Lateral Mobility of Reconstituted Sendai Virus Envelope Glycoproteins on Human Erythrocytes: Correlation with Cell-Cell Fusion†

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ABSTRACT: We have recently employed fluorescence photobleaching recovery (FPR) to demonstrate that the envelope glycoproteins of Sendai virions become laterally mobile on the surface of human erythrocytes following fusion [Henis, Y. I., Gutman, O., & Loyter, A. (1985) *Exp. Cell Res. 160*, 514-526]. In order to investigate whether this lateral mobilization is involved in the mechanism of virally mediated cell-cell fusion, or is merely a result of viral envelope-cell fusion, we have now performed FPR studies on erythrocytes fused with reconstituted Sendai virus envelopes (RSVE). These RSVE, which were prepared by solubilization of Sendai virions with Triton X-100 followed by removal of the detergent through adsorption to SM-2 Bio-beads, fused with human erythrocytes as efficiently as native virions but induced cell-cell fusion to a much lower degree. The fraction of the viral envelope glycoproteins that became laterally mobile in the erythrocyte membrane following fusion was markedly lower in the case of RSVE than in the case of native virions. The lower cell-cell fusion activity of the RSVE does not appear to be due to inactivation of the viral fusion protein, since the envelope-cell fusion and hemolytic activities of the RSVE were similar to those of native virions. Moreover, fusion with RSVE or with native virions resulted in the incorporation of rather similar amounts of viral glycoproteins into the cell membrane. Since the reduced fraction of laterally mobile viral glycoproteins correlates with the lower cell-cell fusion activity of the RSVE, we propose that fusion of the viral envelopes with the cells is not sufficient for the induction of cell-cell fusion and that lateral motion of the viral envelope glycoproteins on the cell surface plays a role in the mechanism of virally mediated cell-cell fusion.

Membrane fusion plays important roles in a variety of biological processes, among which are endocytosis, secretion, fertilization, and the entry of enveloped viruses into mammalian cells (White et al., 1983; Poste & Pasternak, 1978; Evered & Whelman, 1984). In the latter case, the attribution of the fusogenic activities (virus-cell and cell-cell fusion) to specific viral envelope glycoproteins (White et al., 1983; Poste & Pasternak, 1978; Volsky & Loyter, 1978a; Hsu et al., 1979; Florkiewicz & Rose, 1984) makes virally mediated cell fusion a convenient system for studies on the mode of action of fusion proteins.

Paramyxoviruses can penetrate into cells through direct fusion with the plasma membrane at neutral pH values (White et al., 1983; Poste & Pasternak, 1978). Following virus-cell fusion, they induce cell-cell fusion (White et al., 1983; Poste & Pasternak, 1978). In the case of Sendai virus, which is the best characterized of the paramyxoviruses regarding its fusogenic activity, it has been shown that the processes of viral envelope-cell fusion and cell-cell fusion are separable and under specific conditions the first can occur without proceeding to the latter (Miyake et al., 1978; Maeda et al., 1977; Sekiguchi & Asano, 1978; Lalazar & Loyter, 1979). The envelope of Sendai virus contains two glycoproteins: the fusion protein (F),[1] which is believed to promote virus-cell and cell-cell fusion, and the hemagglutinin-neuraminidase (HN) protein, which mediates virus binding to cellular receptors and cell agglutination (Poste & Pasternak, 1978; Choppin & Scheid, 1980). The mechanism by which viral envelope proteins induce virus-cell and cell-cell fusion is yet unknown, although hydrophobic interactions between viral fusion proteins and target membranes were proposed to be involved (White et al., 1983; Gething et al., 1978; Hsu et al., 1981). Sendai virus induced fusion is accompanied by effects which propagate over the entire cell surface and are not limited to the initial viral attachment sites. These include the triggering of cold-induced aggregation of intramembrane particles (Sekiguchi & Asano, 1978; Volsky & Loyter, 1978b,c; Kim & Okada, 1981) and the autocatalytic amplification of virus-cell phospholipid exchange (Kuroda et al., 1980). The propagated nature of these effects led to the proposal that lateral diffusion of the viral glycoproteins in the cell membrane following viral envelope-cell fusion is required in order to perturb the membranes of adjacent cells and to enable cell-cell fusion (Volsky & Loyter, 1978b; Kuroda et al., 1980; Cerry et al., 1976; Bachi et al., 1978).

In a recent study (Henis et al., 1985), we employed fluorescence photobleaching recovery (FPR) to demonstrate that the envelope glycoproteins of native Sendai virions (F and HN) become laterally mobile on the surface of human erythrocytes following fusion. This lateral mobilization during fusion, which was blocked under conditions that do not allow virus-cell and cell-cell fusion, supported the notion that the lateral motion of the viral glycoproteins in the cell membrane may play a role in the mechanism of cell-cell fusion (Henis

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[1] Abbreviations: D, lateral diffusion coefficient; DTT, dithiothreitol; F, fusion protein; FPR, fluorescence photobleaching recovery; HAU, hemagglutinating unit(s); HN, hemagglutinin-neuraminidase protein; N-NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; R_f, mobile fraction; RSVE, reconstituted Sendai virus envelopes; TMR, tetramethylrhodamine; R_{18}, octadecylrhodamine B chloride; Tricine, N-[tris(hydroxymethyl)methyl]glycine.

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et al., 1985). However, the possibility that the lateral mobility of the viral glycoproteins on the cell surface is an immediate consequence of viral envelope–cell fusion, and is not required for the process of cell–cell fusion, could not be excluded (Henis et al., 1985). In the present work, we have investigated this question by studying the lateral mobility of F and HN following fusion of reconstituted Sendai virus envelopes (RSVE), assembled from virions solubilized in Triton X-100, with human erythrocytes. The envelopes fuse with human erythrocytes as efficiently as native virions but induce cell–cell fusion to a much lower degree. The reduced cell–cell fusion does not appear to be due to inactivation of the F protein. Our results demonstrate that the low cell–cell fusion activity of the RSVE correlates with a reduction in the fraction of laterally mobile F and HN proteins (as compared with native Sendai virions) following fusion and support the notion that lateral motion of the viral glycoproteins on the cell surface plays a role in the induction of cell–cell fusion.

Materials and Methods

Reagents. Tetramethylrhodamine (TMR) 5-isothiocyanate was purchased from Research Organics (Cleveland, OH). *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), with the fluorophore attached at the head group, was obtained from Avanti (Birmingham, AL). Its ratio of saturated to unsaturated fatty acids (Henis et al., 1982) suggests that it partitions rather equally into fluid and gel lipid phases. Octadecylrhodamine B chloride (R_{18}) was from Molecular Probes (Junction City, OR). All other chemicals were of the highest purity available.

Virus. Sendai virus (Z strain) was grown in the allanotoic sac of 10–11-day-old embryonated chicken eggs, harvested 48 h after injection, and purified as described earlier (Henis et al., 1985; Hsu et al., 1982; Peretz et al., 1974). The virus was resuspended in 160 mM NaCl/20 mM Tricine, pH 7.4 (solution A), and stored at −70 °C. Viral protein concentration was determined by a modified Lowry procedure (Markwell et al., 1978). Viral hemagglutinating activity was measured in hemagglutinating units (HAU) by Salk's pattern method using chicken erythrocytes (Peretz et al., 1974) and was around 13 000 HAU/mg of viral protein. Hemolytic activity was determined by incubating 2% (v/v) human erythrocytes in solution A with 100 HAU/mL virus for 15 min at 4 °C, followed by 30-min incubation at 37 °C. Released hemoglobin was measured by the optical density at 540 nm, after centrifugation of unlysed cells (Peretz et al., 1974). The optical density obtained by lysis in the same volume of distilled water was taken as 100% hemolysis.

Preparation of Reconstituted Sendai Virus Envelopes. RSVE were prepared as described in detail previously (Vainstein et al., 1984) by solubilization of the virions with Triton X-100, removal of insoluble material by centrifugation, and removal of the detergent by direct addition of SM-2 Bio-beads into the solution containing detergent, viral envelope lipids, and the viral glycoproteins F and HN. The resulting envelopes, which contain F and HN proteins at a ratio similar to that of the native virions (Vainstein et al., 1984), were collected by centrifugation (100000*g*, 60 min) and suspended in solution A. Hemagglutinating and hemolytic activities were determined as described for the native virions. The RSVE typically showed 3–4-fold higher HAU per milligram of protein than the native virions, since they are enriched with the viral envelope proteins. For this reason, the amounts of RSVE and Sendai virions were calibrated by using HAU rather than the amount of viral protein in all the following experiments.

Interaction of RSVE and Sendai Virions with Human Erythrocytes. Fresh human blood (group O, Rh-positive) was obtained from a blood bank and stored with sodium citrate up to 7 days at 4 °C. Prior to use, erythrocytes were washed twice with solution A and diluted to 2% (v/v) with the same solution. Viral adsorption and hemagglutination were obtained by incubating the cells (15 min, 4 °C) with 400 HAU/mL RSVE or Sendai virions, which were subjected earlier to a mild sonication (three 30-s bursts with a Model W-10 sonicator; Heat Systems Ultrasonics, Plainview, NY) and filtration through a 0.45-μm filter (Gelman, Ann Arbor, MI), in order to eliminate large viral aggregates (Henis et al., 1985). After the cells were washed, fusion (mostly envelope–cell fusion in the case of RSVE, and both virus–cell and cell–cell fusion in the case of native virions) was achieved by a further incubation (30 min) at 37 °C (Henis et al., 1985). The degree of cell–cell fusion was evaluated by determining the precentage of fused cells among the total cell population, employing phase-contrast microscopy (Henis et al., 1985; Peretz et al., 1974).

Trypsin and PMSF Treatments. Treatment of RSVE with trypsin was performed essentially as described earlier for Sendai virus (Maeda et al., 1979). RSVE (0.5 mg of protein/mL) were incubated with trypsin (twice recrystallized; 60 μg/mL) in solution A (60 min, 37 °C). Phenylmethanesulfonyl fluoride (PMSF) treatment was carried out as described by Israel et al. (1983), incubating RSVE (3 mg of protein/mL) with 5 mM PMSF (30 min, 37 °C). Both treatments abolish the hemolytic and fusogenic activities of Sendai virions or RSVE, with preservation of the adsorption and hemagglutination activities (Vainstein et al., 1984; Maeda et al., 1979; Israel et al., 1983).

Preparation of Antibodies and Fab′ Fragments against Viral Envelope Proteins. Antibodies to the viral glycoproteins were raised in rabbits as described earlier (Henis et al., 1985), by intracutaneous injections of RSVE or of vesicles containing either F or HN proteins. The latter were prepared by a modification (Nussbaum et al., 1984) of the method of Nakanishi et al. (1982). The IgG fraction was purified by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (Mage, 1980). Fab'_2 fragments were prepared by pepsin digestion (Edelman & Marchalonis, 1967) and tagged with TMR-isothiocyanate by standard procedures (Goldman, 1968). Monovalent TMR–Fab′ fragments (required in order to avoid cross-linking in the FPR experiments) were prepared by reduction of the TMR–Fab'_2 fragments with 2-mercaptoethanol and alkylation witn iodoacetamide (Henis et al., 1985). The TMR–Fab′ fragments were not contaminated by Fab'_2 or IgG, as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. The fluorescent antibody fragments did not stain erythrocytes or erythrocyte ghosts which were not exposed to the virus, and were specific to the viral glycoproteins originally incorporated into the vesicles employed for immunization, as determined by gel electrophoresis (10% polyacrylamide, in the presence of sodium dodecyl sulfate) of whole Sendai virions followed by immunoblotting (Henis et al., 1985). Thus, labeling of both the F and HN proteins was obtained by using anti-RSVE TMR–Fab′ fragments, while with the anti-F and anti-HN preparations labeling of either F or HN, respectively, was observed (Henis et al., 1985).

Fluorescence Photobleaching Recovery. Lateral diffusion coefficients (D) and mobile fractions (R_f) were measured by FPR (Koppel et al., 1976; Axelrod et al., 1976) at 22 °C, using an apparatus described previously (Henis & Gutman, 1983). It was demonstrated recently that the bleaching conditions

employed in FPR studies do not alter the lateral mobilities measured (Wolf et al., 1980; Koppel & Sheetz, 1981). The FPR experiments were performed on erythrocyte ghosts attached to polylysine-coated coverslips and wet-mounted in solution A in order to avoid cell heating due to light absorption by hemoglobin. In experiments where the erythrocytes were fused with RSVE or native virions, the ghosts were formed either by the viral hemolytic activity or by hypotonic lysis and resealing as described by Steck and Kant (1974). The latter procedure was employed for erythrocytes with adsorbed (unfused) viral particles.

The monitoring argon ion laser beam (529.5 nm, 0.5 μW for TMR–Fab′ labeling; 488 nm, 0.03 μW for *N*-NBD-PE) was focused through the microscope to a Gaussian radius of 0.93 μm with an 100× oil-immersion lens. A brief pulse (5 mW for TMR, 0.3 mW for *N*-NBD-PE, 10–40 ms) bleached 50–70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. D and R_f were determined from the fluorescence recovery curves (Axelrod et al., 1976). Incomplete recovery was interpreted as fluorophores which are immobile on the experimental time scale ($D \leq 5 \times 10^{-12}$ cm^2/s). The collected fluorescence was restricted by an image-plane pinhole whose radius was twice that of the beam at the image plane, thus reducing the contribution of out-of-focus membranes (e.g., the bottom membrane while focusing on the top membrane of a cell) to the collected fluorescence (Petersen & McConnaughey, 1981).

In our experimental system where focusing on the top or bottom of the cells yielded similar D and R_f values, the effects of such a contribution may be at most around 20% on the D values, and essentially none on the R_f values (Petersen & McConnaughey, 1981).

Removal of RSVE or Sendai Virions Adsorbed to Human Erythrocytes by Dithiothreitol (DTT). Adsorbed RSVE were detached from human erythrocytes by DTT treatment, employing a procedure described in detail earlier (Chejanovsky et al., 1984). After incubation of the erythrocytes with RSVE or Sendai virions (at 4 °C, or at 4 °C followed by 37 °C), the cells were incubated with 50 mM DTT and 2 mM ethylenediaminetetraacetate in solution A for 15 min at 4 °C, and then for 30 min at 22 °C. Unbound viral particles were removed by centrifugation. This treatment removed adsorbed (but not fused) RSVE or Sendai virions from human erythrocytes, as shown by the almost complete removal of RSVE or Sendai virions from the erythrocytes under conditions that do not allow fusion (incubation with cells at 4 °C only, or the use of trypsin-treated Sendai virus particles) (Chejanovsky et al., 1984).

Determination of Viral Envelope–Cell Fusion by Dequenching of R_{18} Fluorescence. Fusion of RSVE and Sendai virions with human erythrocytes was measured by dequenching of R_{18} fluorescence as described by Hoekstra et al. (1984). R_{18} was incorporated directly into the membrane of intact Sendai virions by incubating the virions (0.9 mg of protein/mL) with 18 μM (1 h, 22 °C; the final solution contained 1% ethanol v/v) in solution A. Excess R_{18} was removed by passage on a Sephadex G-75 column (Hoekstra et al., 1984). RSVE containing R_{18} were prepared following the procedure described earlier for the preparation of RSVE containing *N*-NBD-PE (Chejanovsky & Loyter, 1985), by adding the supernatant obtained after Triton X-100 solubilization of Sendai virions (10 mg of viral protein) to a thin layer of 100 μg of R_{18}, prior to the removal of the detergent by SM-2 Bio-beads. This procedure yielded RSVE with about 5 mol % R_{18}. The R_{18}-labeled viral particles (virions or RSVE) displayed a degree of fluorescence quenching (Q) which varied between 60% and 80% [$Q = 100\ (F_T - F_0)/F_t$, where F_0 and F_t are the fluorescence intensities prior to and after the addition of 1% v/v Triton X-100 to the viral particles]. The residual quenching (RQ) after incubation of the viral particles with the cells was determined similarly, from the fluorescence ratio before and after the addition of Triton X-100. The percentage of fused viral particles was determined from the dequenching (DQ) of the fluorescence during the incubation with the cells, by using the equation $DQ = 100\ (Q - RQ)/Q$. The fluorescence (λ_{ex} = 560 nm, λ_{em} = 590 nm) was measured with a Perkin-Elmer MPF-4 spectrofluorometer at 22 °C.

Results

Fusion of RSVE with Human Erythrocytes. The fusogenic activity of Sendai virus particles is expressed initially in envelope–cell fusion (accompanied by hemolysis), which is followed by cell–cell fusion (Miyake et a., 1978; Maeda et al., 1977). We have therefore compared the RSVE with native Sendai virions with respect to all these activities. Experiments based on dequenching of the fluorescence of R_{18} incorporated into RSVE or into the envelope of native Sendai virions upon fusion (Hoekstra et al., 1984) clearly demonstrated that the RSVE employed in the present studies fuse with human erythrocytes as efficiently as native Sendai virions (Table I). In order to verify that lipid exchange processes do not contribute significantly to the above measurements, we performed similar experiments using RSVE or Sendai virions which were pretreated with trypsin or with PMSF (Table I). Both treatments render the viral particles capable of adsorption but not of fusion or hemolysis (Maeda et al., 1979; Israel et al., 1983). In both cases, the fraction of fused viral envelopes dropped to the level observed under conditions that do not allow fusion (incubation at 4 °C only).

In order to further eliminate the possible contribution of lipid exchange to the viral envelope–cell fusion measurements described above, we have also employed another method to evaluate envelope–cell fusion, based on the use of DTT treatment to remove adsorbed (but not fused) viral particles from the erythrocyte membrane (Table I). This measurement did not include contributions from lipid exchange processes, since the fraction of cell-associated viral envelopes that could not be removed from the cells by the DTT treatment was determined by using labeling with anti-RSVE TMR–Fab′ fragments, which bind to the viral envelope proteins (F and HN) and not to lipids. This method yielded results which were very similar to those obtained by dequenching of R_{18} fluorescence (Table I). Comparison of the level of fluorescence on the erythrocytes which were labeled with the anti-RSVE TMR–Fab′ fragments in these measurements has also indicated that there were no significant differences between the RSVE and native Sendai virions in the amounts of viral envelope proteins attached to an erythrocyte either prior to (4 °C incubation) or after (37 °C incubation) fusion (data not shown). These findings are in accord with a previous report on similar binding of RSVE and Sendai virions to, and fusion of their envelopes with, human erythrocytes, as determined by a combination of the DTT treatment with the use of ^{125}I-labeled viral particles (Chejanovsky et al., 1984). The similarity in the viral envelope–cell fusogenic activity of the RSVE and the native virions was also reflected in their similar hemolytic activity (Table I), which is believed to reflect viral envelope–erythrocyte fusion (Homma et al., 1976).

It should be noted that the fraction of cell-associated viral particles (either RSVE or native virions) which fused with the

Table I: Fusion of RSVE and Sendai Virions with Human Erythrocytes[a]

viral preparation	treatment	fraction of fused viral particles: R_{18} fluorescence dequenching	fraction of fused viral particles: DTT treatment	hemolysis (% of total)	cell–cell fusion (% fused cells)
RSVE	4 °C	0.09 ± 0.05	0.04 ± 0.05	3 ± 2	none
	4 °C + 37 °C	0.85 ± 0.04	0.81 ± 0.16	59 ± 5	10 ± 5
	trypsin	0.08 ± 0.06	0.06 ± 0.06	3 ± 2	none
	PMSF	0.09 ± 0.06	0.07 ± 0.05	4 ± 2	none
Sendai virions	4 °C	0.08 ± 0.05	0.07 ± 0.06	4 ± 3	none
	4 °C + 37 °C	0.84 ± 0.04	0.86 ± 0.15	55 ± 4	70 ± 5
	trypsin	0.08 ± 0.06	0.05 ± 0.06	3 ± 3	none
	PMSF	0.07 ± 0.05	0.08 ± 0.07	4 ± 2	none

[a] RSVE or native Sendai virions (400 HAU/mL in fusion measurements, 100 HAU/mL for the determination of hemolytic activity) were incubated in solution A with human erythrocytes (2% v/v) at 4 °C (rows 1 and 5) or at 4 °C followed by 37 °C (all other rows). The results are mean ± SE. In all the measurements of the fraction of fused viral particles, the incubation conditions were identical with those described for the FPR experiments under Materials and Methods, including washing the samples after the 37 °C incubation. All these measurements employed ghosts, formed either during fusion or by hypotonic lysis after incubation with the virus. Hemolysis and the percentage of fused cells (scoring 200–300 cells in the latter measurement) were determined as described under Materials and Methods. DTT treatment was performed as described under Materials and Methods, and the fraction of cell-associated viral envelopes which could not be removed by DTT was obtained by labeling the cells (either prior to or after the DTT treatment) with anti-RSVE TMR–Fab' fragments (100 μg/mL, 30 min, 22 °C, in solution A containing 0.2% bovine serum albumin) and measuring the fraction of the remaining cell-associated fluorescence after the DTT treatment using the FPR instrumentation under nonbleaching conditions (30 cells were scored in each case). The measurement of viral envelope–cell fusion by dequenching of R_{18} fluorescence was performed as described under Materials and Methods.

erythrocyte membrane was rather high (80–90%). Values in the same range were also measured for the fusion of ^{125}I-labeled Sendai virions or RSVE with intact human erythrocytes using the DTT treatment method (Chejanovsky et al., 1984), and for the fusion of RSVE containing *N*-NBD-PE with human erythrocyte ghosts by dequenching of *N*-NBD-PE fluorescence under conditions where most of the RSVE were associated with the cells (Chejanovsky & Loyter, 1985). This is also the situation in our studies, since all the free viral particles are washed away, including those that may be released by the viral neuraminidase activity during the 37 °C incubation (Micklem et al., 1985), prior to the measurement of the fraction of fused viral particles. The absence of the last washing step may be the reason for the lower (0.4–0.5) fraction of Sendai virions fused with human erythrocyte ghosts which was reported previously (Maeda et al., 1981; Hoekstra et al., 1984; Hoekstra & Klappe, 1986), although effects of different incubation conditions and different ghost preparation techniques in the latter studies cannot be ruled out.

True fusion between the RSVE and the erythrocyte membrane should result in dispersal of the viral envelope lipids over the erythrocyte membrane, thus leading to lateral mobilization of the envelope lipids over the entire cell surface. The fate of the viral envelope lipids after fusion with the erythrocyte membrane was followed by FPR. For this purpose, we prepared RSVE containing 0.6–0.8 mol % *N*-NBD-PE, by adding the supernatant obtained after Triton X-100 solubilization of Sendai virions (10 mg of viral protein) to a thin layer of 20 μg of *N*-NBD-PE, prior to the removal of the detergent by SM-2 Bio-beads (Chejanovsky & Loyter, 1985). The low *N*-NBD-PE molar ratio ensures that its fluorescence is not self-quenched in the RSVE membrane. Since the viral particle is much smaller than the size of the laser beam employed in the FPR experiment, lateral motion of *N*-NBD-PE in the viral envelope membrane itself is not detected in these experiments, which measure the motion of the lipid probe to micrometer distances only after its incorporation into the cell membrane. Our results (Figure 1) show that prior to fusion, the RSVE-incorporated *N*-NBD-PE is immobile on the erythrocyte surface but becomes laterally mobile under conditions that induce fusion. The mobile fraction (R_f = 80% ± 3%; n = 20) observed after fusion was high and close to the level obtained for *N*-NBD-PE incorporated directly [as described by Rimon et al., (1984)] into the membrane of human erythrocyte ghosts

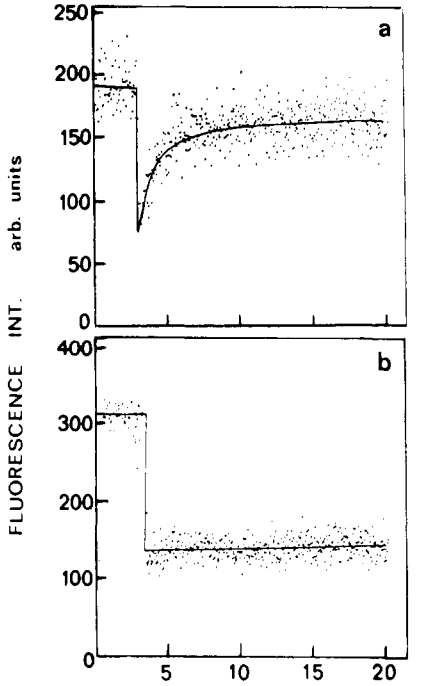

FIGURE 1: Typical FPR curves of *N*-NBD-PE incorporated into RSVE on human erythrocyte ghosts. Erythrocytes were incubated with RSVE containing *N*-NBD-PE, and the ghosts (prepared as described under Materials and Methods) were attached to polylysine-coated coverslips. FPR measurements were performed at 22 °C in solution A. Photons were counted over a 40-ms dwell time (represented as points); solid lines are nonlinear regression best-fit fluorescence recovery curves, obtained for a lateral diffusion process with a single diffusion coefficient, D (Axelrod et al., 1976). (a) Erythrocyte ghosts fused with RSVE containing *N*-NBD-PE (4 °C incubation, followed by 30 min at 37 °C). The specific curve shown yielded $D = 4.2 \times 10^{-9}$ cm^2/s and R_f = 84%. (b) Erythrocyte ghosts with adsorbed RSVE (incubation at 4 °C only). No lateral mobility could be detected ($D \leq 5 \times 10^{-12}$ cm^2/s). Similar results were obtained with RSVE pretreated with trypsin or PMSF, even after incubation at 37 °C.

(87% ± 3%; n = 20). This indicates that the RSVE lipids become incorporated into the cell membrane following fusion, as demonstrated also by the similarity of the D values obtained for *N*-NBD-PE incorporated directly into the erythrocyte membrane or inserted following fusion with RSVE containing *N*-NBD-PE [$D = (2.8 \pm 0.2) \times 10^{-9}$ and $D = (2.5 \pm 0.3) \times 10^{-9}$ cm^2/s, respectively, 20 cells measured in each case]. The

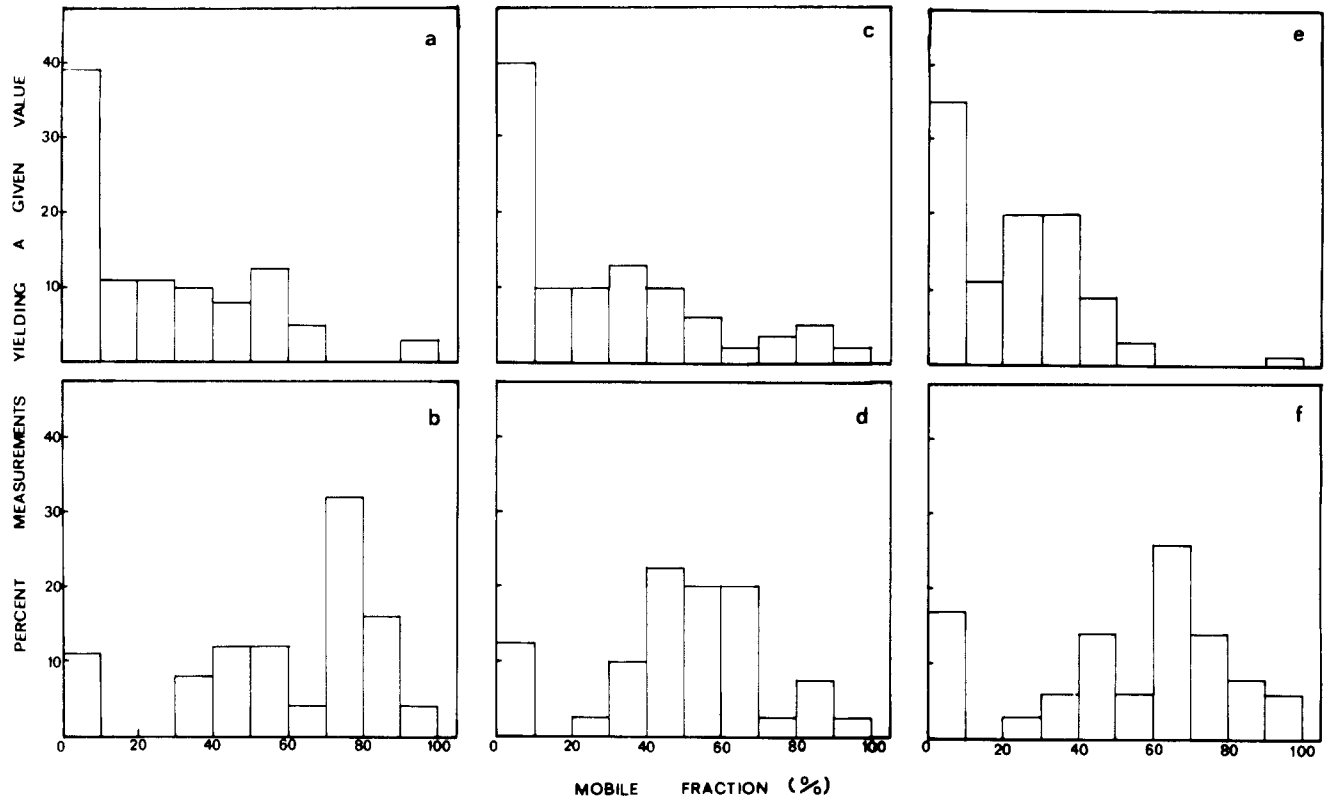

FIGURE 2: Distribution of the mobile fractions of F and HN over the population of erythrocyte ghosts following fusion with RSVE or native Sendai virions. Following treatment of whole erythrocytes with RSVE or Sendai virions, the ghosts (formed by the virally mediated hemolysis) were attached to polylysine-coated coverslips as described under Materials and Methods. TMR–Fab′ fragments (100 μg/mL) directed against F, HN, or F + HN (anti-RSVE) proteins were employed for the labeling (30 min, 22 °C, in solution A containing 0.2% bovine serum albumin). FPR measurements were conducted in solution A at 22 °C. A total of 50–60 cells were measured in each case. Upper row (a, c, and e), fusion with RSVE. Lower row (b, d, and f), fusion with native Sendai virions. (a and b) Labeling with anti-F TMR–Fab′ fragments. (c and d) Labeling with anti-RSVE (directed against HN + F) TMR–Fab′ fragments. (e and f) Labeling with anti-HN TMR–Fab′ fragments.

notion that the lateral mobilization of the RSVE-incorporated *N*-NBD-PE on the cell surface reflects a fusion process is supported by the lack of such mobilization in the case of nonfusogenic RSVE, which were treated with trypsin or PMSF.

Unlike the similarity in the viral envelope–cell fusion capability of RSVE and native Sendai virions, the RSVE were considerably less active than the native virions in the induction of cell–cell fusion (Table I). The system of RSVE and human erythrocytes therefore provides an opportunity to separate between envelope–cell and cell–cell fusion using the same cellular system.

Lateral Motion of Viral Components in the Cell Membrane following Fusion. In order to explore whether the lateral mobilization of the viral envelope glycoproteins in the cell membrane plays a role in the mechanism of cell–cell fusion, we studied their lateral motion in the erythrocyte membrane following fusion with RSVE and with native Sendai virions. Marked differences were observed between the two viral preparations in the mobile fractions of the viral envelope glycoproteins (Figure 2, Table II). The average mobile fractions of both F and HN were reduced over 2-fold on cells fused with RSVE as compared with cells fused with native Sendai virions (Table II). The differences between the two preparations are even more pronounced when one examines the distribution of the R_f values of the viral glycoproteins within the cell population (Figure 2). While a rather normal distribution around the mean is observed for the R_f values of F and HN following fusion of native virions with the erythrocytes, a skewed distribution with a significant increase in the percentage of cells exhibiting undetectable mobile fractions of the two viral glycoproteins is obtained following fusion with RSVE. The two viral glycoproteins, F and HN, appear to behave similarly in these measurements, in accord with our previous report (Henis et al., 1985) on the similarity in their dynamic parameters (D and R_f) following fusion of native Sendai virions with human erythrocytes. In the absence of fusion (incubation at 4 °C only, or the use of virions or RSVE pretreated with trypsin or PMSF), none of the viral envelope components exhibited measurable lateral motion on the cell surface (data not shown).

The differences between the RSVE and native Sendai virions in the distribution of the R_f values of the viral glycoproteins following fusion with human erythrocytes are in sharp contrast with the distribution of the mobile fraction of RSVE-incor-

Table II: Lateral Mobility of the Envelope Glycoproteins of RSVE and of Native Sendai Virions on Human Erythrocyte Ghosts after Fusion[a]

viral preparation	envelope protein labeled	mobile fraction (%)	D ($cm^2/s \times 10^{10}$)
RSVE	F	25 ± 4	3.5 ± 0.2
	HN	22 ± 3	3.2 ± 0.2
	F + HN	26 ± 3	3.6 ± 0.1
Sendai virions	F	60 ± 4	3.2 ± 0.1
	HN	55 ± 5	3.6 ± 0.2
	F + HN	55 ± 3	3.2 ± 0.2

[a] RSVE or native Sendai virions (400 HAU/mL) were fused with human erythrocytes as described under Materials and Methods. The ghosts formed were prepared for the FPR experiments as described in Figure 2. The envelope proteins were labeled with 100 μg/mL anti-F, anti-HN, or anti-RSVE (directed against F + HN) TMR–Fab′ fragments (see Figure 2). FPR measurements were conducted in solution A at 22 °C. The results shown are mean ± SE of 50–60 measurements in each case.

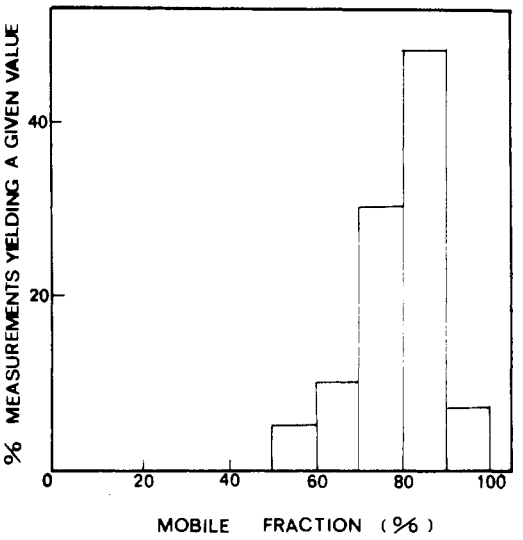

FIGURE 3: Distribution of the mobile fractions of RSVE-incorporated *N*-NBD-PE on human erythrocyte ghosts following RSVE–erythrocyte fusion. RSVE containing *N*-NBD-PE were prepared as described in the text, incubated with human erythrocytes under fusion-promoting conditions, and prepared for the FPR experiments as described in Figure 1. The FPR measurements were performed at 22 °C. A total of 50 cells were scored.

porated *N*-NBD-PE on the erythrocyte population after fusion (Figure 3). The R_f values of the lipid probe, which was originally incorporated into the RSVE membrane, were distributed normally around the mean; this suggests that the appearance of a skewed distribution of the R_f values of F and HN following fusion with RSVE is not due to an analogous distribution of RSVE–cell fusion (namely, the appearance of cells with high ratios of unfused RSVE) within the erythrocyte population. This notion is supported by the finding that DTT treatment, which removes unfused RSVE from the erythrocyte surface, did not result in higher R_f values for the glycoproteins of fused RSVE.

Unlike the marked differences observed between RSVE and native Sendai virions fused with human erythrocytes regarding the R_f values of the viral envelope proteins, no differences were observed between the two preparations in the D values of F and HN (Table II). Moreover, the distribution of the D values of the viral glycoproteins within the erythrocyte population was similar for RSVE–cell fusion and for Sendai virus–cell fusion (Figure 4). Since the R_f values are higher in the latter case, these findings suggest that envelope glycoproteins that become mobile in the cell membrane following fusion attain the same mobility (i.e., demonstrate similar lateral diffusion coefficients), regardless of the value of the mobile fraction.

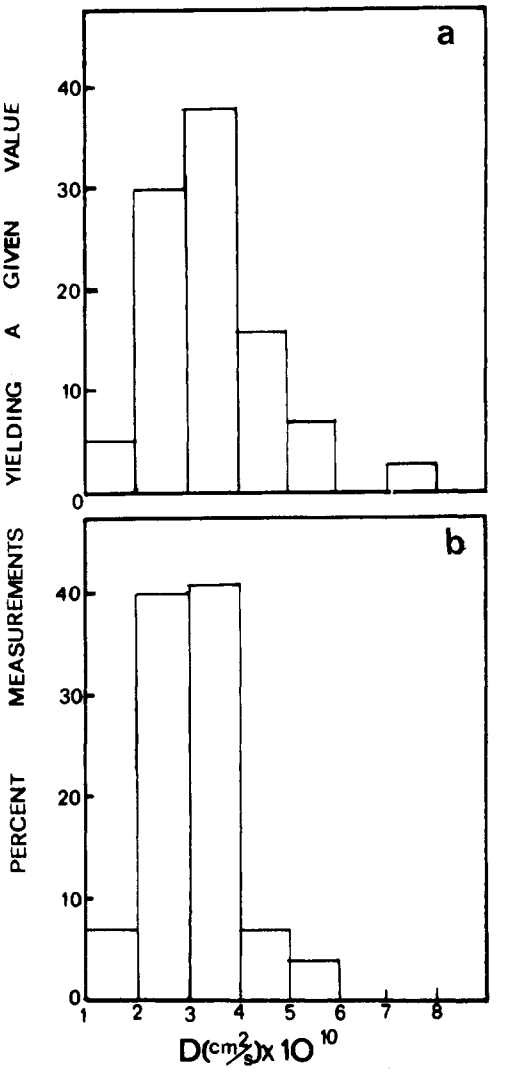

FIGURE 4: Distribution of the D values of the F protein over the population of erythrocyte ghosts following fusion with RSVE or native Sendai virions. Erythrocytes were incubated with RSVE or with Sendai virions and prepared for the FPR experiments as described in Figure 2. The F proteins were labeled by anti-F TMR–Fab′ fragments (see Figure 2). FPR measurements were conducted in solution A at 22 °C, measuring 50 cells in each case. Similar results were obtained with the HN or HN + F viral glycoproteins. (a) Erythrocytes fused with RSVE. (b) Erythrocytes fused with native Sendai virions.

Discussion

An immediate conclusion from the results summarized in Table I is that the RSVE fuse with the erythrocyte membrane as efficiently as native Sendai virions but induce cell–cell fusion to a much lower degree. The similar envelope–cell fusion activity of the two preparations is also reflected in their identical hemolytic activities (Table I). These findings are in accord with the recent report (Citovsky et al., 1985) on similarity in the fusion of RSVE (prepared as described in the present work) and native Sendai virions with human erythrocytes and with phosphatidylcholine/cholesterol liposomes. The selective reduction in cell–cell fusion by RSVE demonstrates that the processes of envelope–cell and cell–cell fusion are separable and the occurrence of the first is not necessarily followed by the second. This notion is supported by former studies on Ehrlich ascites tumor cells, which demonstrated that cells treated with cytochalasins (Asano & Okada, 1977; Miyake et al., 1978) or fused with Sendai virions in the presence of high saccharide concentrations (Maeda et al., 1977) could fuse with the viral envelopes but did not undergo cell–cell fusion. Moreover, the reduction in cell–cell fusion by RSVE in spite of their high envelope–cell fusion activity argues against the possibility that bridging between cells due to fusion of a single viral particle with membranes of two adjacent cells (Maeda et al., 1977) is a major mechanism for the mediation of erythrocyte–erythrocyte fusion by Sendai virus.

What are the possible explanations for the reduced cell–cell fusion activity of the RSVE? The most plausible explanations are as follows. (a) The fusogenic activity, which resides in the viral envelope proteins, could have been damaged during the preparation of the RSVE. However, the high envelope–cell fusion activity and the high hemolytic activity of the RSVE, which are similar to those of native Sendai virions, argue against this explanation. It is also possible that specific

cell–virion interactions are required to activate the cell–cell fusion activity of the viral glycoproteins and that the RSVE are deffective in these interactions; however, no indications for such an activation process are yet available. (b) The amounts of viral spike proteins inserted per cell following fusion with RSVE could be smaller than in the case of fusion with native virions. Again, the results of the fluorescence measurements with the FPR instrumentation following labeling of the viral proteins with fluorescent antibodies (see first section under Results) suggest that this is not the case and that comparable amounts of viral glycoproteins are associated with the erythrocytes when either RSVE or Sendai virions are adsorbed to or fused with the cells. A similar observation was reported by Chejanovsky et al. (1984) employing RSVE or Sendai virions whose envelope proteins were labeled directly with ^{125}I. (c) Part of the RSVE population could be active in cell agglutination but defective in their fusion capability. However, the fact that the majority of the cell-associated RSVE (80–90%) fuse with the erythrocytes under the experimental conditions employed (Table I), and most of the lipids of the viral envelopes become laterally mobile on the cell surface following fusion with *N*-NBD-PE-containing RSVE (Figure 1; see Results), argues against this possibility. (d) The concomitant reduction in the mobile fraction of the fused viral glycoproteins and in the cell–cell fusion activity of RSVE as compared with native Sendai virions raises the possibility that mobilization of the viral envelope proteins in the cell membrane after viral envelope–cell fusion is a necessary step for the induction of cell–cell fusion. The low level of such a mobilization in the case of the RSVE could therefore be the cause for their low cell–cell fusion activity.

Of all these explanations, the last one seems to be the most plausible. In a recent study (Henis et al., 1985), we have demonstrated that the envelope glycoproteins of Sendai virions become laterally mobile in the membrane of human erythrocytes following incubation at 37 °C, which leads to both virus–cell and cell–cell fusion. Moreover, treatments that blocked fusion prevented this mobilization. However, since both viral envelope–cell and cell–cell fusion took place in these experiments, it was not clear whether the lateral mobility of the viral glycoproteins on the cell surface is required for the induction of cell–cell fusion and involved in its mechanism or is merely the result of the fusion of viral envelopes with the cell membrane. The results in Figures 1–3 and in Table II rule out the latter possibility: they suggest that the lower mobilization of the RSVE envelope proteins, which is reflected in their lower R_f values and in the skewed distribution of these values, occurs *in spite* or the almost complete fusion and lipid intermixing between the RSVE and the erythrocyte membrane. These findings demonstrate that the lateral mobilization of the viral glycoproteins in the cell membrane is not a trivial result of viral envelope–cell fusion and under certain conditions (e.g., the use of RSVE instead of native virions) the majority of the envelope proteins may remain immobile even after envelope–cell fusion has occurred. This result is in line with reports on fusion in other experimental systems, which showed that free lateral motion of fused membrane proteins does not necessarily follow the fusion event. Thus, Wojcieszyn et al. (1983) demonstrated that human erythrocyte membrane proteins do not spread over the surface of human fibroblasts after fusion between the two cell types by poly(ethylene glycol). Similarly, restricted diffusion of proteins from reconstituted phospholipid vesicles was observed on the surface of mouse L cells following fusion by poly(ethylene glycol) (Bauman et al., 1980).

In view of the high degree of envelope–cell fusion and the low level of cell–cell fusion induced by the RSVE, viral envelope–cell fusion appears to be a necessary but not a sufficient condition for cell–cell fusion. We propose that lateral motion of the viral glycoproteins on the cell surface after the occurrence of envelope–cell fusion is an additional requirement for the induction of cell–cell fusion. The lateral mobility of these proteins in the cell membrane may be required to perturb the membranes of adjacent cells, thus leading to cell–cell fusion and to the generation of accompanying events that propagate over the entire cell surface, such as the triggering of cold-induced aggregation of intramembrane particles (Sekiguchi & Asano, 1978; Volsky & Loyer, 1978b; Kim & Okada, 1981). The proposed role of lateral motion of the viral glycoproteins in the cell membrane in the induction of cell–cell fusion is in line with the observations that treatments which restrict the lateral motion of fused Sendai virus glycoproteins in the cell membrane (Maeda et al., 1977) or the mobility of both viral and cellular membrane proteins (Volsky & Loyter, 1978b; Bachi et al., 1978) also inhibit cell–cell fusion. Since we cannot measure the lateral motion of the viral glycoproteins in the viral envelope itself (due to the small size of the viral particles relative to the radius of the laser beam), we could not examine whether lateral motion of these proteins in the viral envelope itself is required for envelope–cell fusion.

The reasons for the differences between RSVE and native Sendai virions in the lateral mobility of the viral glycoproteins after fusion with the cell membrane are still unclear. The RSVE lack the matrix protein (M), which interacts with the viral membrane in native virions. The lack of this protein in the RSVE may result in different organization or modified interactions with cellular structures such as the cytoskeleton and could lead to inhibition of the lateral motion of the viral glycoproteins in the cell membrane. Alternatively, modified interactions between F and HN in the RSVE membrane could result in increased interactions of the viral glycoproteins with each other and in mutual aggregation, a phenomenon which could also lead to lateral immobilization. This possibility is currently under study in our laboratory.

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