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STUDIES ON THE INTERACTION OF SENDAI VIRUS WITH LIPOSOMAL MEMBRANES

SENDAI VIRUS-INDUCED AGGLUTINATION OF LIPOSOMES CONTAINING GLYCOPHORIN

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Liposomes constituted with the major sialoglycoprotein of human erythrocytes, glycophorin, were used as models for studies on cell-virus interactions. Liposomes composed of egg yolk phosphatidylcholine, cholesterol and glycophorin were found to interact with the paramyxovirus HVJ to form aggregates. The aggregation process was temperature dependent: it was maximal at 0°C and decreased with increase of the incubation temperature. The activity of viral neuraminidase is also temperature dependent, and it increases with increase of the incubation temperature; release of *N*-acetylneuraminic acid was negligible at 0°C. Shift-up of the incubation temperature immediately cancelled HVJ-induced agglutination of liposomes. Viruses attached to liposomes seemed to be released into the supernatant when the 'virus-liposome' complex formed at 0°C was incubated at 37°C, possibly as a result of breakdown of the 'binding' site by neuraminidase. The characteristics of the interaction of HVJ with liposomes containing glycophorin appeared to be phenomenologically similar to those of HVJ-cell interaction.

Introduction

It is well known that the envelopes of paramyxoviruses have neuraminidase, hemagglutinating, hemolytic and fusion activities. These viruses have glycoprotein spikes that adsorb to receptors containing *N*-acetylneuraminic acid on animal cells. The interactions that subsequently bring about agglutination of cells through various viral activities are poorly understood. The receptors on membranes contain *N*-acetylneuraminic acid, as shown by the fact that cells pre-treated with neuraminidase are insensitive to the virus. Recently, it was found that the major sialoglycoprotein of human erythrocytes (glycophorin), which has been shown to be an integral protein, contains receptors for myxoviruses and paramyxoviruses. Grant and McConnell [1] showed by electron-microscopy that liposomes reconstituted with sialoglycoproteins of human erythrocytes were agglutinated by influenza viruses. MacDonald and MacDonald [2] observed hemagglutination inhibition activity of liposomes constituted with glycophorin, and they emphasized that free and membrane-associated glycophorin have similar receptor activities to HVJ.

However, there have been few systematic studies on the molecular events occurring on the membrane in the presence of HVJ, leading to agglutination, lysis and fusion. In this paper, we describe the interactions of HVJ with model membranes containing glycophorin, and discuss the results in relationship to cell-HVJ interactions.

Abbreviation: HVJ, hemagglutinating virus of Japan, also called Sendai virus.

Materials and Methods

Chemicals. Cholesterol, β,γ-dipalmitoyl-D,L-α-glycerophosphocholine and dicetyl phosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Egg yolk phosphatidylcholine was prepared by chromatography on Aluminium Oxide Neutral and Unisil. Lipid preparations gave single spots on silica gel thin-layer chromatography. *N*-Dansylphosphatidylethanolamine was prepared by the method of Shechter et al. [3], using dansyl chloride and phosphatidylethanolamine of *Escherichia coli*. Egg yolk [*N-methyl*-^{3}H]phosphatidylcholine (212 Ci/mol) was prepared by introducing a ^{3}H-labeled methyl group into the choline moiety with [^{3}H]methyl iodide (Radiochemical Centre, Amersham, U.K.) by the method of Stoffel et al. [4]. Glycophorin was prepared as follows: human erythrocytes were washed three times with phosphate-buffered saline (pH 7.4). Then hemoglobin-free erythrocytes ghosts were prepared by the method of Steck and Kant [15]. Glycophorin was extracted from the ghosts using 0.3 M lithium diiodosalicylate, and purified as described by Marchesi and Andrews [6]. Neuraminidase from *Vibrio cholera* was obtained from Behringwerke AG. Marburg, F.R.G. Concanavalin-A covalently bound to Sepharose 4B was purchased from Pharmicia Fine Chemicals, AB. Uppsala, Sweden.

Virus. The Z train of hemagglutinating virus of Japan (HVJ, Sendai virus) was grown in the allantoic cavity of 10-day-old embryonated eggs, and purified by differential centrifugation. The hemagglutinating titer was determined by the pattern method of salk [7] except partial modification. In brief, HVJ preparation (50 μl) was diluted serially 2-fold. Then 50 μl of 0.5% chick erythrocytes was added to each dilution, and the mixtures were incubated for 2 h at 4°C. One hemagglutinating unit (HAU) corresponds to 50% agglutination of the erythrocytes. The number of HAU was determined as the highest dilution of the virus preparation capable of agglutinating 50% of the erythrocytes. The hemagglutinating titer of purified virus was 50 000 HAU/ml. ^{14}C-labeled HVJ was obtained by growing viruses in the allantoic cavity with [^{14}C]leucine (3 000 dpm/100 HAU).

Preparation of liposomes. Liposomes containing glycophorin were prepared by the method of MacDonald [12]. A solution of egg yolk phosphatidylcholine (3 μmol), dicetyl phosphate (0.3 μmol), cholesterol (1.5 μmol) and glycophorin (150 μg) in $CHCl_3$/MeOH/H_2O (150 : 75 : 1, v/v) was dried in a rotatory evaporator and stored for more than 1 h in vacuo, and then the protein containing lipid film was swollen in 300 μl of phosphate-buffered saline (pH 7.4). For removal of the lipid-free protein, the liposomes were subjected to Sepharose CL-4B column chromatogrphy (15 mm × 450 mm, diameter × length).

Quantitative measurement of agglutination of liposomes. Agglutination of liposomes was measured spectrophotometrically in a spectrophotometer (Shimadzu UV-140). The change of absorbance was measured at 340 nm.

Fluorometric study. Liposomes were prepared with egg yolk phosphatidylcholine/dicetyl phosphate/cholesterol/*N*-dansylphosphatidylethanolamine (molar ratio, 100 : 10 : 50 : 1), and glycophorin (50 μg/μmol phosphatidylcholine). Liposomes (50 nmol as phosphatidylcholine) were incubated with 125 HAU of HVJ at 0°C for 30 min, and then observed under a fluorescence microscope (Olympus, model BHB).

Low angle light scattering study. Liposomes (50 nmol as phosphatidylcholine) were incubated with 125 HAU of HVJ in phosphate-buffered saline at 0°C or 37°C for 2 h. The sizes of liposomes and aggregated liposomes were examined in a low angle light scattering photometer (apparatus, LS-8, Toyo Soda, Manufacturing Co., Ltd.).

Assay of the amount of N-acetylneuraminic acid released from liposomes by viral neuraminidase. Liposomes containing glycophorin were incubated with HVJ in 100 μl of phosphate-buffered saline at various temperature for 15 min or at 37°C for various times. The reaction was terminated by putting the mixtures into boiling water for 3 min, and the amount of *N*-acetylneuraminic acid released was determined by the method of Aminoff [8]. Treatment with *Vibro cholera* neuraminidase was done by incubating the liposomes with 10 mU of enzyme at 37°C for 30 min.

The total sialic acid content was determined fluorometrically using 3,5-diaminobenzoic acid by the method of Hess and Rolde [9].

Results

Agglutination of liposomes by HVJ

Egg yolk phosphatidylcholine liposomes contain-

ing glycophorin and a trace amount of *N*-dansylphosphatidylethanolamine were incubated with HVJ at 0°C and then observed by fluorescence microscopy. HVJ and glycophorin dependent agglutination of liposomes (clumps of 5–10 liposomes) was observed. The agglutination was determined quantitatively by measuring the change of absorbance of the suspension at 340 nm. The increase in absorbance resulted from agglutination of liposomes, since measurement of scattered light intensity at an angle as low as 5° indicated that the liposomal size increased about 10 times under the experimental conditions (data not shown). The results obtained from light scattering experiments agree well with the observations by microscopy. As described above, we observed that clumps of 5 to 10 liposomes were formed during incubation with excess HVJ. This clumping was dependent on the amount of glycophorin incorporated. Without glycophorin, liposomes showed only a slight increase of absorbance. About 150 HAU/ml of HVJ was required for 50% of the maximum agglutination.

Requirements for virus-induced agglutination of liposomes

Agglutination of liposomes by virus was almost completely inhibited by a sialoglycopeptide, fetuin (Table I). Neither Ca^{2+} nor EDTA had any appreciable influence on the reaction. Preincubation of protein-free liposomes with glycophorin did not sensitize the liposomes to HVJ. Incorporated glycophorin may be essential for liposomes to show agglutinability. When the liposomes were treated with neuraminidase from *Vibrio cholera* (2 mU) for 10 min at 37°C, about 50% of their *N*-acetylneuraminic acid was released into the supernatant. On treatment with neuraminidase, liposomes lost their sensitivity to HVJ, indicating that the *N*-acetylneuraminic acid residue of glycophorin is important for the reaction (Table IC).

Temperature dependence of virus-induced agglutination of liposomes

Egg yolk phosphatidylcholine liposomes containing glycophorin were incubated with an excess of HVJ at various temperatures (Fig. 1A). The maximum density increase was obtained at 0°C. With increase of the incubation temperature, the reaction was suppressed and almost no agglutination was observed

TABLE I

TURBIDITY CHANGES OF LIPOSOMES BY HVJ

(A) Liposomes composed of 50 nmol of egg yolk phosphatidylcholine, 5 nmol dicetyl phosphate, 25 nmol cholesterol and 2.5 μg of glycophorin (3.04 nmol sialic acid) were incubated with 125 HAU of HVJ for 30 min at 0°C in 500 μl of phosphate-buffered saline, containing EDTA or fetuin. The same experiment was also performed in veronal buffered saline containing $CaCl_2$. (B) Liposomes, with the same composition as for (A) but without glycophorin, were pre-incubated with 2.5 μg of glycophorin for 10 min at 37°C. The mixture was then incubated with 125 HAU of HVJ. (C) Similar liposomes to those described in (A) were treated with 2 mU of *Vibrio cholera* neuraminidase for 10 min at 37°C. Then they were incubated with 125 HAU of HVJ at 0°C for 30 min. The reaction was done in 500 μl of veronal buffered saline containing 2 mM Ca^{2+} instead of phosphate-buffered saline.

Liposomes	Incubation conditions	ΔA_{340} ($\times 10^3$)
(A) With glycophorin	control (–HVJ)	1
	+HVJ	93
	+HVJ + EDTA (2 mM)	87
	+HVJ + $CaCl_2$ (2 mM)	91
	+HVJ + fetuin (5 mg/ml)	2
(B) Without glycophorin	control (–HVJ)	6
	+HVJ	16
	+glycophorin (2.5 μg) + HVJ	18
(C) With glycophorin, pretreated with *Vibrio cholera* neuraminidase	control (–HVJ)	5
	+HVJ	20

at 40°C. In the temperature range from 0°C to 30°C, the initial rate of the reaction was fast and almost reached the plateau within 10 min. At 30°C, the density decreased slightly after reaching a maximum. Similar temperature-dependence was observed with dipalmitoylglycerophosphocholine liposomes (Fig. 1B).

The virus-induced increase of absorbance of egg yolk phosphatidylcholine liposomes was immediately cancelled on shifting up the incubation temperature (Fig. 2); the absorbance became constant within 10 min after shifting up the temperature. The final absorbance depended on the temperature, and the increase of absorbance at all temperatures was completely cancelled by shifting up the temperature to

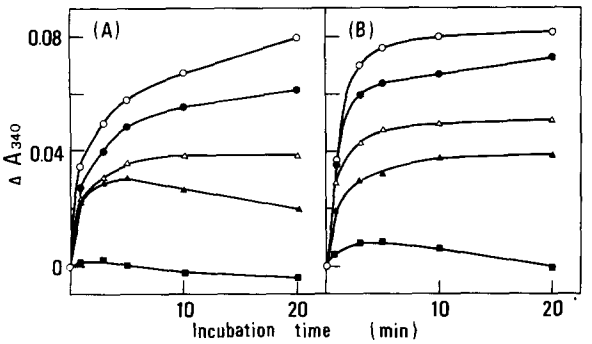

Fig. 1. Temperature-dependence of HVJ-induced increase in absorbance of liposomes. Liposomes composed of 50 nmol egg yolk phosphatidylcholine, 5 nmol dicetyl phosphate, 25 nmol cholesterol and 2.5 μg glycophorin (A), or 50 nmol dipalmitoylglycerophosphocholine, 5 nmol dicetyl phosphate, 25 nmol cholesterol and 2.5 μg glycophorin (B) were incubated with 125 HAU of HVJ in 0.5 ml phosphate-buffered saline at various temperatures. ○——○, 0°C; ●——●, 10°C; △——△, 20°C; ▲——▲, 30°C; ■——■, 40°C.

40°C. It is noteworthy that the final values obtained at each temperature were almost the same as those obtained by incubating liposomes without pre-incubation at 0°C.

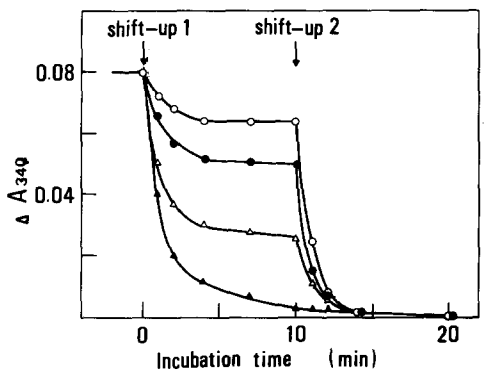

Fig. 2. Change of liposomal absorbance by shift-up of the incubation temperature. Liposomes composed of 50 nmol egg yolk phosphatidylcholine, 5 nmol dicetyl phosphate, 25 nmol cholesterol and 2.5 μg glycophorin were preincubated with 125 HAU of HVJ for 30 min at 0°C. Then the mixtures were further incubated at the indicated temperatures. ○——○, 10°C; ●——●, 20°C; △——△, 30°C; ▲——▲, 40°C. After incubation for 10 min, the temperature was shifted up to 40°C.

Release of N-acetylneuraminic acid from liposomes containing glycophorin by viral neuraminidase

The amount of *N*-acetylneuraminic acid released from liposomes containing glycophorin by incubation with HVJ at various temperatures was determined (Table II). The enzyme activity depended on the incubation temperature, and was almost negligible at 0°C. The rate of *N*-acetylneuraminic acid release was rather slow even at 37°C and did not reach a plateau within 3 h (data not shown). Only 24% of the original *N*-acetylneuraminic acid of glycophorin in liposomes was released after 3 h, whereas almost 50% was released by treatment with neuraminidase of *Vibrio cholera*. When liposomes were incubated with HVJ first at 37°C and then 0°C, rapid agglutination was observed upon shift down of the temperature (Fig. 3). The increase of absorbance observed upon shift down of the temperature after incubation for 3 h at 37°C was almost the same as that observed when the incubation was performed at 0°C without pre-incubation at 37°C, though viral neuraminidase should destroy parts of receptor sites on liposomal membranes. Thus 50% reduction of available *N*-acetylneuraminic acid may not be enough for effective inhibition of HVJ-induced agglutination.

TABLE II

N-ACETYL NEURAMINIC ACID RELEASE FROM LIPOSOMES CONTAINING GLYCOPHORIN BY *VIBRIO CHOLERA* AND HVJ NEURAMINIDASE

Liposomes were prepared from 500 nmol egg yolk phosphatidylcholine, 50 nmol dicetyl phosphate, 25 nmol cholesterol and 25 μg of glycophorin. In (A), these liposomes were incubated with *Vibrio cholera* neuraminidase (10 mU) at 37°C for 30 min in 100 μl of veronal-buffered saline containing 2 mM Ca^{2+}. In (B), liposomes were incubated with 1250 HAU of HVJ in 100 μl phosphate-buffered saline at various temperatures for 15 min. In both (A) and (B), the reaction was stopped by incubating the mixtures at 100°C for 3 min. The amount of *N*-acetylneuraminic acid released and the total neuraminic acid were determined as described in the text.

Incubation with	Temperature (°C)	% Release
(A) *Vibrio cholera* neuraminidase	37	43.7
(B) HVJ	0	0.8
	20	3.9
	40	7.4

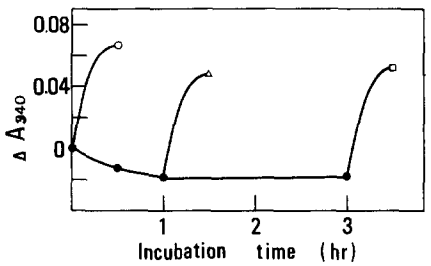

Fig. 3. HVJ-induced increase of liposomal absorbance upon shift-down of incubation temperature to 0°C after incubation of liposomes with the virus at 37°C. Liposomes of the composition described in Fig. 1A were first incubated with 125 HAU of HVJ in 500 μl phosphate-buffered saline at 37°C for various times (•). Change of absorbance was measured when the mixtures were further incubation for 30 min after shift-down of the temperature to 0°C. Shift-down of temperature was performed after incubation at 37°C for 1 h (△) and 3 h (□). HVJ-induced increase of liposomal absorbance at 0°C without preincubation at 37°C was also measured (○).

Release of virus from the liposomal surface

Next we examined whether virus adsorbed at 0°C could be released from the liposomal membranes by incubation at 37°C. First we established a method for separating HVJ-bound liposomes from free liposomes. This method is based on the fact that free liposomes can be separated from HVJ-bound liposomes using conjugates of HVJ and Con A-Sepharose beads instead of HVJ, since only HVJ has receptor sites for concanavalin A [10] and can attach to Con A-Sepharose beads. Free liposomes were recovered in the supernatant fraction, while liposomes associated with HVJ were recovered in the pellet (Con A-Sepharose beads) fraction. As shown in Table III, liposomes with glycophorin became attached to Con A-Sepharose through a bridge of HVJ, while only a small amount of liposomes without protein were found in the Con A-Sepharose beads. The liposomes bound to Con A-Sepharose could not be removed by washing with buffer at 0°C. About 40% of the liposomes (1.32 nmol of 3.42 nmol of phophatidylcholine) could be recovered in the supernatant after incubation for 30 min at 37°C, but under the same incubation conditions, a negligible amount of HVJ bound to Con A-Sepharose was released (data not shown). Thus, release of liposomes from Con A-Sepharose beads at 37°C indicates that at least some of the liposomes are free from HVJ virions when incubated at 37°C. The results at 0°C suggest that liposomes could not be freed from HVJ virions at 0°C.

TABLE III

BINDING OF LIPOSOMES TO CON A-SEPHAROSE-HVJ AT 0°C AND THEIR RELEASE FROM CON A-SEPHAROSE-HVJ ON INCUBATION AT 37°C

	Temperature (°C)	Liposomes	
		with glycophorin	without glycophorin
		nmol phosphatidylcholine/50 μl of Con A-Sepharose (wet volume)	
(A) Amount of phosphatidylcholine bound	0°C	3.42	0.65
(B) Amount of phosphatidylcholine released into supernatant	0°C	0.12	0.00
	37°C	1.32	0.10

Con A-Sepharose bound to HVJ (Con A-Sepharose-HVJ) was prepeared as follows: Con A-Sepharose (wet volume, 1 ml) was mixed with 50 000 HAU of HVJ and incubated for 1 h at 0°C. Then it was washed three times with excess phosphate-buffered saline by centrifugation at 300 × *g* for 5 min, and the pellet obtained was used directly without further characterization. Liposomes were prepared from 200 nmol egg phosphatidylcholine, 20 nmol dicetyl phosphate, 100 nmol cholesterol and a trace amount of ^{3}H-labeled egg yolk phosphatidylcholine, with and without 10 μg glycophorin. These liposomes were incubated with Con-A-Sepharose-HVJ (wet volume, 50 μl) for 1 h at 0°C. The Con-A-Sepharose-HVJ was then washed with phosphate-buffered saline, and counted (A). In (B), further incubation was performed at 0°C or 37°C for 30 min. An aliquot of the 'Con A-Sepharose-HVJ' bound to liposomes was suspended in 500 μl by phosphate buffered saline and the radioactivity in the supernatant obtained by centrifugation (300 × *g* for 5 min) was determined. Results are expressed as nmol of phosphatidylcholine recovered in 'Con A-Sepharose-HVJ' or released into the supernatant.

Discussion

Glycoproteins from erythrocytes and other animal cells are believed to have receptor activity for HVJ, because of their ability to inhibit hemagglutination.

Sialoglycoproteins incorporated into liposomes also bind the virus since they have hemagglutination inhibiting activity [2]. Gangliosides are also thought to be receptor for HVJ, because the virus was adsorbed competitively to liposomes containing gangliosides [11]. It should be stressed here that most previous observations on interaction of virus with liposomes have been rather indirect, except for those by electron microscopy [1,12,13]. In the present study we obtained direct evidence for the interaction of HVJ with liposomal membranes. Binding of virus to liposomal membranes caused agglutination of the liposomes, and this agglutination could be measured as increase in absorbance increase in particle size (with a low angle light scattering photometer) and morphologically by fluorescence microscopy. The HVJ-induced formation of aggregates of liposomes is reminiscent of cell agglutination by HVJ. The agglutination of the liposomes was dependent on the prescence of glycophorin. Sialic acid residues of glycophorin are important for the susceptibility of our liposomes to virus, since treatment of the liposomes with *Vibrio cholera* neuraminidase prevented the agglutination. The agglutination of liposomes was dependent on temperature. The possibility that the agglutination was affected by membrane fluidity can be neglected by the findings that agglutination of egg yolk phosphatidylcholine liposomes showed almost the same temperature dependence as those of dipalmitoylglycerophosphocholine liposomes. The temperature dependence of agglutination may be due to that of neuraminidase in HVJ: at 0°C, where agglutination was maximal, no appreciable neuraminidase activity was detected.

Virus adsorbed to the liposomal membrane may not be released at 0°C, because of lack of neuraminidase activity. In fact, release of virus did not occur during incubation of a mixture of liposomes and HVJ at 0°C. But upon shift up of temperature to 37°C, release of virus from the liposomal membranes and dissociation of aggregates was observed. This dissociation of liposome aggregates observed on shift up of temperature is analogous to that of erythrocytes and other cell agglutinated by HVJ. The present findings strongly indicate a correlation between neuraminidase activity and release of virus from membranes. With living cells, lysis or fusion as well as dissociation of aggregates is often observed when incubation is carried out first at 0°C and then at 37°C. It is quite possible that liposomes treated with the virus are damaged or fused during incubation at higher temperatures. Slight decrease of absorbance of liposomes was observed when they were incubated above 37°C (Fig. 1 and Fig. 3), and this decrease may reflect some morphological change of liposomes during incubation as higher temperature. In preliminary experiments, we observed release of markers trapped in liposomes induced by virus at 37°C. When liposomes were incubated with HVJ first at 0°C and then at 37°C, some of them seemed to be freed from HVJ virion, but almost 60% of liposomes were not freed from virus, suggesting that parts of the viral envelope remained on the liposomal membrane, very likely because the virions of HVJ fused with the liposomal membrane. At present there is no quantitative method for measuring the amount of virus released from liposomal membranes at 37°C, and so it is not possible to determine how much virus remains on the membrane. It is certain, however, that the amount remaining on the liposomes at 37°C is not enough to cause agglutination of liposomes.

Acknowledgments

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