Fluorescence Photobleaching Recovery as a Method To Quantitate Viral Envelope–Cell Fusion: Application To Study Fusion of Sendai Virus Envelopes with Cells†

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ABSTRACT: A method to quantitate viral envelope–cell fusion at the single-cell level is presented. The method is based on the incorporation of nonquenching concentrations of a fluorescent lipid probe into the viral envelope; fluorescence photobleaching recovery (FPR) is then applied to measure the lateral mobilization of the probe in the cell membrane following fusion. In adsorbed (unfused) viral envelopes, the probe is constricted to the envelope and is laterally immobile on the micrometer scale of FPR. After fusion, the envelope lipids intermix with the plasma membrane, the probe becomes laterally mobile, and the fraction of fused viral envelopes can be extracted from the fraction of mobile probe molecules. The method has several advantages: (i) It clearly distinguishes fused from internalized envelopes, as probes in the latter are immobile in FPR studies; (ii) focusing the laser beam on specific regions of the cell enables region-specific measurements of the fusion level; (iii) one cell is measured at a time, enabling studies on the distribution of the fusion level within the cell population. The new method was employed to study fusion of reconstituted Sendai virus envelopes (RSVE) containing *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine with several cell types. Experiments with human erythrocytes demonstrated that the lateral mobilization measured is due to fusion and not the result of exchange processes. The extent of RSVE–erythrocyte fusion determined by FPR was similar to that measured by two other independent methods (fluorescence dequenching and removal of adsorbed RSVE by dithiothreitol). FPR experiments on other cell types (BHK-21, HeLa, GM-22 rat hepatoma cells, and mouse spleen lymphocytes), which are active in endocytosis, showed different degrees of RSVE–cell fusion depending on the cell region examined. There were also cell type dependent differences in the distribution of RSVE–cell fusion within the cell population. These results are discussed in light of the scope of the FPR method for studies on viral envelope–cell fusion at the single-cell level.

Fusion between viral envelopes and cellular membranes is involved in the penetration of enveloped viruses into animal cells. The envelopes may fuse either with the cellular plasma membrane, as in the case of paramyxoviruses, or with membranes of endocytic vacuoles (Choppin & Scheid, 1980; White et al., 1983). Viral envelope–cell fusion is also important as an efficient means for introducing various agents into recipient cells (Uchida et al., 1977; Loyter & Volsky, 1982).

In spite of the importance of virus–cell fusion, the fusion mechanism is still poorly understood. The development of quantitative methods for the measurement of viral envelope–cell fusion is essential in order to enable a thorough investigation of these processes. Electron microscopy (Shimizu et al., 1976) and virus-induced leakage of intracellular material (Homma et al., 1976) have been employed to study virus–cell fusion; however, these methods are at best semiquantitative (as in the case of virus-induced hemolysis of red blood cells) (Homma et al., 1976). Methods which involve the chemical or enzymatic removal of adsorbed (but not fused) virions from cells may be used to quantitate virus–cell fusion, as demonstrated by the removal of adsorbed Sendai virions from human erythrocytes by dithiothreitol (DTT)[1] treatment (Chejanovsky et al., 1984a). However, this method lacks the ability to distinguish between fused and internalized virions and is therefore limited to cell types which exhibit low levels of endocytic activity.

Biophysical techniques have proved to be very useful in quantitating membrane fusion. Thus, electron spin resonance was employed to evaluate virus–cell fusion (Lyles & Landsberger, 1979; Maeda et al., 1981), and fluorescence methods based on energy transfer (Struck et al., 1981; Chejanovsky et al., 1984b; Eidelman et al., 1984; van Meer et al., 1985) or on fluorescence dequenching of fluorescent lipid analogues incorporated into lipid vesicles or viral envelopes (Hoekstra et al., 1984; Chejanovsky & Loyter, 1985) have gained wide acceptance in the study of fusion. However, even the fluorescence methods may suffer from artifacts which result from endocytosis and degradation of the fluorescent viral envelopes or vesicles (Chejanovsky & Loyter, 1985). Moreover, both the biophysical methods mentioned above and the "chemical removal" approach yield average values for the fusion of viral envelopes (or vesicles) with cells and lack the ability to determine the distribution of the extent of fusion over the cell population examined.

The distribution of viral envelope–cell (or vesicle–cell) fusion over the cell population and over the surface of single cells (considering that different regions on the same cell may reveal different fusion levels) can be explored only by methods which enable the quantitative determination of fusion at the level of single cells. In the present work, we have adapted the technique of fluorescence photobleaching recovery (FPR), which

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[1] Abbreviations: DTT, dithiothreitol; FPR, fluorescence photobleaching recovery; RSVE, reconstituted Sendai virus envelope(s); *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; HN, hemagglutinin/neuraminidase glycoprotein; F, fusion glycoprotein; HAU, hemagglutinating units; PMSF, phenylmethanesulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; *D*, lateral diffusion coefficient; R_f, mobile fraction; R_{18}, octadecyl rhodamine B chloride; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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measures the lateral mobility of fluorescence-labeled membrane components on single cells (Axelrod et al., 1976; Koppel et al., 1976), to determine the distribution of viral envelope–cell fusion over cell populations and over the surface of single cells. In these studies, we employed reconstituted Sendai virus envelopes (RSVE) assembled from virions solubilized in Triton X-100 in the presence of low (nonquenching) levels of the fluorescent lipid probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE). Apart of lipids, the RSVE contain the two envelope glycoproteins of Sendai virus—the hemagglutinin/neuraminidase (HN) and the fusion (F) proteins, which mediate Sendai virus binding and fusion, respectively (Poste & Pasternak, 1978; Choppin & Scheid, 1980). The RSVE are as active as Sendai virions in terms of hemolytic activity and envelope–cell fusion (Vainstein et al., 1984; Chejanovsky et al., 1984a; Citovsky et al., 1985) and are therefore suitable for studies on the latter process. Our findings demonstrate that the *N*-NBD-PE incorporated into RSVE becomes laterally mobile on the cell surface following envelope–cell fusion and that the mobile fraction of the fluorophore may be used to determine quantitatively the level of viral envelope–cell fusion and its distribution within the cell population and on the surface of single cells.

Experimental Procedures

Virus. Sendai virus (Z strain) was grown in the allantoic sac of 10–11-day-old embryonated chicken eggs, harvested 48 h after injection, and purified as described earlier (Peretz et al., 1974; Hsu et al., 1982; Henis et al., 1985). The virus was resuspended in 160 mM NaCl and 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). 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 containing the fluorescent phospholipid *N*-NBD-PE (Avanti, Birmingham, AL) were prepared as described previously (Vainstein et al., 1984; Chejanovsky & Loyter, 1985). A pellet of Sendai virions (10 mg of protein) was dissolved in 500 μL of 4% (w/w) Triton X-100 in 100 mM NaCl and 50 mM Tricine, pH 7.4. After centrifugation (100000*g*, 60 min), the supernatant (containing viral envelope lipids, and about 2.5 mg of viral glycoproteins) was added to a thin layer of 20 μg of *N*-NBD-PE, formed by drying an ethanolic solution of the fluorescent lipid under nitrogen. The mixture, which contained 0.6–0.8 mol % *N*-NBD-PE relative to the viral lipid, was shaken vigorously for 5 min, and the detergent was removed by direct addition of SM-2 Bio-Beads (Vainstein et al., 1984). The low ratio of *N*-NBD-PE to viral lipids was chosen to avoid self-quenching of its fluorescence, which occurs at higher fluorophore densities (Chejanovsky et al., 1985). Addition of Triton X-100 (0.1%) to the fluorescent RSVE revealed no significant increase in the fluorescence (below 10%), even after correction for the effect of the detergent on the quantum yield of NBD fluorescence (Struck et al., 1981). The fluorescent RSVE thus obtained were collected by centrifugation (100000*g*, 60 min), suspended in solution A (0.5 mL), and stored at −70 °C. Hemagglutinating and hemolytic activities were determined as described for the native virions.

Trypsin and PMSF Treatments. Fluorescent RSVE particles were trypsinized as described earlier for native Sendai virions (Maeda et al., 1979), with minor modifications. RSVE (1 mg of protein/mL) were incubated with trypsin (100 μg/mL, twice recrystallized; Sigma, St. Louis, MO) in solution A (30 min, 37 °C). The reaction was stopped by the addition of soybean trypsin inhibitor (200 μg/mL; Sigma, St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF) treatment was performed by using established methods (Israel et al., 1983), incubating fluorescent RSVE (3 mg of protein/mL) with 5 mM PMSF (30 min, 37 °C). Both treatments abolish the hemolytic and fusogenic activities of Sendai virus, but not its adsorption and hemagglutinating activity (Maeda et al., 1979; Israel et al., 1983).

Cells. Fresh human blood (type 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 by centrifugation with solution A.

Mouse spleen lymphocytes were prepared from 12–16-week-old AKR/Cu mice in Dulbecco's modified Eagle's medium (DMEM) with low (1000 mg/L) bicarbonate (Beth Haemek, Israel), buffered with 20 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4, as described earlier (Ryan et al., 1975; Henis et al., 1981). They were employed in FPR studies within 8 h.

Baby hamster kidney cells BHK-21 and human HeLa cells were grown as tissue cultures on glass coverslips placed in 35-mm tissue culture dishes (NUNC, Roskilde, Denmark), in DMEM containing 10% fetal calf serum (from Sera-Lab, Sussex, England).

Rat hepatoma cells (clone GM-22) were grown in a spinner bottle using Swim's-77 medium containing 10% bovine calf serum, up to a density to 5×10^5 cells/mL.

Labeling of Cells with N-NBD-PE. Cells were labeled with the lipid probe *N*-NBD-PE as described earlier (Henis & Elson, 1981). Briefly, cells were washed twice with the appropriate incubation medium (or solution A for erythrocytes) without bovine serum albumin and incubated (15 min, 22 °C) with a 1:100 dilution of 2 mg/mL *N*-NBD-PE in ethanol. Excess *N*-NBD-PE was removed by washing twice with the same media. Erythrocytes, lymphocytes, and rat hepatoma cells were labeled in suspension (6×10^6 cells/mL), while tissue-cultured cells were labeled directly on the coverslips.

Interaction of RSVE with Cells. Human erythrocytes (2% v/v) were incubated in solution A with 400 HAU/mL fluorescent RSVE (15 min, 4 °C) to allow viral envelope adsorption and hemagglutination. After the solution was washed to remove unattached RSVE, fusion of RSVE with the erythrocytes was achieved by a further incubation (30 min) at 37 °C (Henis et al., 1985).

Mouse spleen lymphocytes and rat hepatoma cells were also incubated with the fluorescent RSVE in suspension (10^7 cells/mL with 2000 HAU/mL RSVE), using the appropriate media (DMEM for lymphocytes and Swim's-77 medium for hepatoma cells) buffered with 20 mM HEPES and supplemented with 0.2% bovine serum albumin (referred to as incubation media). After 30 min at 4 °C, the cells were washed by centrifugation and incubated 60 min at 37 °C to induce fusion.

Tissue-cultured cells (BHK-21 and HeLa cells) were incubated with the fluorescent RSVE directly on the coverslips. Semiconfluent monolayers (1–2 days after plating) were washed twice with the DMEM incubation medium and layered with 100 μL containing 1000 HAU RSVE. After 30 min at

4 °C, the monolayers were washed twice with the incubation medium and transferred for 60 min to 37 °C in the same medium.

Fluorescence Photobleaching Recovery. Lateral diffusion coefficients (D) and mobile fractions (R_f) of *N*-NBD-PE in cell membranes were determined by FPR (Axelrod et al., 1976; Koppel et al., 1976), using an apparatus described earlier (Henis & Gutman, 1983). Cells were incubated with RSVE containing *N*-NBD-PE as described in the preceding section, washed with the appropriate incubation medium, and taken for the FPR experiments. In the case of tissue-cultured cells, the experiments were performed on the coverslips carrying the cells, wet-mounted with the appropriate incubation medium. Experiments on lymphocytes and hepatoma cells were performed similarly, following attachment of the cells to polylysine-coated coverslips. Erythrocytes were also attached to the polylysine-coated coverslips (wet-mounted in solution A containing 0.2% bovine serum albumin), following the preparation of ghosts in order to avoid light absorption by hemoglobin (Bloom & Webb, 1983). In experiments where the erythrocytes were fused with RSVE, the ghosts were formed either by the viral hemolytic activity or by hypotonic lysis and resealing (Steck & Kant, 1974). The latter procedure was employed for erythrocytes which were not fused with RSVE. In all cases, the cells were washed with the appropriate incubation medium after the incubation at 37 °C in order to remove RSVE released from the cell surface during the incubation (Micklem et al., 1985).

The monitoring argon ion laser beam (488 nm, 0.03 μW) was focused through the microscope to a Gaussian radius of 0.93 μm with a 100× oil immersion lens. A brief pulse (30–80 ms, 0.3 mW) bleached about 50–70% of the fluorescence in the illuminated region, and the fluorescence recovery due to the entry of unbleached fluorophores was monitored with the attenuated beam. D and R_f were derived from the fluorescence recovery curves (Axelrod et al., 1976). It has been shown that the bleaching conditions employed in FPR experiments do not alter the diffusion measurements, yielding values similar to those derived from experiments which do not employ bleaching (Wolf et al., 1980; Wey et al., 1981; Koppel & Sheetz, 1981).

Removal of Adsorbed RSVE from Cells by DTT. Adsorbed RSVE were detached from human erythrocytes and from mouse spleen lymphocytes by employing DTT, as described earlier for human erythrocytes (Chejanovsky et al., 1984a). After incubation with RSVE (at 4 °C only, or at 4 °C followed by 37 °C), the cells were incubated with 50 mM DTT and 2 mM ethylenediaminetetraacetate in solution A (erythrocytes) or in the DMEM incubation medium (lymphocytes) for 15 min at 4 °C, and then for 30 min at 22 °C. Unbound viral envelopes were removed by centrifugation. This treatment removes adsorbed (but not fused) RSVE from human erythrocytes (Chejanovsky et al., 1984a) and from mouse spleen lymphocytes, as shown by the almost complete removal of RSVE from these cells under conditions that do not allow fusion (incubation with cells at 4 °C only, or the use of trypsin- or PMSF-treated RSVE).

Fusion Measurements by Dequenching of Octadecyl Rhodamine B Chloride (R_{18}) Fluorescence. Fusion of viral envelopes with human erythrocytes was measured by dequenching of R_{18} (Molecular Probes, Junction City, OR) fluorescence, following the procedure of 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 R_{18} (1 h, 22 °C; the final solution contained 1% ethanol v/v) in solution A and removing excess R_{18} by passage on a Sephadex G-75 column (Hoekstra et al., 1984). RSVE containing R_{18} were prepared as described for RSVE containing *N*-NBD-PE, except that a higher amount (100 μg) of R_{18} was employed, yielding 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% [$Q = [(F_T - F_0)/F_T]100$, 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 R_{18}-labeled viral particles with the cells were determined similarly, from the ratio of the fluorescence 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 (Chejanovsky & Loyter, 1985), 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

Quantitation of RSVE Fusion with Human Erythrocytes by FPR. Fusion of viral envelopes or lipid vesicles containing fluorescent lipid probes with cell membrane is accompanied by dispersal of the fluorescent probes over the cellular plasma membrane (Struck et al., 1981; Eidelman et al., 1984; Hoekstra et al., 1984; van Meer et al., 1985; Chejanovsky et al., 1985). It therefore follows that prior to fusion, when the *N*-NBD-PE containing RSVE are merely adsorbed to the cells, the lateral motion of the fluorescent lipids will be limited to the viral envelopes, while after fusion has occurred they will be free to diffuse laterally over the plasma membrane. The FPR experiments employ a laser beam considerably larger than the size of the RSVE (0.93-μm Gaussian radius in the present studies) and thus measure lateral motion over an area of several square micrometers. It is therefore expected that the fluorescent lipids of the RSVE will be immobile in FPR experiments prior to fusion and become laterally mobile following envelope–cell fusion.

In order to prove these points and develop them into a quantitative method for the measurement of viral envelope–cell fusion, we performed FPR experiments on RSVE adsorbed to and fused with human erythrocytes. The latter cells were chosen for the initial studies since they lack endocytic activity, which may compete with fusion and complicate the interpretation of the data.

Representative FPR curves obtained for the lateral diffusion of *N*-NBD-PE originally incorporated into RSVE on the surface of human erythrocyte ghosts are shown in Figure 1. The mean values of R_f and D obtained for the RSVE-incorporated *N*-NBD-PE of adsorbed and fused RSVE are depicted in Table I. As can be seen, the above-mentioned expectations of the FPR experiments are indeed met. No lateral mobility of the RSVE-incorporated *N*-NBD-PE could be detected on the cell surface under conditions that lead to adsorption without fusion of the RSVE (incubation at 4 °C). On the other hand, under conditions that lead to RSVE–cell fusion (a further 30-min incubation at 37 °C), the *N*-NBD-PE became interally mobile in the erythrocyte membrane, demonstrating relatively high R_f values (Figure 1, Table I). The lateral mobilization of the RSVE-incorporated *N*-NBD-PE in the erythrocyte membrane upon incubation at 37 °C is accompanied by a transfer of the fluorescence observed on the cells from patches to a homogeneous labeling pattern (Figure 2).

The above findings suggest that the RSVE-incorporated *N*-NBD-PE becomes laterally mobile on the erythrocyte

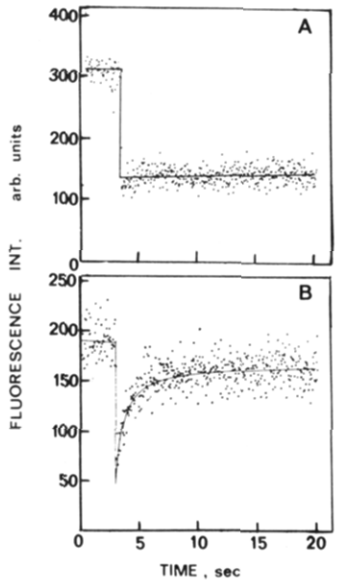

FIGURE 1: Representative FPR curves of RSVE-incorporated *N*-NBD-PE on human erythrocyte ghosts. Following treatment of whole erythrocytes with RSVE containing *N*-NBD-PE, ghosts (prepared as described under Experimental Procedures) were attached to polylysine-coated coverslips. FPR measurements were performed at 22 °C. The points represent the experimental fluorescence intensity (photons counted/40-ms dwell time). Solid lines are computer-generated, 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 with adsorbed (4 °C incubation) RSVE. No mobility could be detected ($D \leq 5 \times 10^{-12}$ cm^2/s). (B) Erythrocyte ghosts fused with fluorescent RSVE (4 °C incubation, followed by 30 min at 37 °C). The specific curve shown yielded $D = 4.8 \times 10^{-9}$ cm^2/s and $R_f = 0.85$.

surface following RSVE–cell fusion. Thus, the fraction of cell-associated RSVE that fuses with the cellular plasma membrane is given by the ratio R_f/R_f^0, where R_f is the mobile fraction measured for the RSVE-incorporated *N*-NBD-PE on the cell surface after fusion and R_f^0 is the mobile fraction of *N*-NBD-PE incorporated directly into the cell membrane. The latter value, which is in the range of 0.9 for most cell types at temperatures of 20 °C and above (Edidin, 1981; Helmreich & Elson, 1984; Rimon et al., 1984), represents the highest mobile fraction to be obtained if all the RSVE-incorporated *N*-NBD-PE is inserted into the cell membrane. The fractions of fused RSVE derived in this manner under different conditions (which do and do not allow fusion) are given in Table I.

Table I: Lateral Motion of RSVE-Incorporated *N*-NBD-PE on the Surface of Human Erythrocytes[a]

treatment	D (cm^2/s × 10^9)	R_f	R_f/R_f^0
4 °C + 37 °C	2.8 ± 0.2	0.80 ± 0.03	0.92 ± 0.07
4 °C	≤0.005	0.05 ± 0.03	0.06 ± 0.04
trypsin	≤0.005	0.05 ± 0.05	0.06 ± 0.06
PMSF	≤0.005	0.06 ± 0.05	0.07 ± 0.06

[a] Erythrocytes (2% v/v) were incubated with RSVE containing *N*-NBD-PE (400 HAU/mL) under fusion-promoting conditions (4 °C followed by 37 °C, rows 1, 3, and 4) or at 4 °C only (row 2). RSVE were pretreated with trypsin or PMSF as described under Experimental Procedures in the cases so designated. The preparation of the cells for the FPR experiments (performed at 22 °C, in solution A) was as described under Experimental Procedures and in Figure 1. The results are mean ± SE of measurements on 20–30 cells in each case. The lateral mobility of *N*-NBD-PE incorporated directly into the membrane of human erythrocyte ghosts (Rimon et al., 1984) was measured as a control. The R_f value obtained for this measurement (designated R_f^0) was 0.87 ± 0.03, with $D = (2.5 \pm 0.3) \times 10^{-9}$ cm^2/s. R_f/R_f^0 is the fraction of cell-associated RSVE which have fused with the cells (see text).

A possibility which has to be considered is that part of the lateral mobilization of the RSVE-incorporated *N*-NBD-PE in the erythrocyte membrane is due to lipid exchange rather than fusion. The lack of such mobilization after incubation of RSVE with erythrocytes at 4 °C argues against this possibility. In order to verify that lipid exchange processes do not contribute significantly to the mobilization observed following incubation at 37 °C, we performed FPR experiments using *N*-NBD-PE-containing RSVE which were pretreated with trypsin or with PMSF (Table I). Both treatments render the RSVE capable of adsorption but not of fusion or hemolysis (Maeda et al., 1979; Israel et al., 1983; Vainstein et al., 1984). In both cases, the RSVE-incorporated *N*-NBD-PE was laterally immobile even after incubation with erythrocytes at 37 °C (Table I), lending further support to the notion that the mobilization of the viral envelope's *N*-NBD-PE in the erythrocyte membrane is the result of fusion and not of exchange. These findings are in accord with the report that *N*-NBD-PE (labeled at the head group) incorporated into liposomes does not enter liposomes or cell membranes by phospholipid exchange (Struck & Pagano, 1980).

A final step in the establishment of FPR measurements on the mobilization of RSVE-incorporated *N*-NBD-PE as a

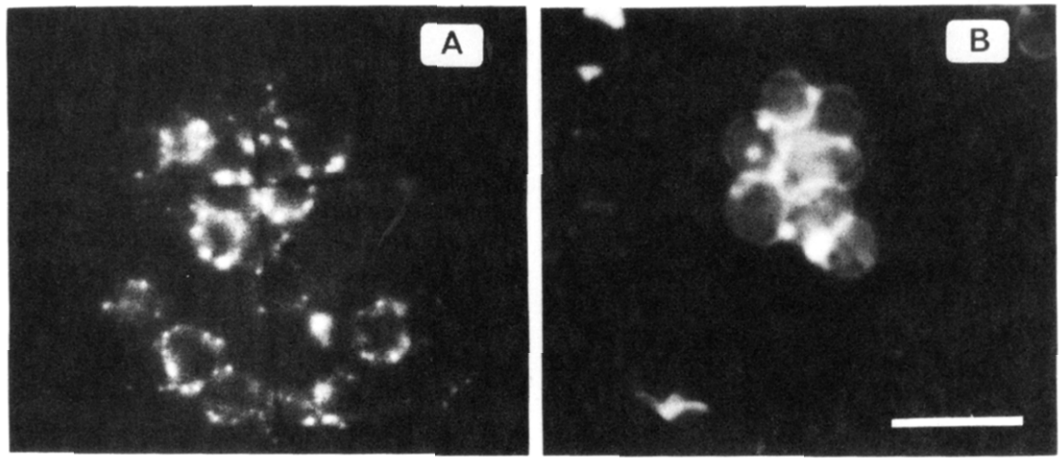

FIGURE 2: Fluorescence micrographs of human erythrocytes incubated with RSVE containing *N*-NBD-PE. Erythrocytes were treated with fluorescent RSVE and attached to polylysine-coated coverslips as described under Experimental Procedures. Photographs were taken with Kodak Tri-X film (400 ASA) under laser illumination (488 nm, 4 mW, beam expanded to 100-μm radius) through an image intensifier (NVC-100, Ni-Tec, Niles, IL) coupled to the microscope. Bar, 10 μm. (A) Erythrocytes with adsorbed fluorescent RSVE (incubation at 4 °C). (B) Erythrocyte ghosts after fusion with the RSVE (4 °C incubation, followed by 30 min at 37 °C).

Table II: Measurements of RSVE–Erythrocyte Fusion by FPR, DTT Treatment, Fluorescence Dequenching, and Hemolysis[a]

	fraction of fused RSVE			
treatment	FPR (*N*-NBD-PE)	DTT treatment (*N*-NBD-PE)	fluorescence dequenching (R_{18})	hemolysis (% of total)
4 °C	0.06 ± 0.04	0.04 ± 0.05	0.09 ± 0.05	3 ± 2
4 °C + 37 °C	0.92 ● 0.07	0.95 ± 0.20	0.90 ± 0.04	59 ± 5
trypsin	0.06 ± 0.06	0.07 ± 0.06	0.08 ± 0.06	3 ± 2

[a] Fluorescent RSVE (containing *N*-NBD-PE in the FPR and DTT treatment experiments and R_{18} in the fluorescence dequenching measurements) were incubated with human erythrocytes at 4 °C (row 1) or at 4 °C followed by 30 min at 37 °C (rows 2 and 3). In all the measurements of the fractions of fused RSVE, the incubation conditions were identical with those described for the FPR experiments under Experimental Procedures (2% erythrocytes with 400 HAU/mL RSVE), including washing of 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. RSVE-induced hemolysis was measured after 30 min at 37 °C, using 100 HAU-mL RSVE and 2% (v/v) erythrocytes (described under Experimental Procedures). FPR measurements were conducted at 22 °C, and the fraction of cell-associated RSVE which have fused with the cells was derived from the R_f/R_f^0 values (see Table I for details). DTT treatment was performed as described under Experimental Procedures, and the fraction of the cell-associated fluorescence which could not be removed by DTT was determined by measuring the fluoresence on the cell surface using the FPR instrumentation under nonbleaching conditions (30 cells were scored in each case). The measurement of fusion by dequenching of R_{18} fluorescence was performed as described under Experimental Procedures.

method to quantitate envelope–cell fusion is the demonstration that similar results are obtained by using other independent methods to quantitate fusion. Comparison of the measurement of RSVE fusion with human erythrocytes by FPR with the results obtained by two other methods under conditions identical with those employed in the FPR measurements is given in Table II. The methods employed are the removal of adsorbed (but not fused) RSVE from the erythrocyte surface by DTT (Chejanovsky et al., 1984a) and the dequenching of the fluorescence of R_{18} incorporated into RSVE upon fusion (Hoekstra et al., 1984). The results (Table II) demonstrate that within experimental error, these methods yield values similar to those of the FPR experiments. Values in the same range were also reported for the fusion of *N*-NBD-PE containing RSVE with human erythrocyte ghosts following dequenching of *N*-NBD-PE fluorescence under conditions where most of the RSVE are associated with the cells (Chejanovsky & Loyter, 1985). Since R_{18} (unlike *N*-NBD-PE) can also be incorporated directly into the membrane of intact Sendai virions (Hoekstra et al., 1984), we compared the level of RSVE–cell fusion with that of R_{18}-labeled Sendai virions by following the dequenching of R_{18} fluorescence. The results were essentially identical with those obtained with the R_{18}-containing RSVE, suggesting that the RSVE are as active as intact Sendai virions in envelope–cell fusion. This situation is also reflected in the hemolytic activity of the native virions (55% hemolysis under the conditions of Table II), which is similar to that of the RSVE. The latter parameter, which reflects viral envelope–erythrocyte fusion (Homma et al., 1976), shows a clear correlation with the fraction of fused RSVE as determined by FPR (Table II).

Quantitation of RSVE–Cell Fusion on Different Regions of Mammalian Nucleated Cells. Unlike erythrocytes, most mammalian nucleated cells are active in endocytosis. Thus, RSVE adsorbed to these cells may either fuse with their plasma membrane or enter via endocytosis. Since endocytic vesicles are immobile on the time scale of the FPR experiments (Schlessinger et al., 1977), internalized RSVE should not contribute to the lateral mobility of RSVE-incorporated *N*-NBD-PE on the cell surface. Moreover, the ability to focus the laser beam in the FPR experiment on a relatively small area of the cell surface enables the quantitation of viral envelope–cell fusion separately for different regions of the cell, a feature of special importance in cell types which are active in endocytosis or exhibit intermediate levels of fusion with adsorbed viral envelopes.

In order to investigate the distribution of fused RSVE on the cell surface, we performed experiments on several cell types, including cell lines growing in monolayers (HeLa and BHK-21) and cells in suspension (rat hepatoma cells clone GM-22 and primary mouse spleen lymphocytes). Adsorption of RSVE containing *N*-NBD-PE to all these cell types at 4 °C resulted in the appearance of random fluorescent patches on the cells, as in the case of human erythrocytes (Figure 2A). No mobility could be detected in FPR experiments under these conditions, indicating the absence of RSVE–cell fusion. After further incubation at 37 °C (conditions which promote fusion and endocytosis), weak homogeneous fluorescence was detected in certain regions of the cells, while fluorescent patches were observed in other cell regions (Figure 3). This situation is contrasted with the rather uniform homogeneous fluorescence observed on human erythrocytes following fusion with RSVE containing *N*-NBD-PE (Figure 2B). It should be noted that a large portion of the fluorescent patches appeared in the perinuclear region, especially in the case of HeLa cells (Figure 3); this type of labeling is thought to represent internalized viral particles, as described earlier for Semliki Forest virus and BHK-21 cells (Helenius et al., 1980; Helenius & Marsh, 1982). The heterogeneous labeling pattern following incubation of the cells (except erythrocytes) at 37 °C with RSVE containing *N*-NBD-PE was also reflected in the FPR experiments on the lateral mobility of *N*-NBD-PE on the cell surface. Unlike the situation in erythrocytes, where rather similar results were obtained by focusing the laser beam on different regions of the cell, regional dependence of the FPR results was observed on the other cell types investigated. Thus, the *N*-NBD-PE associated with the fluorescent patches on (or inside) the cells was completely immobile ($R_f = 0$) in all cases and therefore represents unfused RSVE (either adsorbed or internalized aggregates). On the other hand, focusing the laser beam on membrane areas where no visible patches were detected demonstrated the existence of mobile *N*-NBD-PE populations (Table III).

These findings suggest the existence of "regional heterogeneity" on the cell in the fraction of fused viral envelopes. In order to compare the measurements of the extent of RSVE–cell fusion by FPR to those obtained by other methods, which yield only average values (e.g., spectroscopic or "chemical removal" methods), one has to calculate the *average* fraction of fused RSVE in the FPR experiments. To achieve this, the relative fluorescence levels associated with the homogeneously labeled regions (demonstrating a definite R_f value) and with the patchy regions (demonstrating $R_f = 0$) over the entire cell surface have to be calculated. The fluorescence levels associated with these two types of regions were determined on many cells by measuring the fluorescence with the FPR apparatus under nonbleaching conditions and calibrated according to the respective proportions of the cell surface areas which they covered; the latter were estimated from fluorescence micrographs (for details of the calibration, see legend to Table III). Employing these values, the average fraction of fused RSVE on the cells could be derived (Table

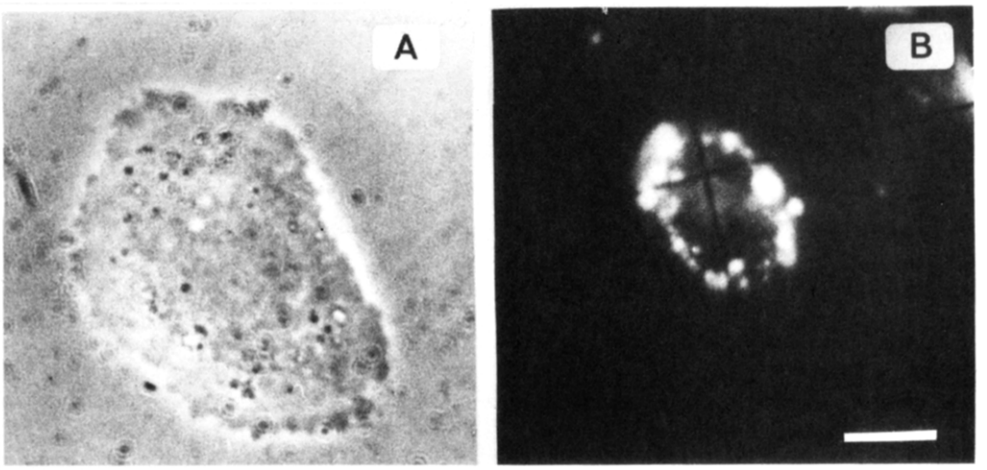

FIGURE 3: HeLa cells fused with RSVE containing *N*-NBD-PE. HeLa cells were incubated with RSVE containing *N*-NBD-PE under fusion-promoting conditions, as described under Experimental Procedures. Photographs were taken as described in Figure 2. Bar, 5 μm. (A) Phase contrast. (B) Fluorescence of the cell shown in (A). The focus was adjusted on the perinuclear fluorescent aggregates; the weak homogeneous fluorescence observed over the other cell regions was therefore somewhat out of focus.

Table III: Measurement of the Fusion of RSVE with Several Cell Types by FPR[a]

cell type	treatment	D ($cm^2/s \times 10^9$)	R_f	R_f/R_f^0	$F_H/(F_H + F_P)$	$(R_f/R_f^0)_{av}$
lymphocytes	4 °C	≤0.005	0.06 ± 0.03	0.08 ± 0.04		
	4 °C + 37 °C	9.0 ± 0.7	0.55 ± 0.04	0.63 ± 0.08	0.30 ± 0.1	0.19 ± 0.09
hepatoma	4 °C	≤0.005	0.04 ● 0.03	0.05 ± 0.04		
	4 °C + 37 °C	3.8 ● 0.4	0.59 ● 0.04	0.70 ± 0.08	0.42 ± 0.11	0.29 ● 0.11
HeLa	4 °C	≤0.005	0.03 ± 0.04	0.04 ± 0.05		
	4 °C + 37 °C	3.5 ± 0.6	0.41 ± 0.04	0.48 ± 0.07	0.32 ± 0.09	0.15 ± 0.06
BHK-21	4 °C	≤0.005	0.03 ± 0.04	0.03 ± 0.05		
	4 °C + 37 °C	5.4 ± 0.5	0.54 ± 0.05	0.63 ± 0.08	0.69 ± 0.19	0.43 ± 0.17

[a] RSVE containing *N*-NBD-PE were adsorbed to the cells at 4 °C, or fused with them by a further incubation at 37 °C, as described under Experimental Procedures. The FPR experiments were performed at 22 °C in the appropriate HEPES-buffered incubation media. The values in the table are means ± SE of 20–30 measurements in each case. The R_f^0 values (R_f of *N*-NBD-PE incorporated directly into the cell membrane) were 0.86 ± 0.04, 0.85 ● 0.04, 0.86 ± 0.05, and 0.86 ± 0.05 for lymphocytes, hepatoma, HeLa, and BHK-21 cells, respectively. The D values of the directly incorporated *N*-NBD-PE were in all cases within 10% of the values observed after fusion of RSVE containing *N*-NBD-PE with the cells. F_H and F_P designate the fluorescence intensity associated with the homogeneously labeled and with the patchy regions, respectively; the fluorescence levels in these regions were measured by the FPR apparatus under nonbleaching conditions and normalized according to the relative size of these areas on the cell surface. $F_H/(F_H + F_P)$ yields the fraction of fluorescence in the homogeneously labeled regions, where R_f/R_f^0 was determined (in the patchy regions, $R_f = 0$). $(R_f/R_f^0)_{av} = (R_f/R_f^0)F_H/(F_H + F_P)$ is the fraction of the fused RSVE averaged over the entire cell surface.

III). Contrary to the situation with human erythrocytes, where almost all of the cell-associated RSVE were found to be fused with the cells after incubation at 37 °C, in all the other cell types significant fractions of the cell-associated RSVE have not fused with the cellular plasma membrane. The average value of fused RSVE observed with rat hepatoma cells after 37 °C incubation (Table III) is in the same range reported for the fusion of RSVE with the same cells as measured by fluorescence dequenching (Chejanovsky & Loyter, 1985).

In the case of mouse spleen lymphocytes, we have also compared the estimation of RSVE–cell fusion by FPR with that obtained by DTT treatment; the latter treatment failed to remove over half of the adsorbed RSVE from the other cell types in the absence of fusion (e.g., after incubation at 4 °C) and thus could not serve to evaluate fusion with these cells. The results obtained from the DTT treatment (performed as described in Table II) for RSVE–lymphocyte fusion (a fused fraction of 0.22 ± 0.08 after 60 min at 37 °C) were very similar to those obtained by FPR experiments to quantitate viral envelope–cell fusion.

Distribution of RSVE–Cell Fusion over Cell Populations. Individual cells within cell population studied differ in the amount of viral particles that they bind and may exhibit different degrees of fusion with cell-associated viral envelopes. A major advantage of the application of FPR to measure viral envelope–cell fusion is that the FPR experiments are performed on single cells (one cell at a time) and can therefore provide information on the distribution of viral envelope–cell fusion within the cell population. The results of such FPR experiments performed on human erythrocytes, BHK-21 cells, and HeLa cells are depicted in Figure 4. In the case of the latter two cell types (but not for human erythrocytes, where similar R_f values are obtained on all cell regions), the results shown are those obtained on the cell regions exhibiting homogeneous fluorescence. This representation was preferred to showing the fraction of fused RSVE averaged over the entire cell surface, since the latter is an average value calculated from measurements on 20–30 cells (Table III) and therefore does not reflect the distribution of RSVE–cell fusion within the cell population. The values for BHK-21 and HeLa cells in Figure 4 are therefore higher than the fraction of fused RSVE averaged over the cell, which may be obtained by multiplying R_f/R_f^0 by the appropriate $F_H/(F_H + F_P)$ values (given in Table III).

The distribution of the R_f/R_f^0 values (Figure 4) appeared to be symmetric and suggests the existence of heterogeneity in the cell population regarding fusion with RSVE. It is worthwhile pointing out that in the case of human erythrocytes (which were the most susceptible to fusion with RSVE), all of the individual cells measured exhibited significant levels of fusion with RSVE (the lowest R_f/R_f^0 values found were around 0.50). On the other hand, in the case of BHK-21 and HeLa cells, which exhibited lower fusion levels with the fluorescent RSVE, the existence of cells which did not fuse

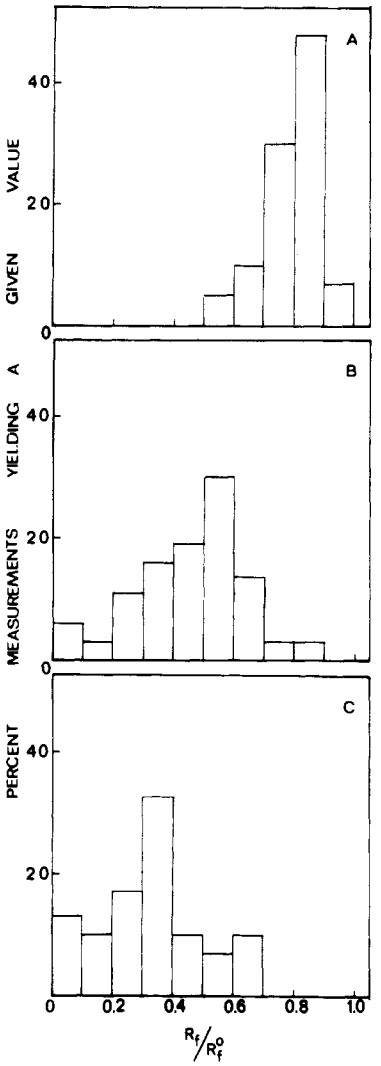

FIGURE 4: Distribution of RSVE–cell fusion over cell populations. Human erythrocytes, BHK-21 cells, and HeLa cells were incubated with RSVE containing *N*-NBD-PE under fusion-promoting conditions and prepared for the FPR experiments as described under Experimental Procedures. In the case of BHK-21 and HeLa cells, the FPR measurements were performed on homogeneously labeled regions. In parallel, the mobile fraction of *N*-NBD-PE incorporated directly into the cell membrane (R_f^0) was determined (given in Table III). A total of 50 cells were measured in each case. (A) Distribution of R_f/R_f^0 in the human erythrocyte population. (B) Distribution of R_f/R_f^0 in BHK-21 cells. (C) Distribution of R_f/R_f^0 in HeLa cells.

at all with RSVE ($R_f/R_f^0 \approx 0$) was detected (Figure 4).

Discussion

Principle and Advantages of the Use of FPR To Quantitate Viral Envelope–Cell Fusion. In the current paper, we have developed a sensitive method based on FPR to quantitate the fusion of viral envelopes with cells. The method is based on the ability of a fluorescent lipid probe originally incorporated into the viral envelope to diffuse laterally over the cell surface following fusion (detailed under Results). These features were clearly verified in the FPR experiments using RSVE containing *N*-NBD-PE with all the cell types investigated: negligible R_f values were observed when the RSVE were merely attached to the cells, and significant increases in the R_f values were obtained under fusion-promoting conditions (Figure 1; Tables I and III). The similarity between the *D* values observed for RSVE-incorporated *N*-NBD-PE after fusion and the *D* values for *N*-NBD-PE incorporated directly into the cell membranes (Tables I and III), as well as the appearance of a homogeneous fluorescence pattern under fusion-promoting conditions (Figure 2), provides further support for the interpretation described above.

The measurement of viral envelope–cell fusion by FPR has several advantages over other methods to evaluate fusion. First, the FPR measurements can clearly distinguish between fused and internalized viral envelopes, since fluorescent probes in the latter are immobile in the FPR experiments (Schlessinger et al., 1977; see legend to Table III). This feature is lacking in other methods to quantitate fusion: methods based on chemical or enzymatic removal of adsorbed viral envelopes from the cell surface measure both fused and internalized viral particles, and methods based on fluorescence dequenching or energy transfer can be complicated by signals arising from the degradation of internalized viral envelopes (Chejanovsky & Loyter, 1985) or from fusion between internalized viral particles and endosomal membranes (White et al., 1983; Hoekstra et al., 1984). Second, due to the ability to perform FPR measurements on single cells and on relatively small regions on the cell surface, the FPR method enables both the "mapping" of single cells regarding fusion with viral envelopes and determination of the distribution of the fusion level within the cell population. The "chemical removal" and spectroscopic methods, which are based on measurements performed on a large mass of cells, do not allow these possibilities and yield only average values.

Measurement of RSVE Fusion with Human Erythrocytes. In order to establish the FPR measurements as a method to quantitate viral envelope–cell fusion, it was required to demonstrate that fusion is the only process contributing to the measurement and that other established methods to evaluate fusion yield results similar to those of the FPR experiments. We have chosen to conduct these experiments with human erythrocytes, since they lack endocytic activity which may complicate the comparison between the methods.

The measurements were performed using RSVE containing 0.6–0.8 mol % *N*-NBD-PE. *N*-NBD-PE was chosen due to the insignificant levels of its exchange with cell membranes (Struck & Pagano, 1980). The RSVE were as active as native Sendai virions in hemolysis and in envelope–cell fusion (Table II; see text), in accord with a recent report (Citovsky et al., 1985) that RSVE prepared by this procedure have properties similar to those of intact Sendai virions regarding fusion with cells and with liposomes of various compositions. The lateral mobilization of the RSVE-incorporated *N*-NBD-PE on the erythrocyte membrane following incubation at 37 °C appears to be due to fusion and not due to phospholipid exchange, since the RSVE-incorporated *N*-NBD-PE from nonfusogenic (trypsinized or PMSF treated) RSVE did not become mobile on the erythrocyte surface under identical conditions (Table I). The latter results also demonstrate that traces of Triton X-100 are not involved either in *N*-NBD-PE mobilization on the cell surface or in the hemolytic activity of the RSVE, since otherwise those phenomena should occur also with trypsinized or PMSF-treated RSVE.

Comparison of the FPR data on RSVE–erythrocyte fusion with other independent methods (chemical removal of adsorbed RSVE by DTT and fluorescence dequenching of R_{18}) under identical experimental conditions shows that essentially identical results were obtained by all methods (Table II), further demonstrating that the FPR experiments indeed provide a quantitative measure for viral envelope–cell fusion.

An important point that should be emphasized is that the laser beam in the FPR experiment is focused on the cell surface and the contribution of fluorescence from free viral envelopes

in the solution is negligible. Thus, the FPR experiment measures the fraction of fused RSVE from the population of the *cell-associated* (fused, adsorbed, or internalized) RSVE. On the other hand, spectroscopic measurements of viral envelope–cell fusion (such as fluorescence dequenching) are performed on suspensions containing cells and viral particles and include a contribution from free viral envelopes. For this reason, in order to enable direct comparison of the results obtained by such methods and by FPR (Table II), care has to be taken to wash away free viral particles—not only after the incubation at 4 °C but also after the 37 °C incubation—since additional viral particles are released due to the viral neuraminidase activity, which proceeds at approximately the same rate as the fusion reaction at 37 °C (Micklem et al., 1985). In the absence of this washing step, the fraction of fused viral particles measured by the spectroscopic techniques decreases due to the presence of free viral particles. This is most likely the reason for the lower fraction (around 0.4–0.5) of Sendai virions fused with human erythrocyte ghosts determined previously by dequenching of R_{18} fluorescence and by electron spin resonance (Hoekstra et al., 1984; Maeda et al., 1981).

Measurements of RSVE Fusion with Mammalian Nucleated Cells. Unlike human erythrocytes, the fluorescence pattern observed on the nucleated cells employed in the present experiments following incubation at 37 °C with RSVE containing *N*-NBD-PE was rather heterogeneous (Figure 3). This is to be expected in view of their endocytic activities, which result in the internalization of part of the cell-associated RSVE during the 37 °C incubation. This situation emphasizes the advantages of the FPR method for the quantitation of viral envelope–cell fusion, since internalized viral particles do not contribute to the lateral mobility measurements, and since the ability to focus the laser beam on specific regions of the cell surface enables separate determinations of viral envelope–cell fusion on different cell regions. However, it should be emphasized that since endocytic vesicles can migrate away from the initial endocytosis sites, the ratio of fused to adsorbed and internalized RSVE on a specific region reflects the situation after the occurrence of fusion, endocytosis, and migration events. The results of such studies performed with RSVE containing *N*-NBD-PE on several cell types (Table III) demonstrate that the fraction of cell-associated RSVE which have fused with the cell varies depending on the location on the cell surface. Such conclusions could not be drawn from spectroscopic methods (e.g., fluorescence dequenching or energy transfer), which measure the average fusion level on the bulk of cells in suspension. The average values obtained by the latter methods cannot be compared directly with the region-specific values determined by FPR; if such a comparison is desired, it has to be made with the values of the fused RSVE fraction averaged over the entire cell surface (Table III). Such a comparison can be made in the case of the rat hepatoma cells, whose fusion with RSVE prepared exactly as in the present studies has been measured by *N*-NBD-PE fluorescence dequenching (Chejanovsky & Loyter, 1985). The latter study reported 55% dequenching, of which about half was contributed by endocytosed RSVE; thus, the fused RSVE fraction in this study was around 0.25–0.30, in accord with the calculation of the averaged fraction of fused RSVE from the FPR measurements (Table III).

The data in Table III indicate that the fraction of fused RSVE depends on the properties of the host cells. This dependence stems, most likely, from different levels on each cell type of the three pathways open to viral particles adsorbed to the cells, namely, fusion with the plasma membrane, endocytosis, or detachment from the cell due to the viral neuraminidase activity. Clear differences between the various cell types studied are also evident in the distribution of RSVE–cell fusion among the cell population. Thus, the data in Figure 4 demonstrate that individual cells within each cell population have different susceptibilities to fusion with RSVE and that each cell type has a characteristic mean value of the fraction of cell-associated RSVE that undergo fusion. The determination of viral envelope–cell fusion by FPR may therefore be useful for distinguishing heterogeneity in the capability of fusion with viral envelopes within a given cell population (e.g., the existence of two groups of cells which fuse with viral envelopes to different degrees).

Finally, it should be noted that the application of FPR to quantitate viral envelope–cell fusion is not limited to *N*-NBD-PE as a probe; essentially any fluorescent lipid probe that does not undergo exchange between the viral envelope and the cellular plasma membrane may be used, provided that its fluorescence is not quenched in the viral membrane at the concentration range employed. Unfortunately, this was not the case with R_{18}, whose fluorescence was significantly quenched in the envelope of Sendai virions at the concentration range which enabled FPR studies (B. Aroeti and Y. I. Henis, unpublished results). However, future development of fluorescent probes that can be incorporated directly into the membranes of native virions (like R_{18}) and which are not quenched in the viral envelope will enlarge the scope of the FPR experiments to encompass the fusion of native virions with cells.

Registry No. *N*-NBD-Pe, 64205-19-2.

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Low Concentrations of Bile Salts Increase the Rate of Spontaneous Phospholipid Transfer between Vesicles†

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ABSTRACT: The rate of 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine (P-C_{12}-NBD-PC) transfer between dioleoylphosphatidylcholine vesicles was measured by a technique based on resonance energy transfer between P-C_{12}-NBD-PC and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine [Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720–1726]. Addition of bile salts at concentrations below their critical micelle concentrations increased the rate of spontaneous P-C_{12}-NBD-PC transfer without disrupting the vesicles. The effectiveness in increasing the transfer rate was dependent on the structure of the bile salt. In general, conjugated bile salts were more effective than unconjugated, and mono- and dihydroxy bile salts were more effective than trihydroxy. The kinetics of intervesicular P-C_{12}-NBD-PC transfer in the presence of cholate were found to be consistent with a mass action kinetic model based on the premise that bile salts bind to the vesicles, alter the dissociation and/or association rate constants for phospholipid monomer–vesicle interaction, and increase the rate of phospholipid transfer via the diffusion of soluble monomers through the aqueous phase. Temperature dependence studies indicated that cholate binding to vesicles is an entropy-driven process and that cholate binding lowers the free energy of activation for phospholipid monomer–vesicle dissociation by producing compensatory decreases in both the enthalpy and entropy of activation.

Phospholipids spontaneously transfer between membranes by the diffusion of soluble monomers through the aqueous bulk phase (Duckwitz-Peterlein et al., 1977; Roseman & Thompson, 1980; McLean & Phillips, 1981, 1984; Nichols & Pagano, 1981, 1982; Massey et al., 1982a,b; Decuyper et al., 1983; Ferrell et al., 1985). This paper demonstrates that concentrations of bile salts below their critical micelle concentrations bind to membranes and increase the spontaneous rate of intervesicular phospholipid transfer. The simplest explanation for this effect is that bile salts increase the rate of spontaneous transfer by partitioning into the membranes and altering the dissociation and/or association rate constants for phospholipid monomer–vesicle interaction. This paper presents a model based on this premise using the principles of mass action kinetics and demonstrates that this model can predict the initial

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