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An Efficient Method for Introducing Defined Lipids into the Plasma Membrane of Mammalian Cells

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ABSTRACT An efficient method has been devised to introduce lipid molecules into the plasma membrane of mammalian cells. This method has been applied to fuse lipid vesicles with the apical plasma membrane of Madin-Darby canine kidney cells. The cells were infected with fowl plague or influenza N virus. 4 h after infection, the hemagglutinin (HA) spike glycoprotein of the virus was present in the apical plasma membrane of the cells. Lipid vesicles containing egg phosphatidylcholine, cholesterol, and an HA receptor (ganglioside) were then bound to the cells at 0°C. More than 85% of the vesicles were released by external neuraminidase at 0°C or by simply warming the cells to 37°C for 10 s, probably because of the action of the viral neuraminidase at the cell surface. However, when the cells were warmed to 37°C in a pH 5.3 medium for 30 s, 50% of the bound vesicles could no longer be released by external neuraminidase. This only occurred when the HA protein had been cleaved into its HA_1 and HA_2 subunits. When we used influenza N virus, whose HA is not cleaved in Madin-Darby canine kidney cells, cleavage with external trypsin was required. The fact that the HA protein has fusogenic properties at low pH only in its cleaved form suggests that fusion of the vesicles with the plasma membrane had taken place. Further confirmation for fusion was obtained using an assay based on the decrease of energy transfer between two fluorescent phospholipids in a vesicle upon fusion of the vesicle with the plasma membrane (Struck, D. K., D. Hoekstra, and R. E. Pagano. 1981. *Biochemistry*, 20:4093–4099).

The interaction between liposomes and living cells has been widely studied (11) and one aim has been to develop methods of fusing liposomes with the plasma membrane. If this were possible, one could introduce the liposome contents into the cell and implant lipid molecules into the plasma membrane to study their fate and effect on the cell. So far, controlled fusion of liposomes with the plasma membrane has not been achieved. Conditions for inducing liposomes to fuse with one another (see references 35 and 44) or with intracellular membranes (6, 40) have been devised, but difficulties have been encountered in adapting these to fuse liposomes with the cell-surface membrane. The contents of the liposomes have been reported to reach the cytoplasm, but even under the best conditions the amounts introduced have been exceedingly small. Liposomes added to cells seem either to adhere to the cell surface or to become endocytosed (7, 8, 10, 39, 44, 45), but the mechanism by which contents enter the cytoplasm has either not been elucidated or has been shown not to be simple fusion (26, 43, 46, 57).

A number of enveloped viruses are known to possess a fusogenic activity. Fusion of paramyxoviruses into the plasma membrane has been convincingly demonstrated (4, 36). Togaviruses (13, 49, 52, 54), orthomyxoviruses (16, 24, 28, 33, 56, 59), and rhabdoviruses (29, 32, 56) have a fusogenic activity, which is expressed only at low pH. The latter viruses enter cells by endocytosis and then gain entry into the cytoplasm by fusion in an acid intracellular compartment (13, 28, 29, 59). Fusion of these viruses can also be induced with the plasma membrane of tissue culture cells (28, 29, 54, 56) or that of erythrocytes (16, 22, 25, 32, 33, 47–49, 58) simply by lowering the pH of the medium. Also, liposomes can be used as target membranes (13, 24, 52, 55). The fusion is mediated by the viral spike glycoproteins. In the case of influenza virus, the hemagglutinin (HA)[1] molecule has been identified as the fusion factor (16, 17, 24, 33, 41, 53, 56). The HA protein is

[1] *Abbreviations used in this paper:* FPV, influenza A strain fowl plague virus; G_{Dla}, IV^3 NeuAc, II^3 Neu Ac-$GgOse_4Cer$; G_{Dlb}, II^3 $(NeuAc)_2$-$GgOse_4Cer$; G_{Tlb}, IV^3 NeuAc II^3 $(NeuAc)_2$-$GgOse_4Cer$; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) dioleoyl phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine-rhodamine-B-sulfonyl) dioleoyl phosphatidylethanolamine; PC, phosphatidylcholine.

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synthesized as a precursor that has to be proteolytically cleaved to yield the fusogenic form consisting of two disulfide-bonded glycosylated polypeptides, HA_1 and HA_2 (20, 21, 42).

In this study, we exploited the fusogenic properties of the influenza virus HA protein in an attempt to develop a method of fusing lipid vesicles with the plasma membrane of living cells. We introduced the viral HA protein into the plasma membrane by infecting the cells with influenza virus (37). Lipid vesicles containing a ganglioside receptor for the HA protein (2) were shown to bind to the infected cells, and these could be induced to fuse with the plasma membrane at low pH.

MATERIALS AND METHODS

Preparation of Lipid Vesicles: As a standard procedure (essentially according to reference 12) 1 μmol of egg phosphatidylcholine (PC), 1 μmol of cholesterol, 100 nmol of the ganglioside G_{D1a}, 8×10^5 cpm of cholesteryl [^{14}C]-oleate, and 6 mg octyl-β-D-glucoside were taken from the stock solutions in $CHCl_3/CH_3OH$ (2:1, vol/vol), and mixed and dried under a stream of N_2 at 37°C. The lipid mixture was then suspended in 2 ml of PBS (Dulbecco's formulation) without Ca^{2+} or Mg^{2+} and dialyzed at 4°C for 36 h against three changes of 0.8 liter of Ca^{2+}-, Mg^{2+}-free PBS containing 2 mM sodium azide. After Sepharose CL-2B gel filtration (15), the elution pattern of the vesicles showed two peaks. One peak contained 30–40% of the lipid radioactivity and eluted with the void volume, and 60–70% eluted with the included volume. The void volume peak, which probably contained vesicle aggregates, could be removed selectively from the vesicle suspension by centrifugation at 4°C for 60 min at 100,000 g_{max} (1) in a 75Ti rotor (Beckman Instruments, Inc., Palo Alto, CA), and the yield of vesicles in the supernatant varied from 50 to 70%. Electron microscopy of negatively stained vesicles (12) in the resulting preparation showed a homogeneous population of 35-nm-diam vesicles, and lipid analysis showed no enrichment of cholesteryl [^{14}C]oleate, cholesterol, or any of the fluorescent lipids (see below) relative to PC in this fraction, in contrast to what has been reported for sonicated vesicles (38). This was irrespective of the lipid composition used. The centrifugation was performed <3 h before addition of the vesicles to cells. The resulting suspension was stable for at least 8 h, since re-centrifugation after 8 h did not sediment any more lipid radioactivity. After the centrifugation, 1.5 ml of the supernatant was taken, kept on ice, and, immediately before addition to the cells, diluted to the desired vesicle concentration with binding medium (see below). Vesicles of this composition, prepared in this way, were termed "standard vesicles."

Cells: MDCK cells were grown at 37°C in 5% CO_2 and routinely passaged twice a week in 75-cm^2 plastic bottles (Falcon Plastics, Oxnard, CA, or Nunc, Algade, Denmark) in Eagle's minimal essential medium with Earle's salts (Earle's MEM) supplemented with 10 mM HEPES, pH 7.3, 5% (vol/vol) fetal calf serum, penicillin (110 U/ml), streptomycin (100 μg/ml), and fungizone (0.025 μg/ml). Cells were released from the plastic using trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/liter in Puck's saline). For all experiments the released cells were plated at a density of 8×10^4 cells/cm^2 in 40- or 93-mm-diam plastic dishes and grown as described above for 48 h, at which time the cells were confluent.

Viruses: The influenza A strains fowl plague (A/FPV/Rostock; FPV) and influenza N (A/chick/Germany/49) were obtained from H.-D. Klenk (Institut für Virologie, Universität Giessen) (20). Virus stocks were prepared, and plaque titrations for FPV were performed as described previously (28). Influenza N virus was plaque titrated on MDCK cells in the presence of 10 μg/ml trypsin (20) and without BSA in the overlay. In the absence of trypsin no plaques were detected on dishes infected with an amount of influenza N virus, which with trypsin gave rise to 4×10^5 plaques.

Infections: Confluent 2-d-old monolayers of MDCK cells were infected with 20 plaque-forming units of FPV or influenza N virus per cell suspended in 0.2 ml (40-mm dishes) or 1.3 ml (93-mm dishes) of Earle's MEM supplemented with 10 mM HEPES, penicillin, streptomycin, and 0.2% BSA. The viruses were allowed to absorb for 1 h at 37°C, after which the inoculum was replaced by the medium containing fetal calf serum.

Binding of Lipid Vesicles to Virus-infected Cells: After infection with FPV or with influenza N virus, the 40-mm petri dishes (for 93-mm petri dishes, all volumes were scaled up 6.5 times) were washed with 1 ml ice-cold "binding medium," which consisted of Earle's MEM without bicarbonate, supplemented with 10 mM morpholinopropane sulfonic acid, 10 mM *N*-tris[hydroxymethyl] methyl 2-amino ethane sulfonic acid, 15 mM HEPES, 2 mM NaH_2PO_4, and 0.2% BSA, pH 7.4, and cooled on a metal plate on ice with 1 ml of binding medium. After 15 min the binding medium was taken off and 200 μl of a suspension of standard lipid vesicles (see above) was spread over the cell monolayer. For routine experiments, vesicles containing 2 nmol PC were added per 40-mm dish containing 2×10^6 cells and 100 nmol total cellular phospholipid. The dishes were gently shaken on ice; after 30 min, the unbound vesicles were aspirated and the cell monolayer with the bound vesicles was washed twice with 1 ml of ice-cold binding medium. Subsequently, the cells were solubilized on the dish with 1 ml of 2% SDS in bi-distilled water, preheated to 80°C, and left for 10 min at 20°C, and cell-associated radioactivity was quantitated in a Mark III liquid scintillation spectrometer (Searle Analytic, Des Plaines, IL). Alternatively, the cells were scraped from the plastic at 0°C with a rubber policeman into 3 ml of binding medium and sedimented at 200 g_{max} for 5 min at 4°C, and the cell pellet was solubilized in Triton X-100 containing scintillation fluid (Carl Roth KG, Karlsruhe, Federal Republic of Germany [FRG]) and counted for cell-associated radioactivity.

The HA protein of influenza N virus is not proteolytically cleaved in MDCK cells (30). Cleavage of the HA protein on the cell surface into the HA_1 and the HA_2 subunits was brought about by the addition of trypsin (30). At 4 h after infection, the cells were washed with ice-cold binding medium, allowed to cool in this medium on ice for 15 min, and washed twice with 1 ml of ice-cold PBS. Ice-cold trypsin solution (100 μg/ml) was added in 0.5 ml of PBS to one series of dishes, whereas the control series received only 0.5 ml of PBS. After gentle shaking on ice for 15 min, 50 μl of soybean trypsin inhibitor solution (1 mg/ml) in PBS was added and shaking continued for 5 min. The dishes were then washed with 1 ml of a soybean trypsin inhibitor solution of 100 μg/ml PBS. Under these conditions the trypsin was observed to cleave a minimum of 84% of the influenza N HA into HA_1 and HA_2 (30), whereas under control conditions no cleaved HA was present. After trypsin treatment the lipid vesicle suspension was added to the cells, and subsequent operations to measure binding were identical to those described above.

Low-pH Treatment: After vesicle binding and two washes with 1 ml of ice-cold binding medium as described above, 1.5 ml of prewarmed pH 5.3 medium consisting of bicarbonate-free Earle's MEM supplemented with 20 mM succinate and 0.2% BSA, pH 5.3, was added to the cells, and the petri dish was quickly placed in a 37°C water bath. After 30 s, unless stated otherwise, the dish was placed back onto the ice-cold metal plate and washed twice with 1 ml of ice-cold binding medium. Subsequently, the cells were scraped, pelleted, and solubilized, or solubilized directly for liquid scintillation counting as described above.

Fusion Assay: The resonance energy transfer assay was carried out essentially as described previously by Struck et al. (44).

INCUBATIONS: Vesicles were prepared from 1 μmol egg PC, 1 μmol cholesterol, 0.1 μmol G_{D1a}, and a trace amount of cholesteryl [^{14}C]oleate (8×10^5 cpm), to which had been added 20 nmol *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) dioleoyl phosphatidylethanolamine (N-NBD-PE; 1 mol%) and 20 nmol *N*-(lissamine-rhodamine-B-sulfonyl) dioleoyl phosphatidylethanolamine (N-Rh-PE; 1 mol%), as described above. These vesicles (200 nmol egg PC) were added to a 93-mm-diam petri dish containing cells infected with influenza N virus (3×10^7 cells, 900 nmol total cellular phospholipid) at 5 h after infection and subsequent activation of the HA by trypsin. They were allowed to bind for 30 min at 0°C as they were being shaken. The unbound vesicles were removed and one set of dishes was incubated with the pH 5.3 medium for 30 s at 37°C and washed twice with ice-cold binding medium. A second set of dishes was only washed twice with ice-cold binding medium. In the first experiments the cells were subsequently released from the dish by a wash with Ca^{2+}- and Mg^{2+}-free PBS, followed by an incubation with 5 ml of trypsin-EDTA at 37°C for 10 min, after which they were rapidly cooled to 0°C. The cells from two dishes were combined and sedimented at 4°C for 5 min at 200 g_{max}. In later experiments the cells were scraped on ice from two dishes into 12 ml of ice-cold PBS with a rubber policeman and sedimented at 4°C for 5 min at 200 g_{max}. 3 ml of PBS was added to the cell pellet and the tube was left on ice. Directly before the fluorescence measurement, the cells were resuspended into an ice-cold, 4-ml cuvette, and the measurement was performed immediately at room temperature (20–23°C). It took 2 min to record the spectrum, so that little warming of the sample occurred during the measurement. After the experiments, an aliquot of the sample was counted for lipid radioactivity in order to quantitate the amount of lipid vesicles associated with the cells.

FLUORESCENCE MEASUREMENTS: Steady state emission spectra were obtained by using an MPF-44A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). All samples were excited at 470 nm and the emission was recorded 480–650 nm. The excitation and emission band slits were set to obtain a resolution of 10 nm and the apparatus was in the ratio mode. Spectra were recorded at 120 nm/min.

CALCULATIONS: The efficiency of energy transfer (E) in a sample was calculated from the relative intensities of the N-NBD-PE emission at 530 nm

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in that sample (F) and the total N-NBD-PE emission in the sample (F_0) after abolishing energy transfer by the addition of Triton X-100 (1% final concentration); the relationship between the parameters was: $E = 1 - (F/F_0)$ (9, 44). F_0 had to be corrected for sample dilution by the addition of Triton X-100 (5%) and the quenching effect of Triton X-100 on the quantum yield of N-NBD-PE (−39%; Figs. 5, *a* and *b*). Because in our studies the N-Rh-PE emission signal during complete energy transfer was of about the same size as the emission signal of total N-NBD-PE in that sample (calculated from the N-NBD-PE emission after Triton X-100 addition and the necessary correction; see above and Fig. 5, *b* and *c*), the efficiency of energy transfer could also be calculated directly from the relative intensities of the N-NBD-PE emission at 530 nm and the N-Rh-PE emission at 590 nm. The two methods were in good agreement.

Miscellaneous Techniques: Cell-cell fusion was measured as described (53) after Giemsa staining of the cell monolayer followed by light microscopy. Electron microscopy was performed as described previously (28). Fluorescence light microscopy was performed on a Zeiss photomicroscope III equipped with a Planapo 63 oil immersion objective. Glass coverslips with a monolayer of cells were mounted in 90% (vol/vol) glycerol in PBS and viewed immediately.

Sources of Reagents: N-NBD-PE and N-Rh-PE were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Egg PC, phosphatidylethanolamine (PE), stearylamine, cholesterol, buffer salts, and *Clostridium perfringens* neuraminidase (1 IU/mg protein) were obtained from Sigma Chemical Co. (St. Louis, MO). Dioleoyl phosphatidic acid was a gift from G. Warren in this laboratory. G_{D1a}, G_{D1b}, and G_{T1b} (protein-free and at least 95% pure) were kindly given to us by L. Svennerholm (University of Göteborg, Sweden). G_{D1a} was also a kind gift of H. Rauvala (University of Helsinki, Finland). G_{T1b} was obtained commercially from Supelco, Inc. (Bellefonte, PA); cholesteryl [1-^{14}C]oleate (50.8 Ci/mol) was purchased from Amersham International (Amersham, UK). Octyl-β-D-glucoside was from Calbiochem-Behring Corp. (La Jolla, CA). Trypsin-L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone and soybean trypsin inhibitor were obtained from Millipore Corp. (Freehold, NJ). ^{125}I protein A from *Staphylococcus aureus* (100 Ci/g) was obtained from New England Nuclear (Boston, MA). Cell culture media and reagents were purchased from Gibco Biocult (Glasgow, UK). FCS and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid were from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany [FRG]). Sepharose CL-2B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Chemicals and solvents were of analytical grade and obtained from Merck (Darmstadt, FRG).

RESULTS

Binding of Lipid Vesicles to Infected Cells at 0°C

To examine the binding of lipid vesicles to MDCK cells that had been infected with FPV, at different times after infection, a 40-mm petri dish with a monolayer of infected cells was cooled to 0°C and standard lipid vesicles (2 nmol PC) were added as described in Materials and Methods. The lipid vesicles contained 5 mol% of the ganglioside G_{D1a}, which has been reported to act as a receptor for FPV (2), and a trace amount of cholesteryl [^{14}C]oleate was incorporated as a marker for the vesicles because it does not spontaneously exchange between membranes (38). When standard vesicles with or without the ganglioside G_{D1a} were incubated with uninfected cells, binding was 2.5% of the added vesicles if after two washes the cell monolayer was directly solubilized with SDS. Binding was only 0.1% when the cells were scraped and washed by centrifugation, and the pellet was solubilized for scintillation counting (for details see Materials and Methods). Similar binding values were obtained with standard vesicles lacking G_{D1a} added to cells infected with FPV.

A specific increase in the binding of the G_{D1a}-containing vesicles was observed in cells that had been infected with FPV for >3 h (Fig. 1). Binding was loose and had to be assayed by SDS solubilization of the washed monolayer. Only background levels were measured if the cells were released by scraping and washed by centrifugation. The specific binding of the vesicles to the cell could be stabilized by a brief

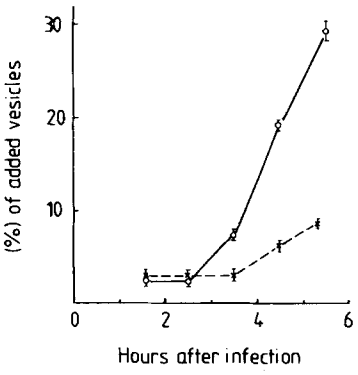

FIGURE 1 Binding of standard lipid vesicles (G_{D1a} - PC - cholesterol) to virus-infected cells at different times after infection. 40-mm petri dishes with MDCK cells were infected with FPV (×) or influenza N virus (○) (20 pfu per cell) and standard vesicles (2 nmol PC, 1.6 × 10^3 cpm) were added to the cells (100 nmol total cellular phospholipid) at different times after infection. After binding for 30 min at 0°C, the cell monolayer was washed twice with ice-cold binding medium, directly solubilized with 2% SDS and counted for cell-associated radioactivity as described under Materials and Methods. Binding is expressed as percentage of the amount of added vesicles. In all graphs each point represents the mean of a duplicate measurement. The error bar indicates the difference between the two measurements.

TABLE I

*Comparison of Three Ganglioside Species in Their Efficiency to Promote Lipid Vesicle-Cell Association (After Low-pH Treatment)**

Ganglioside	Association to MDCK cells infected with FPV	Association to MDCK cells infected with Influenza N virus
	× 10^{-2} nmol PC	× 10^{-2} nmol PC
None	3.5 ± 0.8	2.7 ± 1.0
G_{D1b}	3.6 ± 0.6	2.4 ± 0.0
G_{D1a}	9.6 ± 0.8	27.9 ± 3.0
G_{T1b}	6.0 ± 0.1	35.7 ± 0.1

* Standard vesicles (3 nmol PC), in which G_{D1a} was replaced by an identical amount (5 mol%) of the ganglioside mentioned in the first column of the table, were added to MDCK cells (40-mm dishes) that had been infected with the appropriate virus 4 h before. The influenza N virus-infected cells had been treated with trypsin before vesicle binding (see Materials and Methods). After binding for 30 min at 0°C, the cells were directly subjected to a low-pH step and processed for scintillation counting, as described in the legend to Fig. 1 and in Materials and Methods. The individual numbers are the mean of two independent experiments in duplicate ± SD.

treatment (30 s) with pH 5.3 medium at 37°C before release of the cells by scraping (Table I). The stabilized binding is due to fusion of the vesicles with the plasma membrane, as we will show later.

More G_{D1a}-containing vesicles could be bound to cells infected with influenza N virus. At 5.5 h after infection, ~30% of the added vesicles were bound (Fig. 1). The binding was also more stable; the same results were obtained when the cells were scraped and washed by centrifugation, as after SDS solubilization of washed monolayers.

To optimize the conditions for the fusion procedure, we compared three different gangliosides for their ability to promote vesicle-cell association after low-pH treatment in cells infected either with FPV or with influenza N virus (Table I). G_{D1a} and G_{T1b} were equally efficient, FPV-infected cells having a preference for G_{D1a} and cells infected with influenza N virus for G_{T1b}. In contrast, G_{D1b} did not promote association after low-pH treatment as compared with control vesicles without ganglioside. The low-pH incubation in Table I was applied to stabilize the binding of vesicles to FPV-infected cells, as mentioned above. However, the specificity of the binding to cells infected with influenza N virus was confirmed by omit-

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ting the low-pH step before measuring binding (not shown).

On the basis of these findings, cells infected with influenza N virus were used in the following experiments. Specific binding of ganglioside-containing vesicles was four to six times higher and more stable than the binding to cells infected with FPV. Time points between 4 and 5 after infection were selected as optimal because, at this time, significant binding of the lipid vesicles could be demonstrated, but virus budding from the cell surface had not yet started (51; and unpublished results). The ganglioside G_{D1a} was chosen as the HA receptor. Binding of such standard vesicles to cells infected with influenza N virus showed saturation kinetics with increasing vesicle concentration; maximally, ~7 nmol of vesicle PC could be bound to 40-mm petri dishes (2×10^6 cells, 100 nmol of total cellular phospholipid) at 5 h after infection. In the subsequent experiments, a limited amount (2 nmol) of vesicle PC was routinely added to 100 nmol of total cellular phospholipid.

The time dependence of vesicle-cell association is shown in Fig. 2. When standard vesicles were allowed to bind to MDCK cells, 4 h after infection with influenza N virus, 24% of the vesicles were cell associated after 60 min. The level of binding varied between experiments, but the variation in any one experiment was very low (Figs. 1 and 2). Binding was nearly maximal after 30 min, and this time was therefore selected for further experiments.

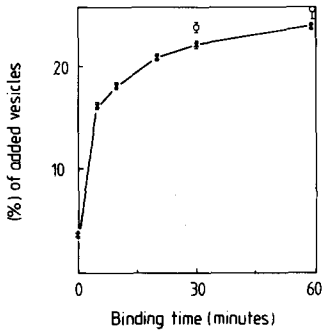

FIGURE 2 Time dependence of vesicle-cell association. At 4 h after the infection of 40-mm petri dishes of MDCK cells with influenza N virus, standard lipid vesicles (1.9 nmol, 1.5×10^3 cpm) were added to the cells and allowed to bind at 0°C. After different time intervals unbound vesicles were aspirated, the monolayer of cells washed twice with ice-cold binding medium, and solubilized with SDS for scintillation counting. Prior to the addition of the vesicles one set of dishes had been treated with trypsin (×) and a control set incubated in PBS (○), as described under Materials and Methods. Results are expressed as in Fig. 1.

Release of Cell-associated Lipid Vesicles by Neuraminidase

In the first binding experiments, we observed that the cells readily lost the cell-associated radioactivity when the temperature was not strictly maintained at 0°C. The effect of temperature on the stability of vesicle binding to cells infected with influenza N virus was tested (Fig. 3*a*), and the release of cell-associated vesicles by a 37°C incubation turned out to be very rapid indeed: 85% were released within 10 s. The vesicles were also released when the cells with bound vesicles were incubated with a bacterial neuraminidase at 0°C (Fig. 3*b*). The latter assay has been previously used to release influenza virus bound to the plasma membrane (28). Since it is known that, in addition to the HA protein, influenza A virus has another spike glycoprotein, a neuraminidase (see reference 3) that is also present on the apical surface of infected MDCK cells, we tested the influence of a neuraminidase inhibitor, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (31), on the vesicle release. The release of lipid vesicles both by the *C. perfringens* neuraminidase and by the 37°C incubation were partially inhibited by the sialic acid analogue (Table II), which implies that activation of the viral neuraminidase is indeed responsible for the vesicle release induced by an increase in the temperature. The vesicle-cell binding could be increased 50% by performing the binding step in the presence of the neuraminidase inhibitor. This shows that the viral neuraminidase is active also at 0°C, which was already clear from Table II. Also, the presence of EGTA (10 mM) increased binding by 50%; the viral neuraminidase is a Ca^{2+}-dependent enzyme (3).

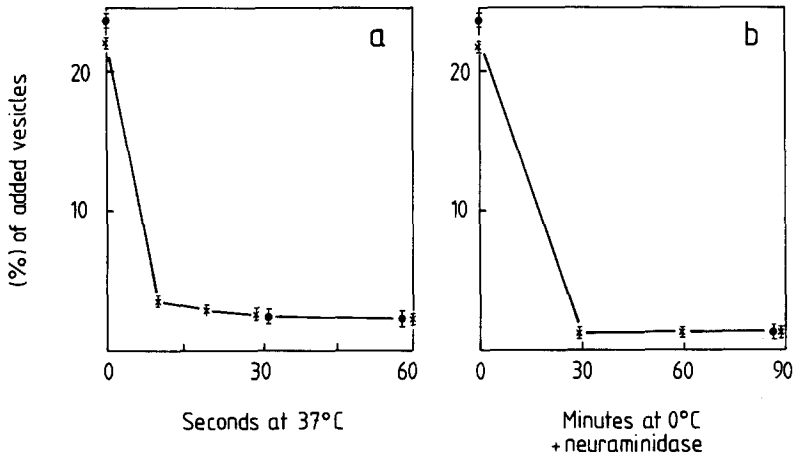

FIGURE 3 Release of cell-associated vesicles by incubation at 37°C or neuraminidase treatment at 0°C. MDCK cells, infected with influenza N virus, were incubated for 30 min at 0°C with standard lipid vesicles as described in the legend to Fig. 2. Unbound vesicles were aspirated and the cells washed twice with ice-cold binding medium. (*a*) Prewarmed binding medium was then added to the cells and the dish placed on a 37°C water bath. After different time intervals dishes were taken from the water bath, the cells washed three times with ice-cold binding medium, solubilized with hot SDS and counted for cell associated counts. (*b*) An ice-cold solution of *C. perfringens* neuraminidase (2 mg in 0.5 ml PBS per 40-mm diameter dish) was added to the cells with the bound vesicles, and the dishes left gently shaking on ice. After different time intervals, cells were taken from the ice, washed three times with ice-cold binding medium and solubilized with hot SDS. Trypsin-treated cells (×), untreated cells (●). For details see Materials and Methods, results are expressed as in Fig. 1.

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Induction of Fusion of the Lipid Vesicles with the Plasma Membrane by Low pH

To test whether the lipid vesicles could be induced to fuse with the plasma membrane by low-pH treatment, two assays were used.

Low pH-induced Neuraminidase Resistance of Vesicle-Cell Association

Infected MDCK cells with bound vesicles (for details, see the legend to Fig. 4) were incubated at 37°C in a medium at pH 5.3, which is near the pH optimum for fusion of influenza N virus after cleavage of its HA protein (16). This treatment resulted in the release of 92% of the vesicle radioactivity from the cells (Table III). However, when the influenza N HA proteins on the cell surface had been cleaved into their HA_1 and HA_2 subunits by the addition of trypsin before the vesicles were bound (see Materials and Methods), low-pH incubation at 37°C produced a very different result: 45–50% of the vesicles were still associated with the cells after 1 min of low-pH treatment and could no longer be removed by either incubation at 37°C or by bacterial neuraminidase (Table III; Fig. 4). The binding step was necessary; no protection against neuraminidase was observed when infected and trypsin-treated cells were directly incubated with vesicles for 1 min at 37°C and pH 5.3. It has been shown previously (28) that influenza virions bound to MDCK cells become resistant to release by bacterial neuraminidase after a low-pH treatment at 37°C and that this is due to fusion of the viral membrane

TABLE II

*Influence of the Neuraminidase Inhibitor 2-Deoxy-2,3-dehydro-N-acetylneuraminic Acid on the Release of Bound Lipid Vesicles**

Treatment	Release	Release + inhibitor (10 mM)
	%	%
90 min, 0°C in PBS	29	19
90 min, 0°C + bacterial neuraminidase	93	65
60 s, 37°C in pH 7.4 binding medium	92	39
60 s, 0°C + 120 s 37°C in pH 7.4 binding medium	94	33

* MDCK cells infected with influenza N virus were treated with trypsin and incubated with standard lipid vesicles (1.9 nmol PC, 1.5 × 10^3 cpm) at 0°C for 30 min (see Materials and Methods). Binding was then measured directly by washing the cells three times with ice-cold binding medium and solubilizing the monolayer with hot SDS for scintillation counting. Other plates were subjected to further treatment, after washing away the unbound vesicles twice with ice-cold binding medium, as indicated in the first column of the table. After this, the radioactivity that remained bound to the cells was measured and the results are expressed as the percentage of cell-associated vesicle counts per minute that was released during the different treatments. Each number is the mean of a duplicate measurement. The difference of the individual measurements from the mean was always <3%.

TABLE III

Cleavage of the HA Protein Decreases Release of the Bound Lipid Vesicles after Low-pH Treatment

	Cell-associated radioactivity* after		
	Binding	Binding + 60 s at 37°C, pH 5.3	Binding + 60 s at 37°C, pH 7.4
HA cleaved	19.1 ± 0.3	8.7 ± 0.6	0.6 ± 0.1
HA uncleaved	20.8 ± 0.6	1.4 ± 0.2	0.5 ± 0.2

* Monolayers of MDCK cells were infected with influenza N virus, treated with trypsin or PBS, and incubated with vesicles as described in Materials and Methods, except that 3.55 nmol PC was added per 40-mm-diam petri dish. After binding of the vesicles and two washes with ice-cold binding medium, some of the dishes were incubated with pH 5.3 and others with pH 7.4 medium at 37°C for 60 s. They were subsequently washed three times with ice-cold binding medium, scraped from the plate, and solubilized for scintillation counting (second and third column) or directly solubilized with hot SDS (first column). All results represent the percentage of total vesicles added to the cells and are followed by the standard deviation (n = 6).

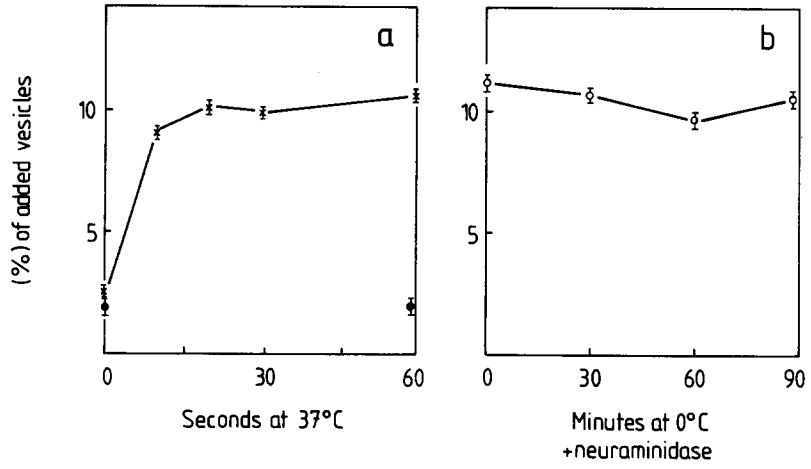

FIGURE 4 Resistance of vesicle-cell association to neuraminidase after low pH treatment. MDCK cells infected with influenza N virus were incubated for 30 min at 0°C with lipid vesicles as described in the legend to Fig. 1. Unbound vesicles were aspirated and the cell monolayer washed twice with ice-cold binding medium. (*a*) Prewarmed pH 5.3 medium was then added to the cells, the dish placed on a 37°C waterbath, and after different time intervals dishes were placed back on ice and washed with ice-cold binding medium. This was followed by addition of prewarmed binding medium and incubation for 30 sec at 37°C. Finally, the cell monolayer was washed twice more with ice-cold binding medium, solubilized with hot SDS, and counted for cell-associated radioactivity. (*b*) In this experiment, fusion was performed for 30 s at pH 5.3. The cells were washed twice, treated with neuraminidase, washed, and solubilized as described in the legend to Fig. 3 *b*. Cells pretreated with tryspin (×, ○), untreated cells (●). For details see Materials and Methods; results are expressed as in Fig. 1.

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with the plasma membrane. In analogy, we propose that the neuraminidase resistance of the vesicle-cell association after the low-pH treatment is caused by the fusion of the vesicle with the plasma membrane. The fusion efficiency would thus be 45–50% of the bound vesicles after 10 s at 37°C in a medium at pH 5.3 (Table III; Fig. 4). We have to subtract from this value the background of unfused vesicles that could not be removed by neuraminidase. This corresponded to 7% of the bound vesicles (measured by omitting the low-pH step before neuraminidase treatment; see Table II and Fig. 3), resulting in a fusion efficiency of 38–43% of the bound vesicles.

The lipid composition of the vesicles influenced the fusion efficiency. As shown by White et al. (55), inclusion of 22 mol% of egg PE into the lipid vesicles containing 28 mol% PC and 50% mol% cholesterol increased the fusion efficiency by a factor of 2. However, we noted a 20-fold increase in nonspecific binding to infected or noninfected cells as compared with our standard PC-cholesterol vesicles. Incorporating extra negative charges into the vesicles (5 mol% dioleoyl phosphatidic acid) also increased both fusion (1.3 times) and nonspecific binding (5 times). Net positive charges (stearylamine 5 mol%) decreased both the fusion efficiency and nonspecific binding by 50% as compared with standard vesicles.

Low pH-induced Loss of Energy Transfer Between Fluorescent Lipid Molecules in the Vesicle

We also used a more direct assay (44) for fusion. This assay is based on the fact that the lipids from the vesicle should intermix with those of the target membrane after fusion and become diluted. For this purpose two fluorescent phospholipids, N-NBD-PE and N-Rh-PE, were incorporated into the standard vesicles. The emission band of the N-NBD-PE overlaps with the excitation band of the N-Rh-PE, and when N-NBD-PE is excited at 450–470 nm, where the excitation spectrum of N-Rh-PE is at a minimum (Fig. 5*a*), the emission energy of the N-NBD-PE is transferred to the N-Rh-PE, resulting in N-Rh-PE emission (Fig. 5*c*). This resonance energy transfer occurs only when the two fluorescent phospholipids are present in the same vesicle (cf. Fig. 5, *b* and *c*) and when the concentration of the probes in the vesicles is high enough. When a mixture of 1 mol% *N*-NBD-PE and 1 mol% N-Rh-PE was incorporated in standard vesicles, excitation at 470 nm resulted in efficient resonance energy transfer (E = 0.95), and when the fluorescent phospholipids were diluted by addition of Triton X-100, the energy transfer was abolished completely and only the direct excitation of the two probes occurred (cf. Fig. 5*c* with Fig. 5*b*).

To perform the assay, standard lipid vesicles containing N-NBD-PE plus N-Rh-PE (1 mol% of each; Fig. 5*c*) were bound to and fused with infected, trypsin-treated cells as described before. About 11 times as many vesicles as were used in previous experiments (Figs. 1–4) were added to the cells in order to increase the fluorescence signal. After the binding step, 10% of the vesicles (20 out of 200 nmol PC) were found to be associated with the cells in the 93-mm petri dish (900 nmol total phospholipid) as compared with 22% when 2 nmol of PC was added to 100 nmol total cellular phospholipid (Fig. 2). The efficiency of fusion as measured with the neuraminidase assay was 47% of the bound vesicles: 4.7% of the added vesicles were resistant to neuraminidase. Before fusion, 1.4% of the bound vesicles were resistant to release by neuraminidase, so in these experiments 71% of the lipid that was cell-associated after the fusion step consisted of fused vesicles. For the fluorescence measurements, the cells were released from the petri dish after the fusion step using trypsin-EDTA at 37°C for 10 min, and the emission spectrum was recorded (Fig. 6*a*).

A significant loss of energy transfer was observed: 50% of the N-NBD-PE no longer transferred its energy to N-Rh-PE and had therefore been diluted into the plasma membrane. A control experiment was done to exclude the possibility that trypsinization at 37°C was alone responsible for the change in signal and not the low-pH-induced fusion event. Standard vesicles containing 1 mol% of N-Rh-PE and N-NBD-PE (10 nmol PC) were added to a 93-mm-diam petri dish of infected MDCK cells and the cells were trypsinized for 10 min at 37°C. The presence of trypsin-EDTA-released cells (10^7 cells/ml) by itself had no influence on the efficiency of energy transfer in the vesicles. Pretreatment of cells or vesicles with low pH did not cause any change either. We could not test

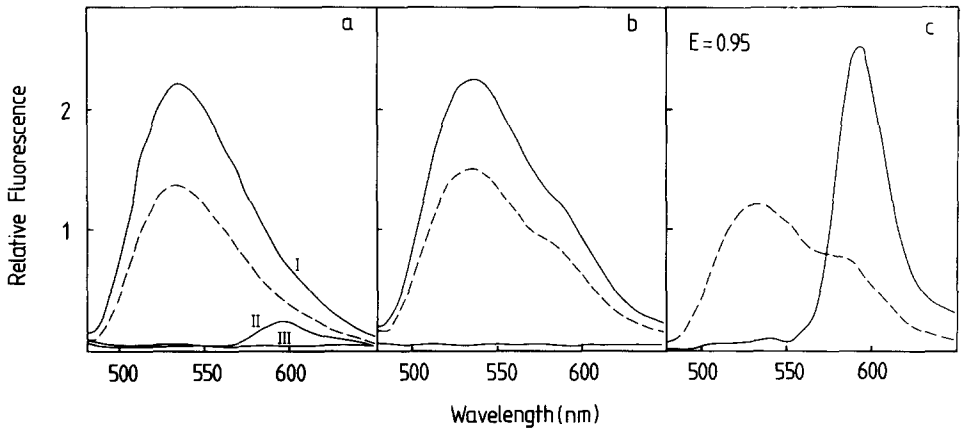

FIGURE 5 Emission spectra of lipid vesicles with fluorescent probes excited at 470 nm. The spectra were recorded in the absence (—) or presence (- - -) of 1% Triton X-100. Standard vesicles (8 μM PC) containing either 1 mol% N-NBD-PE (I) or 1 mol% N-Rh-PE (II) or no fluorescent lipid (III), were measured individually (*a*). Standard vesicles containing 1 mol% N-NBD-PE (8 μm PC), mixed with standard vesicles containing 1 mol% N-Rh-PE (8 μM PC) were measured (*b*). The spectrum of standard vesicles (8 μM PC) containing both 1 mol% N-NBD-PE + 1 mol% N-Rh-PE showed 95% efficiency of resonance energy transfer (*c*). All vesicles were prepared, the fluorescent measurements performed and the efficiency of energy transfer (E) calculated by the formula $E = 1 - (F/Fo)$ as described under Materials and Methods.

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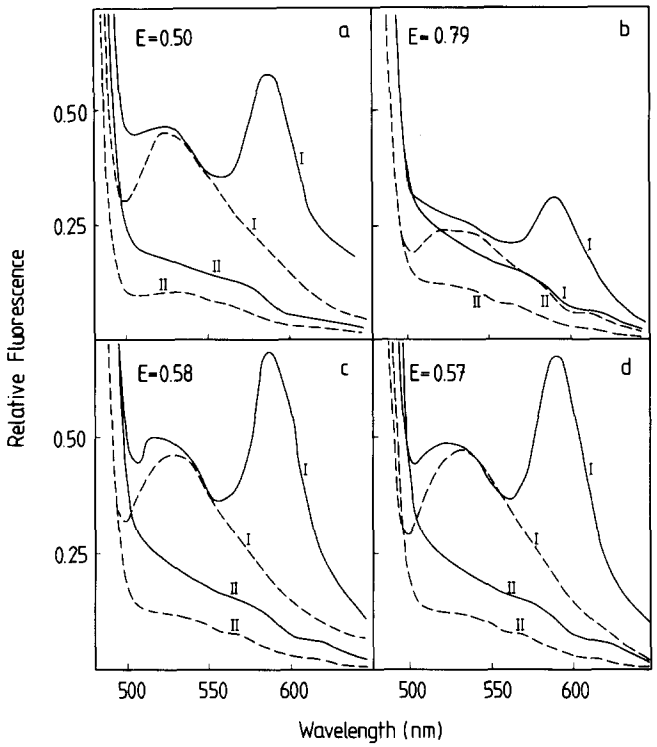

FIGURE 6 Emission spectra of fluorescent lipids in lipid vesicles before and after fusion with MDCK cells infected with influenza N virus and treated with trypsin. The samples were excited at 470 nm and the spectra recorded in the absence (—) or presence (- - -) of 1% Triton X-100. In *a*, curve I shows the loss of energy transfer when standard vesicles (200 nmol PC) containing both 1 mol percent N-NBD-PE + 1 mol% N-Rh-PE (Fig. 5 *c*) were bound to and fused with infected, trypsin-treated cells (900 nmol total cellular phospholipid) at 5 h after infection. After the fusion the cells were released from the dish by a treatment with trypsin-EDTA for 10 min at 37°C. Vesicles were prepared and the experiment performed as described under Materials and Methods. Curve II shows the background spectrum of infected cells released by trypsin-EDTA. The experiment is representative of four experiments with a mean loss of energy transfer of 45 ± 5% (mean ± SD). The panels *b–d* show the emission spectra of vesicles containing 1 mol% N-NBD-PE + 0.4 mol% N-Rh-PE after binding (*b*) or binding and subsequent fusion (*c* and *d*) of these vesicles with MDCK cells infected with influenza N virus. The cells were released from the dish either by scraping (*b* and *c*) or by trypsin-EDTA (*d*). The lipid vesicles (200 nmol PC) were bound to the cells (900 nmol total cellular phospholipid) at 4 h after infection for 30 min at 0°C and the cells were scraped from the dish at 0°C, the spectrum was recorded (*b*, curve I) and corrected for the background of the cells (curve II). This was identical (E = 0.79) to the spectrum of the original vesicles (not shown). When the binding was followed by a treatment with pH 5.3 at 37°C for 30 s and the cells were released by scraping, the energy transfer was drastically decreased (E = 0.58; *c*) and an identical decrease was observed when after the fusion the cells were released by a treatment with trypsin-EDTA for 10 min at 37°C (E = 0.57; *d*). In both panels curve II represents the background of the cells released by the respective method. For experimental details see Materials and Methods.

whether vesicles that were cell bound but not fused gave a change in signal after incubation at 37°C for 10 min; the vesicles were immediately released from the cells by the viral neuraminidase at 37°C (Fig. 3*a*).

To compare the emission spectrum of bound vesicles with that of fused vesicles, we used an alternative method of releasing the cells. Immediately after binding or after fusion, the cells were scraped from the plastic at 0°C. The spectra were recorded and compared with the spectrum of cells that had been released from the plate by the trypsin treatment after the fusion step (Fig. 6, *b–d*). Whereas the signal of the cell-bound vesicles (E = 0.79) was identical to that of the vesicles before addition of the cells (data not shown), low-pH treatment resulted in an identical decrease in energy transfer when the cells were released either by scraping (E = 0.58) or by trypsinization (E = 0.57). This confirms that the fusion induced by low pH is responsible for the spectral changes observed.

The total fluorescence in Fig. 6*b* is much lower than that in Fig. 6, *c* and *d*. This is due to the fact that during the preparation of the cells for the spectrophotometer (washing, scraping, and centrifugation at 4°C; see Materials and Methods) in Fig. 6*b*, the viral neuraminidase steadily released liposomes from the cell surface, which results in a loss of lipid radioactivity and fluorescence in the final cell pellet. This does not occur in Fig. 6, *c* and *d*, because after the fusion the vesicle-cell association has become neuraminidase resistant.

The loss of energy transfer never exceeded 50%. To test whether the concentration of the fluorescent probes in the plasma membrane after the fusion remained high enough to cause some energy transfer between the implanted molecules, vesicles were prepared that contained less fluorescent phospholipid, 0.5 mol% N-NBD-PE and N-Rh-PE (E = 0.88). They were bound to and fused with MDCK cells as before and the resulting decrease in energy transfer was compared with that observed in a parallel experiment with vesicles containing 1 mol% of each probe (E = 0.95). The loss of energy transfer using the vesicles containing 0.5 mol% of each probe, 41% (E = 0.52), was slightly higher than observed for the vesicles containing 1 mol%, 36% (E = 0.61), which suggests that some transfer still occurs in the plasma membrane.

Cell-Cell Fusion

When nonpolarized cells that express viral proteins all over their surface were treated with low pH, cell-cell fusion was observed (16, 32, 53). In our system, cell-cell fusion, tested as described by White et al. (53), was found not to occur. This is probably because the influenza HA protein in the polarized MDCK cells is mainly expressed at the apical surface (37) and because there is little or no HA protein at the sites where the cells in the monolayer are in contact with one another. Electron microscopy confirmed that there was no fusion between cells after fusion of lipid vesicles with the apical plasma membrane. The tight junctions were still intact after fusion. The fusion of lipid vesicles with the plasma membrane was therefore limited to the apical plasma membrane.

Fluorescence Light Microscopy

The fluorescent image of a MDCK cell monolayer with bound N-Rh-PE-containing vesicles is shown in Fig. 7*a*. The dots of fluorescence probably represent microvilli (cf. reference 37). Fig. 7*c* shows the image obtained after fusion of the vesicles with the plasma membrane. Compared with Fig. 7*a*, the differences in fluorescence intensity between individual cells are somewhat more pronounced, and also the cell borders are more apparent. Exactly the same pictures were obtained

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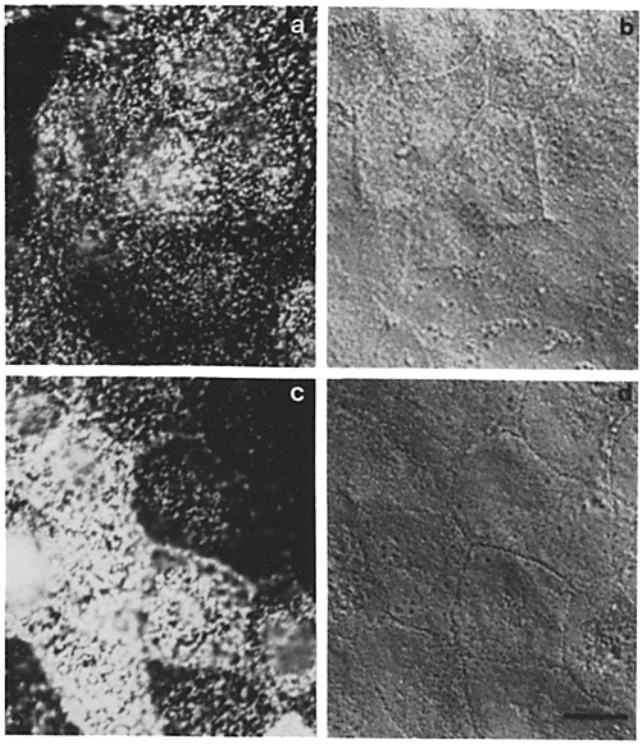

FIGURE 7 Binding and fusion of N-Rh-PE containing liposomes with MDCK cells. A two day old monolayer of MDCK cells was infected with influenza N virus and 4 h after infection N-Rh-PE containing liposomes (1 mol% in standard vesicles) were bound to (*a*) or fused with (*c*) the cells and the fluorescent images recorded, all as described under Materials and Methods. The corresponding Nomarski images are given in *b* and *d*. Bar, 10 μm. × 850.

when N-NBD-PE, instead of N-Rh-PE, was included in the vesicles.

DISCUSSION

In this paper we have shown that lipid vesicles containing the appropriate ganglioside can be bound to MDCK cells infected with influenza virus at 0°C and released from the cells simply by incubating at 37°C for 30 s or fused within 30 s with the plasma membrane of the cell after decreasing the pH to 5.3 at 37°C.

Binding of the Lipid Vesicles to the Cells

Egg PC/cholesterol (1:1, mol/mol) was chosen as the lipid mixture for the vesicles because PC is a neutral phospholipid and membranes of this composition are not fusogenic (see reference 34). Also, in our experiments, these vesicles had a very low, nonspecific binding to the cells. The lipid mixture mimics plasma membranes in its fatty acid composition with intermediate unsaturation and its high cholesterol content (50), and its physicochemical properties have been well characterized (23).

When the receptor ganglioside was included in the lipid vesicles, binding to MDCK cells expressing influenza virus proteins on their plasma membranes occurred; uninfected cells gave background levels of binding. An *N*-acetylneuraminic acid on the terminal galactose of the ganglioside appeared to be required to promote binding (Table I; binding monitored as vesicle-cell association after low pH). A similar minimal requirement has also been reported for the binding of Sendai virus to cellular receptors (14, 27). Bergelson et al. (2) have studied the role of gangliosides in the binding of FPV to Ehrlich ascites carcinoma cells, and they found, as we did, that G_{D1a} and G_{T1b} promoted FPV binding. However, they also found binding with G_{M1}, which has no terminal *N*-acetylneuraminic acid. We did not directly test G_{M1}, but since the influenza virus neuraminidase causes conversion of G_{D1a} into G_{M1} (5), and since this resulted in release of the bound vesicles (Fig. 3), we would assume that the latter ganglioside does not promote binding to the influenza virus HA in our system.

Binding required the presence of HA at the cell surface. The first HA proteins appeared on the apical plasma membrane at ~2.5 h after infection (M. Pesonen and G. van Meer, unpublished data), as measured by a protein A-binding assay (19) using specific antibodies to the HA protein. About 1 h later in infection, specific binding of the lipid vesicles could be demonstrated. Cleavage of the HA protein on the cell surface was not needed for the binding of the lipid vesicles (Table III). This is in agreement with previous studies of hemagglutination and virus-cell binding (20, 21).

Release of the Lipid Vesicles from the Cell Surface by Neuraminidase

The lipid vesicles bound to the cells at 0°C were very rapidly released from the cells at 37°C by the viral neuraminidase because the elution could be partially inhibited by a neuraminidase inhibitor (Table II). At 0°C, some release of vesicles apparently also took place. The weak vesicle binding of FPV-infected cells compared with cells infected with influenza N virus may therefore be due to a more active neuraminidase. Previous studies have shown that the viral neuraminidase causes elution of bound influenza virus from erythrocytes and other cells (see reference 3). This property could be used as a convenient method to release unfused vesicles from the cell.

Fusion of Lipid Vesicles with the Plasma Membrane

Low-pH -induced fusion is now an established property of influenza virus (16, 24, 28, 33, 55, 56, 59). We used this activity to induce fusion of the cell-bound vesicles with the plasma membrane. During the fusion at 37°C and pH 5.3, two processes were competing with each other: a conformational change in the viral HA molecule (41) leading to fusion, and, at the same time, activation of the viral neuraminidase resulting in vesicle release. When ice-cold pH 5.3 medium was added and the cells were warmed up to 37°C for 60 s to induce fusion, 20%-higher fusion values were obtained than when fusion was induced by direct addition of prewarmed pH 5.3 medium. The latter procedure was routinely used, however, because lower temperature might lead to membrane leakage during fusion (55).

Viral fusion with liposomes and cell membranes requires the proteolytic cleavage of the HA protein into the HA_1 and HA_2 polypeptide chains (16, 17, 24, 25, 56). This was also the case in our experimental system (Table III: Fig. 4). Occasionally, some fusion was observed with cells in which the influenza N HA protein had not been cleaved with trypsin (Table III). This was probably due to a small fraction of cleaved HA protein caused by proteases in the medium, perhaps the serum.

The most convincing proof for fusion in our system came from studies with the fluorescent probes N-NBD-PE and N-Rh-PE. Previous studies (10, 44) have used assays based on fluorescence energy transfer to show liposome-liposome fusion, but with these assays no evidence of fusion between liposomes and plasma membranes was found. In our experiments a dramatic change was observed in the fluorescence spectrum after the low-pH treatment as compared with the spectrum seen before the treatment. From this we conclude that extensive fusion between the lipid vesicles and the plasma membrane has taken place. It is highly unlikely that exchange of the fluorescent phospholipids into the plasma membrane is responsible for the spectral changes, since Struck et al. (44) have shown for these lipids that even after 30 min at 37°C the rate of lipid exchange or transfer was too low to lead to changes in transfer efficiencies. The loss of resonance energy transfer between the two fluorescent phospholipids after fusion in our study never exceeded 50%, whereas under similar conditions, ~70% of the lipid that was still cell-associated after the fusion appeared to be fused as measured by the neuraminidase assay. The loss of energy transfer could, of course, be limited by the concentration of the fluorescent lipids in the apical membrane, which might still be high enough after fusion to allow some energy transfer. This possibility was tested by including twofold-lower probe concentrations into the vesicles. However, only a small increase in the loss of energy transfer after fusion did occur. The precise correlation between the neuraminidase assay and the fluorescence assay remains to be determined.

In the experiments described (Figs. 1 and 2), 20% of the lipid vesicles bound to the cells. Of that, 50% were fused with the apical plasma membrane (Fig. 4), amounting to 10% of the added lipid vesicles. Both the neuraminidase and the resonance energy transfer assay show that of these 10%, after the fusion, at least 50% (5% of the added vesicles) are incorporated in the apical plasma membrane domain of the MDCK cells. In this way an absolute amount of 0.1 nmol of PC was incorporated per 100 nmol cellular phospholipid. Estimating that ~5% of the cellular phospholipids are present in the apical plasma membrane domain, the fused liposomes add 2% new phospholipid into the membrane. This amount can, if desired, be increased by a factor of ~50.

The method that we used to fuse lipid vesicles into MDCK cells should be applicable to all cells that can be infected with influenza virus. One limitation of the method is that influenza virus infection usually shuts off host protein synthesis. However, membrane functions in the cell continue for many hours before the cell dies. If necessary, the problem can be overcome by transforming the cell with the gene coding for the influenza virus HA protein (53). In this case, possible cytopathic effects caused by viral infection should not interfere. We are going to use the method described here to implant lipids in the apical plasma membrane domain of MDCK cells for studies of lipid redistribution between the apical and the basolateral surface domains. Our previous studies (51) have shown that these two membrane domains have dramatic differences in phospholipid compositions, and nothing is known of how these differences are generated and maintained in epithelial cells.

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