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Lipids and Cell Fusion *in Vitro*: Effect of Amphotericin B[1] (38825)

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Since the discovery that fusion of somatic cells *in vitro* could be mediated by virus (1), attempts have been made to elucidate the fusion mechanism (2–5). Recently, much attention has been focused on various aspects of the involvement of lipids in cell fusion (6–11). This paper reports the involvement of lipids, primarily of the plasma membrane of the somatic cells, in Sendai virus-mediated cell fusion. The possibility that some change in fluidity of the plasma membrane regulated by cholesterol is a prerequisite for somatic cell fusion is discussed.

Materials and Methods. Cells. Cl-1D mouse cells resistant to bromodeoxyuridine (BrdU) (12) were maintained in Eagle's minimal essential medium (MEM) (13) supplemented with 0.2 m*M* of each nonessential amino acid, 2 m*M* glutamine, 10% calf serum, and BrdU (30 μg/ml). Cells were harvested at confluency by trypsinization, washed once by the supplemented chilled medium, once by chilled phosphate-buffered saline (PBS), and finally suspended in organic buffer adjusted to pH 8.0 or pH 8.2 (14) with cell population at 1×10^7/ml.

Sendai virus. Egg-grown Sendai virus was purified and concentrated by differential centrifugation, and inactivated by β-propiolactone (15), and kept frozen at −30°C until use. PBS was used as a diluent if necessary. Hemagglutination testing using adult chicken red blood cells in PBS gave a titer range from 2,000 to 20,000 hemagglutination units (HAU)/ml (8).

Chemicals. Fungizone (amphotericin B, 250 μg/ml) was purchased from Grand Island Biological Company, Grand Island, NY. Lysolecithin and lecithin were purchased from Pierce Chem. Co., Rockford, IL. Lysolecithin was solubilized in PBS before use. Lecithin (640 μg) was solubilized in 20 μl ethanol with and without cholesterol (202 μg) (kindly supplied by Dr. G. Rothblat) and abruptly mixed with 3 ml of the organic buffer at pH 8.2 (14). Before use, this was diluted 5-fold with the same buffer.

Cell viability test. Viability was calculated from trypan blue uptake after incubation of the cells at 37°C for 20 min in the presence of 0.1% trypan blue (7).

Protein measurement. The Folin method was used with slight modification (16).

Fusion procedure. The procedure was essentially the same as reported elsewhere (7), and the fusion rate was expressed in terms of the percentage of multinucleated cell per total of over 500 cells counted.

Results. Treatment of Sendai virus and Cl-1D cell with lysolecithin and its effect on fusion. Lysolecithin is known to interact with various kinds of biological and artificial membranes (17, 18). Its effect on fusion was examined, therefore, from the standpoint of its effect on the membranes of both the virus and the cell.

Figure 1 shows the effect of the various amounts of lysolecithin on hemaglutination activity, fusion activity, and the protein content in Sendai virus. Sendai virus treated with 100 μg of lysolecithin retained its hemagglutination activity and the original amount of viral protein but the fusion activity was lost and not recovered in the supernatant after sedimentation of the virus particle. It is likely that a disturbance of the viral envelope which is not accompanied by any loss of viral materials results in the loss of fusion activity.

Figure 2 shows the effect of different amounts of lysolecithin on the viability and fusion capability of Cl-1D cells. Cl-1D cells treated with 10 μg of lysolecithin lost the capacity for fusion while maintaining viability (primarily the permeability of the plasma membrane). This suggests that a minute but

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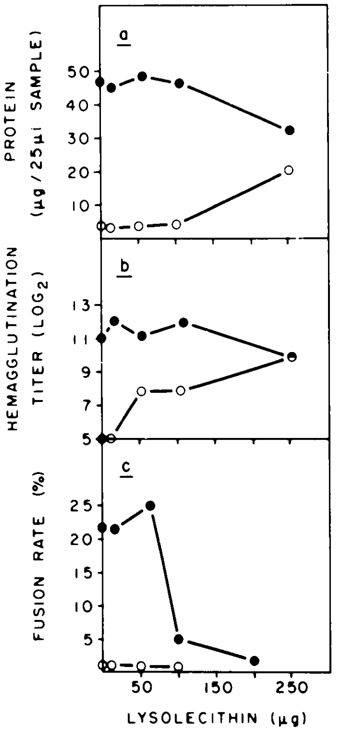

FIG. 1. The effect of lysolecithin on Sendai virus. The virus suspension (2400 HAU) was incubated for 15 min at room temperature in the presence of various amounts of lysolecithin (abscissa). Final volumes were adjusted to 0.6 ml by PBS. After cold centrifugation for 30 min at 27,000g, sediments were suspended in 0.3 ml PBS and both the sediment (●——●) and supernatant (○——○) were assayed for protein (a), hemagglutination activity (b), and fusion activity (c). The assay of supernatant fusion rate could not be carried beyond 100 μg lysolecithin because of toxicity.

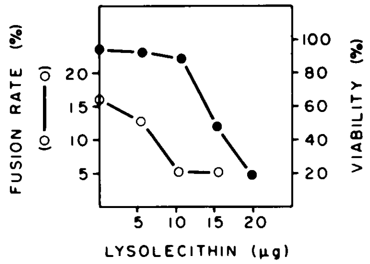

FIG. 2. The effect of lysolecithin on Cl-1D cells. The cell suspension (5 × 10^6 cells) was incubated for 10 min at room temperature in the presence of various amounts of lysolecithin (abscissa); further incubated for 5 min in ice, and the fusion experiment started by mixing with 100 μl of Sendai virus (1000 HAU/ml). Viability was tested immediately after incubation at 37°C for 20 min.

critical disturbance of the cytoplasmic membrane of the Cl-1D cell not accompanied by a change in permeability results in the loss of fusion capacity of Cl-1D cells. Although lysolecithin which is not bound to the cells is present throughout this experiment, its effect on the viral fusion activity can be excluded on the basis of data shown in Fig. 1.

The above result suggests that in studying cell fusion a primary emphasis must be placed on the structural state of the membranes of the virus and the cell rather than compositional changes. Taking account of the fact that lysolecithin interacts with the hydrophobic region of the cell membrane (18) and also that dinitrophenylated derivatives of lysolecithin, lysophosphatidylethanolamine, and lipidic substances of similar physicochemical nature, interact with artificial membranes which are composed exclusively of lipids (17, 19, 20), the disturbances of fusion observed above might be attributed primarily to an interaction between lysolecithin and lipids of the viral envelope or plasma membrane which produce some change in the structure of those membranes.

The effect of treatment of Cl-1D cell with amphotericin B on fusion mediated by Sendai virus. In the structural state of the artificial membrane which is composed exclusively of lipids, cholesterol is known to play a regulatory role (21–25). From this and the above argument it was assumed that the fusion capacity of the cell would be altered through modification of the cholesterol molecules in the plasma membrane. To test this, amphotericin B, a polyene antibiotic whose mechanism of action has been shown to involve a specific interaction with cholesterol (26), was employed as a reagent.

Figure 3 shows that preincubation of Cl-1D cell with amphotericin B increases the fusion rate over a wide range of Sendai virus concentrations. This increase was dependent on the concentration of amphotericin B (Fig. 4). Under these experimental conditions, significant toxic effects on Cl-1D cells were not observed.

The association of amphotericin B with Cl-1D cells rather than with Sendai virus was demonstrated to be responsible for this increase (Table I). Increases in the fusion

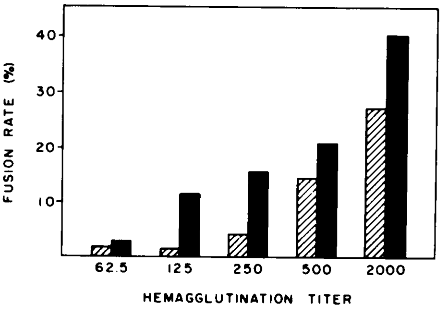

FIG. 3. The effect of amphotericin B on the fusion rate at various concentrations of Sendai virus. Cl-1D cells (5×10^6 cells), preincubated with 2.5 μg of amphotericin B for 10 min at room temperature were cooled in ice for 5 min prior to the addition of 100 μl of Sendai virus suspension at the hemagglutination titer specified in abscissa. See the text for details. Closed bar and hatched bar represent the presence and absence of amphotericin B, respectively.

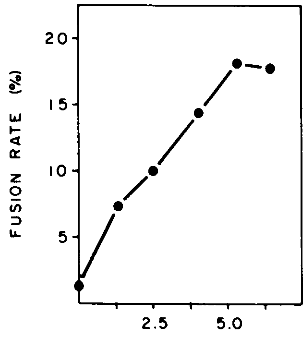

FIG. 4. Titration curve of fusion rate with amphotericin B. The experiment was done under the same condition as in Fig. 3 except that hemagglutination titer of Sendai virus was fixed to 125 HAU/ml and the amount of amphotericin B is specified in abscissa.

rates were observed when Cl-1D cells were pretreated with amphotericin B, independently of whether Sendai virus was suspended in the supernatant of the preincubation mixture of Cl-1D cell alone or Cl-1D cells with amphotericin B.

To determine which component of the plasma membrane of Cl-1D cells interacts with amphotericin B, the inhibitory effect of cholesterol was studied (Table II). To have a stable and homogeneous suspension of cholesterol, lecithin was codispersed. Amphotericin B preincubated with lecithin suspension is as active as a control with no lecithin. Preincubation with a codispersion of lecithin and cholesterol inactivated amphotericin B down to a level where no amphotericin B was present. In view of the demonstrated binding of cholesterol by amphotericin B, the data suggest that the association of amphotericin B with cholesterol molecules in the plasma membrane of Cl-1D cells may be partially responsible for the increase of Sendai virus-mediated fusion.

Discussion. This study has shown that some change in the structural state of the plasma membrane favors Cl-1D cell fusion mediated by Sendai virus. This change occurred with amphotericin B (Figs. 3 and 4) but not with lysolecithin (Fig. 2) which suppressed the fusion capacity of Cl-1D cell. The fact that both of these reagents are considered to interact primarily with the lipids in biological membranes (17, 18, 26) and the fact that they work in opposite ways with regard to fusion, strongly indicates that during the course of fusion the lipids of the plasma membrane are not inert but play a regulatory role.

In contrast to lysolecithin which is considered to be a nonspecific reagent, amphotericin B has been shown to interact specifically with cholesterol in biological membranes and artificial membranes (26). This was confirmed by the data in Table II where increase of Sendai virus-mediated fusion by amphotericin B was completely reversed after preincubation of amphotericin B with cholesterol–lecithin, but not with lecithin. These findings suggest that the cholesterol molecule in the plasma membrane of Cl-1D cells may be partially responsible for the positive effect on fusion by amphotericin B. It is possible that amphotericin B modifies the mobility of the plasma membrane of Cl-1D cell by interacting with the cholesterol in the membrane. It has been demonstrated (27) that cholesterol in the artificial membrane is bifunctional in its influence on the mobility of the membrane. That is, cholesterol fluidizes the membrane below and rigidifies the membrane above the liquid crystal transition temperature. Under our experimental condition, the incubation temperatures, ice-cold for the first 20 min and 37°C for the following

TABLE I. THE EFFECT OF AMPHOTERICIN B ON SENDAI VIRUS-INDUCED FUSION OF Cl-1D CELLS.[a]

HAU/ml Sendai virus used in the experiment	Lot	Treatment of Cl-1D cells	Fusion rate of cells treated with Sendai virus diluted in medium derived from	
			Cl-1D cells alone	Cl-1D cells treated with amphotericin B
310	A	None	3.0	4.9
		Amphotericin	9.4	7.3
	A	None	4.8	4.5
		Amphotericin	8.1	10.3
	B	None	22.2	25.2
		Amphotericin	32.3	34.3
620	B	None	10.9	15.0
		Amphotericin	25.2	28.6

[a] Cl-1D cells (5 × 10⁶ cells) were preincubated in the presence or absence of amphotericin B (5 μg) for 10 min at room temperature. After centrifugation for 5 min at 100g, the supernatant was mixed with 100 μl virus (310 or 620 HAU/ml) and added back to one of the cell pellets. Four incubation mixtures thus prepared were examined for their fusion rate as described in the text.

TABLE II. THE EFFECT OF CHOLESTEROL ON THE INCREASE OF SENDAI VIRUS-INDUCED FUSION RATE BY AMPHOTERICIN B.[a]

Cells preincubated prior to fusion in	Fusion rate (%) of cells after exposure to Sendai virus	
	Expt. 1	Expt.2
Medium alone	10.7	12.6
Medium containing		
Amphotericin alone	19.4	19.4
Amphotericin and lecithin	21.4	21.5
Amphotericin, lecithin, and cholesterol	9.9	11.3

[a] Cl-1D cells (5 × 10⁶ cells) were incubated for 10 min at room temperature with and without amphotericin B (5 μg) which had been preincubated with the suspension of lecithin (64 μg) or a mixture of lecithin (64 μg) and cholesterol (20 μg) for 10 min at room temperature. The cells were cooled and used for the fusion experiment in the presence of 100 μl of Sendai virus (310 HAU/ml).

20 min, are apparently below and above the liquid crystal transition temperature of the lipids of plasma membrane (25). On the other hand, amphotericin B did not show any increase of the virus-mediated fusion rate, when it was added 10 min after the incubation mixture of Cl-1D cell and Sendai virus was transferred from ice-cold temperature to 37°C (Kataoka and Koprowski, unpublished result). This suggests that the effect of amphotericin B on fusion is related to its effectiveness at ice-cold temperature, but not 37°C, although amphotericin B is likely to interact with cholesterol equally at 4°C and 37°C, (28; Kataoka and Koprowski, unpublished result). Perhaps amphotericin B removes the fluidizing effect of cholesterol from the cytoplasmic membrane through some specific interaction which rigidifies the membrane.

Summary. Fusion rate of Cl-1D cells mediated by Sendai virus was decreased by preincubating the cells or the virus in the presence of lysolecithin. However, a significant increase occurred when the cells were preincubated in the presence of amphotericin B. This increase was suppressed by exogenous cholesterol.

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