepared free of magnesium and calcium except as otherwise specified. Following dispersion at 37 °C, 2–3 μmol of lipid were distributed into individual sample tubes to which was added 5–10 ml of the solution to be studied.

Differential scanning calorimetry

Following a period of incubation of at least 3 h at 37 °C, the lipid suspension was centrifuged at 2000 rev./min for 30 min and the wet pellet transferred to a sample pan and covered. Using a phosphate buffered saline reference, the phase transition temperature (T_c) of the phospholipid vesicle suspension was determined using a differential scanning calorimeter (DSC-2, Perkin-Elmer, Norwalk, Conn.). In all the studies the sensitivity scale of 1 mcal per s, a chart speed of 20 mm per min and a scanning rate of 5 °C per min were used. The T_c, defined as the midpoint of the transition temperature, was found by heating the lipid samples at the indicated rate.

RESULTS

Effects of dimethylsulfoxide on the phospholipid membrane transition temperatures (T_c)

We have recently reported [13] that dimethylsulfoxide and a number of other chemical inducers of differentiation alter the normal phase transition and cause the appearance of a previously unrecognized endothermic transition at much higher temperatures. This upper T_c was associated with floculation of the phospholipid dispersion similar to that observed in the presence of divalent cations [21]. Fig. 1 demonstrates that there is a nearly linear correlation between increasing concentration

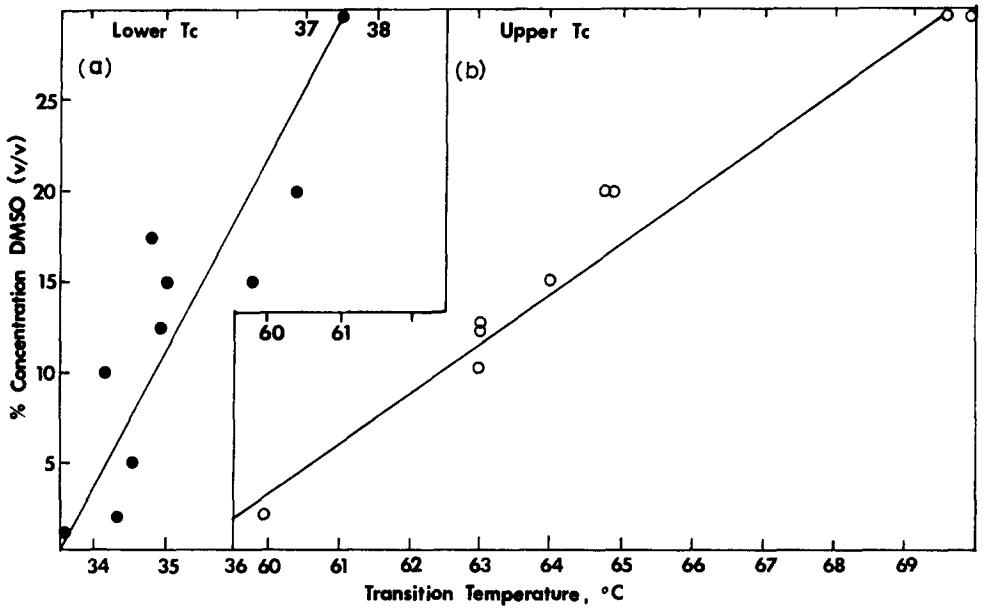

Fig. 1. The effect of increasing concentrations of dimethylsulfoxide (DMSO) on the transition temperature of dimyristoyl phosphatidylglycerol determined by differential scanning calorimetry. Vesicles were prepared in phosphate buffered saline containing 5 mM magnesium. (a) The effects of different concentrations of dimethylsulfoxide on the lower (normal) transition temperature with the control T_c at 33.6 °C. (b) The effects of different concentrations of dimethylsulfoxide on the appearance and position of the upper transition temperature. No upper T_c was observed in the absence of dimethylsulfoxide.

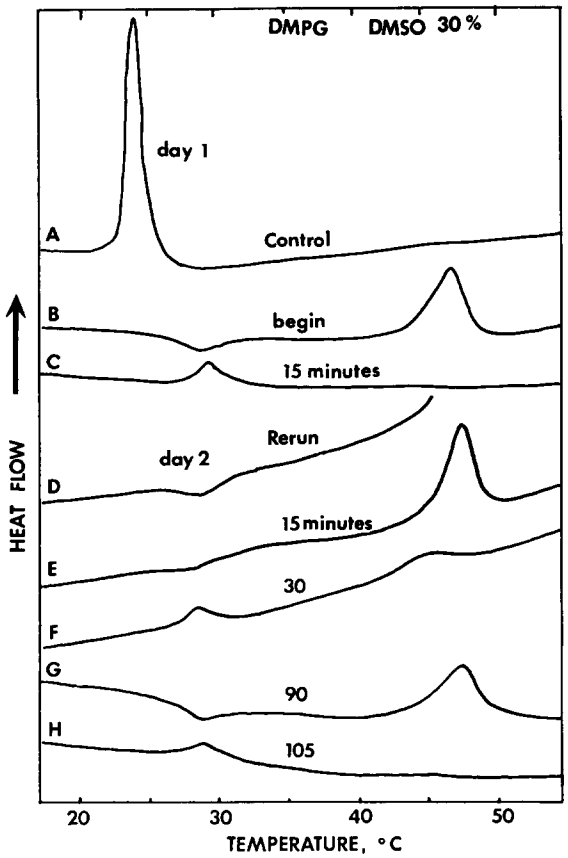

Fig. 2. Differential scanning calorimetry of dimyristoyl phosphatidylglycerol (DMPG) vesicles in the presence of dimethylsulfoxide (DMSO) on repeated heating and cooling. (A) Control vesicles prepared in the presence of phosphate buffered saline. All further calorimetry thermograms are on the same sample of dimyristoyl phosphatidylglycerol vesicles incubated initially with 30 % dimethylsulfoxide. (B) Initial calorimetry thermogram; (C) after cooling and reheating within fifteen min; (D) the following day after 24 h at room temperature, heated to within a few degrees of the upper T_c; (E) after cooling and reheating within fifteen min through the entire range; (F) after cooling and reheating again within 30 min of the original thermogram; (G) cooled to room temperature and aga n heated 1 h later; (H) after cooling and reheating again within fifteen min of the last thermogram.

of dimethylsulfoxide and the degree of elevation of both the lower (a) and the upper (b) T_c. In general, there was an inverse correlation between the area under the upper peak and that of the lower peak. The upper T_c first appears at 2 % (v/v) concentration of dimethylsulfoxide, it becomes predominant at about 20 % (v/v), and the only peak at 30 % (v/v).

Fig. 2 demonstrates that the process of heating the sample through the temperature range of the upper peak results in a dramatic decrease or disappearance of this peak, as shown when the sample is immediately cooled and reheated (curves B, C). Heating to within a few degrees of the upper T_c does not eliminate the peak on subsequent scanning (curves D, E). If the sample is left at room temperature for a

period of 30–90 min, there is a spontaneous return of the upper peak on subsequent scanning (curve G). Fig. 2 also demonstrates the changes in the lower T_c, which, in the presence of high concentations of dimethylsulfoxide, is indicated only by a small exothermic peak during the initial scan (curves B, D, E, G). If the sample is heated through the upper T_c and then cooled immediately and rescanned, the exothermic peak disappears with the appearance of a typical endothermic peak at the same temperature (curves C, F, H). Additional studies demonstrated that at high concentrations of dimethylsulfoxide (30 %) the enthalpy of the upper transition was approximately twice that of the normal transition without dimethylsulfoxide.

The effects of other chemical inducers of differentiation on phospholipid membrane transition temperatures

We have reported elsewhere [13] that, although their effects on the lower T_c varied, all of the various agents that are effective in inducing erythroid differentiation of Friend leukemic cells were found to cause the appearance of a new (high temperature) transition in dimyristoyl phosphatidylglycerol membranes.

The urea family of compounds have been found to have varying effects on the induction of erythroid differentiation. Tetramethylurea is an excellent inducer while dimethylurea is only a fair inducer and urea is a very poor inducer [10]. Fig. 3a compares the effects of the different members of the urea family on the phase transition of dimyristoylphosphatidylglycerol. Urea has little effect at any concentration

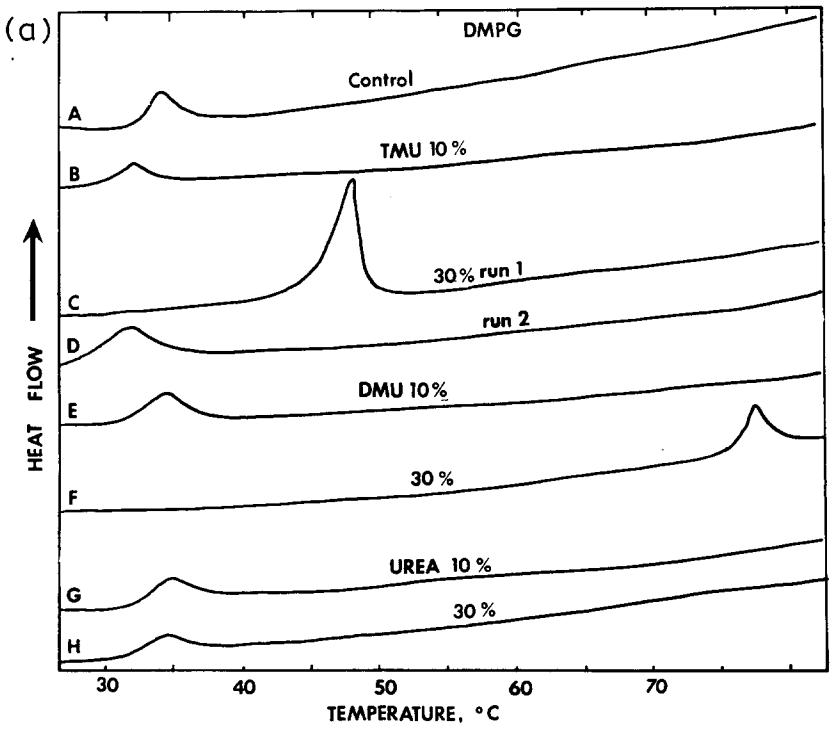

Fig. 3. See opposite page for legend.

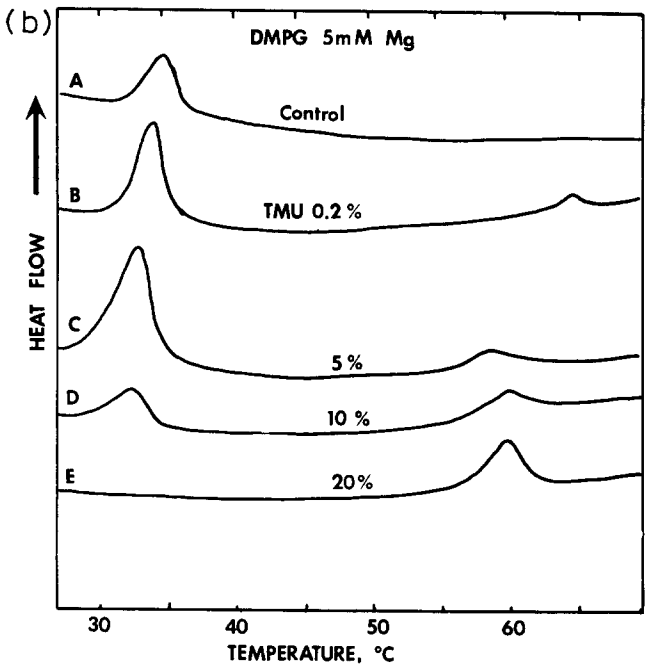

Fig. 3. (a) Differential scanning calorimetry of dimyristoyl phosphatidylglycerol (DMPG) vesicles in the presence of different concentrations of three members of the urea family of inducing compounds. (A) Control thermogram obtained from vesicles prepared without an inducing agent and in the presence of phosphate buffered saline only. The remaining calorimetry thermograms were obtained with vesicle populations under the same conditions except for the presence of; (B) tetramethylurea (TMU) 10 % (v/v), 0.86 M; (C) tetramethylurea 30 % (v/v), 2.58 M first run; (D) tetramethylurea 30 % (v/v), 2.58 M after rapid cooling and reheating of the same sample as in (C); (E) dimethylurea (DMU) 10 % (v/v) of stock, 1.0 M (stock dimethylurea, 10 M, 8.81 g/10 ml); (F) dimethylurea 30 % (v/v of stock), 3.0 M; (G) Urea 10 % (v/v of stock), 1.0 M; (H) urea 30 % (v/v of stock), 3.0 M (stock urea, 10 M, 6 g/10 ml water). (b) Differential scanning calorimetry of dimyristoyl phosphatidylglycerol (DMPG) vesicles in the presence of different concentrations of tetramethylurea (TMU). (A) Thermogram of control sample with no tetramethylurea added and prepared in the presence of phosphate buffered saline with 5 mM magnesium chloride. The remaining thermograms were obtained with vesicle populations under the same conditions except for the presence of: (B) tetramethylurea 0.2 % (v/v), 0.0172 M; (C) tetramethylurea 5 % (v/v), 0.43 M; (D) tetramethylurea 10 % (v/v), 0.86 M; (E) tetramethylurea 20 % (v/v), 1.72 M.

tested on the lower T_c and did not produce any other transition at higher temperatures (curves G, H). Both tetramethylurea and dimethylurea produce a transition at high concentrations but at different temperatures (curves C, F). Fig. 3b illustrates the effect of increasing concentrations of tetramethylurea in the presence of 5 mM $MgCl_2$. Tetramethylurea, one of the most effective inducers of erythroid differentiation, gives rise to an upper T_c at concentrations as low as studied, i.e., 0.2 % (v/v) or .0172 M (curve B). The greater potency of this agent in altering the T_c (compared to dimethylsulfoxide) is also evident in the prominence of the upper T_c at 10 % (v/v) or 0.86 M (curve D) and the complete loss of the lower T_c at 20 % (v/v) or 1.72 M (curve E). As with dimethylsulfoxide, tetramethylurea, demonstrated an inverse relationship

between the enthalpy of the lower and the upper peaks with increasing concentrations and with the same sample on reheating. Tetramethylurea differs from dimethylsulfoxide in that there is no apparent rise in the upper T_c with increasing concentrations while the lower T_c is shifted to slightly lower temperatures.

The effects of inducing agents on different phospholipids

The effects of dimethylsulfoxide on vesicles prepared from phospholipids of differing head groups are illustrated in Fig. 4. There was little effect on the T_c of the neutral phospholipid dimyristoyl phosphatidylcholine except at higher concentrations (curves A, B), at which point there was only a slight shift upward in the lower T_c and no appearance of an upper transition. This contrasts with the effects of dimethylsulfoxide on the acidic phospholipid dimyristoylphosphatidylglycerol, (curves C and D) which were shown to occur at lower concentrations (see Fig. 1). Fig. 4 also demonstrates a small effect of dimethylsulfoxide on the pre-melt of the phosphatidylcholine membranes even at low concentrations which have little effect on the main endothermic peak (data now shown).

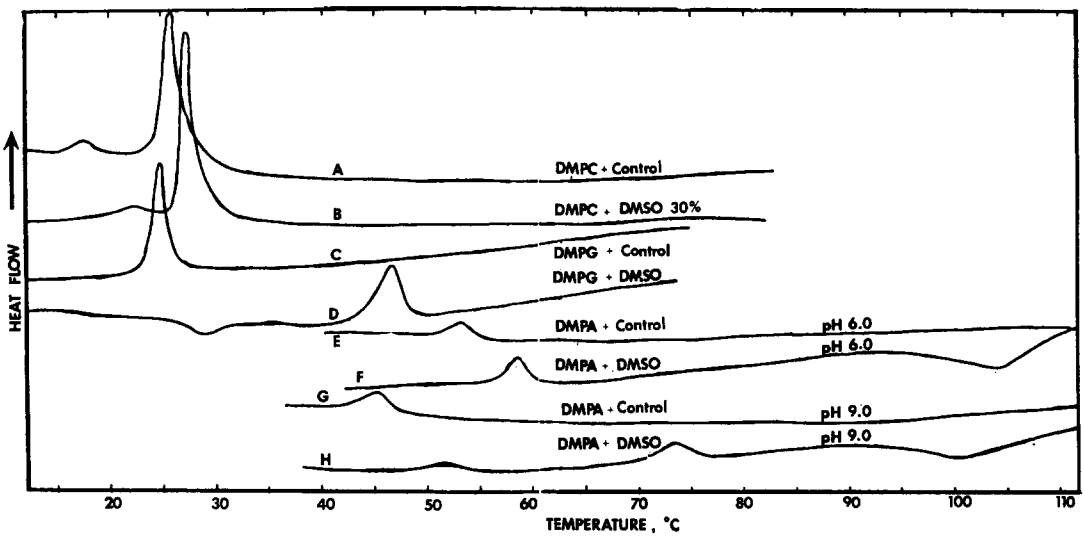

Fig. 4. Differential scanning calorimetry of vesicles prepared from different phospholipids in the presence of dimethylsulfoxide (DMSO); Samples (A) and (B) were prepared from vesicles composed of the neutral phospholipid dimyristoyl phosphatidylcholine (DMPC) (A) in the presence of phosphate buffered saline alone; and (B) in the presence of dimethylsulfoxide 30 % (v/v), 3.9 M. Thermograms (C) and (D) were obtained from vesicles prepared from the acidic phospholipid dimyristoyl phosphatidylglycerol (DMPG) under the same conditions (C) in the presence of phosphate buffered saline alone, and (D) In the presence of dimethylsulfoxide 30 % (v/v), 3.9 M. Thermograms (E) to (H) were obtained from vesicles prepared from the acidic phospholipid, dimyristoyl phosphatidic acid (DMPA). Tracings (E) and (F) were obtained from vesicles prepared at a pH of 6.0 by titrating 1 M HCl into the buffered solution. The vesicles were then prepared (E) in the presence of the titrated buffer alone and (F) in the presence of titrated dimethylsulfoxide 30 % (v/v), 3.9 M. Tracings (G) and (H) were obtained from vesicles prepared at a pH of 9.0 by titrating 1 N NaOH into the buffer solution. The vesicles were then prepared (G) in the presence of the titrated buffer alone and (H) in the presence of titrated dimethylsulfoxide 30 % (v/v), 3.9 M.

As shown in Fig. 4, a significant effect on differential scanning calorimetry patterns was also obtained with the other acidic phospholipid studied, dimyristoylphosphatidic acid (curves E–H). In addition to the rise in T_c of the lower transition seen before, dimethylsulfoxide also produces the appearance of a large exothermic peak at least 50 °C above the normal T_c (curves F, H). This exothermic transition was not present on immediate reheating of a previously studied sample. Leaving the samples at room temperature or at 4 °C for up to 72 h did not result in restoration of this exothermic peak as shown before with the endothermic peak of the phosphatidyl glycerol. The effect of dimethylsulfoxide on phosphatidic acid phase transitions differed with the pH of the dispersion media. At lower pH (6.0) the normal T_c of the untreated control occurs at 53 °C, which is 8 °C higher than the T_c of the same sample at pH 9.0. Addition of dimethylsulfoxide at 6.0, in addition to producing an exothermic upper T_c, shifted the lower T_c by 6 °C toward higher temperatures (curve F). At

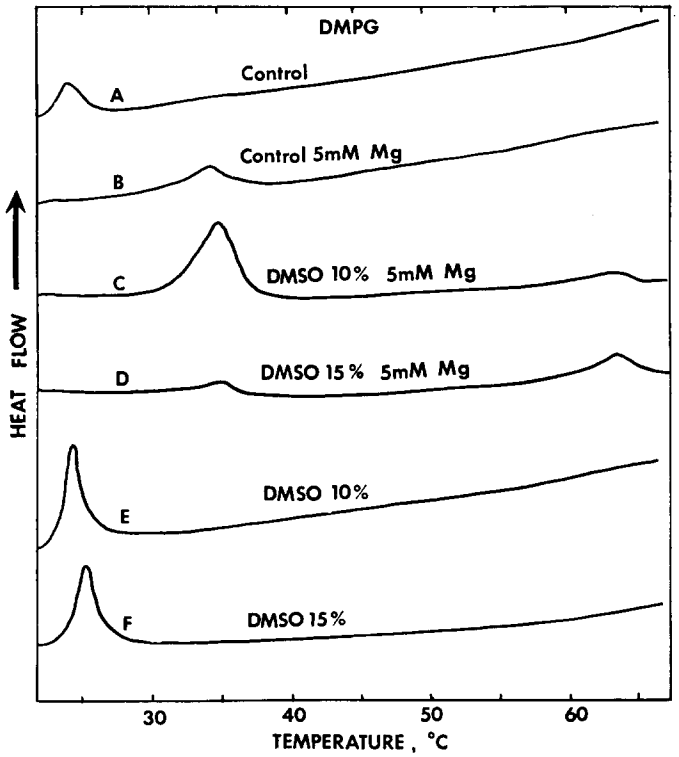

Fig. 5. Differential scanning calorimetry of dimyristoyl phosphatidylglycerol (DMPG) vesicles in the presence or absence of magnesium and dimethylsulfoxide (DMSO). Thermograms (A) and (B) were obtained from vesicle populations to which no dimethylsulfoxide was added and were prepared in the presence of: (A) Mg-free and Ca-free buffer alone; (B) buffer with 5 mM magnesium chloride. Thermograms (C) and (D) were obtained from vesicle populations prepared in the presence of 5 mM magnesium chloride to which was added: (C) dimethylsulfoxide 10 % (v/v), 1.3 M; (D) dimethylsulfoxide 15 % (v/v), 1.95 M. Thermograms (E) and (F) were obtained from vesicle populations prepared in the presence of Mg-free and Ca-free buffer alone and to which was added: (E) dimethylsulfoxide 10 % (v/v), 1.3 M; (F) dimethylsulfoxide 15 % (v/v), 1.95 M.

pH 9.0, the addition of dimethylsulfoxide also produces a shift of the normal T_c toward higher temperatures (from 45 to 52 °C) and an exothermic peak at much higher temperatures (curve H). In addition to these two peaks, however, there was another endothermic transition at 73 °C (curve H) approximately 28 °C above the normal T_c, which appeared only at high concentrations of dimethylsulfoxide and was also not apparent on subsequent reheating up to 72 h later.

Enhancement of the dimethylsulfoxide effect on phospholipid membranes by calcium and magnesium

Since the divalent cations had been previously shown to elevate the T_c of acidic phospholipids [21–24], we studied their effects on the ability of inducers to modify phospholipid membranes. Fig. 5 demonstrates the effect of the addition of magnesium to the solution, prior to the phospholipid dispersion. The main transition of the control samples of dimyristoylphosphatidylglycerol demonstrated the expected rise of 10 °C (curves A, B). The addition of dimethylsulfoxide in the presence of magnesium (curves C, D) elicits the appearance of the higher temperature endothermic peak at concentrations of dimethylsulfoxide that do not give this effect in the absence of magnesium (curves E, F). Magnesium alone did not induce the appearance of the upper T_c under the same conditions (curve B). Similar "enhancing" effects by

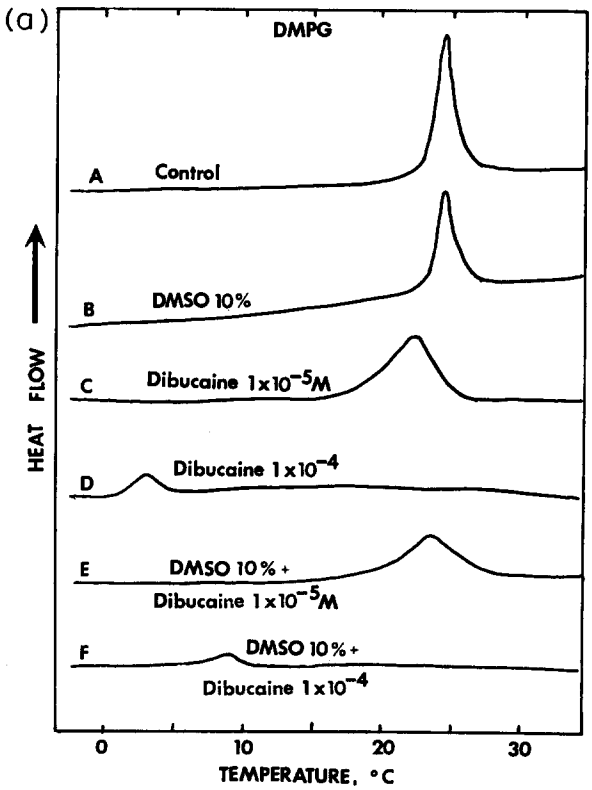

Fig. 6. See opposite page for legend.

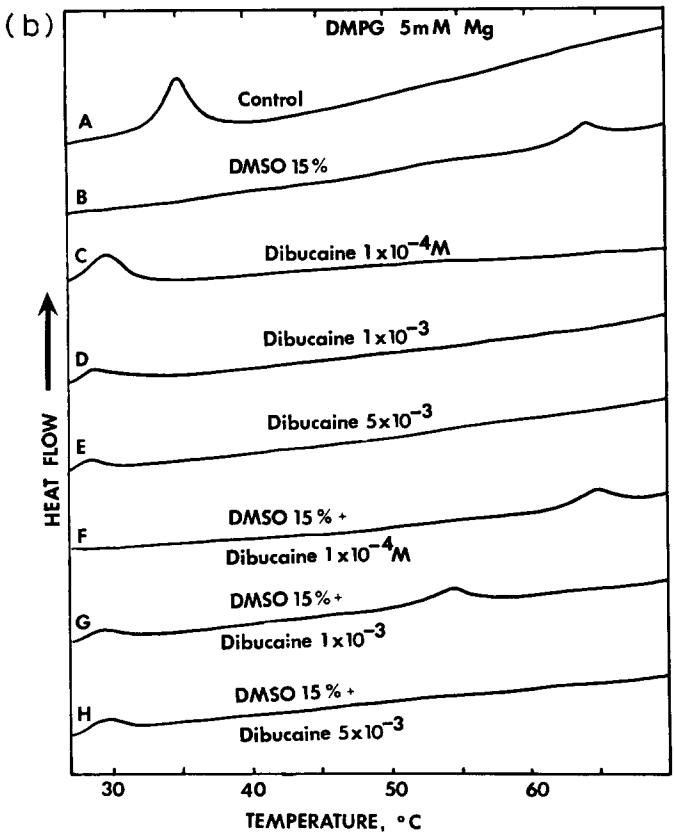

Fig. 6. (a) Differential scanning calorimetry of dimyristoyl phosphatidylglycerol (DMPG) vesicles in the presence of dimethylsulfoxide (DMSO) and different concentrations of dibucaine. (A) Control thermogram obtained from vesicles prepared in the presence of phosphate buffered saline alone. The following thermograms were obtained from vesicle populations under the same conditions except for the addition of: (B) dimethylsulfoxide 10 % (v/v), 1.3 M; (C) dibucaine hydrochloride $1 \cdot 10^{-5}$ M; (D) dibucaine hydrochloride $1 \cdot 10^{-4}$ M; (E) dimethylsulfoxide 10 % (v/v), 1.3 M and dibucaine hydrochloride $1 \cdot 10^{-5}$ M; and (F) dimethylsulfoxide 10 % (v/v), 1.3 M and dibucaine hydrochloride $1 \cdot 10^{-4}$ M. (b) Differential scanning calorimetry of dimyristoyl phosphatidylglycerol vesicles in the presence of dimethylsulfoxide and different concentrations of dibucaine. (A) Thermograms obtained from vesicles prepared in the presence of buffer with 5 mM magnesium chloride only. The remaining curves were prepared from vesicle populations under the same conditions except for the addition of: (B) Dimethylsulfoxide 15 % (v/v), 1.95 M; (C) dibucaine hydrochloride $1 \cdot 10^{-4}$ M; (D) dibucaine hydrochloride $1 \cdot 10^{-3}$ M; (E) dibucaine hydrochloride $5 \cdot 10^{-3}$ M; (F) dimethylsulfoxide 15 % (v/v), 1.95 M and dibucaine hydrochloride $1 \cdot 10^{-4}$ M; (G) dimethylsulfoxide 15 % (v/v), 1.95 M and dibucaine hydrochloride $1 \cdot 10^{-3}$ M; and (H) dimethylsulfoxide 15 % (v/v), 1.95 M and dibucaine hydrochloride $5 \cdot 10^{-3}$ M.

magnesium were significantly less pronounced if magnesium was added after the dispersion of the phospholipid into vesicles. We have also reported [13] that the addition of calcium appears to have a similar enhancing effect on the effects of dimethylsulfoxide.

Inhibition of the dimethylsulfoxide effect on phospholipid membranes by local anesthetics

It has previously been demonstrated by others [25, 26], and by us [18], that local anesthetics act to increase the fluidity of phospholipid membranes. Since dimethylsulfoxide and the other inducing agents appear to have the opposite effect on artificial membranes, we examined their interaction with phospholipid vesicles by calorimetry. Fig. 6a demonstrates the action of these agents on the normal endothermic peak in the absence of divalent cations. As expected, dibucaine hydrochloride lowers the T_c of phosphatidylglycerol and the effect is related to the concentration of the anesthetic in solution (curves C, D). The effects of dimethylsulfoxide and dibucaine appear to antagonize each other when added simultaneously, resulting in an intermediate peak somewhere between that expected for dibucaine alone and for dimethylsulfoxide alone (curves E, F). The greater the concentration of dibucaine used, the lower the temperature at which the intermediate peak occurred.

Fig. 6b illustrates the effects of these agents on the T_c of phosphatidylglycerol in the presence of Mg^{2+}. As expected, Mg^{2+} alone produces an increase in the T_c from 24 °C (curve A, Fig. 6a) to 34 °C (curve A, Fig. 6b); dimethylsulfoxide (15 %) with Mg^{2+}, induced the appearance of the upper peak (curve B, Fig. 6b). Dibucaine in the presence of Mg^{2+} had a smaller effect on the main T_c (compare curves C, D and E of Fig. 6b with curves C and D of Fig. 6a). On the other hand, dimethylsulfoxide in the presence of Mg^{2+} (curves F, G, Fig. 6b) was much more effective in reversing the dibucaine effect. Increasing concentrations of dibucaine do, however, decrease and finally eliminate the dimethylsulfoxide-induced upper peak (curves G, H). This inhibition of the dimethylsulfoxide effect by dibucaine corresponds with a decrease and then disappearance of the dimethylsulfoxide-induced floculation mentioned earlier, which is associated with the appearance of the upper T_c.

DISCUSSION

Considerable evidence has been obtained recently indicating the occurrence of alterations in membrane structure and function during neoplastic transformation [12, 27–29]. It has been suggested that neoplastic transformation may be associated with an increased lateral mobility of surface receptors attributable to increased membrane fluidity [30–32].

Very little work has been done up to now on the effects of dimethylsulfoxide on membranes. Apart from physiological studies on erythrocyte cryoprotection [33], effects on lysosomal membrane integrity [34, 35], possible effects on lipid peroxidation [38], dimethylsulfoxide has been shown to produce contraction of lipo-protein monolayers [39], to cause a pronounced decrease in the deformability of erythrocytes [40], and more recently to produce a more contracted, highly ordered structure with nerve myelin [41].

As reported here, chemical inducers of differentiation generally raise the temperature (T_c) of melting of the acyl chains in phospholipid membranes, indicating a stabilization of the bilayers perhaps related to a decrease in fluidity. This elevation of the T_c is inhibited by local anesthetics and restored by divalent cations. These observations strongly suggest that dimethylsulfoxide and the other inducing agents might decrease the fluidity of cell membranes. Our results with artificial membranes show a good correlation with observations in suspension culture where anesthetics inhibit and

divalent cations enhance [13, 42] the heme synthesis induced by dimethylsulfoxide and tetramethylurea. This correlation suggests that the membrane effects may be related to the agent's mechanism of action in inducing Friend leukemic cell differentiation.

While some of the effective chemical inducing agents studied produced a rise in the temperature of the main endothermic transition of dimyristoyl phosphatidyl glycerol, all of these agents induced the appearance of a new endothermic peak at much higher temperatures. Increasing concentrations of the agents increased the relative heat of transition associated with the upper peak at the expense of the lower peak. Although high concentrations of inducing agents were used for these effects, detectable effects on membrane fluidity were obtained with concentrations in the range used to induce differentiation in suspension culture. Dimethylsulfoxide clearly resulted in an elevation of both the lower and upper T_c with increasing concentrations while some agents produced only the appearance of the upper peak with little change in the actual temperature of the lower transition. Studies with the urea family of agents have demonstrated differences in their ability to induce the appearance of the upper peak. These differences correlate with previous evidence that tetramethylurea is an extremely effective inducer, dimethylurea a good inducer and urea a very poor inducer of erythroid differentiation [9].

Local anesthetics have been shown to have a significant "fluidizing" effect on phospholipid bilayers as indicated by nuclear magnetic and electron spin resonance [25], fluorescence polarization [18], differential scanning calorimetry [18, 43]. They have also been shown to increase the agglutination of untransformed cells by concanavalin A [44]. On the other hand, low concentrations of the divalent cations, Ca^{2+} and Mg^{2+}, have been demonstrated to increase the transition temperature of acidic phospholipids suggesting a stabilization effect on the bilayer [21–24]. Local anesthetics and divalent cations have been shown to antagonize each other's effects on membrane fluidity [18] as we have confirmed in this study. In addition we have demonstrated that the "stabilizing" effect of the inducing agents and the "fluidizing" effect of local anesthetics also appear antagonistic to each other, with dibucaine lowering the T_c of both transitions of the dimethylsulfoxide treated samples. The presence of divalent cations clearly augmented the "stabilizing" effect of dimethylsulfoxide while it reduced the anesthetic's inhibition of dimethylsulfoxide effects.

The actual mode of interaction of dimethylsulfoxide and the other agents with the phospholipids and the mechanisms by which the various changes on the thermotropic transitions occur are not clearly understood at present. It is apparent from the studies described earlier in this paper that inducing agents, like local anesthetics and divalent cations, are more effective in their interaction with acidic phospholipid membranes, suggesting that ionic charge interactions with the polar phospholipid headgroup are important. The elevation of the normal T_c, and the appearance of a discrete new transition at high temperature associated with an increased enthalpy, all indicate a significant stabilizing effect on the phospholipid membranes. This could occur through ionic interactions with the polar headgroup leading to closer packing and restricted hydrocarbon chain motion, or more likely it could involve the agent's interaction with the molecules of water at the interface creating ice-water clusters through hydrogen bonds such as those observed in bulk water with dimethylsulfoxide [45]. The disappearance of the upper peak after melting suggests a similarity with the

metastable state and supercooling observed with divalent cations and acidic phospholipids [46]. With dimyristoylphosphatidic acid we have observed a large exothermic process which disappears for an indefinite period after heating, for which we have no explanation at present.

The studies reported here are consistent with, but do not prove the possibility that the cell membranes are the primary site of action of inducing cryoprotective agents. However, the observed excellent correlation between the effects of these agents on the thermotropic properties of phospholipid membranes and biological effects on cell differentiation suggests that the interactions responsible for the phase transition changes are similar to those involved in the induction of differentiation. Similarly, the fact that most inducers are effective cryoprotective agents suggests that similar physical properties probably underlie this action as well. Preliminary studies suggest that local anesthetics inhibit the ability of these agents to cryopreserve red blood cells (Preisler, H. D. and Walzak, I., in preparation). Studies are currently underway to determine if appropriate concentrations of divalent cations might allow cryopreservation of cells at lower and safer concentrations of these agents.

If the primary site of action of agents that induce Friend leukemic cell differentiation is at the cell membrane, the question still remains as to how such action is reflected at the transcriptional level, which must be the final control stage. It is possible that interaction of these agents with membrane components, such as the stabilization of phospholipid bilayer reported here could alter the microviscosity and rheology of the cell membranes with consequent changes in the topological distribution of specific membrane components, resulting in modifications in transport properties and receptor interactions. Alternatively, the interactions of the membranes with cytoskeletal structures such as microfilaments and microtubules might also be affected, thus initiating intracellular events that in turn are responsible for the transcription of the mRNA's involved in differentiation. Direct membrane effects by these compounds with concomitant changes in membrane lipid fluidity could explain observed effects on lipid-dependent membrane enzymes such as adenyl cyclase and various ATPases resulting in the "release" of intracellular "messages". This effect could explain recent observations on alterations of intracellular acid soluble nucleoside pools during culture of cells in the presence of cryoprotective agents [47]. It is also, of course, possible that these agents, because of their strong interactions with water [45] could change the hydration of other macromolecules within the cell (including proteins and nucleic acids) with a resultant alteration in local hydrogen bonding and hydrophobic interactions leading to various conformational changes [48, 49].

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