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Transmembrane Phospholipid Motions Induced by F Glycoprotein in Hemagglutinating Virus of Japan

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Transfer of phospholipid from the envelope of hemagglutinating virus of Japan (HVJ) to erythrocyte (RBC) membrane and the virus-induced transfer of phospholipid between RBC membranes were studied using spin-labeled phosphatidylcholine (PC*). The transfer of PC* from membranes labeled densely with PC* to unlabeled membranes was followed by the peak height increase in the electron spin resonance spectrum. The two kinds of transfer reactions took place very rapidly as reported previously. To obtain further details, the transfer reactions were studied with HVJ, HVJ inactivated by trypsin, HVJ harvested early, HVJ grown in fibroblast cells, the fibroblast HVJ activated by trypsin, influenza virus, and glutaraldehyde-treated RBCs. The results demonstrated that the viral F glycoprotein played a crucial role in the transmembrane phospholipid movements as well as in the fusion and hemolysis of RBCs. The transfer from HVJ to RBCs occurred partially through an exchange mechanism not accompanying the envelope fusion. This was shown by a decrease in the exchange broadening of the electron spin resonance spectrum of released spin-labeled HVJ (HVJ*) and also by an increase in the ratio of PC* to viral proteins incorporated into RBC membranes. HVJ modified RBC membrane so as to be able to exchange its phospholipids with those of inactive membranes such as fibroblast HVJ, influenza virus, glutaraldehyde-treated RBCs, and phosphatidylcholine vesicles. HVJ affected the fluidity of RBC membranes markedly, the environments around PC* being much fluidized. The virus-induced fusion was discussed based on close apposition of the membranes by HANA proteins and on the destabilization and fluidization of RBC membranes by F glycoproteins.

The cell fusion induced by hemagglutinating virus of Japan (HVJ) has been the subject of extensive morphological and biochemical studies since its discovery by Okada (15, 17). There are several elementary steps involved in the whole fusion reaction: adsorption of the virus to cells, aggregation of the cells, fusion of the viral envelope with the cell membrane (12), and fusion and hemolysis of the cells. The envelope membrane contains two kinds of proteins: HANA protein possessing hemagglutinating and neuraminidase activities and F glycoprotein responsible for fusion and hemolysis of cells (5, 21).

The molecular mechanism of the fusion and hemolysis, however, has not yet been fully understood. We attempted to study molecular dynamics, especially the lipid dynamics during the fusion process using spin-labeled phosphatidylcholine (PC*), and demonstrated rapid intermixing of phospholipids between the HVJ envelope and erythrocyte (RBC) membrane and also HVJ-induced intermixing between RBC membranes (10). The method utilizes a marked decrease in the exchange broadening of electron spin resonance (ESR) spectrum when densely labeled membranes were incubated with unlabeled membranes (11). In the present paper, we will show the crucial role of F glycoprotein in the transmembrane phospholipid movements and HVJ-induced modifications of RBC membranes.

MATERIALS AND METHODS

Viruses. HVJ, z strain, was used throughout. The virus was usually grown for 72 h in the allantonic cavity of 10-day-old eggs and purified as described previously (10). Early harvested HVJ was prepared by incubation for a shorter period of 24 h. HVJ was also grown in fibroblast cells obtained from a trypsinized 9-day-old whole chicken embryo. This virus was abbreviated as HVJ (fib). Influenza virus A_0PR8 was prepared and purified as described previously (10). All the viruses were stored at −80°C.

Concentration was expressed as hemagglutinating units (HAU) per milliliter of solution. Assay of the hemagglutinating activity was done according to the pattern method of Salk (18).

Inactivation of HVJ was carried out by incubation with a high concentration of trypsin (20 μg/ml) at 37°C for 20 min in 10 mM phosphate buffer, pH 7.2, according to the method of Shimizu and Ishida (22). At the end of reaction, soybean trypsin inhibitor (type 1-SC; Sigma Chemical Co.) was added at a concentration twice as high as that of trypsin. An analysis by sodium dodecyl sulfate-gel electrophoresis showed that this treatment by trypsin split out almost all F protein without any changes in other proteins. Activation of HVJ (fib) was performed by treating with a low concentration of trypsin (1 μg/ml) at 37°C for 6 min in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (140 mM NaCl, 5.4 mM KCl, 20 mM Tris-hydrochloride, pH 7.6) (5). It was confirmed by sodium dodecyl sulfate-gel electrophoresis that HVJ (fib) and the activated HVJ (fib) differed only in F protein as described previously.

Radioiodination of HVJ. Iodination of HVJ with ^{131}I was performed with slight modifications according to the method of Sefton et al. (20). The twice-washed HVJ was iodinated in 1.0 ml of Tris-buffered saline containing about 10^4 HAU of HVJ, 30 μg of lactoperoxidase (from milk; Boehringer Mannheim Corp.), 2 nmol of NaI, 20 μCi of $Na^{131}I$, and 1 mM ethylenediaminetetraacetic acid. Hydrogen peroxide (10 μmol) was added to initiate the reaction at room temperature. An additional 10 μmol of H_2O_2 was added after 5 min, and the mixture was incubated for 10 min more. About 20 to 30% of the radioactivity was incorporated in HVJ. The labeled HVJ was washed twice with Tris-buffered saline and extensively dialyzed against 3 liters of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) at 4°C for 4 days. To determine the radioactivity covalently bound to the protein, 5 μl of the virus was disrupted by sodium dodecyl sulfate (1%) and precipitated with 1 ml of 10% trichloroacetic acid after addition of 0.4 ml of bovine serum albumin solution (2.5 mg/ml). Eighty-seven percent of the incorporated radioactivity was precipitated together with the proteins. Before dialysis, 54% of the radioactivity was precipitated. Disc gel electrophoresis on a 7.5% acrylamide gel (25) indicated that radioactivity was incorporated into HANA (60%), F (30%), and M protein (10%). NP protein was also labeled occasionally.

RBCs and ghosts. Chicken and human RBCs were obtained from a local slaughterhouse and blood bank, respectively, and used within 2 weeks after drawing. RBCs were rendered free of buffy coat by at least three washings in 150 mM NaCl and 15 mM sodium citrate for chicken and Tris-buffered saline for human RBCs. Ghosts were prepared by a hypotonic hemolysis in 5 mM Tris-hydrochloride (pH 7.6) and collected by centrifugation for 30 min at 2×10^4 *g*. The volume of the initial RBCs was used to represent ghost concentration. Treatment of RBCs with glutaraldehyde (0.1%) was performed at 37°C for 20 min. Glutaraldehyde (70%) was obtained from Ladd Research Industries.

Preparation of spin-labeled virus and RBCs. This was done in essentially the same way as described previously (10). PC* with 12-nitroxide stearic acid attached at the 2-position was suspended in Tris-buffered saline at a concentration of 2 mg/ml, subjected to sonic treatment in an ice bath for 10 min under a nitrogen stream, and centrifuged at 4°C for 60 min at $10^5 \times g$. The virus (about 2×10^4 HAU/ml) and the supernatant (1 mM) were mixed, with the mole ratio of the viral lipids to PC* being about 0.2, and incubated at 37°C for 6 h. The spin-labeled virus was washed with Tris-buffered saline containing bovine serum albumin (10 mg/ml) and then with Tris-buffered saline only. In one experiment, the labeled virus was negatively stained with 1% uranyl acetate and examined by an electron microscope. The photographs shown in Fig. 1 indicated almost no morphological changes of the virus by the spin-labeling and no contamination of PC* vesicles absorbed on the surface of the virus envelope. Human RBCs were labeled by incubation of 0.1 ml of 100% (vol/vol) RBC with 0.1 ml of PC* dispersion (2 mM) for 3 h at 40°C. The molar ratio of phospholipids of RBCs to PC* was calculated to be about 1.5 in the incubation mixture. The spin-labeled RBCs (RBCs*) were washed in a similar way to the virus.

Incubation time taken for the spin-labeling was deliberately long. The amount of PC* incorporated into the membranes was therefore rather large, and the ESR spectrum was broadened by the spin-spin exchange interaction. For example, the spin-labeled HVJ (HVJ*) contained PC* amounting to 20% of total lipid. The densely labeled HVJ and RBCs were used for assay of phospholipid transfer reactions. Lightly labeled RBCs were prepared by incubating for 1 h and used to study the fluidity change of the cell membrane, since the ESR had not suffered from the exchange broadening.

Assay of phospholipid transfer between membranes. ESR spectra of densely labeled membranes are broadened by the spin-spin exchange interaction, since the average distance between the labels is smaller or the collisional frequency is higher (14). When the labels are transferred to nonlabeled membranes, the extra broadening diminishes due to dilution, resulting in an increase in the peak height of the ESR spectrum. Typically, 0.1 ml of HVJ* at a concentration of 2×10^4 to 4×10^4 HAU/ml was added to 1 ml of 10% RBCs at 0°C. The mixture, in which the mole ratio of the virus lipids to those of RBCs was about 0.06, was diluted to a final volume of 4 ml with Tris-buffered saline and left for 15 min at 0°C. A part of the mixture was taked into a quartz capillary tube for ESR measurement and incubated at 37°C for various lengths of time, and the ESR spectrum was recorded at 22°C on a JEOLCO model ME-X spectrometer. Increase in the central peak height was measured and plotted against incubation time (Fig. 2A and 3A). The transfer rate of PC* was estimated from the initial slope. The remaining mixture was used for assay of hemolysis and fusion as described previously (10). Phospholipid transfer between RBC membranes was assayed by the increase in the ESR peak height on incubation of the densely labeled RBCs with unlabeled RBCs in the

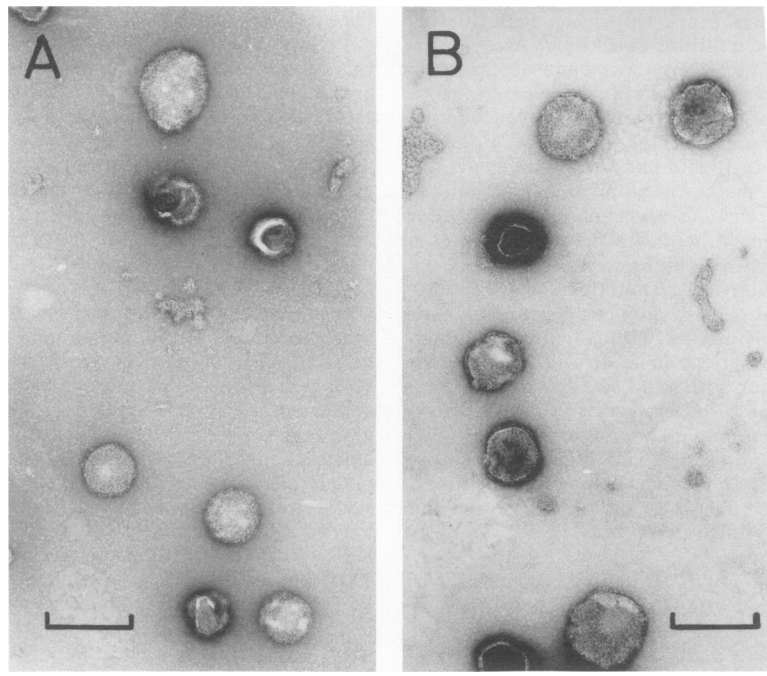

FIG. 1. *Electron micrographs of (A) non-spin-labeled and (B) spin-labeled HVJ. The virus (about 2 × 10⁴ HAU/ml) was incubated with vesicles of spin-labeled phosphatidylcholine (1 mM) at 37°C for 6 h and twice washed to remove unincorporated vesicles. The virus preparation was negatively stained by 1% uranyl acetate and examined in an electron microscope. Bar, 500 nm.*

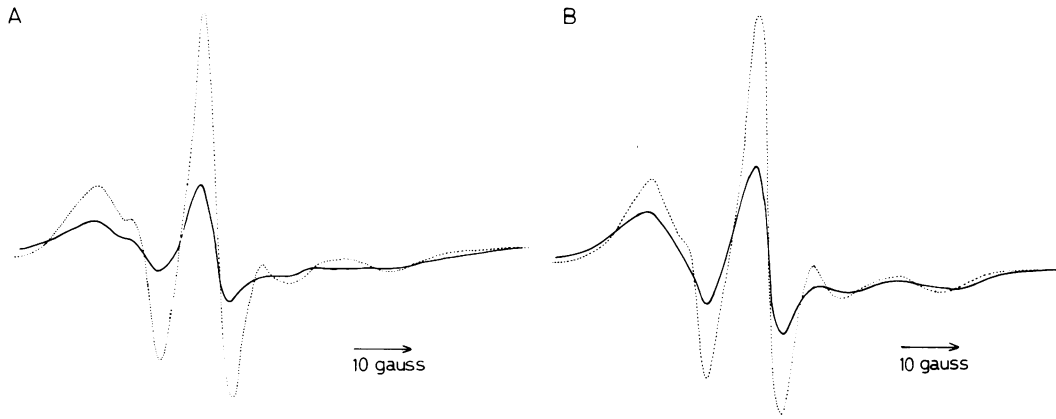

FIG. 2. *Change in the ESR spectrum when (A) HVJ* and RBCs and (B) HVJ, RBCs*, and RBCs were incubated at 37°C for 0 min (—) and (B) 60 min (···). The densely spin-labeled HVJ* (final concentration, 2,000 HAU/ml) and RBCs (2.5%) or HVJ (500 HAU/ml), densely-labeled RBCs* (0.8%), and RBCs (1.7%) were mixed at 0°C in Tris-buffered saline. The cell aggregates were taken into a quartz capillary tube and incubated at 37°C, and the ESR spectrum was measured at 22°C with the same spectrometer setting.*

presence of HVJ (see Fig. 2B and 3B). Transfer from PC* vesicles to RBC membranes was determined by the increase in the sharp three-line component of the ESR spectrum (11) when 0.15 ml of sonically treated PC* dispersion (2 mM), 50 μl of HVJ (10^4 to 2 × 10^4 HAU/ml), and 0.2 ml of 100% RBCs or ghosts were mixed and incubated. In one experiment, the total lipids were extracted from the mixture of HVJ* and RBCs or HVJ, RBCs*, and RBCs after incubation at 37°C for 30 min according to the method described by Bligh and Dyer (2) and developed by silica gel thin-layer chromatography (Art. 5721, Silica Gel 60; Merck & Co., Inc.) using $CHCl_3$—CH_3OH—CH_3COOH (65:25:8, vol/vol/vol) as a sol-

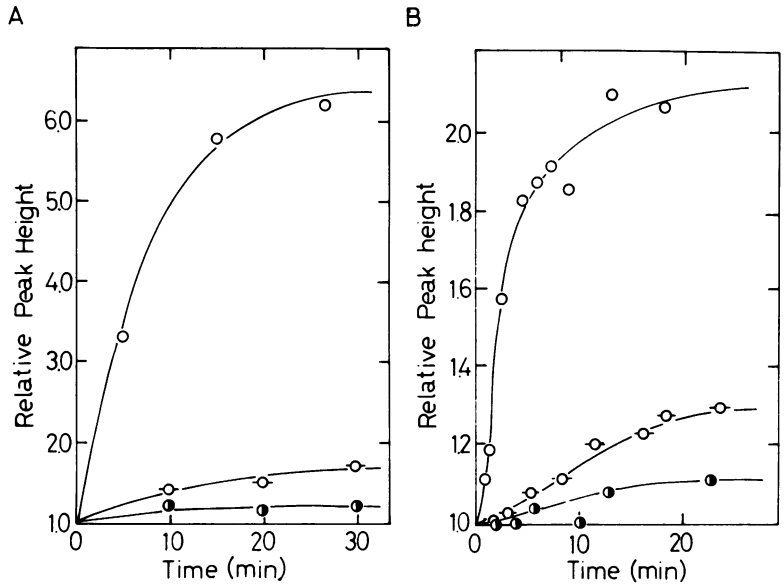

FIG. 3. *Time course of transfer of PC* from (A) HVJ* to RBCs and (B) RBCs* to RBCs. (A) HVJ* (○, 500 HAU/ml), HVJ* inactivated by trypsin (-○-, 500 HAU/ml), and IFV* (◑, 1,000 HAU/ml) were mixed with RBC (2.5%) at 0°C and incubated at 37°C, and the central peak height of the ESR spectrum was measured. (B) HVJ* (○), *HVJ inactivated by trypsin* (-○-), *and IFV* (◑) *(1,000 HAU of each per ml) were incubated with RBCs* (1.25%) and RBCs (1.25%) at 37°C.*

vent system. The analysis followed by ESR measurements indicated almost no degradation of PC* during the incubation.

Preparation of HVJ* released from RBCs. HVJ once adsorbed to cells was partially released again from the cells by the action of the neuraminidase (16). We collected HVJ* released from RBCs and purified by a CsCl equilibrium density gradient centrifugation. HVJ* (0.3 ml, 2.5 × 10^4 HAU/ml) containing a small amount of isotope-labeled HVJ as a marker was added to 17.7 ml of 2.5% (vol/vol) RBC at 0°C and kept for 15 min. After removing unadsorbed HVJ by centrifugation at 700 × *g*, the pellet was resuspended in 18 ml of Tris-buffered saline and incubated for 1 h at 37°C. The mixture was gently homogenized by a Pasteur pipette and centrifuged at 700 × *g* for 15 min to remove any ghosts and RBCs. The released HVJ* was collected by centrifugation of the supernatant at 2.2 × 10^4 × *g* for 60 min. The pellet was suspended in 5 mM Tris-hydrochloride (pH 7.6) containing 22% CsCl and centrifuged for 20 h at 1.5 × 10^5 × *g*. For reference, the original HVJ* and fused and hemolyzed RBCs were centrifuged. The released HVJ*, original HVJ*, and hemolyzed ghost were equilibrated at buoyant densities of 1.21, 1.21, and 1.16 respectively (Fig. 6). The buoyant densities were determined by an Abbe refractometer (Erma Optical Co.).

Assay of viral PC* and proteins integrated in RBC membranes. The spin- and isotope-labeled HVJ (0.1 ml, 2.5 × 10^4 HAU/ml) was added to 0.9 ml of 10% (vol/vol) RBC at 0°C, and unattached HVJ was removed after 15 min by centrifugation. Cold Tris-buffered saline (3.5 ml) was added to the pellet and mildly homogenized by Pasteur pipette. The homogenate was divided into six portions and incubated at 37°C for various intervals. The mixture was then chilled, gently homogenized, and centrifuged at 700 × *g* for 15 min to remove the released HVJ. The pellet contained HVJ* integrated as well as adsorbed to RBCs. Dodecyltrimethylammonium chloride (50 μl, 200 mM) was added to the pellet, and the PC* content was determined by the ESR signal intensity. The ESR spectrum was measured soon after addition of the detergent, since the nitroxide spin labels were gradually reduced in the presence of a high concentration of detergent. After ESR measurement, 50 μl of the sample was diluted to 2 ml with water and precipitated by the addition of 4 ml of 10% trichloroacetic acid. The amount of proteins in the precipitant was counted by a scintillation counter. For comparison, HVJ* inactivated by trypsin or glutaraldehyde-treated RBCs were used in some experiments.

RESULTS

Participation of F glycoprotein in the lipid intermixing. To identify the viral protein(s) responsible for the lipid intermixing between the HVJ envelope and RBC membrane and between the RBC membranes, the transfer reactions were studied using HVJ, HVJ inactivated by trypsin, HVJ (fib), HVJ (fib) activated by trypsin, and influenza virus. When densely labeled HVJ was incubated with RBCs at 37°C, the ESR spectrum changed rapidly and mark-

edly as shown in Fig. 2A, in agreement with the previous results (10). The peak height grew rapidly and became 6.4 times larger after 30 min (Fig. 3A). However, when the labeled HVJ was trypsinized and incubated with RBCs, only very little increase in the peak height was observed (Fig. 3A). The transfer rate decreased to one-eighth of intact HVJ (Fig. 5). HVJ-induced transfer of phospholipid between RBC membranes is shown in Fig. 2B and 3B. The ESR peak height markedly increased when unlabeled HVJ was added to a mixture of densely labeled and unlabeled RBCs. However, the trypsin treatment of HVJ negated the enhanced transfer. The transfer rate decreased to 1/18 of intact HVJ (Fig. 5). The trypsin treatment split only the F glycoprotein as reported by Shimizu and Ishida (22) and greatly decreased its hemolysis and fusion activities without loss of hemagglutinating and neuraminidase activities.

When HVJ* (fib) was incubated with RBCs, the ESR spectrum changed only slightly (Fig. 4A). The spectral change on mixing of HVJ (fib), RBCs*, and RBCs was also only slight (Fig. 4B). However, when the virus was lightly trypsinized, it became active in the two kinds of transfer reactions, i.e., from the virus to RBCs and from RBC to RBC (Fig. 4A and 4B). The transfer rates became 10 times larger than those for intact HVJ (fib) (Fig. 5). HVJ (fib) had only low hemolysis and fusion activities, whereas its hemagglutinating and neuraminidase activities were normal. The limited digestion by trypsin cleaved the precursor gly-

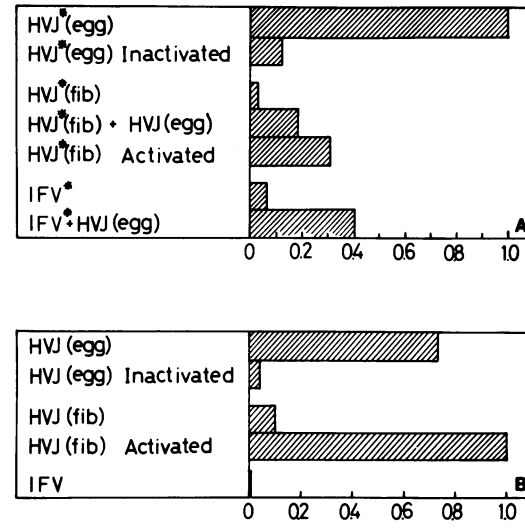

FIG. 5. *Comparison of the rate of (A) transfer of PC* from the viral envelope to RBC membrane and (B) virus-induced transfer of PC* from RBCs* to RBC membranes. The rates are presented on a relative scale. Those in (A) and (B) are not directly comparable.*

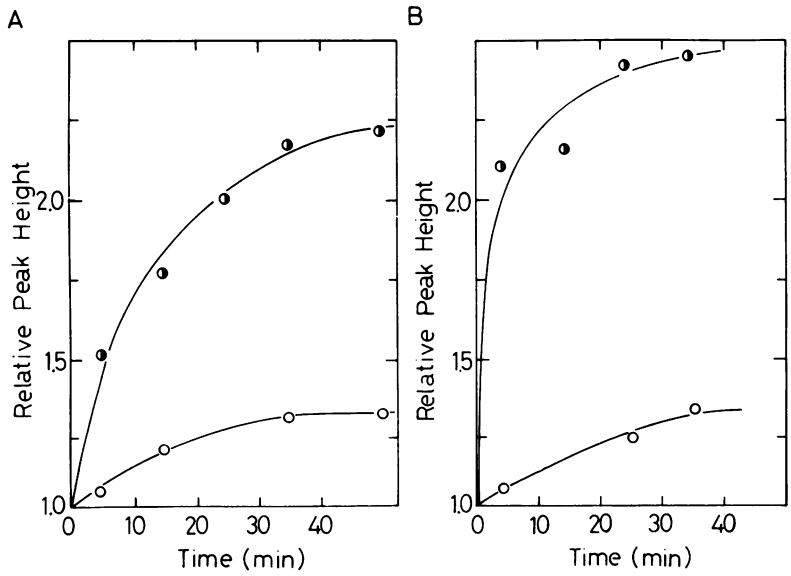

FIG. 4. *Effect of trypsin treatment on the transfer of PC* from (A) HVJ* (fib) to RBCs and (B) from RBCs* to RBCs. (A) HVJ* (fib) (○) and HVJ* (fib) activated by trypsin (◑; 125 HAU/ml of each) were mixed with RBCs (2.5%) at 0°C and incubated at 37°C. (B) HVJ (fib) (○) and HVJ (fib) activated by trypsin (◑; 62 HAU/ml of each) were incubated with RBCs* (0.8%) and RBCs (1.7%) at 37°C. The central peak height of the ESR spectrum was plotted on a relative scale against incubation time.*

coprotein F_0 into two active fragments, having respective molecular weights of 5.3×10^4 and 1.5×10^4 (5, 21) and activated fusion and hemolysis activities. Hemolysis was increased 10 times as much as that before trypsin treatment.

Influenza virus hardly enhanced the lipid intermixing between its envelope and RBC membrane and also between RBC membranes (Fig. 3A and B). The virus lacks a protein functionally corresponding to F protein and possesses only hemagglutinating and neuraminidase activities (19). All the results in this section indicate the crucial role of viral F glycoprotein in the transmembrane lipid intermixing as well as fusion and hemolysis of RBCs. The relative transfer rate in various systems is summarized in Fig. 5.

Lipid intermixing between HVJ and RBCs without envelope fusion. The lipid intermixing can occur simply as a result of envelope fusion followed by lateral diffusion in the membrane. To study the other intermixing mechanism, the released HVJ* was collected and purified as described in Materials and Methods. The ESR spectrum of the released HVJ* showed a definite decrease in the exchange broadening as compared with the original HVJ* (Fig. 6A). The decrease indicates that some lipids were intermixed between the virus and RBC membranes by an exchange mechanism without any accompanying envelope fusion. The fraction at the buoyant density of 1.16 contained fused and hemolyzed RBCs, which showed the ESR spectrum with no exchange broadening (Fig. 6A).

The second experiment to explore the intermixing mechanism was to analyze the ratio of PC* to viral proteins incorporated into RBCs. HVJ* was incubated with RBC, and the released HVJ* was removed by centrifugation. The pellet containing RBCs and HVJ adsorbed and incorporated into RBCs was analyzed for PC* and viral proteins. The ratio of PC* to viral proteins increased with incubation time and then tended to saturate (Fig. 7). The ratio remained constant in control experiments where inactivated HVJ* or glutaraldehyde-treated RBCs were used (Fig. 7). It can be concluded that at least some lipids (~30%) were exchanged between the virus and RBCs without accompanying fusion and the F glycoprotein and mobility of RBC proteins were required for this exchange.

HVJ-induced intermixing of lipids between inactive membranes. The transfer reactions between HVJ (fib) and RBCs and between influenza virus and RBCs were only to limited extents. HVJ (egg) was found to greatly enhance the transfer reactions between these inactive membranes (Fig. 8). The transfer rate became larger with an increase in the amount of added HVJ, being nearly proportional to the amount. The transfer rate from influenza virus to RBC in the presence of 1,500 HAU of HVJ per ml was 6.5 times larger than that in its absence (Fig. 5). The transfer was not due to direct intermixing between the labeled virus and nonlabeled HVJ.

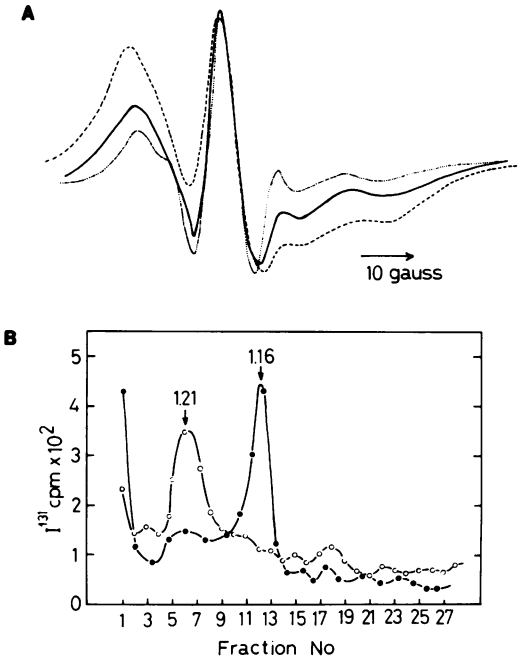

FIG. 6. *(A) ESR spectrum of HVJ* (---), HVJ* once adsorbed and released from RBCs (——), and HVJ* incorporated into RBCs (· · ·). HVJ*, containing a trace amount of isotope-labeled HVJ, was added to RBCs at 0°C, and the aggregated RBCs were incubated at 37°C for 60 min. The released HVJ* was collected and purified by a CsCl (22%) density gradient centrifugation for 20 h at 1.5 × 10^5 × g. As shown in (B), the released HVJ (○) and RBC membrane (●) were equilibrated at ρ = 1.21 and 1.16, respectively. Each peak was spun down, and the ESR spectrum was measured at 22°C.*

The transfer reaction between the phosphatidylcholine vesicle and RBC membrane was also markedly enhanced by HVJ. The transfer rate became six times larger when 2,000 HAU of HVJ per ml was added to a mixture of PC* vesicle and RBCs and incubated at 37°C. The enhancement was not due to lysis of RBCs, since the transfer to ghosts was nearly the same as that to unlysed RBCs. These results indicate that HVJ (egg) modified RBC membranes so as to allow the exchange of lipids with those of inactive membranes.

HVJ-induced lipid intermixing between in-

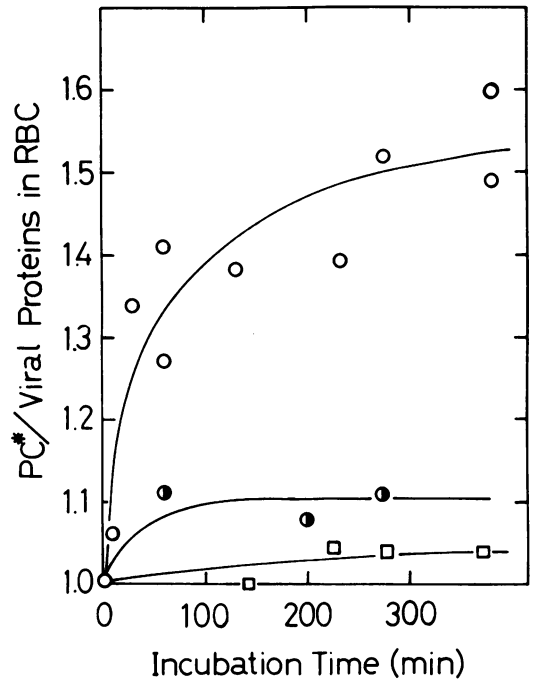

FIG. 7. *Change in the ratio of PC* to viral proteins adsorbed and integrated into RBC membranes during incubation time. HVJ doubly labeled with PC* and ^{131}I (○) was added to RBCs at 0°C, and the cell aggregates were incubated for various lengths of time at 37°C. After removing the released HVJ, the final pellet was assayed for PC* and ^{131}I. HVJ* inactivated by trypsin (◑) was incubated with RBC. HVJ* was incubated with glutaraldehyde-treated RBCs (□). The initial value before incubation on the ordinate corresponds to about 0.05 μmol of PC*/1 mg of virus protein.*

tact and glutaraldehyde-treated RBCs. RBCs were treated with glutaraldehyde, and the effect of the treatment on the lipid intermixing was studied. The treatment did not interfere with the hemagglutinating and neuraminidase activities of HVJ; the treated RBCs were aggregated by HVJ, and the aggregates were then disaggregated on further incubation at 37°C. However, the treatment completely inhibited the transfer reactions from HVJ to RBC and between RBCs. The ESR spectrum did not change at all when HVJ* was incubated with glutaraldehyde-treated RBCs and when HVJ was incubated with a mixture of glutaraldehyde-treated RBCs and glutaraldehyde-treated RBCs* (Fig. 9). No hemolysis and fusion were observed.

Surprisingly, HVJ was able to induce rapid intermixing of lipids between glutaraldehyde-treated and untreated RBCs. The ESR spectrum markedly changed when HVJ was incubated with a mixture of either RBCs* and glutaraldehyde-treated RBCs or RBCs and glutaraldehyde-treated RBCs* (Fig. 9). The rate and extent of the transfer were almost the same as those between untreated RBCs. HVJ caused fusion and hemolysis of untreated RBCs only in these systems. This was directly shown by an experiment done with human and chicken RBCs. Hemoglobins released from these RBCs can be distinguished by a disc gel electrophoresis at pH 8.3 (3). When glutaraldehyde-treated human RBCs and untreated chicken RBCs were incubated with HVJ, only hemoglobin from chicken RBCs was detected. The second evidence is that hemolysis hardly exceeded 50% in a 1:1 mixture of glutaraldehyde-treated and untreated RBCs even with high doses of HVJ (52% hemolysis at 2,500 HAU/ml). The results indicate that the structural modification in RBC membranes made the lipid transfer even to glutaraldehyde-treated partner possible.

Fluidization of RBC membrane induced by HVJ. The HVJ-induced structural modification in RBC membranes was studied using lightly spin-labeled RBC. When RBCs* were mixed at 0°C with the early harvested HVJ, the cells were aggregated but the ESR spectrum was unchanged. When the cell aggregates were incubated at 37°C, the ESR spectrum changed markedly (Fig. 10). The overall splitting value decreased from 53 to 50 G, indicating a marked fluidization of the membranes around the incorporated PC*. The cells were fused extensively by the incubation, but hemolysis was only to a limited extent (15 to 20%). It is known that the early harvested HVJ has normal fusion activity but lower hemolysis activity (6). This virus was used to show that the spectral change was not due to hemolysis, since hemolysis caused similar fluidization of RBC membranes (23).

DISCUSSION

The present spin-label study was confirmed and substantiated the hypothesis that the lipid intermixings between HVJ and RBC membranes and between RBC membranes are crucial steps in the virus-induced cell fusion and hemolysis (10). It is the viral F glycoprotein that was responsible for the lipid intermixings as well as fusion and hemolysis.

The transmembrane lipid intermixing can occur by exchange of lipids during contact of the membranes and also by fusion of membranes followed by lateral diffusion. The HVJ envelope and RBC membrane are brought into contact by HANA proteins. The enhanced lipid intermixing would result from interaction of the viral F protein with lipids and/or proteins in RBC membranes. It appears likely that the

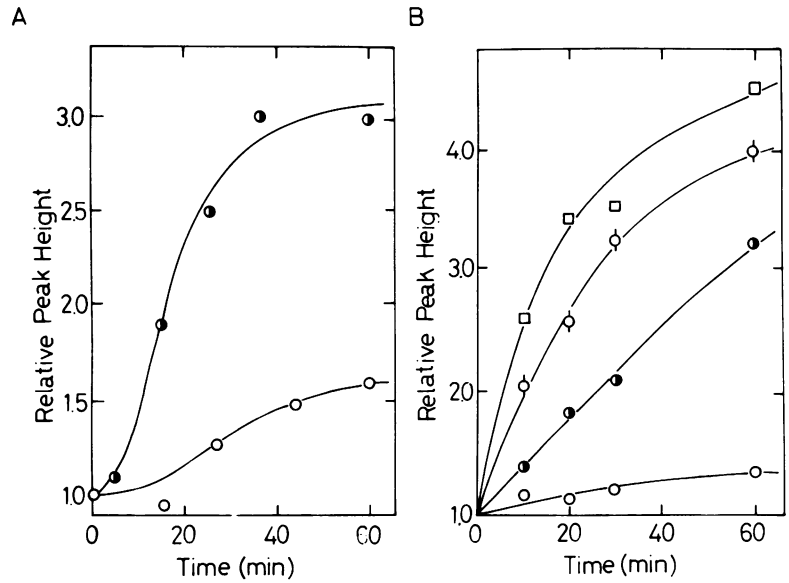

FIG. 8. *HVJ-induced transfer of PC* from (A) HVJ* (fib) to RBCs and (B) IFV* to RBCs. (A) HVJ* (fib) (125 HAU/ml) and RBCs (2.5%) were incubated at 37°C in the absence* (○) *and presence* (◑) *of HVJ (500 HAU/ml). (B) IFV* (1,000 HAU/ml) and RBCs (2.5%) were incubated at 37°C in the presence of HVJ at 0* (○), *200* (◑), *800* (ϕ), *and 1,500 HAU/ml* (□). *The ordinate plotted the relative ESR peak height.*

interaction perturbs the lipid bilayer portion of RBC membranes and causes transient fluctuation in its density. Such destabilization of membranes would affect various properties of the membranes, including increase in the permeability (7, 9, 14), enhancement of lateral and transverse mobilities of lipids, modification of enzyme activities, etc. When the two membranes are in close contact, the lipid exchange would be enhanced (11), and when larger area is contacted, the membranes would fuse (8, 24). There is another possibility for the lipid intermixing. The fused virus may be split off again before being stabilized in RBC membrane and some lipids are exchanged during the stay.

After the envelope fusion, the foreign molecules would further modify the RBC membrane. The rigid phosphatidylcholine portion in intact RBC membrane was markedly fluidized by the action of HVJ. This is equivalent to the production of a lipid bilayer region that is less influenced by the proteins. When the membranes are in contact at such a fluid lipid-rich area, the lipid intermixing would be a natural tendency. A rapid lipid intermixing was actually observed between lipid bilayer vesicles (11). When the contact area is large, the membranes would fuse. The production of a lipid-rich area may be compatible with a morphological observation of the virus-induced aggregation of proteins in RBC membranes (1). The viral F protein implanted in the membranes could play a more direct role in the fusion of cell membranes. However, in view of no transfer of lipids from HVJ to glutaraldehyde-treated RBCs and a rapid transfer between glutaraldehyde-treated and untreated RBCs, it is more likely that the F proteins give effects through the production of a fluid lipid-rich area in RBC membranes.

The fluidization of RBC membranes may be caused by modification of the interaction between the membrane constituents and the intracellular fibrous proteins by the F glycoprotein. A quite similar fluidity change was observed in osmotic hemolysis where some evidence showing that the fluidization was due to the disruption of the interaction of the membrane constituents with the fibrous proteins spectrin and actin was presented (25). In the immune attack the complement component C9 caused similar fluidity change in RBC membranes pretreated with an antibody and the complement components C1 through C8 (M. Nakamura, S. Ohnishi, H. Kitamura, and S. Inai, Biochemistry, in press). Several morphological studies have shown modification in the distribution of the intramembraneous particles that was probably caused by disruption of the interaction with the fibrous proteins (4, 13).

In conclusion, close apposition of the membranes and destabilization and/or fluidization of the membranes are necessary for the transmembrane phospholipid intermixing and fu-

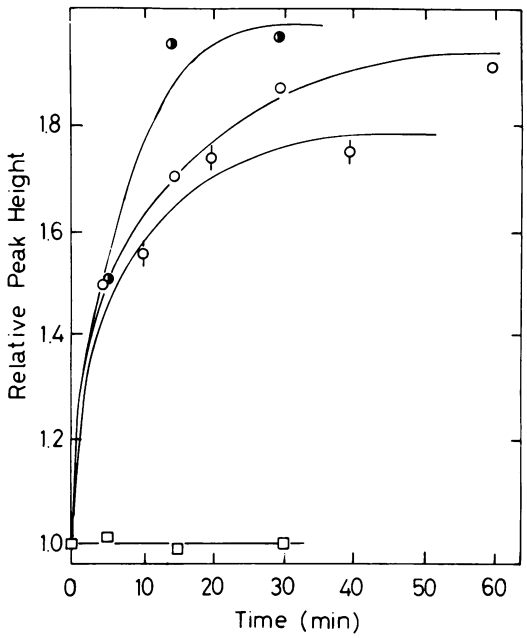

FIG. 9. *HVJ-induced transfer of PC* between glutaraldehyde-treated and nontreated RBCs. The following mixtures were incubated at 37°C: RBC*, RBC, and HVJ (○); glutaraldehyde-treated RBCs*, RBCs, and HVJ (◑); RBCs*, glutaraldehyde-treated RBCs, and HVJ (◑); and glutaraldehyde-treated RBCs*, glutaraldehyde-treated RBCs, and HVJ (□). The central peak height of the ESR spectrum was plotted against incubation time. Concentrations were 0.8% for labeled RBCs, 1.7% for RBCs, and 1,000 HAU/ml for HVJ.*

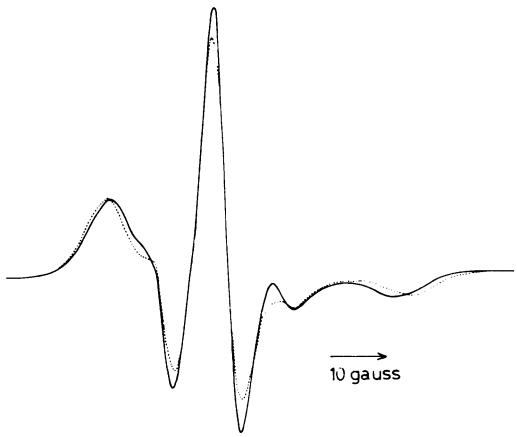

FIG. 10. *Change in the ESR spectrum of spin-labeled RBC on incubation with HVJ. The dotted and full lines represent the spectra before and after the incubation, respectively. The lightly labeled RBCs (2.5%) were incubated with early harvested HVJ (2,500 HAU/ml) at 37°C for 13 min.*

sion of the membranes. It may be said that intact RBCs are protected from the lipid intermixing and fusion, and HVJ breaks the protection to lead spontaneous membrane reactions.

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