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The activity of membranes reconstituted from HVJ envelope proteins and lipids to induce hemolysis and fusion between liposomes and erythrocytes

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A simple method for preparation of lipid-free envelope proteins (HN protein and F protein) of HVJ (Sendai virus) was developed. Reconstituted 'envelopes' were then prepared from envelope proteins and various lipids by the detergent dialysis method, and the activity to induce hemolysis and fusion between liposome and erythrocyte was studied. Lipid-free envelope protein aggregates could induce hemolysis and liposome-erythrocyte fusion. The activity was however greatly augmented by incorporation of envelope proteins into membrane of viral total lipids. Hemolytic and fusogenic activity was somewhat augmented by incorporation of envelope proteins into dipalmitoylphosphatidylcholine/cholesterol (1:1, molar ratio) and dimyristoylphosphatidylcholine/cholesterol (1:1), though the augmentation was lower than that observed with viral total lipids. When 'envelopes' were reconstituted with the proteins and viral total lipids supplemented with phosphatidylethanolamine, two kinds of 'envelopes' were prepared; one was permeable to Dextran (M_r 75000) and hemolytic, and the other was impermeable to Dextran and nonhemolytic. The latter acquired hemolytic activity after subjection to freezing and thawing, and its barrier function was lost concomitantly. The study suggests that envelope proteins (HN protein and F protein) could function without lipids but their activity was greatly influenced by not only the composition of additional lipids but also mode of arrangement of components on the reconstituted membranes.

Introduction

The envelope of HVJ (Sendai virus) consists of a lipid membrane with two glycoproteins (HN and F protein) which form spike-like projections and are anchored in lipid membrane by their hydrophobic peptide segments. HN protein has hemagglutinating and neuraminidase activities [1] and is involved in the interaction between virus and receptors on host cell membrane [2–4]. The events leading to envelope fusion (hemolysis) and cell-cell fusion are caused by F protein [5,6]. These proteins should function cooperatively in the form of molecular assembly with lipids.

Using the reconstituted system, useful information can be obtained on the relation between function and structure of the envelopes. Although reconstitutions of HVJ envelope [7–12] as well as other envelope viruses [13–15] have been described previously from several laboratories, detailed studies on the relation between fusogenic activity and lipid composition of the reconstituted envelope have not yet been performed.

In the present paper, we developed a simple method for preparation of lipid-free envelope proteins. Using 'envelopes' reconstituted from envelope proteins thus prepared and various lipids,

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the activity to induce hemolysis and fusion between liposome and erythrocyte was studied. The correlation between hemolytic activity and barrier function of reconstituted envelopes will be also discussed.

Materials and Methods

Chemicals. Egg yolk phosphatidylcholine was prepared by chromatography on Aluminum Oxide Neutral and Unisil. A stearic acid derivative (12′-(*N*-oxyl-4′,4′-dimethyloxazolidine)stearate), which has a nitroxide-containing ring in the C-12 position ((5,10) fatty acid), was synthesized by the method of Waggoner et al. [16]. The (5,10) spin-labeled phosphatidylcholine was synthesized by the method of Hubbell and McConnell [17]. Egg yolk phosphatidyl[*N-methyl-* ^{3}H]choline 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. [18]. Dimyristoryl-L-α-phosphatidylcholine, dipalmitoyl-L-α-phosphatidylcholine, cholesterol and dicetylphosphate were purchased from Sigma, St. Louis, MO, U.S.A. All lipid preparations showed a single spot on silica-gel thin-layer chromatography. Viral total lipids were extracted by the method of Bligh and Dyer [19]. Phospholipid composition of viral total lipids were as follows; 32.6% phosphatidylethanolamine, 8.6% phosphatidylcholine, 23.2% phosphatidylserine, 19.2% sphingomyelin, 1.9% phosphatidylinositol and 14.5% unidentified substances. Cholesterol content in viral total lipids was 0.91 in the molar ratio to phospholipids. Other chemicals were purchased from the following companies: Pharmacia Fine Chemicals, A.B., Uppsala, Sweden (ficoll 70, Sepharose CL-4B); Shering A.G., Berin/Bergkampen, F.R.G. (urografin 60); Bio-Rad Laboratories, Richmond, CA (Bio-Beads SM-2 20–50 mesh); BDH Chemicals, Poole, U.K. (Dextran grade C); Wako Pure Chemicals, Osaka (Triton X-100, calcium chloride, magnesium chloride); Nakarai Chemicals, Kyoto (sucrose); Iwai Kagaku Yakuhin Co., Tokyo (cesium chloride); Radiochemical Centre, Amersham, U.K. (glycerol tri[9,10(n)-^{3}H]oleate); New England Nuclear, Boston, MA, U.S.A. ([*carboxyl-*^{14}C]dextran carboxyl); Japan Atomic Energy Radioisotope (inorganic [^{32}P]phosphate, [^{51}Cr]Na_2CrO_4).

Buffer. Tris-buffered saline (10 mM Tris-HCl (pH 7.4)/150 mM NaCl) was used throughout, except when otherwise noted.

Erythrocyte. The human erythrocytes used were from freshly drawn, heparinized blood of healthy donors. The blood was centrifuged at $300 \times g$ for 5 min and plasma and buffy coat were discarded. The precipitated cells were then washed three times with buffer and used for experiments within 48 h.

Virus. The Z strain of hemagglutinating virus of Japan (HVJ, Sendai virus) was grown in the allantoic cavity of 10-day-old embryonated chicken eggs by differential centrifugation and subsequent sedimentation through a sucrose gradient. To obtain [^{32}P]phosphate-labeled virus, 10 μCi of [^{32}P]phosphate was injected into the allantoic cavity at the time of infection.

Isolation of envelope protein and reconstitution of envelope. [^{32}P]Phosphate-labeled virus (10 mg protein/ml) was solubilized at 25°C for 1 h in 50 mM Tris-HCl (pH 7.4) 100 mM NaCl containing 2% (v/v) Triton X-100. The solubilized virus was then centrifuged (1 h, 4°C, $100\,000 \times g$, Hitachi rotor RPS40T-2) to remove M protein and nucleocapsid. The supernatant (1 ml) was layered onto 3.6 ml of 10–25% (w/v) sucrose linear gradient containing 2% (v/v) Triton X-100 which was layered over 0.5 ml of 60% (w/v) sucrose containing 2% (v/v) Triton X-100. After the centrifugation (24 h, 4°C, $250\,000 \times g$, Hitachi rotor RPS65T), 0.25-ml fractions were collected from the bottom and aliquots of each fraction were assayed for radioactivity and protein [20]. When nonlabeled virus was used in place of [^{32}P]-phosphate-labeled virus, a trace amount of ^{3}H-labeled egg yolk phosphatidylcholine was added to the supernatant of the solubilized virus. The envelope protein fraction designated in Fig. 1 was pooled and dialyzed against buffer containing 2% (v/v) Triton X-100 to remove sucrose. Reconstituted envelopes were prepared as follows: organic solution of either viral total lipids or an appropriate mixture of phospholipids and cholesterol was evaporated with a trace amount of ^{3}H-labeled egg yolk phosphatidylcholine under a stream of nitrogen gas, then dried in vacuo for 1 h. The dried lipids were dispersed in 0.1 ml of 2% (v/v) Triton X-100 solution with a brief sonication and envelope pro-

tein fraction (300 μg protein) was added to the lipid dispersion. The mixture was stirred vigorously with a vortex mixer and then dialyzed in Spectrapor membrane tubing 2 against 2 l of buffer containing 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 8 g Bio-Beads SM-2 at room temperature for 5 h, and subsequently at 4°C for 72 h [8]. The dialyzed solution was used as reconstituted envelope. The molecular weight of the protein in the evelope protein fraction was approximated to be 70000, since they were composed of HN protein (70000) and F protein (66000). Based on the approximation, molar ratio of lipid/protein in reconstituted enveloped was calculated, and such proximate molar ratio was used throughout the present study.

Assay of hemagglutinating and hemolytic activity. Hemagglutinating activity was determined by the pattern method of Salk [21]. Hemolysis was measured using human erythrocytes labeled with radioactive chromate by the method of Inoue et al. [22]. Chromate release from erythrocytes was almost parallel to hemoglobin release measured spectrophotometrically at 412 nm, suggesting that almost all chromate was bound to hemoglobin under the present experimental condition. An appropriate amount of reconstituted 'envelope' (100 μl) was mixed with 900 μl of ^{51}Cr-labeled erythrocyte suspension ($1.1 \cdot 10^7$ cells/ml), and the mixture was incubated at 37°C for 30 min after standing on ice for 20 min. After incubation, the mixture was centrifuged at $300 \times g$ for 5 min, 500 μl of the supernatant was carefully removed for counting in an auto-well gamma counter (Aloka, JDC-751 apparatus). The percentage of hemolysis was calculated from the following formula: [(counts in 500 μl of supernatant) × 2/total counts in one assay tube] × 100.

HLD_{50} (50% hemolytic dose) was determined from the dose-dependence curve of hemolysis induced by reconstituted envelope.

Assay of liposome-erythrocyte fusion. Liposomes were prepared by the method of Szoka and Papahadjopoulas [23] with a slight modification. A typical preparation contained egg yolk phosphatidylcholine (1.32 μmol), dicetylphosphate (0.33 μmol), cholesterol (1.65 μmol) and spin-labeled egg yolk phosphatidylcholine (0.33 μmol). The ether solution of lipids (0.6 ml) was mixed with 0.1 ml of 0.3 M glucose solution, and the resulting two-phase system was sonicated for 3 min in a bath-type sonicator. Liposomes were then prepared by exactly the same procedure as that for preparation of 'reverse-phase evaporation vesicles'. Assay of liposome-erythrocyte fusion was carried out as previously described [24,25]. In brief, reconstituted envelope (80 μg protein) was added to 0.5 ml of 10% (v/v) erythrocyte suspension. The mixture was incubated at 0°C for 20 min, then centrifuged down ($300 \times g$ for 5 min) at 4°C. Liposome suspension, which was obtained by centrifugation at $12000 \times g$ for 15 min, (0.22 μmol as lipids) were added to the mixture. Aliquots of the reaction mixture (50-μl) were taken in a quartz capillary tube and further incubated at 37°C for various times. ESR spectra were measured with a JES-FE3X, X band, 100 kHz field modulator (JEOL, Tokyo, Japan) at 22°C. The central peak height of ESR spectra was increased when liposome-erythrocyte fusion occurred, since the occurrence of the fusion could result in the dilution of spin-labeled egg yolk phosphatidylcholine to erythrocyte membranes and disappearance of exchange broadening as described by Maeda et al. [26]. Quantitative measurement of transfer of [^{14}C]-Dextran entrapped in an aqueous compartment of liposome and tri[^3H]acylglycerol incorporated into liposomal membrane to erythrocyte induced by reconstituted envelope or intact HVJ was determined by the method using a discontinuous ficoll-urografin gradient as discribed previously [25]. When uranyl acetate was involved in the reaction mixture to avoid hemolysis, radioactive Dextran and triacylglycerol were almost simultaneously transfered to erythrocyte [24,25]. Under the same conditions except that uranyl acetate was omitted from the reaction mixture, complete hemolysis was observed and no appreciable transfer of Dextran to eyrythrocyte could be detected though the transfer of triacylglycerol to erythrocyte membrane efficiently occurred (see Table II). Since HVJ could not fuse with or lyse receptor-free liposome [27], it can be concluded that an aqueous marker might be once transferred to the inside of erythrocyte by fusion process and then released into the medium through the perturbed erythrocyte membrane. It is thus rational that the transfer of radioactive triacylglycerol to erythrocyte membrane is indicative of the efficiency of fusion of

liposome to erythrocyte. In this paper, we mostly compared the fusogenic activity of various reconstituted envelopes on the basis of the results of ESR measurements. Quantitatively, the same results were obtained by measuring the transfer of triacylglycerol to erythrocyte membrane.

Dextran or sucrose-cushion centrifugation. Reconstituted envelope was layered onto a Dextran cushion. The Dextran cushion was made by layering of 3 ml of 8% (w/v) Dextran in buffer over 0.5 ml of 60% (w/v) sucrose. After the centrifugation (12 h, 4°C, 50000 × *g* Hitachi rotor RPS40T-2), 0.28-ml fractions were collected from the bottom and aliquots were assayed for radioactivity, hemagglutinating activity and hemolytic activity. When sucrose-cushion centrifugation was carried out, reconstituted envelope was layered onto a sucrose cushion. The sucrose cushion was made by layering of 3 ml of 10% (w/v) sucrose in distilled water over 0.5 ml of 60% (w/v) sucrose. Centrifugation and subsequent fractionation were carried out under the same experimental conditions as described above. Either 8% Dextran or 10% sucrose sulution used in this experiment was osmotically equivalent to the buffer.

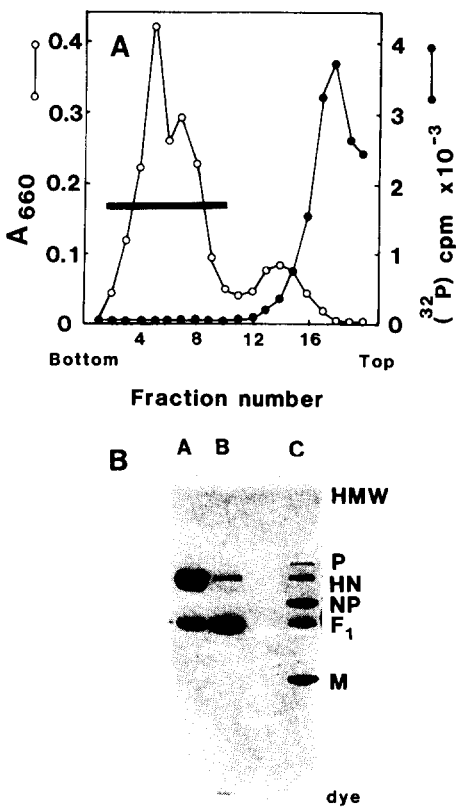

Fig. 1. Separation of envelope proteins from lipids. (A) The supernatant of the solubilized [^{32}P]phosphate-labeled virus was centrifuged into a sucrose gradient under the experimental conditions as described in Materials and Methods Fractions were then collected and aliquots were assayed for radioactivity (●) and protein (○). The horizontal bar shows envelope protein fraction pooled for reconstitution. (B) Fraction 5 (lane A), fraction 7 (lane B) and total proteins of HVJ (lane C) were electrophoresed on 10% polyacrylamide slab gel containing sodium dodecyl sulfate as described in Materials and Methods.

Cesium chloride centrifugation . Reconstituted envelope was mixed with CsCl solution (final concentration, 20% (w/v)).

After the centrifugation (24 h, 4°C, 200000 × *g*, Hichachi rotor RPS65T), 0.3-ml fractions were collected from the bottom and aliquots were assayed for radioactivity.

Freezing and thawing of reconstituted envelope. Freezing and thawing of reconstituted envelope was accomplished by immersing the tube containing reconstituted envelope alternatively for three times into a mixture of solid CO_2 and acetone for 3 min and into a water-bath at 37°C for 3 min.

Polyacrylamide gel electrophoresis. Samples were dissolved by boiling for 3 min in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, and were then electrophoresed on 10% polyacrylamide slab gel containing sodium dodecyl sulfate as described by Laemmli [28]. The gel was then stained with Coommasie brilliant blue.

Results

Separation of envelope proteins from lipids by sedimentation through a sucrose density gradient

Virus was solubilized with Triton X-100 and the solubilized fraction was centrifuged to remove nucleocapsid and M protein. The supernatant obtained after centrifugation contained viral envelope proteins (HN protein and F protein) and envelope lipids. In order to separate envelope proteins from lipids, the supernatant was centrifuged into a sucrose gradient containing Triton X-100 (Fig. 1A). Proteins were recovered from lower fractions of the gradient as two major peaks (fraction 5 and fraction 7) and from upper fractions as one minor peak (fraction 14), whereas lipids were

almost completely recovered from the top fractions of the gradient. Fraction 5 was rich in HN protein and fraction 7 was rich in F protein (Fig. 1B). Fraction 14 contained M protein judging from the mobility in 10% SDS-polyacrylamide gel electrophoresis (data not shown).

Fractions from 2 to 10 were used as envelope protein fraction and no protein band was observed other than HN protein and F protein in this fraction. It was calculated from the radioactivity of phospholipids that more than 98% of lipids were removed from this fraction. The ratio of HN protein and F protein in this fraction was almost equivalent to that of intact virion judging from the density of the Coomassie brilliant blue staining of 10% SDS-polyacrylamide gel electrophoresis.

Activity of envelope reconstituted with lipids to induce hemolysis and liposome-erythrocyte fusion

To investigate the effect of lipid composition and lipid/protein molar ratio on hemagglutinating and fusogenic activity of reconstituted envelope, we prepared various reconstituted envelopes by dialysis of the Triton X-100-solubilized mixture of envelope proteins and lipids of various compositions. Hemolytic activity of envelopes reconstituted with various lipids are shown in Table I. Lipid-free protein aggregates generated by removal of detergent could induce hemolysis, though the activity ($HLD_{50} = 8.0$ μg protein) was much weaker than that of intact virion ($HLD_{50} = 0.04$ μg protein). The hemolytic activity was augmented by reconstitution of envelope with the proteins and viral total lipids; the activity was increased about 50-fold when the lipid/protein molar ratio was 75. The value was rather close to that of intact virion. Some augmentation of the activity was also observed when envelope was reconstituted with the proteins, phosphatidylcholine and cholesterol. The activity of envelope reconstituted with egg yolk phosphatidylcholine and cholesterol was, however, suppressed when the lipid/protein molar ratio was over 150. It should be stressed here that the augmentation by these lipids was much lower than that by viral total lipids. The activity was completely suppressed when proteins were incorporated into egg yolk phosphatidylcholine or dimyristoylphosphatidylcholine membranes. Dipalmitoylphosphatidylcholine had little stimula-

TABLE I

HEMOLYTIC ACTIVITY OF VARIOUS RECONSTITUTED ENVELOPES

^{51}Cr-labeled erythrocytes were incubated with various amounts of reconstituted envelopes at 0°C for 20 min, and the mixture was further incubated at 37°C for 30 min. Hemolytic activities were expressed as 50% hemolytic dose (HLD_{50}) determined as described in Materials and Methods. Abbreviations used: egg PC, egg-yolk phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Chol, cholesterol.

Lipids in reconstituted envelopes	HLD_{50} (μg protein)			
	Lipid/protein (molar ratio)			
	37.5	75	150	300
None	8.0			
Egg PC	> 40	> 40	> 40	> 40
DMPC	> 40	> 40	> 40	> 40
DPPC	10.6	> 40	> 40	> 40
Egg PC/Chol (1 : 1) [a]	9.0	18.8	> 40	> 40
DMPC/Chol (1 : 1) [a]	4.5	3.8	4.5	4.5
DPPC/Chol (1 : 1) [a]	5.3	4.9	7.2	5.6
Viral total lipids	1.7	0.15	1.7	1.0

[a] Molar ratio.

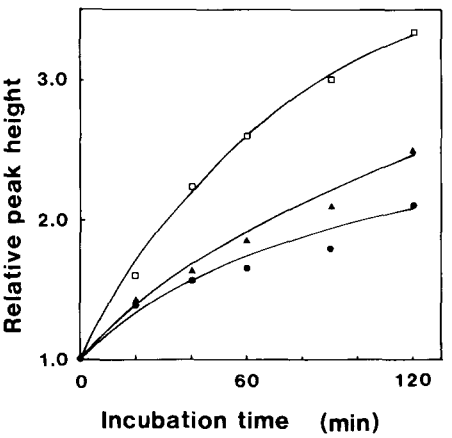

Fig. 2. Time-course of the fusion of liposome to erythrocyte induced by various reconstituted envelopes and HVJ. Erythrocytes were incubated with liposomes containing spin-labeled phosphatidylcholine in the presence of lipid-free protein aggregates (80 μg protein) (●), envelope reconstituted with envelope proteins and viral total lipids at lipid/protein molar ratio of 75 (80 μg protein) (□) or HVJ (80 μg protein as envelope proteins) (▲) at 37°C for various times under the experimental conditions as described in Materials and Methods.

tory or suppressive effect on the activity.

Lipid-free protein aggregates could induce liposome-erythrocyte fusion, and the activity was augmented by reconstitution with viral total lipids (Figs. 2 and 3). Similar augmentation was observed with dipalmitoylphosphatidylcholine/cholesterol. Dipalmitoylphosphatidylcholine also augmented the activity at the lipid/protein molar ratio of 37.5, whereas egg yolk phosphatidylcholine or egg yolk phosphatidylcholine/cholesterol was not stimulatory but rather inhibitory. The fusogenic activity of envelope reconstituted with protein and viral total lipids was even higher than that of intact virion when compared at the same protein concentration.

Hemagglutinating activities of various reconstituted envelopes were 80 000–100 000 HAU/mg protein, irrespective of lipid composition or lipid/protein molar ratio of the reconstituted envelope (data not shown).

Reconstitution of nonhemolytic envelope which could induce liposome-erythrocyte fusion

When the lipid/protein ratio was 2400, hemolytic activity of the envelope reconstituted with envelope proteins and viral total lipids was suppressed, whereas the activity to induce liposome-

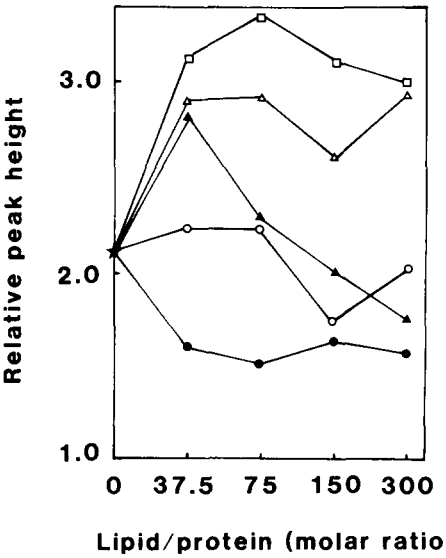

Fig. 3. Activities of various reconstituted envelopes to induce liposome-erythrocyte fusion. Erythrocytes were incubated with liposomes containing spin-labeled phosphatidylcholine in the presence of various reconstituted envelopes (80 μg protein) as described in Materials and Methods. The increase of the peak height of the ESR spectra after 2 h incubation at 37°C was plotted against lipid/protein molar ratio in reconstituted envelopes. Envelopes were reconstituted without lipids (★) or with dipalmitoylphosphatidylcholine (▲), egg yolk phosphatidylcholine (●), viral total lipids (□), dipalmitoylphosphatidylcholine/cholesterol (1 : 1, molar ratio) (△), or egg yolk phosphatidylcholine/cholesterol (1 : 1, molar ratio) (○).

TABLE II

ACTIVITY OF VARIOUS RECONSTITUTED ENVELOPES AND INTACT HVJ TO INDUCE HEMOLYSIS AND LIPOSOME-ERYTHROCYTE FUSION

	Lipid/protein (molar ratio)	HLD_{50} [a] (μg protein)	Liposome-erythrocyte fusion		
			relative peak height [b]	% transfer [c]	
				[^{3}H]TG	[^{14}C]Dextran
Reconstituted envelopes [d]	0	8.0	2.1	– [e]	– [e]
	150	1.7	3.1	– [e]	– [e]
	2400	100	2.5	10.0	9.1
Intact HVJ	150	0.04	2.5	15.0	– [f]

[a] ^{51}Cr-labeled erythrocytes were incubated with various amounts of reconstituted envelopes and intact HVJ at 0°C for 20 min, and the mixture was further incubated at 37°C for 30 min. 50% hemolytic dose (HLD_{50}) was determined as described in Materials and Methods.

[b] Erythrocytes were incubated with liposomes containing spin-labeled egg yolk phosphatidylcholine in the presence of reconstituted envelopes or intact HVJ at 37°C for 2 h. The increase of the peak height of ESR spectra after 2 h incubation at 37°C was then determined as described in Materials and Methods.

[c] Erythrocytes were incubated with liposomes which entrapped [^{14}C]Dextran in their aqueous compartments and contained tri[^{3}H]acylglycerol (TG) in their membranes in the presence of reconstituted envelopes or intact HVJ at 37°C for 2 h. The amount of transfer of [^{14}C]Dextran and [^{3}H]triacylglycerol to erythrocytes was then determined as described in Materials and Methods.

[d] Envelopes were reconstituted without lipids or with viral total lipids as described in Materials and Methods.

[e] Not done.

[f] This value could not be determined owing to the complete hemolysis.

erythrocyte fusion was not affected significantly (Table II). Transfer of [^{14}C]Dextran entrapped in an aqueous compartment of liposome and tri[^{3}H]acylglycerol incorporated into liposomal membrane to erythrocyte were next measured in the presence of nonhemolytic envelope which could induce liposome-erythrocyte fusion. The percentage of transferred [^{14}C]Dextran and tri-[^{3}H]acylglycerol were almost the same, suggesting that liposomes could fuse to erythrocytes without appreciable membrane damage, since the transfer of triacylglycerol is indicative of the efficiency of liposome-erythrocyte fusion [25].

Hemolytic activity of reconstituted envelope

When the envelope reconstituted with envelope proteins and viral total lipids (lipid/protein molar ratio = 150) was centrigured into a Dextran cushion, hemolytic activity, hemagglutinating activity and lipids were recovered from the bottom fractions as a single peak (Fig. 4A). Supplement of extra equimolar *Escherichia coli* phosphatidylethanolamine to viral phospholipids during reconstitution gave two peaks in a Dextran cushion. (Fig. 4B). Fraction I which was recovered from the bottom fractions had both hemagglutinating and hemolytic activity, whereas fraction II which was recovered from the top fractions showed hemagglutinating activity but not hemolytic activity. Table III shows the lipid/protein ratio and density of various reconstituted envelopes determined by CsCl centrifugation. The lipid/protein ratio of fraction I was lower than that of fraction II and was rather close to that of the envelope reconstituted without extra *E. coli* phosphatidylethanolamine. The density of fraction II was lower than that of fraction I or envelope reconstituted

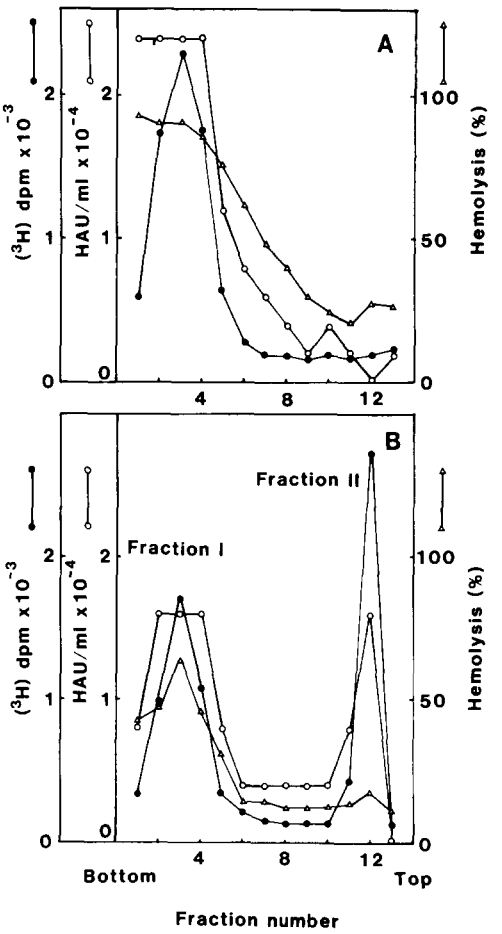

Fig. 4. Sedimentation patterns of the reconstituted envelopes in a Dextran solution. Envelope was reconstituted from envelope protein fraction (300 μg protein), viral total lipids (300 nmol as phospholipid) and a trace amount of ^{3}H-labeled egg-yolk phosphatidylcholine (A), or from the same component as those of (A) and *E. coli* phosphatidylethanolamine (300 nmol) (B). Envelope was then layered onto a Dextran cushion and centrifuged under the experimental conditions as described in Materials and Methods. Fractions were collected and were assayed for radioactivity (●), hemagglutinating activity (○) and hemolytic activity (△) as described in Materials and Methods.

TABLE III

LIPID/PROTEIN MOLAR RATIO AND DENSITY OF VARIOUS RECONSTITUTED ENVELOPES

	Lipid/protein [b] (molar ratio)	Density [c] (g/cm^{3})
Reconstituted envelope prepared without extra phosphatidylethanolamine [a]	150	1.21–1.22
Fraction I (in Fig. 4B) [a]	160	1.20–1.21
Fraction II (in Fig. 4B) [a]	330	1.14–1.16

[a] Preparation of reconstituted envelopes was performed as described in Fig. 4.

[b] Lipids were extracted from each reconstituted envelope by the method of Bligh and Dyer [15] and lipid/protein molar ratio was then determined.

[c] Each reconstituted envelope was centrifuged in 20% (w/v) CsCl solution as described in Materials and Methods, and density was then determined from the distribution of reconstituted envelopes in the fraction.

without extra *E. coli* phosphatidylethanolamine.

The result obtained by CsCl centrifugation (density of fraction II = 1.14–1.16 g/cm^3) is apparently inconsistent with floatation of fraction II in Dextran solution the density of which was 1.05 g/cm^3. Fraction II might form closed vesicles which were impermeable to the Dextran molecule (M_r = 60 000–90 000), floating in the Dextran cushion. In fact, fraction II could trap Dextran (M_r = 75 000) (Fig. 5). Fraction I did not trap any appreciable Dextran under the same condition and was sedimented in the Dextran cushion.

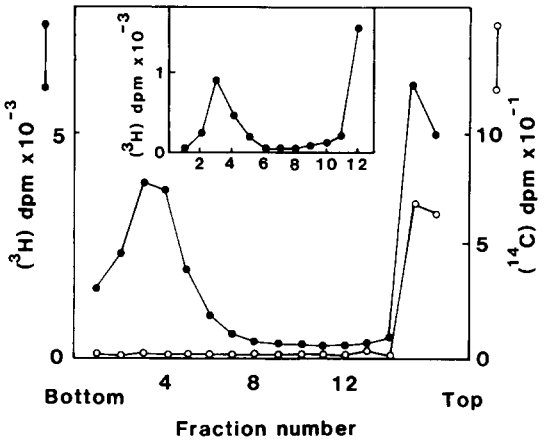

Fig. 5. Barrier function of envelope reconstituted with envelope proteins, viral total lipids and *E. coli* phosphatidylethanolamine against Dextran or sucrose. [^{14}C]Dextran was added to the mixture of envelope protein fraction (300 μg protein), viral total lipids (300 nmol), *E. coli* phosphatidylethanolamine (300 nmol) and a trace amount of ^{3}H-labeled egg-yolk phosphatidylcholine, and the mixture was then dialyzed against buffer to remove detergent as described in Materials and Methods. The dialyzed solution was applied onto Sepharose CL-4B column (1.4×32.5 cm) to separate reconstituted envelope from free [^{14}C]Dextran. Reconstituted envelope and [^{14}C]Dextran entrapped in an aqueous compartment of reconstitutied envelope were eluted in void volume fractions, and free [^{14}C]Dextran was included in the column. Void volume fractions were mixed with a 8% Dextran solution and were then recentrifuged under the experimental conditions as described in Materials and Methods. After the centrifugation (12 h, 4°C, 50000×*g*, Hitachi rotor RPS40T-2), 0.3-ml fractions were collected from the bottom and aliquots were assayed for radioactivity of ^{3}H labeled egg-yolk phosphatidylcholine (●) and [^{14}C]Dextran (○). Inset: Fraction II was pooled and layered onto a sucrose cushion, then recentrifuged under the experimental conditions as described in Materials and Methods. 0.3-ml fractions were collected from the bottom and were assayed for radioactivity of ^{3}H-labeled egg-yolk phosphatidylcholine.

When fraction II vesicles were further centrifuged into a sucrose cushion whose density was 1.06 g/cm^3, about 50% of fraction II vesicles were recovered from the bottom fractions (Fig. 5 inset), indicating that fraction II was consisted of heterogeneous populations and a half had barrier function against sucrose molecule (M_r = 342).

When bovine brain phosphatidylserine was added in place of *E. coli* phosphatidylethanolamine, all of the reconstituted envelopes were recovered from the bottom fractions of the Dextran cushion. They showed hemolytic as well as hemagglutinating activity. When egg yolk phosphatidylcholine was used in place of *E. coli* phosphatidylethanolamine, two peaks were observed, but hemolytic activity of both two peaks was negligible.

Effect of freezing and thawing of reconstituted envelopes on their hemolytic activity

Fraction II was originally recovered from the top fractions of the Dextran cushion, but it was mostly recovered from the bottom fractions when subjected to freezing and thawing for three times

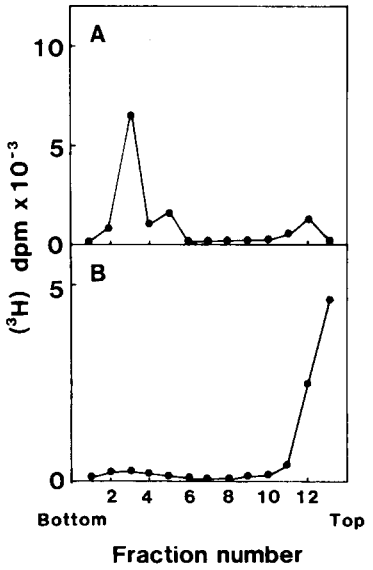

Fig. 6. Effect of freezing and thawing on barrier funtion of fraction II vesicles. Fraction II vesicles were pooled and recentrifuged onto a Dextran cushion before (B) and after freezing and thawing for three times (A) as described in Materials and Methods. After the centrifugation, fractions were collected and assayed for radioactivity of ^{3}H-labeled egg-yolk phosphatidylcholine.

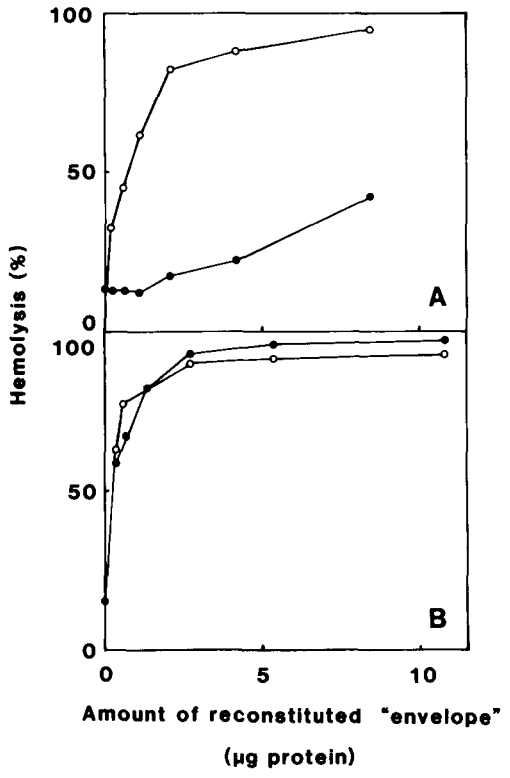

Fig. 7. Effect of freezing and thawing on hemolytic activity of fraction I and that of fraction II. ^{51}Cr-labeled erythrocytes were incubated with various amounts of fraction II (A) or fraction I (B) at 0°C for 20 min and the mixture was further incubated at 37°C for 30 min. Hemolytic activity before (●) and after subjection to freezing and thawing for three times (○) were assayed. The percentage of hemolysis was determined as described in Materials and Methods.

prior to centrifugation (Fig. 6). Fraction II acquired hemolytic activity after subjection to freezing and thawing (Fig. 7a). Freezing and thawing of fraction I did not have any appreciable effect on its hemolytic activity (Fig. 7B). Dextran originally entrapped in fraction II was completely lost after freezing and thawing (data not shown). It is noteworthy here that hemagglutinating activity of both fraction I and fraction II was not varied by this treatment.

Discussion

Using reconstituted HVJ envelope, we have tried to clarify the function of viral envelope during evoking cell-cell fusion as well as cell lysis. As a model of cell-cell fusion, we measured the activity to induce liposome-erythrocyte fusion. We have previously found that liposomes could fuse to erythrocyte membranes in the presence of HVJ [4,24,25]. The similarity between the results obtained with the liposome-erythrocyte system and those obtained with the erythrocyte-erythrocyte system indicates that liposome-erythrocyte fusion might be a reasonable model of erythrocyte-erythrocyte fusion by HVJ. In fact, in our preliminary experiments, erythrocyte-erythrocyte fusion could be always observed under the conditions where liposome-erythrocyte fusion was observed.

When the HVJ envelope was extracted with Triton X-100, both lipids and proteins were solubilized. Envelope proteins (HN and F protein) could be easily separated from lipids by sucrose gradient centrifugation. Envelope proteins formed aggregates when Triton X-100 was removed by dialysis. These protein aggregates showed hemolytic activity as well as activity to induce liposome-erythrocyte fusion. These findings suggest that lipids were not absolutely required for the functioning of F protein. Kruse et al. [12] reported that their isolated HN and F protein fraction which contained only 7% of the initial levels of envelope lipid had strong fusogenic activity. We could not deny the possibility that a small amount of lipid which existed in our envelope protein fraction might activate F protein.

Augmentation of the activity was observed when lipids were added during removal of Triton X-100. Among lipids, viral total lipids extracted from intact virion showed the highest augmentative effect. Total lipids were composed mainly of phosphatidylethanolamine, phosphatidylserine, sphingomyelin and cholesterol. Composition of lipids may be important for the augmentation. The alternative possibility is that some specific molecular species of lipids play an important role in the augmentation. Phosphatidylcholine could not stimulate the function of envelope proteins but was rather inhibitory. Cholesterol incorporation as well as fatty acid composition of phosphatidylcholine affect the activity of reconstituted envelope. Changes in the lateral distribution or comformational structure of envelope proteins may be involved in regulating the hemolytic or fusogenic

activity of the reconstituted envelope. The requirement of lipids observed in the present study is rather inconsistent with the previous observations by Ozawa and Asano [11]. They reported that envelopes reconstituted with a single species of phospholipid and envelope proteins had hemolytic activity but not fusogenic activity even when cholesterol was included in the reconstitution mixture. The discrepancy may be due to the procedure for protein purification. In our procedure, HN and F proteins were almost quantitatively recovered and the mixture of the proteins were directly used without further separation. Ozawa and Asano [11] reconstituted envelopes using purified HN and F proteins. Some populations of envelope proteins may be lost during purification of each component.

The activity of reconstituted envelope was also affected by the lipid/protein ratio. The molar ratio of 150 (the weight ratio of lipid/protein, 1) was close to that observed with intact virion. When the lipid/protein ratio was increased, hemolytic activity was greatly suppressed. The envelopes reconstituted with viral total lipids, whose lipid/protein ratio was 2400, showed activity to induce liposome-erythrocyte fusion without accompanying hemolytic activity. In the presence of these reconstituted envelopes, marker entrapped in an aqueous compartment of liposomes could be transferred into erythrocytes. These reconstituted envelopes might be useful for microinjection of foregin water-soluble substances into living cells, since they give rather little damage to cells.

The envelopes reconstituted with viral total lipids at the lipid/protein ratio of 150 were permeable to Dextran and hemolytic, whereas a half of reconstituted envelope (fraction II) prepared with supplement of extra phosphatidylethanolamine were impermeable to Dextran and nonhemolytic. A half of reconstituted envelope (fraction I) were permeable to Dextran and hemolytic. Lipid/protein ratio of fraction II was higher than that of fraction I, indicating that envelopes were reconstituted heterogenously. Heterogenous reconstitution was also shown by separation of fraction II into further two fractions on sucrose-cushion centrifugation. Fraction II may consist of envelopes permeable to sucrose but impermeable to Dextran and those impermeable to both Dextran and sucrose. Permeability property of reconstituted envelopes apparently correlated with their hemolytic activity; the envelopes permeable to Dextran showed hemolytic activity and those impermeable to Dextran did not. Such a correlation was further demonstrated by a different set of experiment. When envelopes impermeable to Dextran (fraction II) were subjected to freezing and thawing, envelopes lost almost all entrapped Dextran. Judging from the pattern of the Dextran-cushion centrifugation, envelopes subjected to freezing and thawing were permeable to Dextran. The envelopes acquired hemolytic activity by the treatment. It is quite possible that the composition of envelopes was not changed during freezing and thawing. Rearrangement of the components in envelopes may occur during freezing and thawing, resulting in loss of barrier function to Dextran and acquirement of hemolytic activity. The addition of phosphatidylserine instead of phosphatidylethanolamine did not affect the barrier function or hemolytic activity of reconstituted envelopes under the same experimental conditions. Phosphatidylcholine also could not replace phosphatidylethanolamine. The presence of extra phosphatidylethanolamine evoked such unique properties to reconstituted envelopes by some unknown mechanisms. It was reported that early harvested virus showed activity to induce fusion (envelope fusion, cell-cell fusion) without accompanying hemolysis. When these virions were subjected to freezing and thawing, hemolytic activity was introduced [29,30], though the mechanism of the change of envelopes has not been clarified yet. The similar events could be reproduced on reconstituted envelopes.

Using such an artificial system, elucidation of the mechanisms of the change of envelopes during freezing and thawing may be possible.

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